Nucleic Acids Research, 1993, Vol. 21, No. 23 ***5301–5307***

Yeast open reading frame YCR14C encodes a DNA β-polymerase-like enzyme

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Received October 1, 1993; Accepted October 12, 1993

ABSTRACT

We have shown by activity gel that overexpression in *E.coli* of a yeast chromosome 3 open reading frame (ORF) designated YCR14C and bearing homology to mammalian DNA polymerases β results in a new DNA polymerase in the host cells. The molecular mass of this enzyme corresponded to the YCR14C-predicted 67 kDa protein, and NH_2-terminal amino acid sequencing confirmed that the expressed protein was encoded by the yeast ORF. This new yeast DNA polymerase was purified to homogeneity from *E.coli*. In a fashion similar to that of mammalian β-polymerases, the purified yeast enzyme exhibited distributive DNA synthesis on DNA substrate with a single-stranded template and processive gap-filling synthesis on a short-gapped DNA substrate. Activity of this yeast β-polymerase-like enzyme was sensitive to the β-polymerase inhibitor ddNTP and resistant to both 1mM NEM and neutralizing antibody to *E.coli* DNA polymerase I. These results, therefore, indicate that YCR14C encodes a DNA β-polymerase-like enzyme in yeast, and we name it DNA polymerase IV. Yeast strains harboring a deletion mutation of the *pol IV* gene are viable, they exhibit no increase in sensitivity to ultraviolet light, ionizing radiation or alkylating agents, and sporulation and spore viability are not affected in the mutant.

INTRODUCTION

DNA polymerase β (β-pol) has been characterized primarily as a ~39 kDa monomeric enzyme from vertebrates, and use of selective inhibitors in permeabilized and intact mammalian cell culture systems has pointed to a role of β-pol in some, but not all, DNA repair pathways(1–6). With DNA substrates containing short gaps, purified mammalian β-pols conduct gap-filling synthesis to completion (7–9) and at least two mammalian *in vitro* DNA repair systems for short gap-filling DNA repair synthesis have been shown to require β-pol (10, 11).

To gain more information about the *in vivo* roles of β-pol in eukaryotic DNA metabolism, the identification of a homologue of β-pol in yeast could prove to be important; however, no such enzyme has yet been identified and characterized among the several DNA polymerases (DNA polymerases I, II, III, mitochondrial, and *REV3* gene product of unknown function) described from the yeast *Saccharomyces cerevisiae* (12–16). Interestingly, Oliver *et al.* (17) reported that a DNA sequence on yeast chromosome 3 potentially encodes a novel 67 kDa protein that has partial sequence homology to the 39 kDa mammalian β-pol (18). This open reading frame (ORF) has been designated as YCR14C and potentially encodes a protein exhibiting 26 % identity and 50 % similarity with the human and rat β-pol homologues. The NH_2-terminal ~170 residues of the YCR14C-deduced protein bear no homology to mammalian β-pol (18).

In the present study, we overexpressed YCR14C in *E.coli* and evaluated the recombinant protein for its ability to carry out DNA β-polymerase-like DNA synthesis *in vitro*. Our results indicate that YCR14C, indeed, encodes a DNA polymerase. This enzyme conducts short gap-filling DNA synthesis in a fashion very similar to that of mammalian β-pol. Thus, these results document the presence of a β-pol-like DNA polymerase in yeast and, along with the initial discovery of the ORF (17), point to further biochemical and genetic studies in this facile eukaryotic system.

MATERIALS AND METHODS

Materials

Radioactive [γ-^{32}P] ATP and [α-^{32}P] dTTP were purchased from ICN (>4500 Ci/mmol). Unlabeled dNTPs, T4 polynucleotide kinase, T4 DNA ligase, DNA polymerase 1, Klenow fragment and the restriction enzymes were from Boehringer Mannheim. M13mpl8(+) ssDNA and the M13 universal sequencing primer were purchased from Pharmacia LKB Biotechnology Inc. HPLC purified synthetic oligonucleotide primers were obtained from Genosys Biotechnologies Inc. The

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expression vector pRSET was purchased from Invitrogen Corp. Activated calf thymus DNA was prepared as described (19). N-ethylmaleimide and aphidicolin were purchased from Sigma.

Buffers

Buffer A consisted of 50 mM Tris−HCl, pH 7.5, 10 mM $Na_2S_2O_5$, 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, and 1 mg/L pepstatin A. Buffer B was 50 mM Tris−HCl, pH 7.5, 150 mM NaCl, 20% glycerol, 100 μg/ml BSA, 1 mM DTT, and 0.1 mM EDTA. Buffer C was 50 mM NH_4HCO_3 and 0.01 % SDS.

Construction of the yeast DNA polymerase expression vector

YCR14C was placed into the pRSET-A bacterial expression vector in two steps. First, a 2.6 kb *Xho*I−*Eco*RI fragment from E3-E4 was inserted into *Xho*I−*Eco*RI cut pRSET-A to make plasmid pRSET-X/R. The XhoI site is ~400 bp upstream of the first ATG codon of YCR14C and the *Eco*RI site is ~400 bp downstream of the YCR14C stop codon. The extra upstream sequences were removed by cleaving with *Nde*I (which cuts at the ATG of the pRSET-A expression vector) and *Nsi*I which cuts 180 bp 3′ to the YCR14C ATG. The YCR14C ATG was changed to an *Nde*I site by PCR amplification using oligonucleotide primers spanning the ATG and the *Nsi*I site. After digestion with *Nde*I and *Nsi*I, the PCR generated 180 bp fragment was purified and inserted into *Nde*I−*Nsi*I cut pRSET-X/R. This plasmid was named pRSET-YβP and the PCR generated 180 bp insert was confirmed by DNA sequencing.

