Purification and Characterization of the *Saccharomyces cerevisiae* DNA Polymerase δ Overproduced in *Escherichia coli**

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In order to further define the enzymatic properties of yeast DNA polymerase δ, the *Saccharomyces cerevisiae POL3* gene, whose expression is highly toxic to bacteria in most cloning vectors, was cloned into a new T7 expression vector (W. C. Brown and J. L. Campbell, submitted for publication) which allowed efficient overexpression in bacteria. Fifteen mg of polymerase were obtained from 3 g of cells. Since the protein is produced in insoluble form, to obtain active polymerase, inclusion bodies were solubilized with urea. DNA polymerase δ (124 kDa) was purified in the presence of urea and then renatured by dialysis against buffers containing decreasing concentrations of urea. Optimal protein concentration for refolding was 5 μg/ml. By several criteria the enzyme obtained is comparable with that from yeast: specific activity, electrophoretic mobility, template preference, sensitivity to inhibitors, and processivity. The electrophoretic mobility suggests that, unlike DNA polymerase α, polymerase δ is not posttranslationally modified in yeast. Polyclonal antibody was raised against the full-length DNA polymerase δ from bacteria and shown to cross-react with the protein purified from yeast on protein blots. The renatured protein also exhibits an exonucleolytic activity. Further examination of this nuclease determined it to be a 3′ to 5′ exonuclease with the characterisitics of a proofreading activity. The presence of this nuclease in the highly purified bacterial polymerase provides biochemical confirmation of earlier genetic evidence (Simon, M., Giot, L., and Faye, G. (1991) *EMBO J.* 10, 2165–2170) that suggested that DNA polymerase δ's core catalytic subunit contains an intrinsic 3′ to 5′ exonuclease.

A truncated form of DNA polmerase δ in which the amino-terminal 220 amino acids were removed was also investigated. The polymerase activity of truncated protein (98 kDa) is similar to the 124-kDa form except that it shows a reduced ability to perform "proofreading."

DNA polymerase δ was originally distinguished from DNA polymerase α by its copurification from rabbit bone marrow with a 3′ to 5′ exonuclease activity (Byrnes *et al.*, 1976). A reversion assay showed calf thymus DNA polymerase δ to be highly accurate and the 3′ to 5′ exonuclease activity was considered to be at least partially responsible for this fidelity

(Kunkel *et al.*, 1987). Bauer *et al.* (1988) were the first to purify DNA polymerase δ from *Saccharomyces cerevisiae*. The purified polymerase consisted predominantly of peptides of 125 and 55 kDa (Bauer and Burgers, 1988). Proteins of this size are observed in DNA polymerase δ from all sources, suggesting they are subunits of the enzyme. Both the polymerase and nuclease activities are thought to reside in the large subunit (Sitney *et al.*, 1989; Boulet *et al.*, 1989; Simon *et al.*, 1991). The function of the 55-kDa subunit is unknown.

Biochemical analysis of *S. cerevisiae cdc2* mutants (Sitney *et al.*, 1989) and DNA sequence analysis (Boulet *et al.*, 1989) both arrived at the conclusion that DNA polymerase δ is encoded by the *CDC2* gene. This gene is now known as *POL3*. In effect, this result simultaneously established DNA polymerase δ as genetically distinct from other polymerases as well as proving it to be essential. The open reading frame codes for a protein comprised of 1097 amino acids with an approximate molecular mass of 124 kDa (Boulet *et al.*, 1989; Morrison and Sugino, 1991). The sequences of human and bovine DNA polymerase δ have been determined recently, and they exhibit 46% homology with the budding yeast sequence (Zhang *et al.*, 1991). These genes are also predicted to encode proteins of molecular mass 124 kDa. The *pol3*⁺ gene isolated from *Schizosaccharomyces pombe* is even more closely related to *S. cerevisiae*, displaying 52% conservation of amino acid residues (Pignede *et al.*, 1991).

An accessory factor was discovered during the purification of DNA polymerase δ from calf thymus that affected the ability of the polymerase to utilize long stretches of single-stranded DNA (Tan *et al.*, 1986). This factor was found to be identical with human proliferating cell nuclear antigen (PCNA)[1] (Prelich *et al.*, 1987). PCNA does not bind to DNA but has been shown to reduce the K_m of DNA polymerase δ for template as well as increasing the V_{max} during DNA synthesis (Ng *et al.*, 1991). PCNA has also been purified from yeast (Bauer and Burgers, 1988), and the gene has been cloned and shown to be essential (Bauer and Burgers, 1990). There is enough functional homology that mammalian and yeast PCNA can substitute for each other *in vitro* in stimulating the respective cognate polymerase δ.

Another accessory factor, activator 1 (A1), which appeared to be specific for DNA polymerase δ, was first identified as being required for reversing the inhibition of SV40 DNA replication by an elongation inhibitor (Lee *et al.*, 1988). Tsurimoto and Stillman (1989) have also purified a multiprotein complex required for coordinated leading and lagging strand synthesis during SV40 replication which they called RF-C. Both factors display DNA-dependent ATPase activity and preferentially bind to primer template DNA (Tsurimoto and

[1] The abbreviations used are: PCNA, proliferating cell nuclear antigen; A1, activator 1; RF-C, replication factor-C; BuPhdGTP, N^2-(*p-n*-butylphenyl)-2′-deoxyguanosine-5′-triphosphate; DTT, dithiothreitol.

Stillman, 1991a; Lee and Hurwitz, 1990). The subunit composition is also quite similar and these proteins are probably identical. The A1 (RF-C) complex interacts with DNA polymerase δ through PCNA. This complex binds the primer terminus and then PCNA binds in the presence of ATP to form a charged primer recognition complex (Lee and Hurwitz, 1990; Tsurimoto and Stillman, 1991b). The ATP is hydrolyzed and DNA polymerase δ binds to the complex and initiates DNA synthesis. It has since been found that the A1 (RF-C)·PCNA complex also interacts with DNA polymerase ε of both yeast and human cells (Burgers, 1991; Lee *et al.*, 1991), although the complexes with this polymerase are much less stable.

The current model for how the three polymerases interact during DNA replication begins with the opening of origin DNA by an initiator protein such as the SV40 T antigen. This is assisted by single-stranded DNA binding protein. DNA polymerase α-primase may then synthesize an RNA primer which it subsequently extends with a short strand of DNA. The A1 (RF-C)·PCNA complex forms at the primer terminus, perhaps displacing DNA polymerase α. Either DNA polymerase δ or ε may be recruited to begin leading strand synthesis (Tsurimoto and Stillman, 1991b; Morrison *et al.*, 1990). This same process is repeated to initiate lagging strand synthesis, and these initiations may be coordinated. The assembly-disassembly cycle of the A1 (RF-C)·PCNA primer recognition complex then participates in the repeated reinitiation of lagging strand synthesis.

