The catalytic subunit of human DNA polymerase δ has been overexpressed in insect cells by a recombinant baculovirus. The recombinant protein has a Mr = 125,000 and is recognized by polyclonal antisera against N-terminal and C-terminal peptides of the catalytic subunit of human DNA polymerase δ. The recombinant protein was purified to near homogeneity (approximately 1200-fold) from insect cells by chromatography on DEAE-cellulose, phosphocellulose, heparin-agarose, and single-stranded DNA-cellulose. The purified protein had both DNA polymerase and 3′–5′ exonuclease activities. The properties of the recombinant catalytic subunit were compared with those of the native heterodimeric DNA polymerase δ isolated from fetal calf thymus, and the enzymes were found to differ in several respects. Although the native heterodimer is equally active with either Mn2+ or Mg2+ as divalent cation activator, the recombinant catalytic subunit is 5-fold more active in Mn2+ than in Mg2+. The most striking difference between the two proteins is the response to the proliferating cell nuclear antigen (PCNA). The activity and processivity of native DNA polymerase δ are markedly stimulated by PCNA whereas it has no effect on the recombinant catalytic subunit. These results suggest that the small subunit of DNA polymerase δ is essential for functional interaction with PCNA.

Although genetic studies in yeast have suggested that three distinct DNA polymerases (α, δ, and ε) are required for nuclear DNA replication in eukaryotes (1–4), only two DNA polymerases (α and δ) were found to be essential components of an in vitro reconstituted SV40 DNA replication system (5); polδα, with its tightly associated primase activity, was shown to be required for the synthesis of primers for both leading and lagging strands, whereas polδ and its accessory proteins, proliferating cell nuclear antigen (PCNA) and replication factor C, also known as activator 1, were found to be required for leading strand synthesis and for completing Okazaki fragments initiated by polα/primase. More recently, elucidation of the roles of DNA polymerases α, δ, and ε in DNA replication was approached by UV cross-linking of polymerases to nascent DNA within replicating chromosomes (6). These studies showed that only polα and polδ were photolabeled by nascent SV40 DNA whereas polα, polδ, and polε were all photolabeled by nascent cellular DNA, suggesting that the replication of SV40 chromosomes may require only a subset of the proteins required for replication of cellular chromosomes. Thus, the roles of polδ and polε in cellular DNA replication may not be resolved by studies of the replication of DNA viruses; rather, studies of the properties of the replicative DNA polymerases and their interactions with other replication proteins will be necessary to answer these questions.

polδ has been purified from a number of eukaryotic sources including calf thymus (7), budding yeast (8), mouse cells (9), and Drosophila melanogaster (10). The enzyme is usually isolated as a heterodimer with subunits of approximately 125 and 50 kDa, although the Drosophila enzyme has a single subunit of 120 kDa (10) and both one- and two-subunit forms of polδ were isolated from mouse cells (9). PCNA was found to stimulate the activity and processivity of heterodimeric forms of polδ (8, 9, 11, 12) but to have no effect on the isolated catalytic subunit (9, 10). The catalytic (125 kDa) subunit has DNA polymerase and 3′–5′-exonuclease activity (13, 14). The function of the small subunit is still unknown. In order to determine the function of the small subunit of polδ, as well as to facilitate studies on the functional properties of polδ and its role in cellular DNA replication, we have constructed recombinant baculoviruses for expression of both the catalytic and small subunits of human polδ in insect cells. Here we report the expression, purification, and characterization of the recombinant human catalytic subunit and compare the properties of the recombinant protein with those of the native heterodimer isolated from calf thymus.