Expression of the recombinant 67 kDa yeast protein in *E.coli*

E.coli BL21 (DE3) pLys S cells were transformed with plasmid pRSET-YβP and grown overnight in LB medium at 37°C. Four two-liter flasks containing 500 ml LB broth were inoculated with 10 ml of an overnight culture of *E.coli* and aerated on a rotary shaker at 37°C until the absorbance at 595 nm reached 0.5. Then isopropyl β-D-thiogalactopyranoside (IPTG, Sigma) was added to a final concentration of lmM and incubation was continued at 37°C for 3 hr. Cells were collected by centrifugation for 15 min at 5,000 rpm and stored at −80°C until used. The cell pellet was suspended in buffer A containing 500 mM NaCl, sonicated on ice with a Braun Sonic U sonifier, 6×30s. An aliquot of the suspension was saved for total extract fraction. The expressed recombinant yeast 67 kDa protein was insoluble under these conditions, and therefore, all of it was collected by centrifugation for 20 min at 15,000 rpm at 4°C. The pellet was washed once in buffer A and resuspended in the same buffer. Aliquots from the total extract, supernatant and pellet fractions were examined by 9.5% SDS−PAGE for expression of recombinant 67 kDa yeast protein and visualized in a Coomassie blue stained gel. DNA polymerase activity was determined by activity gel as described below.

Preparative SDS−PAGE electrophoresis

For preparative SDS-polyacrylamide gel electrophoresis, a Bio-Rad Model 491 Prep Cell apparatus was used to purify the recombinant 67 kDa yeast protein. A 12 cm high, 7.5% acrylamide (29.2:0.8, acrylamide: bis-acrylamide, w/w) separating gel was polymerized in a 28 mm diameter tube of the preparative gel apparatus. After separating gel formation, 1 cm high stacking gel of 3% acrylamide was polymerized on top of the separating gel. The pellet fraction containing the recombinant protein (6 mg) was dissolved in 1 ml of SDS−PAGE sample buffer and incubated at 37°C for 5 min. The sample was loaded and the gel run at 280V constant voltage. When the bromophenol blue marker dye reached the bottom, eluting buffer C was pumped through the elution chamber and fractions of 2.5 ml were collected at a rate of 1 ml/min. A total of 200 fractions were collected. These fractions were first examined on a mini SDS-PAGE to locate the recombinant protein (~67 kDa). Once the elution position of the recombinant protein was determined, 100 μl of every fraction near the peak of eluted recombinant protein was examined. The peak fractions (140−160) were divided into five pools which were renatured for DNA polymerase activity.

Renaturation of the recombinant yeast protein

The proteins were renatured as described (20) with the following modification. The pooled fractions were dried in a speed vac to remove NH_4HCO_3. The samples were dissolved in water and precipitated with four volumes of cold acetone (−20°C) for 30 min in a dry ice−ethanol bath. The tubes were then centrifuged for 10 min, supernatants were poured off, and the precipitates were rinsed once with 1 ml of ice-cold acetone containing 20% buffer B. The precipitates were dried 2−3 min in a speed vac and dissolved in 25 μl of 6M guanidine−HCl prepared in buffer B. The samples were allowed to stand at room temperature for 30 min. The samples were then diluted 5-fold in buffer B and left at room temperature for 2 hr. These samples were then dialyzed against buffer B for 36 hr to allow the proteins to renature and to remove the guanidine−HCl. The renatured samples were analyzed for DNA polymerase activity.

In vitro DNA synthesis

In vitro DNA synthesis was carried out essentially as described (9), except that the concentration of each dNTP was 50 μM. Products were analyzed by electrophoresis on a 12% polyacrylamide−7 M urea gel and visualized by autoradiography.

Activity gel analysis of DNA polymerase

For activity gel analysis, the protocol of Karawya *et al.* (19) was followed. The protein samples were electrophoresed in 9.5% SDS−PAGE gels containing 100 μg/ml of activated calf thymus DNA as template-primer and probed with a reaction mixture carrying [α-^{32}P] dTTP as labeled dNTP. The newly synthesized product DNA was visualized by autoradiography.

