DNA polymerase δ isolated from *Schizosaccharomyces pombe* contains five subunits

Shaojun Zuo*, Emma Gibbs*, Zvi Kelman*, Teresa S.-F. Wang†, Mike O'Donnell‡, Stuart A. MacNeill§, and Jerard Hurwitz*

*Program in Molecular Biology, Memorial Sloan–Kettering Cancer Center, New York, NY 10021; †Department of Pathology, Stanford University, School of Medicine, Stanford, CA 98305-5324; ‡Laboratory of DNA Replication, Howard Hughes Medical Institute, Rockefeller University, New York, NY 10021; and §Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh EH9 3JK, United Kingdom

ABSTRACT   DNA polymerase δ (pol δ) plays an essential role in DNA replication, repair, and recombination. We have purified pol δ from *Schizosaccharomyces pombe* more than 10^3-fold and demonstrated that the polymerase activity of purified *S. pombe* pol δ is completely dependent on proliferating cell nuclear antigen and replication factor C. SDS/PAGE analysis of the purified fraction indicated that the pol δ complex consists of five subunits that migrate with apparent molecular masses of 125, 55, 54, 42, and 22 kDa. Western blot analysis indicated that the 125, 55, and 54 kDa proteins are the large catalytic subunit (Pol3), Cdc1, and Cdc27, respectively. The identity of the other two subunits, p42 and p22, was determined following proteolytic digestion and sequence analysis of the resulting peptides. The peptide sequences derived from the p22 subunit indicated that this subunit is identical to Cdm1, previously identified as a multicopy suppressor of the temperature-sensitive *cdc1*-P13 mutant, whereas peptide sequences derived from the p42 subunit were identical to a previously uncharacterized ORF located on *S. pombe* chromosome 1.

DNA replication in eukaryotes requires three distinct DNA polymerases, α, δ, and ε (1–6). DNA polymerase α, complexed with DNA primase, initiates DNA synthesis by catalyzing the formation of pre-Okazaki fragments (7, 8). A direct role of DNA polymerase δ (pol δ) in the replication of simian virus 40 (SV40) DNA has been shown. This polymerase is involved in the maturation of pre-Okazaki fragments on the lagging strand and the synthesis of leading strand DNA (3). In contrast, no role for pol ε has been demonstrated in SV40 DNA synthesis. However, pol ε is essential for viability of *Saccharomyces cerevisiae* and it has been proposed that pol ε acts at the cellular replication fork and/or plays a role in DNA repair pathways (9). In contrast to pol α, pols δ and ε require two accessory protein factors, proliferating cell nuclear antigen (PCNA) and replication factor C (RFC, also called Activator 1), for processive action during strand elongation of low levels of primer ends (10–12). Complexed with a primed template, RFC recruits PCNA and assembles it onto DNA in the presence of ATP (13–15). PCNA, a ring-shaped homotrimeric protein, encircles DNA, acting as a sliding clamp that tethers the pols to the template DNA (16–18). Subsequent ATP hydrolysis is required for the DNA polymerase to enter the complex to initiate chain elongation in the presence of dNTPs (13, 19, 20–23).

Human (*h*) pol δ has been shown to be a heterodimer consisting of a 125-kDa subunit containing both the polymerase and 3′ to 5′ exonuclease domains and a 48-kDa subunit

essential for the stimulation of polymerase activity by PCNA (24–28). In *Saccharomyces cerevisiae* (*sc*), pol δ includes subunits of 125 kDa (homologous to the *h* pol δ large subunit), 58 kDa (homologous to the *h* pol δ 48-kDa subunit), and other subunits that remain to be defined (2, 29). Studies of *Schizosaccharomyces pombe* (*sp*) have identified genes for the large catalytic subunit of pol δ, *pol3⁺/cdc6⁺*, and two additional genes, *cdc1⁺* and *cdc27⁺*, and their respective proteins of 125, 51, and 43 kDa (30–33). The Pol3/Cdc6 and Cdc1 proteins are homologous to those isolated from *S. cerevisiae,* suggesting that the structure and function of pol δ in these two yeasts may be similar. Genetic and biochemical studies have shown that the p125 subunit of *sp* pol δ (Pol3/Cdc6) interacts with Cdc1, the homologue of the *h* pol δ 48-kDa subunit and the *sc* pol δ 58-kDa subunit. Furthermore, Cdc27 interacts with Cdc1, suggesting that Pol3, Cdc1, and Cdc27 form a complex that constitutes the pol δ holoenzyme (32).