EXPERIMENTAL PROCEDURES

Materials—pGEX-5X-1 and (dT)12,18 were obtained from Pharmacia Biotech Inc. Taq DNA polymerase was from Promega, Vent DNA polymerase was from New England Biolabs, and the Klenow fragment of Escherichia coli DNA polymerase I was from U. S. Biochemical Corp. Poly(dA-dT), poly(dA), and (dT)12–18 were obtained from Midland Certified Reagents. (dA)1000 was from Life Sciences, Inc. Heparin-agarose, single-stranded DNA-cellulose, and aphidicolin were from Sigma. Spodoptera frugiperda (Sf9) wild-type baculovirus AcMNPV, β-galactosidase recombinant baculovirus, linearized AcMNPV DNA, the transfer vector pHBlueBacIII, cationic liposomes, and PCR primers for amplification of inserts in recombinant baculoviruses were obtained from Invitrogen Corp. All other PCR primers were a gift of Dr. Earl W. Dave, University of Washington. Fetal bovine serum, gentamycin, and pleuoronic F-68 were from Life Technologies, Inc. Grace’s insect medium was prepared at the Cell Culture Media Facility, University of Miami. calf thymus DNA polymerase δ was prepared as described in Downey and So (15). PCNA was prepared from fetal calf thymus as described in Tan et al. (11). Rabbit polyclonal antiserum to C-terminal and N-terminal

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Purification and Characterization of the Catalytic Subunit of Human DNA Polymerase δ Expressed in Baculovirus-infected Insect Cells*

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peptides of the large subunit of mammalian pol δ were described previously (16).

Construction of Recombinant Baculovirus—Standard DNA manipulations were carried out according to Sambrook et al. (17). A full-length cDNA clone for the catalytic subunit of human pol δ was constructed in a pGEX vector by combining overlapping cDNA fragments. An 1882-bp DNA fragment (amino acids 1 to 621) and a 1561-bp DNA fragment (amino acids 514 to the stop codon) were obtained by EcoRI digestion of M13mp18 clones HGF5/HHR2-21 and H3/HGRS-10, respectively (18), cut with BspHI, and ligated to pGEX-5X-1 that had been digested with EcoRI and treated with bacterial alkaline phosphatase. The resulting construct was digested with BglII to remove a 1137-bp fragment which was then replaced with a BglII fragment from M13 clone H3/HGRS-15 (18). The resulting plasmid, containing the correct cDNA sequence for human pol δ, was designated pGEX-5X-1-E. Cloning arms were generated by PCR amplification of a 5′-1117-bp fragment and a 3′-315-bp fragment containing engineered NdeI, BamHI, and EcoRI sites (underlined nucleotides) using pGEX-5X-1-E as a template. For the 5′ fragment the sense primer was 5′-TTTGGATCCCATATGGGATGGCAGGCGGCGCA-3′ and the antisense primer was 5′-TTTGAATTCTCTTTCGATCCCTGGGATCCCC-3′. For the 3′ fragment the sense primer was 5′-TTTGAATTCGACCCCCGCGATGTTG-3′ and the antisense primer was 5′-TTTGAATTCGACCCCCGCGATGTTG-3′. Amplifications were carried out with Taq and Vent DNA polymerase (100:1) using a denaturation temperature of 94°C for 1 min, an annealing temperature of 55°C for 2 min, and an extension temperature of 72°C for 3 min. The purified fragments were digested with BamHI and EcoRI and ligated to BamHI-digested pGEX-5X-1. The plasmid with both inserts was designated pGEX-5X-1-ARMS. To generate a full-length clone of the catalytic subunit of human pol δ with BamHI and NdeI cloning sites, pGEX-5X-1-E DNA was digested with BamHI and KpnI, and the resulting 2532-bp DNA fragment was inserted into the pGEX-5X-1-ARMS vector that had been digested with BstXI and KpnI and treated with bacterial alkaline phosphatase. The resulting plasmid was designated pGEX-5X-1-p125. The sequences that had been amplified were verified by dyeoxy sequencing. The coding sequences for p125 were recovered from pGEX-5X-1-p125 by BamHI digestion, and the purified DNA was inserted into the BamHI site of the pBlueBacIII transfer vector. The resulting construct, pBlueBac-p125, was co-transfected with linearized wild type virus AcMNPV DNA into Sf9 cells using cationic liposome-mediated transfection according to the Invitrogen protocol, and recombinant virus was plaque purified. The presence of an insert in a putative recombinant virus was verified by PCR using primers complementary to the polyhedrin locus. Recombinant virus AcN-p125-14 was amplified in Sf9 cells to a titer of 108 plaque-forming units/ml.