Deletion of the YCR14C (*POL IV*) gene in yeast

A 5.2 kb *Eco*RI fragment containing YCR14C, which we named *POL IV*, was obtained in bluescript SK(−) (17), and used as the starting material. Plasmid pJW191, a 3.58 kb pUC19 based plasmid with a unique *Bam*HI site flanked by 415 bp of 5′ sequences of the *POL IV* gene from nucleotide positions −414 to +1, where + 1 has the A of the translation initiation ATG codon, and 504 bp of 3′ sequences from +1656 to +2160 of the *POL IV* ORF (stop codon at + 1747). Two *POL IV* deletion generating plasmids were constructed from pJW191. In one, pJW192, the *LEU2* gene (in which the *Kpn*I and *Eco*RI sites have been removed) was inserted between positions +1 and +1656 (90 nt upstream of the stop codon) of the *POL IV* ORF. To obtain a genomic deletion mutation of the yeast *POL* IV, pJW192 was digested with *Eco*RI and *Pst*I and used to transform yeast to *Leu*$^+$. Alternatively, plasmid pJW193, contains a 3.8 kb *Bam*HI *(His* G-*URA43-HisG)* fragment, that was inserted into the *Bam*HI

site of pJW191. Transformation of yeast to *Ura*$^+$ with *Eco*RI−*Sph*I digested pJW193 results in deletion of the *POL IV* ORF and its replacement by the *URA3* gene. Subsequent growth of cells on 5-fluoro-orotic acid (5-FOA) containing medium selected for *Ura*$^-$ cells.

Southern hybridizations were performed to confirm the generation of a genomic deletion mutation in the *POL IV* ORF. Hybridization probes corresponding to different regions of the *POL IV* and to flanking regions were labeled with ^{32}P using the multi-prime kit from Amersham.

Construction of double mutants of *pol IV*Δ with the other yeast DNA polymerases

Double mutants of *pol IV*Δ with temperature-sensitive (ts) mutations of DNA polymerase I *(pol1-17)*, DNA polymerase II *(pol 2-18)*, and DNA polymerase III *(cdc2)*, were constructed by appropriate matings with *pol IV*Δ strains using standard techniques of yeast genetics. The *rev3*Δ*pol IV*Δ double mutant was generated by making a genomic deletion mutation of the *REV3* gene in the *pol IV*Δ mutant JW315 *(MATαCAN1^s leu 2-3, -112 trp1*Δ*ura3 pol IV*Δ*::LEU2)* by transformation to *Ura3*$^+$ with plasmid pPM292.

Response to various DNA damaging agents

Oxidative damage. Cells were grown in YPD to a density of ~4×10^8 cells/ml, pelleted by centrifugation, washed once with water, and suspended at 10^8 cells/ml in various concentrations of H_2O_2 buffered with 100 mM KPO_4 (pH 7.0). Following a 2 hr incubation period at room temperature, the cells were washed once with water, resuspended in water, and plated onto YPD plates. The surviving colonies were counted after 2 days incubation at 30°C.

UV and γ radiation. A master plate containing the strains of interest was grown overnight at 30°C on YPD medium. Colonies from this master plate were transferred to new YPD plates. The plates were then exposed to various doses of UV or ionizing radiation, incubated at 30°C for 2 days, in the dark for UV irradiated plates, and scored for survival.

MMS. For determining survival after treatment with methyl methanesulfonate (MMS), colonies were transferred onto plates containing MMS at 0.0 1 %, 0.02 %, and 0.03 %, and examined following 2−3 days of growth at 30°C.

Bleomycin, ethyl methane sulfonate (EMS), and N-methyl-N'-nitro-N-nitrosoguamidine (MNNG). These agents were applied to filter discs at the center of YPD plates that were streaked with the various strains. Bleomycin was dissolved in water at 0.5 mg/ml and 50 μl added to the disk. EMS was added to the disk undiluted (20 μl per plate). MNNG was dissolved in acetone to 10 mg/ml. An aliquot of this was diluted 20-fold in 50 mM sodium phosphate (pH 7.4) and 50 μl was added to the disk. Sterile water was added to the disk on the control plate. Plates were incubated at 30°C for 2−3 days before being scored.

Other methods. SDS−polyacrylamide gel electrophoresis was performed according to the method of Laemmli (21), using 9.5 % discontinuous slab gels. NH_2-terminal sequencing was carried out on an Applied Biosystems model 477A protein sequencer. Protein concentration was determined by the Bradford method (Bradford, 1976), using a Bio-Rad protein assay kit.

RESULTS

Expression and purification of yeast β-pol-like DNA polymerase

The expression plasmid pRSET-YβP has YCR14C under the control of a promoter for the bacteriophage *T7* RNA polymerase. The ORF was predicted to encode a polypeptide of 582 amino acid residues and ~67 kDa (67,632) molecular mass. pRSET-YβP was transformed into *E.coli* BL21 (DE3) pLys S, a strain that contains the *T7* RNA polymerase gene under the control of the *lac* promoter. Mid-logarithmic stage cultures of *E.coli* bearing pRSET-YβP were induced with IPTG. Induced cells were found by SDS−PAGE to contain a new major, polypeptide migrating at ~67 kDa (Fig. la, lanes, 2−4). The quantity of this polypeptide appeared to be approximately 5 % of total *E.coli* protein. Similar SDS−PAGE analysis of the uninduced cultures, or cultures containing the vector alone (not shown), did not show a corresponding ~67 kDa major polypeptide (Fig. la, lane 1). Results of fractionation experiments using sonicated lysates revealed that the recombinant 67 kDa polypeptide was almost exclusively in the pellet fraction, rather than the supernatant fraction (Fig. la, lanes 5 and 6, respectively), indicating that the 67 kDa protein remained insoluble under the extraction conditions used.