In order to further dissect their roles, it is advantageous to characterize the individual polymerases more thoroughly. Obtaining DNA polymerase δ in high yield and purifying it to homogeniety from yeast is difficult due to the inherent lability of the enzyme as well as proteolysis. To overcome these problems, we attempted to produce DNA polymerase δ in bacteria. Numerous workers have found it difficult to maintain *POL3*-containing plasmids in *Escherichia coli* (Simon *et al.*, 1991). Instability of plasmids carrying the gene and low levels of expression lead us to construct a new expression vector and develop a novel expression strategy for this gene.[2] As with many proteins overexpressed in bacteria, DNA polymerase δ was found to be insoluble. In this report we describe a purification and renaturation procedure for this protein. The enzymatic activities thought to be associated with the 124-kDa subunit were characterized and compared with a DNA polymerase δ preparation purified from yeast cells. A truncated form of DNA polymerase δ in which the amino-terminal 220 amino acids were missing was also produced, purified, and renatured. The activities associated with this form of DNA polymerase δ were also characterized.

EXPERIMENTAL PROCEDURES

Materials—Aphidicolin was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. N^2-(*p-n*-butylphenyl-2'- deoxyguanosine-5'-triphosphate (BuPhdGTP) was generously provided by Dr. George Wright (University of Massachusetts Medical School). Both poly(dA) and (dT)$_{10}$ were from Pharmacia LKB Biotechnology Inc. Urea was Ultrapure from United States Biochemical Corp., and DEAE-cellulose DE52 was from Whatman.

Enzymes—Both DNA polymerase δ and ε were partially purified from yeast strain BJ3501 (α, *pep4::HIS3, prb1-Δ1.6R, his3-Δ200, ura3-52, GAL, can1*). DNA polymerase δ was purified to Fraction V as in Bauer *et al.* (1988) and then run on a 10–30% glycerol gradient as described (Bauer and Burgers, 1988). DNA polymerase ε was purified to Fraction IV as described (Budd *et al.*, 1989).

Purification of DNA Polymerase δ Produced in E. coli—HMS174 harboring plasmids containing either a full-length copy of the *POL3* gene or a truncated version were grown and induced as described.[2]

Induced cells were spun down and resuspended at 2.8 ml/g in 50 mM Tris-HCl (pH 7.4) with 10% sucrose. These were frozen in 10-ml aliquots and stored at −70 °C. A method for purifying proteins from inclusion bodies was adapted for our use (Kumagai and Dunphy, 1991). One tube each (10 ml, 3.5 g of cells) of full-length and truncated containing cells were thawed on ice. A lysozyme solution and NaCl were added to final concentrations of 2.5 mg/ml and 100 mM, respectively. These were incubated on ice for 15 min and then sonicated at 150 watts four times for 30 s each. The tubes were then spun in a Sorvall SS34 rotor for 15 min at 10,000 rpm and 4 °C. The supernatants were decanted, and the pellets were resuspended in 10 ml each of 0.2 M Tris-HCl (pH 8.0), 0.5 M NaCl, 1 mM dithiothreitol (DTT), and 0.1% Triton X-100. The resuspensions were then sonicated and spun as above. The supernatants were again decanted and the insoluble material resuspended in 25 ml of Tris-HCl (pH 8.0), 1 mM DTT, and 7.2 M urea to disperse the inclusion bodies. This solution is then passed over a 5-ml DE52 column, prepared in a 10-ml tuberculin syringe. The column was washed with 4 column volumes of 0.25 M Tris-HCl (pH 8.0), 0.25 M NaCl, and 6 M urea, followed by 10 column volumes of 25 mM Tris-HCl (pH 8.0), 25 mM NaCl, 1 mM DTT, and 6 M urea. Polymerase δ was eluted with 25 ml of the same buffer containing 0.8 M NaCl.

Renaturation of Purified DNA Polymerase δ—The 0.8 M salt fraction from the purification of full-length DNA polymerase δ and the flow-through and high salt fraction of the truncated form were diluted to 5 μg/ml for renaturation. These were then dialyzed sequentially against the following buffers maintaining a ratio of no greater than 50 for dialysis volume to sample volume. The dilution buffer is 0.2 M Tris-HCl (pH 8.0), 0.5 M NaCl, 1 mM DDT, and 6 M urea. The samples were first dialyzed against 0.2 M Tris-HCl (pH 8.0), 0.5 M NaCl, 1 mM DTT, 100 μM ZnCl₂, 2 mM MgCl₂, and 4 M urea for at least 8 h at 4 °C. The next three dialysis steps employ buffers that contain the same DTT, ZnCl₂, and MgCl₂ concentration but vary in Tris, NaCl, and urea concentrations. The second buffer contains 0.1 M Tris-HCl (pH 8.0), 0.5 M NaCl, and 2 M urea, whereas the third contains 50 mM Tris-HCl (pH 7.4), 0.2 M NaCl, and 1 M urea. The final buffer had 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 10% glycerol and did not contain urea. The final dialysis was stopped after 4 h. Samples were either stored on ice or concentrated on a 0.1-ml DE52 column using 50 mM Tris-HCl (pH 7.4), 0.45 M NaCl, 1 mM DTT, 10% glycerol, and 50% ethylene glycol to elute bound polymerase. These samples were then frozen in dry ice/ethanol and stored at −70 °C.

DNA Synthesis and Inhibitor Assays—The standard DNA synthesis reaction (60 μl) contained 25 mM Tris-HCl (pH 7.4), 8 mM MgCl₂, 0.1 mg/ml bovine serum albumin, 5 mM DTT, 100 μM each of dATP, dCTP, and dGTP and 50 μM [³H]dTTP (105 cpm/pmol). Either poly(dA):oligo(dT) (30:1) or activated salmon sperm DNA were present at 160 μg/ml (Bauer *et al.*, 1988). For aphidicolin sensitivity assays the dCTP concentration was reduced to 10 μM. When BuPhdGTP was present the dGTP concentration was reduced to 10 μM. DNA polymerase δ, either full-length or truncated was added as indicated. Reactions were incubated for 30 min at 37 °C. They were stopped by spotting on Whatman DE81 filter paper (Sitney *et al.*, 1989). Filters were washed four times with 0.5 M Na₂ HPO₄, twice with dH₂O, and once with 95% ethanol. Incorporation of radioactivity was measured on a Beckman LS 5000TD liquid scintillation counter. One unit of enzyme incorporates 1 pmol/min of nucleotide into radioactive product (Bauer *et al.*, 1988).

Processivity Assays—The processivity of the various forms of polymerase δ were compared with each other and polymerase ε using 100 μg/ml (dA)₅₅₀:oligo(dT)₁₀ at a ratio of 20:1 as the template and [α-³²P]dTTP (8,000 cpm/pmol) as the label in the standard DNA synthesis reaction described above. Reactions were started by the addition of 1.0 unit of polymerase activity and incubated for the indicated times at 37 °C. Reactions were stopped by the addition of an equal volume of 1% sarkosyl and 1.2 μg of proteinase K. Incubation was then continued for an additional 20 min. The samples were extracted with phenol/chloroform and ethanol-precipitated. Pellets were resuspended in deionized formamide containing 1 mM EDTA, 0.1% xylene cyanole, and 0.1% bromphenol blue. The samples were placed in a 100 °C heating block for 1 min, cooled on ice, and loaded onto an 8% polyacrylamide gel containing 7 M urea.