Expression of Recombinant Protein—Sf9 cells were grown to confluence in T-25 Falcon flasks at 27°C in Grace’s medium supplemented with 10% fetal bovine serum, 50 μg/ml gentamicin, and 0.1% pleurope F-68, and infected with recombinant virus AcN-p125-14 at a multiplicity of infection 10. Cells were harvested at 55 h postinfection by centrifugation at 27,000 × g, and washed twice with serum-free Grace’s medium. Infected cells were suspended in SDS-PAGE sample buffer. Aliquots of each cell extract were resolved on 10% (A) or 7.5% (B) SDS-PAGE and then stained with Coomassie Blue (A) or analyzed by Western blotting using antisera to the catalytic subunit of pol δ (B). Molecular size markers (in kDa) are indicated on the left. Lane 1, 300 ng; lane 2, 100 ng; lane 3, 30 ng. Molecular weight standards were applied to each gel lane following SDS-PAGE.

Following SDS-PAGE, proteins were electroblotted to a nitrocellulose membrane that was incubated for 30 min with blocking buffer, Tris-buffered saline containing 2% nonfat dry milk and 0.1% Tween 20. The membrane was washed with 3 beds of the same buffer, and the protein was eluted with a 700-ml linear gradient from 20–600 mM NaCl in Buffer A. Fractions containing p125, as identified by activity assays and Western blot analysis, were pooled and precipitated with 55% saturated ammonium sulfate. The precipitate was dissolved in Buffer B (Buffer A with 20% glycerol) supplemented with the mixture of protease inhibitors described above and dialyzed against the same buffer. The dialysate was loaded onto a phosphocellulose (P11) column (1.0 × 12.5 cm) equilibrated with Buffer B containing 100 mM NaCl. After washing with 3 beds of equilibration buffer, protein was eluted with a 300-mllinear gradient of 100–600 mM NaCl in Buffer B. The peak fractions were pooled, diluted with Buffer B to 150 mM NaCl, and loaded onto a heparin-agarose column (1.0 × 10.2 cm) preequilibrated with Buffer B containing 150 mM NaCl. Protein was eluted with a 150-mllinear gradient from 150 to 750 mM NaCl in Buffer B. Active fractions were pooled, dialyzed against Buffer B, and loaded onto a single-stranded DNA-cellulose column (1.0 × 3.7 cm) equilibrated with Buffer B containing 20 mM NaCl and then eluted with a 120-mllinear gradient of 20–250 mM NaCl in Buffer B.

SDS-Polyacrylamide Gel Electrophoresis and Protein Determination—SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (19). Protein concentration was determined by the method of Bradford (20) with BSA as standard.

Immunoblot Analysis—Following SDS-PAGE, proteins were electroblotted to a nitrocellulose membrane that was incubated for 30 min with blocking buffer, Tris-buffered saline containing 2% nonfat dry milk and 0.1% Tween 20. The membrane was washed with 3 beds of the same buffer, and the protein was eluted with a 700-ml linear gradient from 20–600 mM NaCl in Buffer A. Fractions containing p125, as identified by activity assays and Western blot analysis, were pooled and precipitated with 55% saturated ammonium sulfate. The precipitate was dissolved in Buffer B (Buffer A with 20% glycerol) supplemented with the mixture of protease inhibitors described above and dialyzed against the same buffer. The dialysate was loaded onto a phosphocellulose (P11) column (1.0 × 12.5 cm) equilibrated with Buffer B containing 100 mM NaCl. After washing with 3 beds of equilibration buffer, protein was eluted with a 300-mllinear gradient of 100–600 mM NaCl in Buffer B. The peak fractions were pooled, diluted with Buffer B to 150 mM NaCl, and loaded onto a heparin-agarose column (1.0 × 10.2 cm) preequilibrated with Buffer B containing 150 mM NaCl. Protein was eluted with a 150-mllinear gradient from 150 to 750 mM NaCl in Buffer B. Active fractions were pooled, dialyzed against Buffer B, and loaded onto a single-stranded DNA-cellulose column (1.0 × 3.7 cm) equilibrated with Buffer B containing 20 mM NaCl and then eluted with a 120-mllinear gradient of 20–250 mM NaCl in Buffer B.