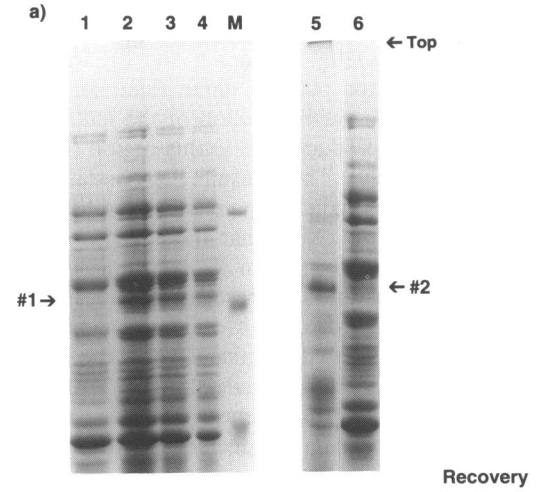

Figure 1. Expression of the yeast ORF YCR14C in *E.coli*. **Panel a**, *E.coli* BL21 (DE3) plyS cells, transformed with plasmid pRSET-YβP, were grown in LB medium to an OD of 0.5 and induced with IPTG (lmM). For a total extract fraction (lanes 1−4), the cells were collected by centrifugation and lysed directly by boiling in the gel-loading buffer for 3 min. In a parallel experiment, the induced culture was fractionated into supernatant and pellet fractions as described under 'Materials and Methods'. (lanes 5 and 6, respectively). Samples were then separated by 9.5 % SDS−PAGE and visualized by staining with Coomassie blue. Lane 1 contains 30 μl of total extract fraction from the uninduced culture, whereas lanes 2, 3 and 4 contain 15, 30, and 50 μl, respectively, of total extract from the induced culture. Lane 5 contains the pellet fraction and lane 6 the supernatant from 50 μl of extract. Arrows refer to 67 kDa polypeptide from the total extract (#1) and the pellet fraction (#2). The protein marker lane is designated by 'M'. **Panel b**, the NH_2-terminal sequences of the predicted YCR14C ORF and the 67 kDa polypeptide of yeast expressed in *E.coli*. Recovery in the 10th cycle of sequencing is indicated in parenthesis.

To confirm the identity of the recombinant 67 kDa protein, the total extract and the pellet fraction were separated by SDS−PAGE, and proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. The Coomassie blue-stained 67 kDa protein band was cut from the membrane and sequenced. The NH_2-terminal sequence of the peptide started at serine 2 of the ORF-predicted sequence and the subsequent 14 residues were in exact agreement with the sequence (Fig. lb) predicted from YCR14C translation. These results taken together indicate that YCR14C ORF had been expressed in *E.coli*. Probing of Western blots of the pellet fraction with our laboratory's polyclonal antibody to rat β-pol or with monoclonal antibodies to rat and human β-pol 10S, 18S, 22S, 35S or 38S (Srivastava, Showalter and Wilson, unpublished) failed to reveal significant cross-reaction with the 67 kDa yeast protein.

Demonstration of DNA polymerase by activity gel analysis

Various fractions (total extract, supernatant and pellet) of induced and uninduced cultures were subjected to the *in situ* DNA polymerase activity gel assay (19). Proteins were separated by SDS−PAGE, and DNA polymerase analysis was then carried out. *E.coli* DNA polymerase I (Pol I) and Klenow fragment were used as reference enzymes, and *E.coli* cells containing the plasmid without YCR14C DNA served as a negative control. It is apparent from the autoradiogram shown in Figure 2 that the total extract and the pellet fraction from the induced culture showed a strong product band indicating DNA polymerase activity at molecular mass of ~67 kDa (Fig 2, lanes 5 and 8). In contrast, cells containing the vector plasmid alone (lane 3), the uninduced culture, or the supernatant fraction of the induced culture did not show a strong product band in this region (Fig. 2, lanes 4, 6 and 7, respectively). These results demonstrate that a 67 kDa DNA polymerase is induced in cells containing the expression plasmid pRSET-YβP. Note that there appeared to be weak product bands of similar size to the induced band (~67 kDa) in virtually all preparations. These polypeptides, were probably due to endogenous proteolysis of Pol I to produce Klenow-like fragments. Therefore, to determine whether the ~67 kDa DNA polymerase activity in the induced cells truly represented recombinant yeast DNA polymerase or was due to contamination by a Klenow-like fragment, we decided to purify the recombinant yeast protein to homogeneity, and then examine its DNA polymerase activity *in vitro*.