Exonuclease Assays—The 40-μl assay contained 25 mM Tris-HCl (pH 7.4), 8 mM MgCl₂, 0.1 mg/ml bovine serum albumin, 5 mM DTT. The uniformly labeled substrate was produced by incubating poly(dA):oligo(dT) with Klenow fragment and [³H]dTTP. This was stopped with EDTA (20 mM) and phenol-extracted. The aqueous

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[2] W. C. Brown and J. L. Campbell, submitted for publication.

phase was ethanol-precipitated in the presence of 2.5 M ammonium acetate. The substrate was added to the assay mix at 1.5 μg/ml (final concentration, 30,000 cpm total) as described (Bauer *et al.*, 1988). DNA polymerase δ was added as indicated, and reactions were incubated at 37 °C for the indicated times. Reactions were stopped by spotting on DE81 paper and washing as above. Exonuclease activity is represented by loss of radioactivity compared with a control incubated without protein.

(dT)₁₀ was labeled at the 3′ end by addition of [³H]dT or [³H]dC using terminal transferase according to Roychoudhury *et al.* (1979). The labeled primers were separately annealed to poly(dA) at a ratio of 15:1 (poly(dA):oligo(dT) (nucleotide concentration) and passed over two successive Sephadex G50 spun comlumns (1 ml). Quantitation of incorporation determined that both labels occur at approximately one per primer. These substrates were added to exonuclease reactions at approximately 200 μg/ml. Reactions were run as above or in the presence of 100 μM unlabeled dTTP as indicated.

Heat Denaturations—These assays were run as described for DNA synthesis and exonuclease using uniformly labeled substrate. The DNA polymerase δ preparations were incubated at 47 °C for the times indicated. At each time, aliquots were withdrawn and placed into reaction buffer on ice. Reactions were started by the addition of substrate and incubated at 37 °C for 30 min (DNA synthesis) or 20 min (exonuclease). Reactions were processed as described above.

Polyclonal Antibodies and Western Analysis—The 124-kDa band of the purified bacterially produced DNA polymerase δ was excised from a 7.5% SDS-polyacrylamide gel (1 mg total protein) and sent to Berkeley Antibody Company. 0.5 mg was used for the initial immunization and two boosts of 0.25 mg per boost each were carried out. Antibody was affinity-purified from serum by the protocol of Harlow and Lane (1988). 100 μg of bound polymerase δ was used to purify 2 ml of serum. A 1:100,000 dilution of serum detects 1 μg on protein blots. A 1:1000 dilution of affinity-purified antibody detects as little as 100 ng of purified protein. A 1:250 dilution detects 35 ng. For protein blots, antibody specifically bound to membrane-bound protein was visualized by alkaline phosphatase according to the directions of the supplier of the reagents (Bio-Rad) following the methodology of Harlow and Lane (1988). Non-fat dry milk was substituted for bovine serum albumin in the blocking steps and for antibody dilution.

RESULTS

Purification of DNA Polymerase δ—We cloned the *S. cerevisiae Pol3* gene into several widely used bacterial expression vectors and found every construct to be unstable. It appeared that even at very low levels of expression, DNA polymerase δ was toxic to *E. coli*. In order to fully repress transcription prior to induction, a new T7-based expression vector was developed. This was used to clone the full-length *POL3* gene and deletions of either the amino terminus or the putative exonuclease domains or both.[2] Only the full-length copy and the gene truncated at the *Nde*I site (Fig. 1), which removes the 220 amino acids of the amino terminus but retains all of the polymerase conserved regions including the exonuclease domains in region IV, were found to be overexpressed. The full-length clone of the gene gives rise to a 124-kDa protein, and the truncated clone gives rise to a 98-kDa form of DNA polymerase δ.

While working out the conditions for expressing the pro-

teins in *E. coli*, we determined that when overexpressed they are insoluble. Sarkosyl (1%) in the extraction buffer (Frankel *et al.*, 1991) released approximately half of the full-length DNA polymerase δ but in an apparently inactive form. Therefore, a procedure developed for purifying *Drosophila* cdc25 protein from bacterial inclusion bodies was adapted for DNA polymerase δ (Kumagai and Dunphy, 1991; see "Experimental Procedures"). In brief, the cells were disrupted by lysozyme and sonication, and the insoluble material was collected and washed. The inclusion bodies were then dispersed in a urea buffer and passed over a DEAE column. The full-length form of DNA polymerase δ was found at low levels in all fractions (Fig. 2), but the bulk of the polymerase eluted from the column in the high salt wash. The truncated form of the protein, in several purifications, was always observed to partition roughly equally between the column flow-through and the eluate (Fig. 2). The consistency of these chromatography results suggests that the amino terminus is involved in the electrostatic interactions of DNA polymerase δ, at least with respect to its chromatographic behavior. Protein concentrations were fairly reproducible from preparation to preparation with 0.6 mg/ml average for the full-length eluate and 0.07 mg/ml for both the truncated flow-through and eluate. Total protein ranged from 12 to 15 mg for the full-length form and 1.5 to 2.0 mg for each fraction of the truncated form.

Refolding of DNA Polymerase δ—When the renaturation protocol of Kumagai and Dunphy (1991) was followed, active protein was obtained but the specific activity was very low. For this reason, a number of variations were tested. First, the buffer compositions were changed at each step so that the final buffer would be compatible with assay conditions and similar to buffers used in purifying DNA polymerase δ from yeast cells. Second, a series of protein concentrations were tested (150–2.5 μg/ml). The specific activity increased as the protein concentration was reduced, peaking at 5 μg/ml. DNA polymerase δ is proposed to contain zinc finger regions, and so zinc was added to the dialysis buffers. Although activity did increase severalfold with this addition, we did not observe an absolute dependence on zinc for activity. However, stringent conditions were not applied to rule out contamination.