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DNA Polymerase Assays—DNA polymerase activity was assayed with either poly(dA-dT) or poly(dA)oligod(T) (10:1 nucleotide ratio) as substrate.
Recombinant Catalytic Subunit of Human DNA Polymerase δ

TABLE I

| Fraction                  | Total protein* | Total activity* | Specific activity | Yield |
|---------------------------|----------------|-----------------|-------------------|-------|
| Crude extract             | 612 mg         | 10,200 units    | 16.7 units/mg     | 100%  |
| DEAE-cellulose            | 31.5 mg        | 5,860 units     | 186 units/mg      | 58%   |
| Phosphocellulose          | 8.4 mg         | 5,240 units     | 624 units/mg      | 51%   |
| Heparin-agarose           | 2.7 mg         | 3,810 units     | 1,410 units/mg    | 37%   |
| DNA-cellulose             | 0.074 mg       | 1,480 units     | 20,000 units/mg   | 14%   |

* Protein concentrations of crude extract, DEAE-cellulose, phosphocellulose, and heparin-agarose fractions were determined by the Bradford assay with BSA as standard. The protein concentration of the DNA cellulose fraction was determined by densitometric analysis of Coomassie Blue-stained gels containing known amounts of BSA as standard.

* Activity was determined as described under "Experimental Procedures" with poly(dA-dT) as the template-primer and 2 mM MnCl₂ as the divalent cation activator. One unit of enzyme incorporates 1 nmol of nucleotide/h at 37 °C.

Results

Construction of Recombinant Virus AcN-p125-14—Comparison of the published cDNA sequences of the catalytic subunit of human pol δ (18, 21) identified 16 differences, 5 of which result in amino acid changes, i.e. Trp-30, Arg-119, Ser-173, His-472, and Arg-776 in Chung et al. (18) and Arg-30, His-119, Asn-173, Try-472, and Gly-776 in Yang et al. (21). The resequencing of a number of partial cDNA clones that had been generated by PCR amplification of poly(A⁺) RNA from HepG2 cells using primers HGF3/HR3 and H3/HGR5 (18) revealed that codons for Arg-30 and Tyr-472 were present in some clones and were likely correct, whereas codons for His-119, Asn-173, and Gly-776 were not present in any of our clones. Furthermore, the sequencing of genomic clones (22) confirmed that the correct sequence is Arg-30, Arg-119, Ser-173, Try-472, and Arg-776. In order to construct a correct cDNA clone, overlapping partial cDNA inserts in M13mp18 were combined and cloned into a pGEX vector as described under "Experimental Procedures." Cloning arms were introduced by PCR amplification of 5′- and 3′-fragments using primers with engineered BamHI and NdeI cloning sites. After verifying the sequence of the amplified portions of the construct, the corrected full-length catalytic subunit of human pol δ was subcloned into a pBlueBac transfer vector. Recombinant virus was then obtained by cotransfection of insect cells (Sf9) with the transfer vector pBlueBac-p125 and linearized wild type virus DNA. The recombinant virus AcN-p125-14 expresses the full-length catalytic subunit of human pol δ from its natural ATG start codon under the control of the polyhedrin promoter. Fig. 1 shows that, at 48 h postinfection, Sf9 cells infected with AcN-p125-14 produced a protein of approximately 125 kDa that was immunoblotted by an antipeptide antibody to mammalian pol δ, whereas unim-
infected cells or cells infected with wild type virus or β-galactosidase recombinant virus did not.

Purification of Recombinant p125—At a multiplicity of infection of 10, a time course of p125 expression in AcN-p125-14-infected Sf9 cells showed that the recombinant protein was detectable by immunoblotting beginning at 36 h postinfection and the amount of protein was maximal at 60 h postinfection (data not shown). Consequently, AcN-p125-14 was used to infect 4 × 10⁶ insect cells at a multiplicity of infection of 10, and cells were harvested at 55 h postinfection. Recombinant p125 was purified by chromatography on DEAE-cellulose, phosphocellulose, heparin-agarose, and single-stranded DNA-cellulose as described under “Experimental Procedures.” The purification is summarized in Table I, and SDS-PAGE and Western blot analysis of each fraction are shown in Fig. 2. Recombinant p125 was followed by both Western blot analysis and activity assays using poly(dA-dT) as the template-primer. The purified protein had a specific activity of 20,000 units/mg and was approximately 92% pure as determined by Coomassie-stained SDS-PAGE (Fig. 2). The protein has both DNA polymerase and 3′- to 5′-exonuclease activity. Fig. 3 shows the elution profile of p125 on single-stranded DNA-cellulose chromatography. Both DNA polymerase and 3′- to 5′-exonuclease activity co-eluted at approximately 130 mM NaCl with a 125-kDa polypeptide that was immunoblotted by antibody to pol δ.