We report here on the purification of *sp* pol δ and demonstrate that both Cdc1 and Cdc27 proteins copurify with the Pol3 catalytic subunit, confirming that these three subunits interact. In addition, we have detected two proteins of 42 and 22 kDa that copurify with *sp* pol δ, suggesting that the *sp* pol δ holoenzyme comprises five distinct subunits. In support of this, the sequence of the 22-kDa protein reported here is identical to the gene product Cdm1, which was previously identified as a multicopy suppressor of the *S. pombe* temperature-sensitive mutant *cdc1*-P13 (32).

Furthermore, we present peptide sequencing data indicating that the p42 subunit is encoded by a previously uncharacterized ORF present in a cosmid c9G1, which is present on chromosome 1 of *S. pombe*.

MATERIALS AND METHODS

Reagents and Enzymes. Poly(dA)$_{4500}$ and oligo(dT)$_{12–18}$ were obtained from Life Sciences (St. Petersburg, FL) and Pharmacia LKB, respectively. [³H]dTTP was from Amersham. *E. coli* single-stranded DNA-binding protein (SSB) was from Pharmacia. The polyclonal antibody against the *sp* pol δ p125 subunit was a generous gift from G. Baldacci (30), and the antibodies for detection of Cdc1 and Cdc27 were as described previously (32).

Assays for *sp* PCNA, *sp* RFC, and *sp* Pol δ. The synthesis of poly(dT) during the elongation of multiply primed poly(dA):oligo(dT) DNA templates is dependent on the presence of pol δ, PCNA, and RFC. The activities of these proteins were measured in reaction mixtures (30 μl) containing 40 mM Tris·HCl (pH 7.8), 7 mM MgCl$_2$, 2 mM ATP, 0.5 mM DTT, 166 μg/ml BSA, 50 ng of poly(dA)$_{4000}$·oligo(dT)$_{12–18}$ (20:1), 33 μM

Abbreviations: pol, DNA polymerase; RFC, replication factor C; PCNA, proliferating cell nuclear antigen; hSSB, human single-stranded DNA-binding protein (also called RPA); *h*, human; *sp*, *Schizosaccharomyces pombe*; *sc*, *Saccharomyces cerevisiae*; PMSF, phenylmethylsulfonyl fluoride.

[^{3}H]dTTP (300 cpm/pmol), and 0.3 μg of *E. coli* SSB. For the assay of PCNA activity, reaction mixtures were supplemented with 0.15 unit of pol δ and 0.13 unit of RFC; for the assay of RFC, reaction mixtures contained 50 ng of PCNA and 0.15 unit of pol δ; for the assay of pol δ, reaction mixtures contained 50 ng of PCNA and 0.13 unit of RFC. Reactions were incubated at 37°C for 60 min, and the incorporation of [^{3}H]dTMP into an acid-insoluble form was measured by liquid scintillation counting. One unit of pol δ or RFC supported the incorporation of 1 nmol of dTMP under the conditions specified above.

sp **PCNA Isolation.** The plasmid construct for overexpression of His-tagged *sp* PCNA (34) was transformed into *E. coli* M15[pREP4] cells. Cell cultures (10 liters) were grown in 2× YT medium (containing 16 g of Bacto-tryptone, 10 g Bacto-yeast extract, 5 g of NaCl per liter) to an OD$_{600}$ of 0.7 prior to induction with 1 mM IPTG for 3 hr. The resulting cells (18 g) were lysed for 1 hr at 0°C in 100 ml of buffer containing 50 mM Tris·HCl buffer (pH 8.0), 20 mM potassium phosphate buffer (pH 8.0), 0.5 M KCl, 3 mg/ml lysozyme, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM EGTA, 0.2 μg/ml aprotinin, 0.2 μg/ml leupeptin, and 0.1 μg/ml antipain. Lysed cells were centrifuged at 18,000 rpm for 30 min, and the supernatant (85 ml) was mixed with 15 ml of Ni^{2+}/NTA beads for 2 hr at 4°C. The beads were then washed three times with 75 ml of buffer containing 50 mM Tris·HCl (pH 8.0), 20 mM potassium phosphate (pH 8.0), and 1 M KCl, followed by washing with the same buffer containing 0.25 M KCl. The beads were then packed into a column, and PCNA was eluted with a solution containing 0.5 M imidazole, 20 mM potassium phosphate (pH 8.0), 0.25 M KCl, and 10% glycerol. Fractions containing PCNA, as identified by SDS/PAGE, were pooled and dialyzed for 12 hr against buffer containing 25 mM Tris·HCl (pH 7.5), 25 mM KCl, 1 mM EGTA, 0.1 mM PMSF, 0.2 μg/ml aprotinin, 0.2 μg/ml leupeptin, and 0.1 μg/ml antipain. SDS/PAGE analysis indicated that PCNA had been purified to ≥95% homogeneity with a yield of 200 mg.