Purification of the recombinant 67 kDa yeast protein

The recombinant ~67 kDa yeast protein was purified using a procedure that relied on preparative SDS−polyacrylamide gel electrophoresis as the final step. Multiple fractions were collected during preparative SDS−PAGE and were analyzed first by 'mini SDS−PAGE' to identify fractions containing the overexpressed 67 kDa polypeptide (data not shown). Once the elution position of the recombinant protein was determined, selected fractions near the peak were analyzed for purity by SDS−PAGE. Photographs of Coomassie-stained and silver-stained gels are shown in Figures 3a and 3b. Several fractions appeared to contain homogeneous 67 kDa protein, as revealed by the absence of any visible polypeptide contaminants (Fig. 3a, b). This interpretation was further supported by the absence of other polypeptides upon NH_2-terminal sequence analysis of the fractions 150 and 151 (data not shown). The lower level of sensitivity of these experiments, for detection of contaminants, was about 1 part in 30. Several of these individual fractions were examined for DNA

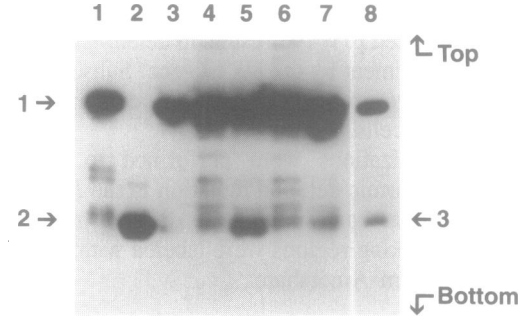

Figure 2. Photograph of autoradiogram illustrating activity gel analysis. Cultures of *E.coli* containing plasmid pRSET-YβP were grown and induced as in Fig. 1. The cell pellet was sonicated in Buffer A with 500 mM NaCl and fractionated into total extract, supernatant and pellet fractions. In lanes 3−7, equal amounts (72 μg) of total extract and supernatant fraction were submitted to 9.5% SDS−PAGE and analyzed for DNA polymerase activity by the *in situ* activity gel assay. Purified *E.coli* DNA Pol I and Klenow fragment were used as reference. The activity gel was performed as described (19), with activated calf thymus DNA as template·primer and [α-^{32}P] dTTP as labeled dNTP. Lanes: 1, *E.coli* Pol I; 2, Klenow fragment; 3, total extract of induced culture of *E.coli* with vector alone (control); 4 and 5, total extract; 6 and 7, supernatant fraction from uninduced and induced cultures of *E.coli* with pRSET-YβP, respectively. Lane 8 contained one-third of the pellet fraction from the extract added to lane 5. Arrows 1, 2, and 3 refer to positions of the Pol I, Klenow fragment and the 67 kDa expressed yeast protein, respectively.

polymerase by activity gel analysis, which confirmed the presence of a 67 kDa polymerase (Fig. 3c). These results indicated that the purified recombinant yeast protein was a DNA polymerase, as expected. To address the question of whether the recombinant yeast protein had β-pol-like DNA polymerase activity, we divided the peak fractions (140−160) into five pools. Pools I to V represent fractions: 140−146; 147−149; 150−153; 154−156; 157−160, respectively. These pools were renatured and examined for *in vitro* DNA synthesis activity.

In vitro DNA synthesis

The renatured pooled fractions (I−V) described above were tested for DNA synthesis gap-filling activity and distributive primer extension activity on DNA template primer. Assays were performed using M13mpl8 ssDNA as template and a 17 residue oligonucleotide primer labeled at the 5′-end. To create a 5-nucleotide gap, another oligonucleotide (51 residues) was annealed downstream of the labeled primer. Reactions were initiated by adding 1 μl of each pooled fraction to a reaction mixture (total volume 5 μl) at 37°C. The primer-extended products were analyzed by urea-polyacrylamide gel electrophoresis and visualized by autoradiography. The results in Figure 4, representing activity of pool III, indicated that the recombinant yeast DNA polymerase conducted distributive DNA synthesis. This activity was very similar to that routinely exhibited by mammalian β-pols and was distinguished by the detailed distribution of extended products, from the activity of Pol I or Klenow fragment under the same reaction conditions. We conclude from these results that the recombinant yeast DNA polymerase is similar to a mammalian DNA β-polymerase, since β-pol is the only distributive DNA polymerase known, under the reaction conditions of the experiment shown in Figure 4.

In a gap-filling assay with a substrate containing a 5-nucleotide gap, a strong product band was formed corresponding to the completely filled gap. This product represented processive filling