Effect of PCNA on the activity and processivity of p125—Fig. 4 shows the effects of PCNA on the activity (panel A) and processivity (panel B) of heterodimeric calf thymus pol δ and recombinant human p125 with poly(dA-oligo(dT)) as template-primer. Calf thymus pol δ has little or no activity on this template-primer in the absence of PCNA, but it is markedly stimulated by the addition of PCNA, 50-fold at 800 ng/ml. Recombinant p125 also has little or no activity on this template-primer in the absence of PCNA, but in contrast to the native heterodimer, the recombinant protein was not stimulated by the addition of the processivity factor. Attempts to find conditions (pH, monovalent cation concentration, divalent cation concentration, MnCl₂, or MgCl₂) that resulted in stimulation of the recombinant protein by PCNA were unsuccessful. The effects of PCNA on the processivity of calf thymus pol δ and recombinant p125 are shown in panel B. In the absence of PCNA, both enzymes synthesized very short products. In the presence of PCNA, calf thymus pol δ synthesized long products, but the recombinant p125 products were essentially unaffected. These results are similar to those reported by Hindges and Hubscher (23) with recombinant mouse p125, expressed in E. coli as a glutathione S-transferase-fusion protein, and by Arroyo et al. (24) with recombinant Schizosaccharomyces pombe p125 expressed in insect cells. However, they are in contrast to the results reported by Brown and Campbell (25) with renatured recombinant Saccharomyces cerevisiae p125, expressed in E. coli, and by Zhang et al. (26) with recombinant human p125 expressed in monkey cells. The recombinant budding yeast protein was
found to be essentially identical to the heterodimeric protein isolated from yeast cells, i.e. both enzymes had activity on poly(dA)-oligo(dT) template-primers in the absence of PCNA and were stimulated 3–6-fold by the addition of PCNA, and the increased activity was accompanied by increased product size. The recombinant human protein expressed in monkey cells was stimulated 4.5-fold by PCNA, somewhat less than the 10-fold stimulation seen with the heterodimeric pol δ isolated from human placenta, and the increased activity was also accompanied by an increase in product size.

Comparison of Recombinant p125 and Calf Thymus pol δ—In addition to its insensitivity to PCNA, recombinant p125 was found to differ from heterodimeric calf thymus pol δ in its divalent cation requirement. Although the Mn²⁺ optima were similar for the two proteins, the Mn²⁺ optimum for recombinant p125 (2 mM) was 10-fold higher than that for native pol δ (data not shown). A comparison of the steady state kinetic parameters for dTTP and poly(dA-dT), determined at optimal Mg²⁺ (2 mM) and Mn²⁺ (0.2 mM for calf thymus pol δ and 2 mM for recombinant p125) concentrations, is shown in Table II. Recombinant p125 was approximately 5-fold more active with Mn²⁺ as the divalent cation activator than with Mg²⁺, whereas calf thymus pol δ had equivalent activity with either Mn²⁺ or Mg²⁺. Furthermore, with calf thymus pol δ, the apparent $K_m$ values for dTTP and poly(dA-dT) did not differ significantly in Mg²⁺ or Mn²⁺, whereas substraction of Mn²⁺ for recombinant p125 resulted in significantly lower apparent $K_m$ values for both dTTP (from 19 to 2.0 $\mu$M) and poly(dA-dT) (from 5.2 to 0.29 $\mu$g/ml).