A number of other variations were also investigated. A single-step dialysis was tested, but activity levels recovered were considerably lower than for stepwise dialysis. The method of Frorath *et al.* (1991) was tested, but no activity was recovered. This method uses a two-step dialysis in glycine-NaOH buffer at pH 9.0. As activity was recovered from single-step dialysis at pH 7.4, but not at pH 9, it would appear that the pH is crucial to effective refolding of DNA polymerase δ. We also experimented with carrying out the first three dialysis changes at room temperature, with slow cooling to 4 °C during the final step, but did not benefit from this change. It had been suggested to us that dialysis at 4 °C would be less
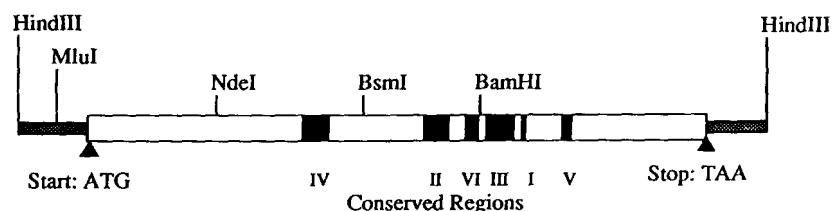

FIG. 1. **Linear map of *S. cerevisiae POL3* gene.** The *POL3* gene is found on a 4,000-base *Hind*III fragment of chromosome IV. The open reading frame is shown as a *thick white box* (3291 base pairs), noncoding sequences are *medium thickness* and *shaded*. The *black boxes* in the coding region correspond to the conserved regions associated with α-like DNA polymerases. The full-length protein is predicted to be 124 kDa. A truncated form of *POL3* was constructed by cloning the *Nde*I to carboxyl-terminal *Hind*III fragment which yields a 98-kDa protein.

FIG. 2. **Purification of overexpressed DNA polymerase δ.** To purify the protein from inclusion bodies, the cells were sonicated and then insoluble material was spun out (*Extract*). The pellet was resuspended in a buffer containing 0.1% Triton X-100 to wash out loosely associated membrane proteins, resonicated, and again insoluble material was spun out (*Wash*). The inclusions were then dispersed in 7.2 M urea, and this was passed over a DE52 column (*Flow-Through*). The column was then eluted with urea buffer containing 800 mM NaCl (*Elution*). Aliquots of each major fraction were run on a 7.5% SDS-polyacrylamide gel and stained with Coomassie Blue. The full-length protein is 124 kDa and the truncated form is 98 kDa. The marker shown is *E. coli* RNA polymerase.
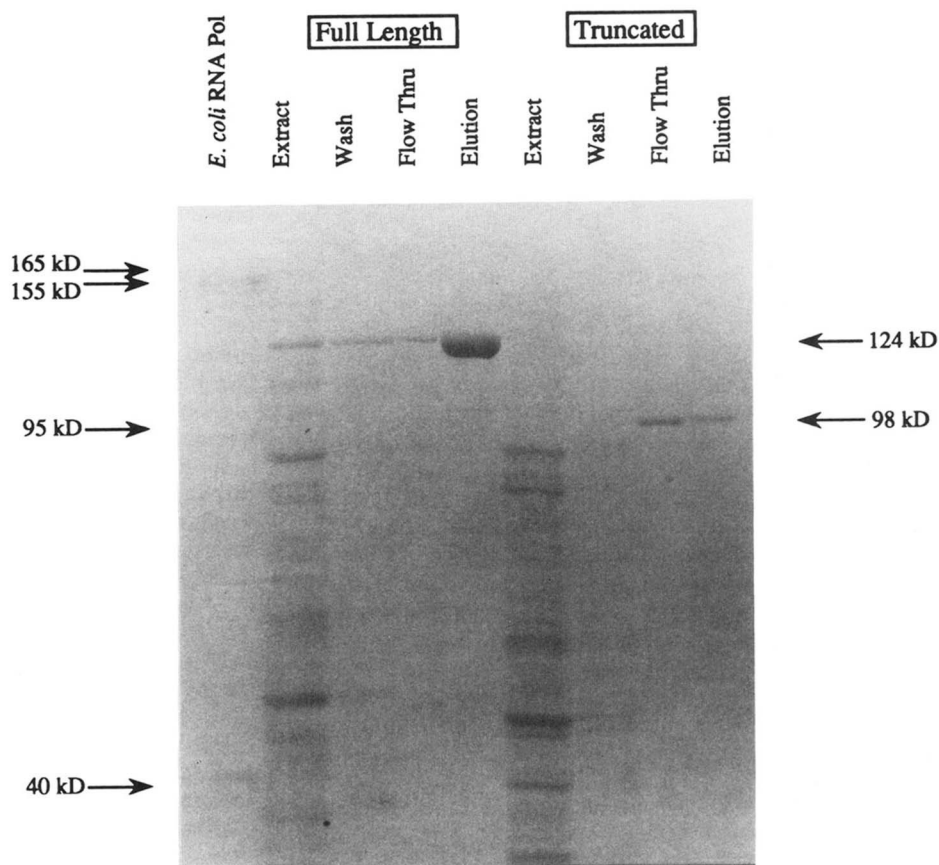
effective because it may force the protein into conformation too quickly. However, since all of the procedures prior to renaturation are carried out in the cold, there is no large drop in kinetic energy occurring as the buffers cool to cause the protein to prematurely assume a low energy conformation. We next investigated several different ratios of dialysis buffer volume to sample volume, with the best results obtained at ratios of 35 and 50, with higher ratios giving very low yields. The increased osmotic pressure at higher ratios may cause the urea to be removed too rapidly, again forcing premature refolding into an inactive conformation. Finally, the time of the last dialysis step was varied from 2 to 10 h. DNA polymerase δ is a labile protein, and it was essential to determine at what point the dialysis should be terminated to maximize recovery. Active protein is detected within 2 h of the final change with the specific activity plateauing from 4 to 6 h and then decreasing.

A stepwise dialysis at 4 °C was adopted and the procedure was terminated 4 h after the final change (see "Experimental Procedures"). Using these conditions we have obtained specific activities for both the full-length (124 kDa) and truncated (98 kDa) versions ranging from 10,000 to 60,000 units/mg. The most frequently obtained values are between 15,000 and 20,000 units/mg. There is no detectable difference in activity between the flow-through and high salt eluate fractions of the truncated form of the protein. Specific activity values obtained for DNA polymerase δ purified from yeast have been reported at 140,000 units/mg (Bauer *et al.*, 1988) for the most purified fraction. The slightly lower specific activity here may be due to decreased stability of single-subunit protein, the lack of posttranslational modification, differences in template efficiency, or lack of an activity associated with the putative 55-kDa subunit. Although both bacterial expressed polymer-

ases have the same stability when stored on ice (active up to 2 weeks), the truncated form is often killed in freeze-thaw cycles.

Comparison of DNA Polymerase δ Produced in Bacteria with That from Yeast—DNA polymerase δ purified either from bacteria or from yeast cells was run on an SDS-polyacrylamide gel to determine if there was any significant difference in electrophoretic mobility. This was examined in two ways. One gel was stained with silver and another was used to transfer the protein to nitrocellulose for probing with affinity-purified polyclonal antibody. Both the silver-stained gel (Fig. 3*A*) and the protein blot (Fig. 3*B*) show that there is no difference in migration between the DNA polymerase δ obtained from yeast and that produced in bacteria. It is interesting to note from the protein blot that the lower molecular weight bands visible in the bacterial DNA polymerase δ are the same size as those observed in the yeast preparation, suggesting that these lower molecular weight proteins are antigenically related and not contaminants. No bacterial proteins are recognized by the antibody (Fig. 3*B*, *lane 3*), confirming that it is specific for DNA polymerase δ. It may be significant that the proteolytic fragments of DNA polymerase δ from yeast are the same size as the fragments detected in the bacterial preparation.