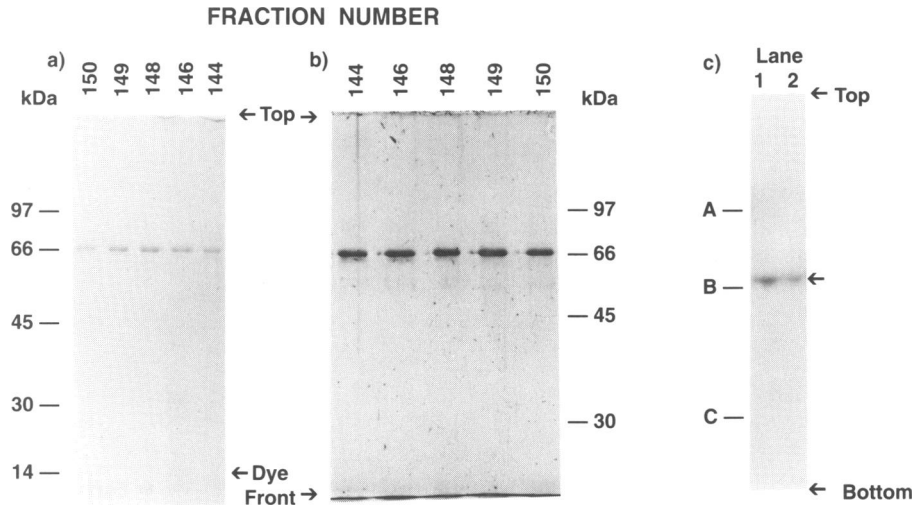

Figure 3. Analysis of purified 67 kDa yeast protein expressed in *E.coli*. Portions of selected fractions eluted from the Prep Cell were analyzed by 9.5 % SDS−PAGE and stained with Coomassie blue (**Panel a**) or SDS−PAGE silver (**Panel b**). Numbers on left (Panel a) and right (Panel b) indicate the positions of protein markers. **Panel c**, photograph of an autoradiogram of the activity gel analysis for the purified fractions 149 (lane 1) and 150 (lane 2); electrophoresis was continued until the 30 kDa protein marker ran out of the gel. Arrow indicates the position of the DNA polymerase activity in the 67 kDa yeast protein expressed in *E.coli*. A, B and C denote the positions of 97, 66 and 45 kDa protein markers.

of the 5-nucleotide gap (9), and the synthesis was very similar to that conducted by a purified mammalian β-pol used as reference enzyme (Fig. 4). Gap-filling by Klenow fragment was also examined as a reference. Klenow fragment was able to fill the 5-nucleotide gap, but this polymerase activity could be readily distinguished from that of β-pol, as Klenow conducts strand-displacement of the downstream polynucleotide to a much greater extent than does β-pol (Fig. 5b, lanes 5, 6 and 6′).

Further comparison of yeast and rat β-pols with Pol I and Klenow fragment

We have shown by amino acid sequencing and other experiments that YCR14C was expressed in *E.coli* and that the purified recombinant yeast protein has DNA polymerase activity. Products made by this yeast DNA polymerase are very similar to those generated by the 39 kDa mammalian β-pol, distinguishing it from *E.coli* Pol I or Klenow-like fragments. In further characterizing the enzyme, we found that it is not inhibited by aphidicolin to 100 μg/ml or by lmM N-ethylmaleimide, and the enzyme was completely inhibited by ddNTP, at a 1:10 and 1:100 ratio of dNTP/ddNTP (Fig. 5a). These properties of the yeast DNA polymerase are very similar to those of rat β-pol used as reference in these experiments. Finally, both yeast and rat β-pols are not inhibited by a neutralizing antibody to Pol I, whereas Klenow fragment, studied as reference, was almost completely inhibited under the same reaction conditions (Fig. 5b). This result confirms that contamination by a Klenow-like fragment of Pol I did not account for the activity of our yeast β-pol samples.

Genetic studies in yeast

Since our studies indicate that YCR14C encodes a yeast homologue of mammalian β-polymerase, although the enzymes differ in size, we have designated the β-pol-like encoding gene as *POL IV*. In order to delete the genomic copy of the *POL IV* gene, we constructed plasmids pJW192 and pJW193 which had nucleotides from positions +1 to +1656 (90 nucleotides upstream

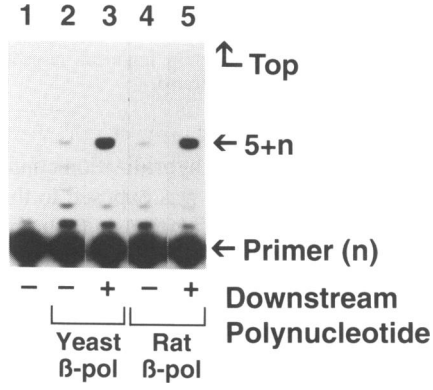

Figure 4. Comparison of primer extension and gap-filling activities of recombinant yeast β-pol and rat β-pol on different DNA substrates. A photograph of an autoradiogram illustrating product distribution in a sequencing gel is shown. *In vitro* DNA synthesis was carried out on M13mp18 template using ^{32}P-labeled primer in the absence or presence of an unlabeled downstream polynucleotide to form a 5 nt gap. Gel electrophoresis and DNA synthesis were performed as described (9). Lanes 2 and 3, primer extension and gap-filling carried out by the recombinant yeast β-pol; lanes 4 and 5, purified rat β-pol activity, as reference. Lane 1, a reaction mixture without enzyme. Positions of the primer (n) and completely filled 5 nt gap (5+n) are indicated.

of the stop codon) of the *POL IV* gene replaced with either the *LEU2* or *URA3* gene, respectively. The *Leu*$^+$ *or Ura*$^+$ transformants were analyzed by Southern blot hybridization to confirm that the *POL IV* gene had been deleted. Unlike deletions of DNA polymerase I, DNA polymerase II, or DNA polymerase III genes, deletion of *POL IV* from haploid strain was not lethal, and had no effect on growth. We tested for duplication of *POL IV* in six unrelated haploid strains, as well as a *pol IV*Δ strain. Southern blot hybridizations were done independently with three different probes corresponding to the 5′, 3′, and central region