sp **Pol δ Isolation.** The isolation of *sp* pol δ was carried out using a procedure modified from that described for the purification of DNA pol III from *S. cerevisiae* (4). *S. pombe* cells (1.2 kg) were resuspended in 1,200 ml of buffer containing 0.2 M Tris·HCl (pH 8.0), 2 mM EDTA, 2 mM DTT, 10% glycerol, 0.01% Brij 58, 2 μM pepstatin A, 10 mM NaHSO$_3$, 0.5 mM PMSF, and 2 mM benzamidine and were broken with glass beads using the DynoMill (Impandex, Clifton, NJ). Saturated ammonium sulfate solution (saturated at 4°C) was added to 5%, and the mixture was centrifuged at 15,000 × *g* for 60 min. The supernatant was adjusted to 55% with solid ammonium sulfate (280 g/ml), and the pellet obtained after centrifugation at 15,000 × *g* for 90 min was dissolved in 300 ml of buffer A (50 mM potassium phosphate, pH 7.0/10% glycerol/2 mM EDTA/2 mM DTT/2 μM pepstatin A/10 mM NaHSO$_3$/0.5 mM PMSF/2 mM benzamidine) and 0.2 M KCl. This pol δ-containing fraction was dialyzed against three changes of 4 liters of the same buffer over a 12-hr period. The dialyzed material was loaded onto a phosphocellulose column (5.5 × 23.5 cm, 500 ml) equilibrated with 16 liters of buffer A with 0.2 M KCl and eluted sequentially with 800 ml of buffer A containing 0.4 M KCl and 500 ml of buffer A containing 0.7 M KCl. The 0.4 M KCl fraction (330 ml) containing pol δ activity was diluted to 0.1 M KCl with 1,170 ml of buffer B (25 mM potassium phosphate, pH 7.0/10% glycerol/2 mM EDTA/1 mM DTT/2 μM pepstatin A/10 mM NaHSO$_3$/0.5 mM PMSF/2 mM benzamidine) and loaded onto a Q Sepharose column (2.5 × 8.5 cm, 40 ml) and eluted with a 1-liter linear gradient of 0.1–0.4 M KCl in buffer B. The active fractions (peaking at 0.2 M) were pooled (225 ml), diluted to 0.15 M KCl with 75 ml of buffer B, and loaded onto a SP-Sepharose column (1.0 × 7.0 cm, 5 ml). A 200-ml linear gradient of 0.15–0.5 M KCl in buffer B was used to develop this column, and active fractions (peaking at 0.2 M KCl) were

pooled (25 ml), diluted to 0.1 M KCl with 30 ml of buffer B, and loaded onto a single-stranded DNA cellulose column (1.0 × 2.8 cm, 2 ml) equilibrated with buffer B and 0.1 M KCl. A 50-ml linear gradient of 0.1–0.4 M KCl in buffer B was applied to this column, and active fractions peaking at 0.2 M KCl were pooled (16 ml) and loaded directly onto a hydroxylapatite column (1.0 × 1.4 cm, 1 ml). A 40-ml linear gradient of 0.025–0.3 M potassium phosphate, pH 7.0, in buffer B containing 0.2 M KCl was used to elute bound proteins, and the pol δ activity eluting at 0.1 M potassium phosphate was pooled (8 ml) and concentrated to 4 ml using a centrifugal filter device (Millipore). A portion of this fraction (0.2 ml) was applied to a 5-ml 15–35% glycerol gradient in buffer B containing 0.2 M KCl. After centrifugation at 45,000 rpm for 18 hr at 4°C, fractions (0.2 ml) were collected from the bottom of the tube. A summary of the purification procedure is presented in Table 1. The yield of *sp* pol δ after the glycerol gradient step has been calculated assuming that this procedure was carried out with the entire fraction isolated after hydroxylapatite chromatography.