Inhibitor Assays and Synthetic Parameters—To ensure that the activity observed after renaturation was due to DNA polymerase δ and not a bacterial contaminant, the sensitivity of the bacterial preparation to several inhibitors of DNA synthesis was determined. The first inhibitor employed was aphidicolin. The only *E. coli* polymerase sensitive to aphidicolin is DNA polymerase II (Chen *et al.*, 1991). The I_{50} of DNA polymerase II (15 μg/ml) is twice that of yeast DNA polymerases α and ε (4 and 6 μg/ml, respectively) and over 20
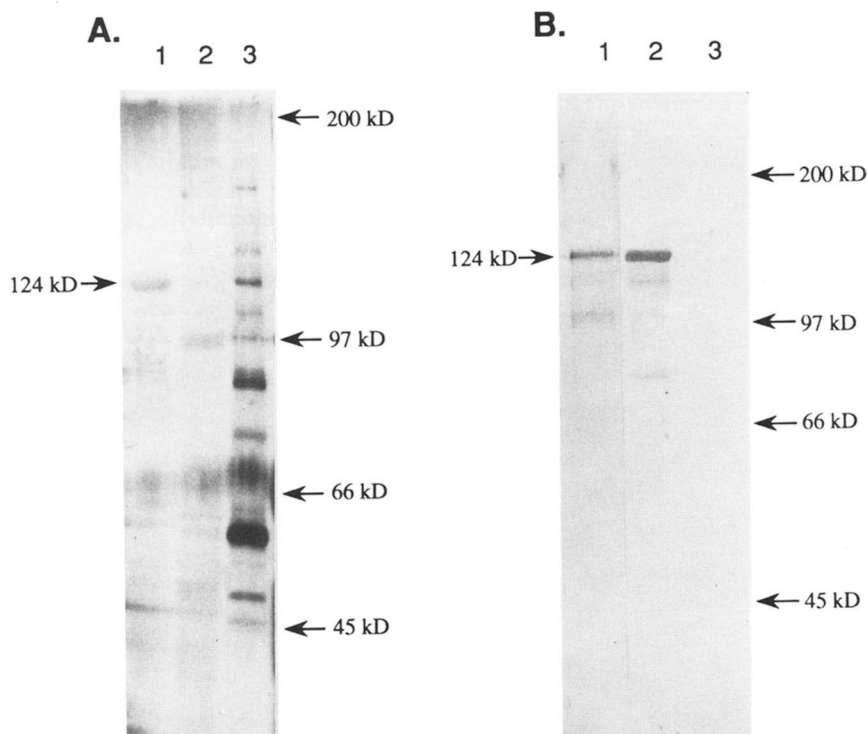
FIG. 3. **Comparison of the electrophoretic mobility of DNA polymerase δ produced in bacteria with DNA polymerase δ produced in yeast.** The purified 124- and 98-kDa forms of DNA polymerase δ produced in bacteria were run on 7.5% SDS-polyacrylamide gels along with protein partially purified from yeast as described under "Experimental Procedures." One gel was stained with silver as described by Wray *et al.* (1981) and another was used for a protein blot. The silver-stained gel is shown in *A* (*lane 1*, full-length from bacteria, 2 μg; *lane 2*, truncated, 1.5 μg; *lane 3*, yeast purified, 2 μg). The Western blot is shown in *B*. The yeast lane and the bacterial 124-kDa form lane were probed separately with affinity purified antibody. The bacterial form (*B, lane 2*, approximately 250 ng of protein) was probed with a 1 to 1,000 dilution, whereas the yeast form (*B, lane 1*) was probed with a 1 to 20 dilution. A whole cell extract of *E. coli* HMS174 infected with T7 was also probed at a 1 to 1000 dilution (*B, lane 3*). Markers used: myosin, 200 kDa; phosphorylase *b*, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa.

times that for DNA polymerase δ (0.6 μg/ml) (Burgers and Bauer, 1988). The curves for the titration of activity with aphidicolin concentration for the bacterial-expressed DNA polymerase δ, both full-length and truncated, are virtually the same as that for yeast-purified DNA polymerase δ (Fig. 4). As reported previously, yeast DNA polymerase ε is less sensitive than the δ polymerases (Burgers and Bauer, 1988). Similar assays were also carried out in the presence of BuPhdGTP with similar results (data not shown). The I_{50} for several inhibitors and synthetic parameters for both forms of bacterial expressed DNA polymerase δ are compared with values determined previously for yeast DNA polymerase δ (Table I). The only significant differences between the bacterial form of the protein and that obtained from yeast are a 2- or 3-fold increase in the K_m for dNTPs and an increased tolerance for KCl. The increased K_m may reflect the lack of the 55-kDa subunit or renaturation leading to slightly less structural integrity or the requirement for some form of posttranslational modification. The KCl tolerance is discussed below.

A titration of polymerase activity versus KCl concentration is shown in Fig. 5. DNA polymerase δ obtained from yeast shows inhibition by KCl at concentrations as low as 60 mM. By contrast both forms of the bacterial polymerase δ are stimulated by KCl at concentrations up to 240 mM. The KCl may be acting to stabilize the refolded proteins in solution and/or reduce self-aggregation. At the optimal KCl concentration, there is a 10–14-fold increase in activity which makes the specific activities of these preparations comparable with the specific activities of DNA polymerase δ obtained from yeast. Stimulation by KCl is also observed when

poly(dA):oligo(dT) is used as template though the enhancement is slightly lower (data not shown). Aphidicolin resistance, K_m for dNTPs and processivity were not altered when reassayed at optimal KCl concentrations.

The processivities of the bacterial forms of polymerase δ were also compared with both the yeast form and yeast polymerase ε. Even at the shortest incubation time (1 min) DNA polymerase ε has produced products corresponding to the longest product possible on this template (Fig. 6). The yeast and bacterial forms of DNA polymerase δ show similarly lower processivity, with the longest product after 5 min being less than 50 nucleotides in length. Taken together, all these results lead us to conclude that the synthetic activity obtained from renaturation is due to DNA polymerase δ and not a bacterial contaminant and that the monomeric enzyme has catalytic properties very similar to the heterodimeric yeast enzyme.