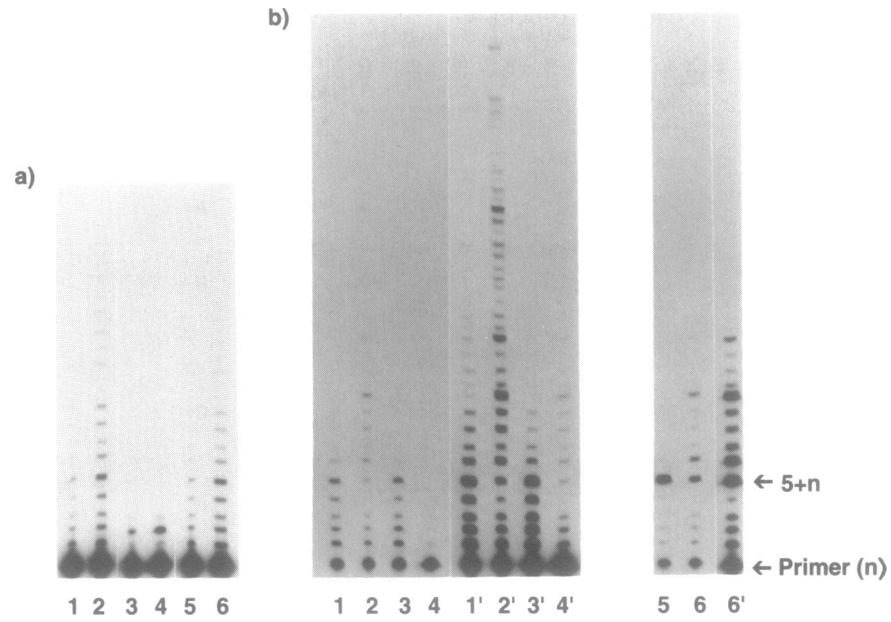

Figure 5. Comparison of gap-filling and effect of inhibitors or antibody to *E.coli* DNA Pol I on primer extension activity of yeast β-pol and Klenow fragment. *In vitro* DNA synthesis was carried out on M13mpl8 template using ^{32}P-labeled primer, and the products were analyzed by gel electrophoresis as described (9). Photographs of the autoradiograms are shown. **Panel a**: Lanes 1 and 2, DNA synthesis by yeast β-pol, with 5 μM dCTP for 10 and 30 min. respectively; 3 and 4, same as lanes 1 and 2 except with addition of 50 μM ddCTP; 5 and 6, same as lanes 1 and 2 except with addition of 1 mM NEM. **Panel b**: Lanes 1 and 2, primer extension DNA synthesis by yeast β-pol and Klenow fragment, respectively; lanes 3 and 4, same as lanes 1 and 2, except that both enzymes were preincubated with antibody to *E.coli* DNA Pol I. Lanes 1′−4′ are the same as lanes 1−4, but with longer autoradiogram exposure time; lanes 5 and 6, 5-nucleotide gap-filling synthesis activity by yeast β-pol and Klenow fragment, respectively; lane 6′, the same as lane 6, but with longer exposure time. Positions of the primer (n) and the completely filled gap (5+n) are indicated.

of *POL IV*, under low stringency hybridization conditions (50°C instead of the usual 65°C). Film was exposed to the hybridized filters after each wash step of increasing harshness. These experiments gave no evidence for duplication of the *POL IV* gene, suggesting that it is present in single copy in yeast.

To examine the effect of the *pol IVΔ* mutation on sporulation, four different *pol IVΔ/pol IVΔ* diploids were transferred to sporulation medium. All diploids showed normal sporulation frequencies. One of them was dissected; out of 15 tetrads, 12 produced 4 viable spores, 2 yielded 3 viable spores, and one gave one viable spore. Similar results were obtained when *pol IVΔ/+* diploids were subjected to tetrad analysis. Normal segregation was observed for the various markers present. Thus, deletion of the *POL IV* gene has no effect on sporulation or spore viability.

Purified mammalian β-pols exhibit a high rate of misincorporated nucleotides ~1/1000 bases inserted). Hence, we examined haploid *pol IVΔ* strains for both spontaneous and UV-induced mutagenesis. We determined the rate of forward spontaneous mutations from *CAN1^s* to *can^r*, as well as UV-induced mutagenesis of the *CAN1^s* locus. No difference in spontaneous mutation rate was observed between isogenic wild type and *pol IVΔ* strains. Likewise, the frequency of UV-induced mutagenesis was unaffected by the *pol IVΔ* mutation.

The primary role of β-pol is believed to be in DNA repair. Therefore, we examined the sensitivities of isogeneic wild type and *pol IVΔ* strains to various DNA damaging agents. The *pol IVΔ* strain showed no altered sensitivity to any of the agents we used (UV radiation, γ radiation, bleomycin, MMS, EMS, MNNG, and H_2O_2). Furthermore, double mutants of *pol IVΔ* and *polII(ts), or polII(ts), or rev3Δ* were no more sensitive to these DNA damaging agents than the single mutants of *polI, polII, polIII, or rev3Δ*, respectively.

DISCUSSION

The entire DNA sequence of chromosome 3 of the yeast *Saccharomyces cerevisiae* has been determined (17) and the sequence reveals 182 open reading frames (ORFS) for proteins longer than 100 amino acids. The biochemical implication or role of 74 of these ORFs has been predicted (18). The ORF termed YCR14C was predicted to encode a type X DNA polymerase, which can be aligned to exhibit 26% amino acid identity with both human and rat DNA β-polymerases (18). However, the biochemical function of this predicted protein had not been determined. To study structure-function relationships of this potential DNA polymerase in yeast, we overexpressed YCR14C in *E.coli*. This yeast DNA sequence directed expression of a 67 kDa polypeptide that remained insoluble under the extraction conditions used. The identity of the protein was confirmed by NH_2-terminal amino acid sequencing; the NH_2-terminal methionine was absent, by virtue of an unknown mechanism, as was observed earlier for intact β-pol, 8 kDa domain of β-pol, hnRNPA1, and HIV-1 RT expressed proteins in *E.coli* (22−25).