The effect of KCl on enzyme activity was found to be dependent on the divalent cation activator (Fig. 5A). With Mn²⁺ as the divalent cation, both recombinant p125 and native pol δ were slightly stimulated by low KCl concentrations (25 mM) and inhibited by higher KCl concentrations. With Mg²⁺ as the divalent cation, both proteins were inhibited by KCl, but the native enzyme was somewhat more sensitive to inhibition than the recombinant protein. Similarly, the effect of aphidicolin was also affected by the divalent cation (Fig. 5B). With Mn²⁺ as the divalent cation, both p125 and native pol δ were inhibited to the same extent by aphidicolin (IC₅₀ of 5 μg/ml) whereas in Mg²⁺, the recombinant protein was more resistant to aphidicolin. At 8 μg/ml, the native enzyme was 90% inhibited whereas recombinant p125 was only 30% inhibited.

Although the effects of KCl and aphidicolin on recombinant p125 have not been reported previously with Mn²⁺ as the divalent cation, the present results with Mn²⁺ as the divalent cation differ from those in the literature. The recombinant yeast protein (27) was found to be markedly stimulated by KCl (10–14-fold at 240 mM) while the native yeast enzyme was inhibited by KCl. Both the native and recombinant yeast proteins were found to be equally sensitive to inhibition by aphidicolin. The recombinant human protein expressed in monkey cells (26) was found to be identical to the enzyme isolated from human placenta in its response to both KCl and aphidicolin.

**DISCUSSION**

Recombinant catalytic subunits of pol δ have now been purified and characterized in several laboratories using a variety of expression systems (Refs. 23 and 25–27 and this report). There are significant differences in the enzymatic properties of the recombinant proteins, and the reasons for these differences are not entirely clear. A major difference among the recombinant proteins is the effect of PCNA, which has been shown to increase the activity and processivity of the recombinant budding yeast protein expressed in E. coli (25) and the recombinant human protein expressed in monkey cells (26) but to have no effect on the recombinant mouse protein expressed in E. coli as a glutathione S-transferase-fusion protein (23), the recombinant fission yeast protein expressed in insect cells (24), or the human protein expressed in insect cells (this report). This discrepancy is unlikely to be due to differences in the expression system, as the catalytic subunits that are stimulated by PCNA were expressed in both E. coli and primate cells. It is important to note that single subunit forms of pol δ have been isolated from mouse cells (9) and D. melanogaster (10). In each case the polymerase was found to be unresponsive to PCNA, implying that the 50-kDa subunit is required for the interaction of pol δ with PCNA. Although the isolated catalytic subunit from mouse cells has not been shown unequivocally to be pol δ and there remains a possibility that the polypeptide is a proteolytic
fragment of pol ε, the Drosophila protein has been cloned and sequenced and shown to be authentic pol δ (28). Thus, it seems clear that the catalytic subunit of pol δ, when isolated from cells or tissues, is incapable of interacting with PCNA.

The recombinant catalytic subunits characterized thus far are also somewhat different in terms of catalytic activity. Although assay conditions vary in the several reports, it appears that the specific activity of the recombinant catalytic subunit is generally lower than that of the native heterodimer. Whether the lower activity is due to the lack of the 50-kDa subunit, to a lack of posttranslational modification is not clear at present. The protein is phosphorylated in vivo (29), but whether and how phosphorylation affects activity is unclear. It is also unclear whether the recombinant proteins are folded properly when expressed in the absence of the small subunit. The observations that the yeast protein is most active in the presence of KCl concentrations that are inhibitory to the native enzyme and that the human protein expressed in insect cells is more active in Mn\(^{2+}\) than in Mg\(^{2+}\) suggest the possibility that the conformation of these recombinant proteins is not optimal for catalysis and that monovalent and divalent cations may help the proteins to assume an active conformation. Under conditions optimal for calf thymus pol δ with poly(dA-dT) as the template-primer, i.e. 2 mM MgCl\(_2\), recombinant human p125 expressed in insect cells is approximately 14% as active as the native heterodimer and similar in activity to the single subunit form of pol δ isolated from Drosophila (10). These results suggest that the small subunit of pol δ is required for optimal activity as well as for interaction with PCNA. In preliminary studies, co-expression of the 125- and 50-kDa subunits of human pol δ in insect cells resulted in the formation of a recombinant heterodimer that was identical to the native calf thymus heterodimer in its response to PCNA.\(^2\)

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\(^2\)J.-Q. Zhou, H. He, C.-K. Tan, K. M. Downey, and A. G. So, manuscript in preparation.

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