Exonuclease Assays—The 124-kDa subunit of DNA polymerase δ from mammalian sources has been shown to contain a 3′ to 5′ exonuclease activity as well as the synthetic activity. There is strong genetic evidence that this is also true of the 124-kDa subunit from *Saccharomyces* (Simon *et al.*, 1991). Both forms of DNA polymerase δ produced in bacteria were examined for exonuclease activity. The results of a time course using a uniformly labeled template as a substrate are shown in Fig. 7. Both the 124-kDa form and the 98-kDa form of DNA polymerase δ display exonuclease activity and digest the substrate to the same extent. The DNA polymerase δ partially purified from yeast cells contains a much more potent exonuclease activity. This may be the result of the presence of
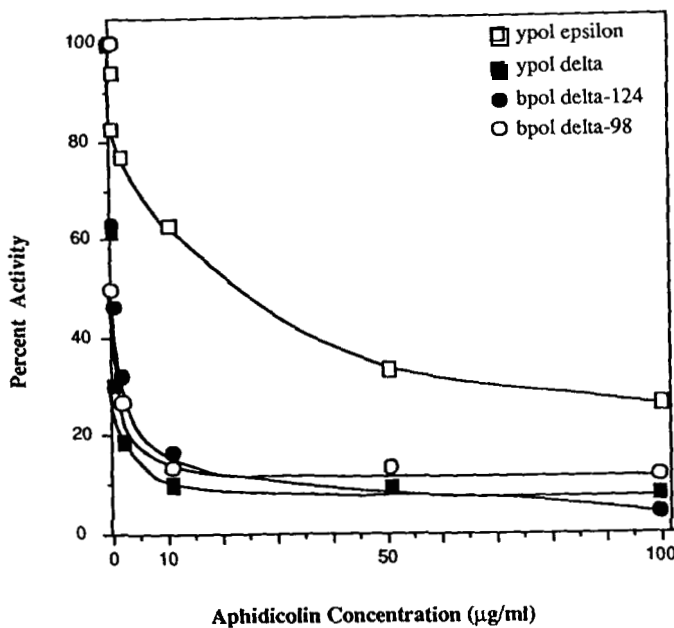
FIG. 4. **Inhibition of renatured DNA polymerase δ by aphidicolin.** Polymerase activity associated with protein purified from bacteria was tested for sensitivity to aphidicolin as described under "Experimental Procedures." DNA synthesis reactions were carried out with increasing concentrations of aphidicolin using 1 unit of bacterially produced protein, and both DNA polymerase δ and DNA polymerase ε purified from yeast were used as controls. The response to the inhibitor is plotted as synthetic activity remaining as a percent of initial activity *versus* the concentration of inhibitor (μg/ml).

TABLE I

Comparison of DNA polymerase δ from bacteria and yeast

	ypolδ[a]	bpolδ-124	bpolδ-98
BuPhdGTP, I_{50} (μg/ml)	80	88	86
Aphidicolin, I_{50} (μM)	0.6	0.1	0.1
KCl, I_{50} (M)	0.12	0.41	0.26
Mg^{2+}, I_{50} (mM)[b]	16	14 (23)[e]	14 (21)[e]
N-ethylmaleimide, $t_{1/2}$ (min)[c]	3.6	3.4	3.5
K_m, dNTPs (μM)[d]	7.1	16.5	21.0

[a] Values reported in Burgers and Bauer (1988).

[b] Compared to optimum concentration (8 mM).

[c] Reactions run as described in Burgers and Bauer (1988).

[d] K_m apparents determined as described in Burgers and Bauer (1988).

[e] Values in parentheses were obtained using Mg(AcO)$_2$ in place of MgCl$_2$.

exonuclease IV which copurifies with DNA polymerase δ through Fraction V (Bauer *et al.*, 1988), the fraction used in our studies (see "Experimental Procedures"). It has been estimated that exonuclease IV accounts for as much as half the nuclease activity in DNA polymerase δ preparations (Bauer *et al.*, 1988; Sitney *et al.*, 1989). If this is taken into account the bacterial forms display approximately 70% of the exonuclease activity of DNA polymerase δ obtained from yeast.

To ensure that the exonuclease and polymerase activities are associated with a single polypeptide, heat denaturation assays were performed. The bacterial preparations were preincubated at 47 °C, and aliquots were removed and assayed for both enzymatic activities. The percent of activity remaining for each function was then plotted against the length of time at 47 °C (Fig. 8, *A* and *B*). The results indicate that both activities are lost at approximately the same rate for both the full-length (Fig. 8*A*) and truncated (Fig. 8*B*) forms of DNA
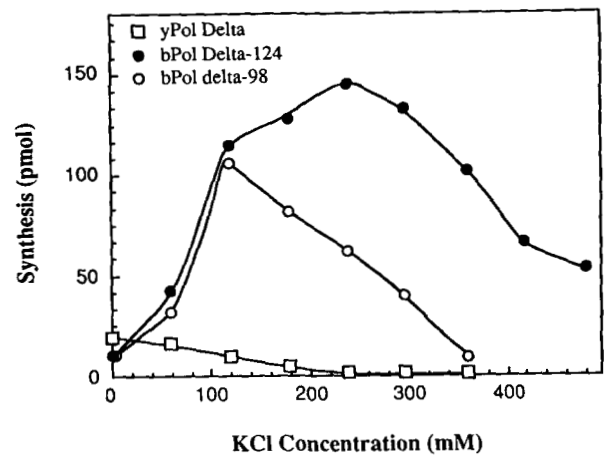

FIG. 5. **Response of the bacterial and yeast forms of DNA polymerase δ to increasing KCl concentration.** Standard DNA synthesis assays were carried out in the presence of increasing concentrations of KCl as described under "Experimental Procedures." Both the full-length and truncated protein were added at 50 ng, whereas DNA polymerase δ purified from yeast was added at 1 unit of polymerase activity.

polymerase δ. This implies that both activities reside in a single polypeptide.

Proofreading Assays—A longstanding method for determining the proofreading potential of an exonuclease is to measure the removal of a mispaired base (Brutlag and Kornberg, 1972). To determine the directionality and proofreading ability of the exonuclease associated with the renatured DNA polymerase δ preparations, 3' end-labeled substrates were prepared. Terminal transferase was used to end-label (dT)$_{10}$ with [³H] dT or [³H]dC. These primers were then annealed to poly(dA) to yield substrates either fully paired or mispaired at the 3' terminus. When incubated with the substrates in the absence of dTTP so that primer extension could not occur, both forms of bacterially produced polymerase remove the paired and mispaired label (Fig. 9, *A* and *B*). Taken together with the fact that in the presence of dTTP there is no degradation, this strongly suggests that the exonuclease functions with 3' to 5' directionality. The full-length DNA polymerase δ shows a distinct preference for the mispaired terminus (Fig. 9*A*), whereas the truncated removes both to the same extent (Fig. 9*B*). When reactions are run in the presence of unlabeled dTTP to allow for primer extension, both forms of polymerase cease to remove the correctly paired base but still excise the mispair. These results argue that the large subunit of yeast DNA polymerase δ does possess a proofreading activity. The rate of removal of the mispair by the full-length form of DNA polymerase δ is unchanged under conditions of DNA synthesis, but the truncated form appears to process the mispaired base more slowly under these conditions. The lack of preferential excision and slowing of excision of the mispair during synthesis suggest that perhaps the truncated form of DNA polymerase δ has a reduced ability to distinguish complementarity at the 3' terminus.