The DNA polymerase activity of the recombinant yeast protein was first examined using the activity gel assay, which clearly suggested that the recombinant 67 kDa protein had DNA polymerase activity. This was evident by analysis of crude extract where we found strong induction of a 67 kDa DNA polymerase activity in IPTG treated cultures, as compared to uninduced or control cultures of cells containing the plasmid vector alone. Since the size of this protein was similar to that of Klenow-like fragments (~68 kDa) of *E.coli* Pol I, it was difficult to assess the validity of these interpretations using crude extract preparations. Therefore, we purified the recombinant yeast protein to homogeneity and then examined detailed properties

of its DNA polymerase activity *in vitro*. The purity and identity of the protein were first confirmed by SDS−PAGE and NH_2-terminal sequence analyses and the purified protein showed DNA polymerase activity in the activity gel assay, confirming that the overexpressed 67 kDa yeast protein was, indeed, a DNA polymerase. To establish that the recombinant protein was a DNA polymerase β-like enzyme, we examined its activity in a second series of *in vitro* DNA synthesis assays. In these assays, β-pol activity can be readily differentiated from that of other DNA polymerases. For example, β-pol fills a 5-nucleotide gapped DNA substrate to completion by a processive mechanism and at low concentration shows only limited strand-displacement activity of the downstream polynucleotide. In addition, β-pol conducts distributive DNA synthesis on a single-stranded DNA template-primer, yielding a typical 'stepladder pattern' upon sequencing gel analysis of products, beginning with primer +1 dNMP. β-pol is further differentiated from other DNA polymerases by its pause site pattern on a heteropolymer template (9).

When we tested the purified, recombinant yeast DNA polymerase in these assays, the gap-filling and distributive primer extension activities were similar to those of the purified rat β-pol (Fig. 4) used as reference. Furthermore, the conclusion that the recombinant yeast DNA polymerase has activity like that of β-pol of vertebrates, was supported by experiments with the inhibitors NEM, and ddNTP, and an antibody to *E.coli* DNA polymerase I.

Taken together, our results demonstrate that YCR14C encodes a 67 kDa β-pol-like enzyme. Although this yeast β-pol differs in size from mammalian β-pol, it shows strong biochemical similarities for DNA synthesis. It will be interesting to know whether the 'extra' NH_2-terminal sequences of this yeast DNA polymerase vis-a-vis mammalian, β-pol carry some other intrinsic activity.

The discovery of a DNA polymerase β-like enzyme in yeast has interesting implications for future genetic and biochemical experiments in this facile eukaryotic system. Genetic experiments indicate that a yeast *pol IV* deletion strain is perfectly viable and exhibits no defect in sporulation or spore viability. Our experiments also have failed, thus far, to reveal a DNA repair phenotype for yeast *pol IV* deletion strains, and similarly *pol IV* deletion mutation in a background of *ts* mutations in other yeast DNA polymerases failed to reveal an enhancement of the phenotype of *ts* mutations. For future biochemical experiments, the development of an *in vitro* crude extract-based system for mismatch repair (26,27) could provide an approach to study of a role of a β-pol-like enzyme in short gap-filling synthesis.

Another important implication of the discovery of the 67 kDa β-like DNA polymerase in yeast is the possibility that mammalian cells also contain a β-pol of similar molecular mass. This idea is not remote, because it is well known that such diverse organisms as Drosophila, fungi, protozoa, and plants have higher molecular mass β-pol-like DNA polymerases (28−33). In addition, there are multiple transcripts in rat tissues (34) larger than the 1.4kb 39kDa β-pol coding transcript. The precise identity of these alternate size β-pol transcripts has remained unclear. We note, on the other hand, that in many human cultured cell types, the only β-pol transcript detected by Northern blot analysis is the 1.4 kb species corresponding to the 39 kDa β-pol. Similarly, in cultured cell systems analyzed by Western blotting with β-pol specific monoclonal antibodies, our recent results have failed to reveal a larger species of β-pol protein (i.e., > 39 kDa) (Srivastava, Showalter, and Wilson, manuscript in preparation). Nevertheless, in view of the 67 kDa size of the yeast β-pol-like enzyme, evaluation of the possibility of similar size β-pol molecules in mammalian cells is underway.

ACKNOWLEDGEMENTS

We thank Dr. A.Goffeau for the generous gift of the yeast clone containing YCR14C, F.B.McClenny for contributions to the protein purification, S.Serabyn for amino acid sequencing, D.Herrera for assistance in preparation of pRSET-YβP and Dr. W.Beard for helpful discussions and critical reading of the manuscript. This work was supported in part by grants to S.H.W. from the National Institute of Environmental Health Sciences (ES06492) and the Robert A.Welch Foundation (H-1265), and by a grant to L.P. from the National Institutes of Health (GM19261).

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