DISCUSSION

In this paper we have described a purification for the 124-kDa catalytic subunit of DNA polymerase δ of *S. cerevisiae* from an overproducing bacterial strain. We have also produced and purified an amino-terminal truncated form of DNA polymerase δ (98 kDa) that still contains all of the polymerase conserved regions. Both forms of this protein have been renatured to yield catalytically active preparations. The specific activity of these preparations is roughly the same as that
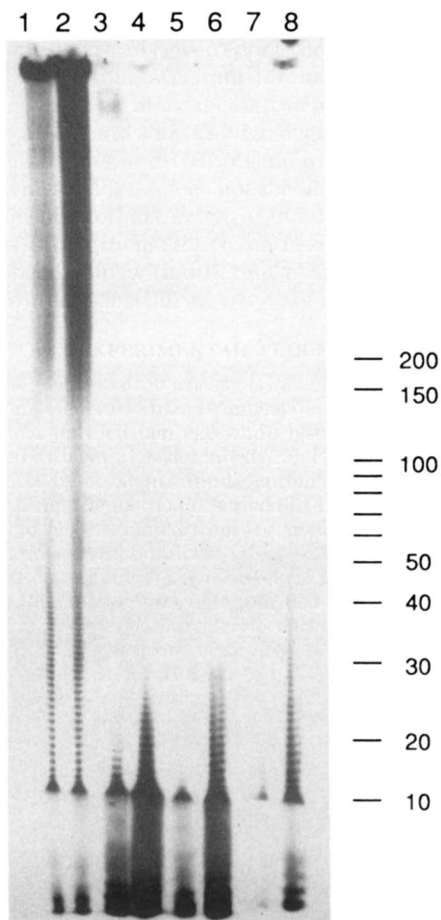
FIG. 6. **Comparison of processivity of the various polymerases.** Reactions were carried out as described under "Experimental Procedures." Polymerases were added at 1 unit of activity and incubations were for 1 or 5 min at 37 °C. *Lanes 1* and *2* are DNA polymerase ε for 1 and 5 min, respectively. *Lanes 3* and *4* (1 and 5 min) are yeast-purified DNA polymerase δ. *Lanes 5* and *6* (1 and 5 min) are full-length bacterial polymerase δ, and *lanes 7* and *8* (1 and 5 min) are the truncated bacterial form. Markers (*far right*) are a labeled 10-nucleotide linker ligation ladder. The 10-base length of the primer has been subtracted from the marker size, and thus the numbers reflect the extent of incorporation directly.

reported for DNA polymerase δ purified from yeast cells; the overall yield from bacteria is considerably higher than from yeast and the purity is greater.

As mentioned previously, the molecular mass predicted from the *POL3* gene is in good agreement with the mass determined experimentally for purified protein, suggesting that posttranslational modifications may be minimal *in vivo*. This was part of our rationale for pursuing bacterial production of DNA polymerase δ. In contrast, the predicted mass for DNA polymerase α is 165 kDa, whereas the purified protein is often observed as a 180-kDa species. DNA polymerase α from human cells has been shown to be phosphorylated throughout the cell cycle (Nasheuer *et al.*, 1991) and may be glycosylated (Hsi *et al.*, 1990). This has necessitated the use of baculovirus cloning to overproduce active human DNA polymerase α (Copeland and Wang, 1991). When the full-length DNA polymerase δ produced in bacteria is compared with DNA polymerase δ obtained from yeast, no difference in electrophoretic mobility is apparent (Fig. 3). Bacteria do not properly modify eukaryotic proteins and so this comparison suggests that DNA polymerase δ is not significantly modified in yeast. This does not rule out minor modifications as a
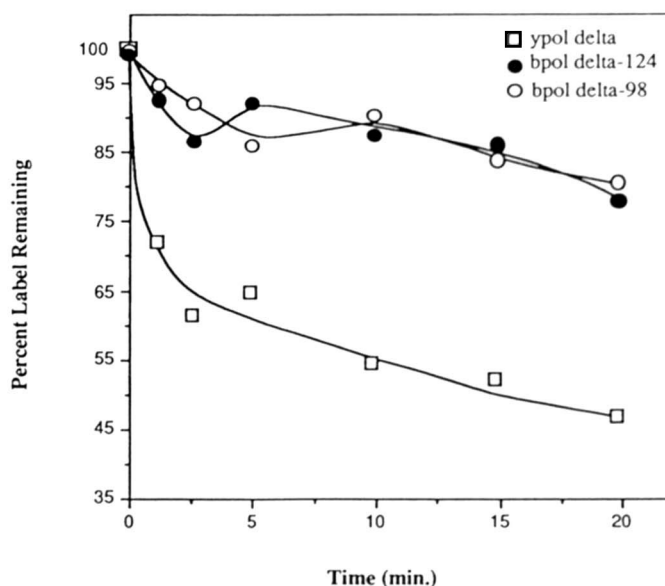
FIG. 7. **Bacterially produced DNA polymerase δ exhibits exonuclease activity.** Exonuclease assays were run as described under "Experimental Procedures." Both the full-length and truncated forms of DNA polymerase δ produced in bacteria were added at 50 ng (0.9 unit), whereas that purified from yeast was added at 1 unit of DNA polymerase activity. Results are plotted as percent of initial radioactive substrate remaining *versus* time of incubation.

means of altering DNA polymerase δ activity. Human DNA polymerase α displays hyperphosphorylation after S-phase (Nasheuer *et al.*, 1991), and a similar mechanism for regulating the active states of DNA polymerase δ may exist. However, the polymerase activity *per se* is apparently not dependent on posttranslational modifications.

When both forms of the single subunit DNA polymerase δ produced in bacteria are compared with each other and to multisubunit DNA polymerase δ purified from yeast with respect to sensitivity to differential inhibitors of DNA synthesis and processivity, there is no distinguishable difference in response. One major difference between the single subunit bacterial and heterodimeric yeast forms of polymerase δ is the K_m apparent for dNTPs, which is 2–3-fold higher in the bacterial forms. The activity of the single peptide form of DNA polymerase α overproduced in insect cells is indistinguishable from the four-subunit complex isolated from human cell lines in every way examined including K_m for dNTPs (Copeland and Wang, 1991). The renaturation of DNA polymerase δ may yield a slightly different nucleotide binding site with lower affinity for dNTPs. Another difference between the bacterial and yeast DNA polymerase δ is a reduced sensitivity to monovalent cations in the bacterial enzyme as compared with the yeast enzyme. Our results also show that the amino-terminal 220 amino acids are not essential for synthetic activity.

Simon *et al.* (1991) constructed site-specific mutants of DNA polymerase δ in which single amino acid changes in one of the putative exonuclease domains led to lower fidelity of DNA replication in yeast. This result strongly suggested that the 3' to 5' exonuclease activity associated with DNA polymerase δ preparations is a function of the 124-kDa subunit. Our results confirm that the 124-kDa subunit contains exonuclease activity, and its behavior is consistent with a proofreading activity (Fig. 9). This is true for both forms of the polymerase, although there does appear to be some difference in the processing of mispairs between them. The full-length form displays a marked preference for the noncomplementary base,
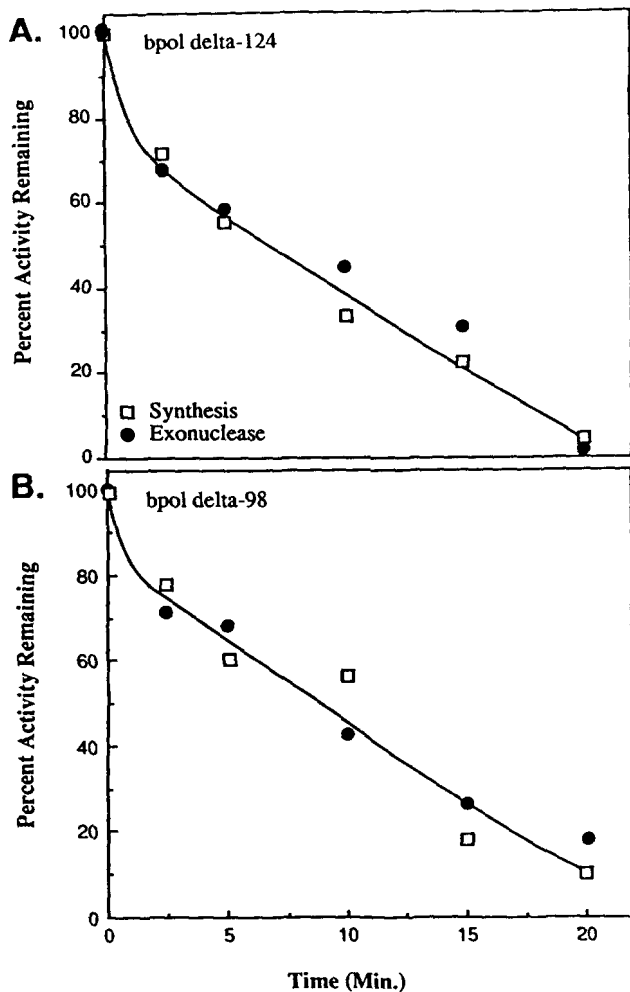
FIG. 8. **Heat denaturation curves for DNA polymerase δ produced in bacteria.** To determine if the exonuclease activity detected in Fig. 5 is associated with the polymerase activity or is a bacterial contaminant, heat denaturation experiments were carried out. Purified protein was incubated at 47 °C, aliquots were removed at varying times, and 50 ng (0.9 unit) was assayed for both synthetic and exonuclease activity. The loss of activity is plotted as a percent of initial activity *versus* the time at 47 °C. The loss of both activities occurs at the same rate for both full-length (*A*) and truncated forms (*B*) of DNA polymerase δ, indicating that they are on the same peptide.

whereas the truncated form processes both paired and mispaired to the same extent. Also, when DNA synthesis is possible, the truncated form shows a reduced rate of processing the mispair, whereas the full-length form shows no difference in rates. The disparity between the truncated and full-length forms of DNA polymerase δ may be the result of improper folding of the exonuclease domain due to the loss of the amino terminus. This is unlikely, however, since the overall activity of the truncated form is not reduced in comparison with the full-length form. Alternatively, the missing portion of polypeptide may have a structural role that allows for efficient discrimination of the complementarity of terminal base pairing by the active site.

The loading of DNA polymerase δ onto the primer terminus is thought to occur through interaction with PCNA which in turn has been shown to complex with A1 (RF-C). The A1 (RF-C) complex contains a DNA binding activity specific for the double strand-single strand DNA junction. Thus the protein-protein interactions of DNA polymerase δ are predicted to be essential for its proper positioning to initiate
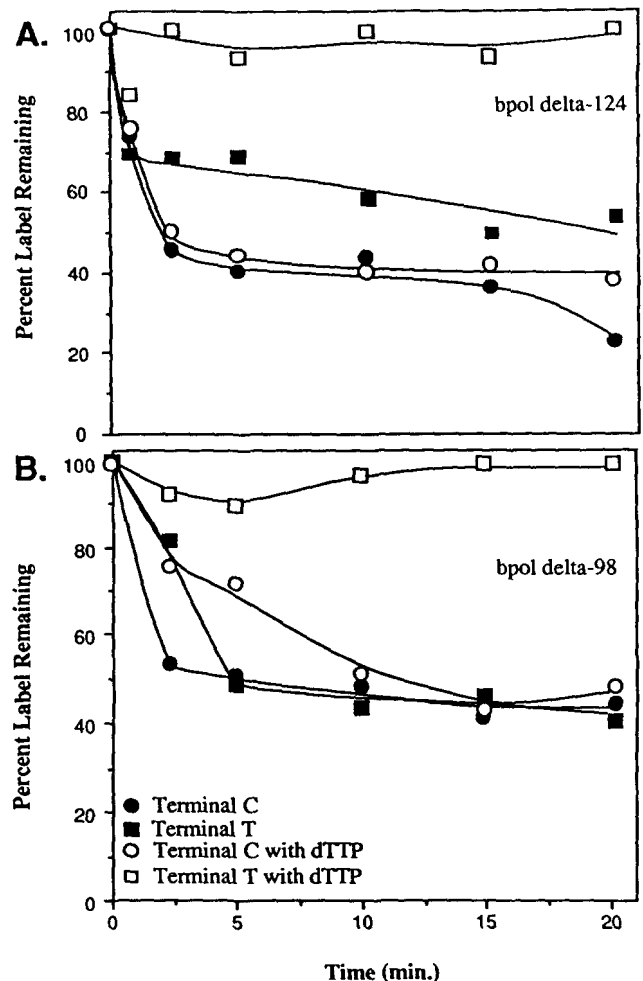

FIG. 9. **Proofreading activity of DNA polymerase δ.** The ability of the exonuclease activity associated with DNA polymerase δ to discriminate between matched and mismatched substrates was determined. Exonuclease reactions were run as described under "Experimental Procedures" using 0.9 unit (50 ng) of full-length (*A*) or truncated (*B*) DNA polymerase δ produced in bacteria, which removes 50–60% of the label. The loss of label is plotted above as a percent of initial label *versus* time of incubation. These reactions were also run with dTTP added to allow for synthesis to occur.

DNA synthesis during replication. To investigate simple structure function relationships, we constructed the amino-terminal truncation. This region of the protein was chosen for both ease of cloning and because it is the region of least homology between DNA polymerase δ and DNA polymerase α. There is some homology in this region between δ and ε, both of which interact with the PCNA-A1 (RF-C) primer recognition complex. We reasoned that perhaps this region is involved in the protein-protein interactions of DNA polymerase δ but is not essential for polymerase function. If this were true we would be able to obtain functional protein for testing this hypothesis. The altered chromatographic behavior of the 98-kDa form of DNA polymerase δ suggests that it is indeed involved in electrostatic interactions and the ability to renature it and obtain activity indistinguishable from the yeast form of the enzyme indicate that it will be useful for further study.

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