sp **RFC Isolation.** For the isolation of *sp* RFC, *S. pombe* cells (600 g) were resuspended in buffer A (600 ml) and subjected to the same procedure described above for the isolation of *sp* pol δ through the phosphocellulose step. *sp* RFC activity eluted from the phosphocellulose column with buffer A containing 0.4 M KCl (260 ml, 4,100 units, 332 mg protein) and was dialyzed against two changes of 4 liters of buffer B containing 50 mM KCl over a 12-hr period. Following application to a Q Sepharose column (2.5 × 10.5 cm, 50 ml), bound proteins were eluted with a 500-ml linear gradient of 0.05–0.5 M KCl in buffer B. Active fractions (eluting at 0.2 M KCl) were pooled (80 ml, 8,080 units, 43 mg protein) and loaded onto a hydroxylapatite column (1.0 × 11.2 cm, 8 ml), which was developed with a 150-ml linear gradient of 0.025–0.4 M potassium phosphate, pH 7.0, in buffer B containing 0.2 M KCl. RFC-enriched fractions (eluting at 0.25 M potassium phosphate) were pooled (16 ml, 2,144 units, 8.8 mg protein), dialyzed against 2 liters of buffer C (25 mM Hepes, pH 7.5/10% glycerol/1 mM DTT/2 μM pepstatin A/2 mM NaHSO$_3$/0.1 mM PMSF/1 mM benzamidine) with 0.2 M KCl, and mixed with 1 ml of *sp* PCNA-linked Affi-Gel 15 beads (containing 10 mg of PCNA per ml of beads) equilibrated with the same buffer in the presence of 2 mM ATP and 7 mM MgCl$_2$ (35). After rocking for 4 hr at 4°C, the mixture was packed in a column and washed with the same buffer prior to elution of bound proteins with 25 ml of 0.5 M KCl in buffer C, and active fractions were pooled (5 ml, 732 units, 36 μg of protein). SDS/PAGE analysis indicated that the RFC complex was purified to near homogeneity.

Protein Sequence Analysis. *sp* Pol δ containing glycerol gradient fractions was pooled (2.4 ml, ≈5 μg protein) and precipitated with 0.1 volume of 0.15% sodium deoxycholate and 0.1 volume of 72% trichloroacetic acid. The mixture was centrifuged at 15,000 rpm for 30 min at room temperature, and the pellet was dissolved in 20 μl of 2× SDS sample buffer, neutralized with 1 M Tris·HCl, pH 8.3, and then resolved by

Table 1. Purification of *S. pombe* DNA polymerase

Step	Protein, mg	Activity, units	Specific activity, units/mg of protein	Yield, %
Crude extract	91,200	—	—	—
Phosphocellulose	930	19,050	21	(100)
Q-Sepharose	59	12,690	215	67
Sp-Sepharose	13	6,116	470	32
SS-DNA cellulose	5.4	4,132	765	22
Hydroxylapatite	0.28	1,378	4,921	7
Glycerol gradient	0.036	731	20,300	3.8

12% SDS/PAGE and transferred to a nitrocelloluse membrane (0.2 μm, Schleicher & Schuell). The 42- and 22-kDa protein bands were excised from the Poinceau S-stained nitrocellulose membrane, subjected to *in situ* tryptic digestion (36, 37) prior to peptide resolution through a 1-mm RP-HPLC column (ODS 1 mm × 150 mm). Chemical sequencing and matrix-assisted laser-desorption ionization time-of-flight mass spectrometry were carried out as described (38–40).

RESULTS

Isolation of *sp* PCNA and *sp* RFC. PCNA, RFC, and SSB all stimulate pol δ-catalyzed DNA synthesis of primed single-stranded DNA templates. To assay for *sp* pol δ activity during purification, PCNA and RFC were purified from *S. pombe* as described in *Materials and Methods*. It has previously been shown that *h* PCNA can substitute for *sp* PCNA *in vivo* (41). To test whether *sp* PCNA and *sp* RFC are able to substitute for *h* PCNA and *h* RFC, respectively, in supporting *in vitro* DNA synthesis, poly(dA):oligo(dT) assays were carried out in which the pol δ holoenzyme was reconstituted with *h* pol δ, *h* or *sp* RFC, and *h* or *sp* PCNA. *sp* RFC and *sp* PCNA supported *h* pol δ-dependent DNA synthesis to an extent similar to that observed with the homologous species pol δ-holoenzyme. Thus, for its isolation, *sp* RFC activity was assayed for its ability to stimulate *h* pol δ-dependent DNA synthesis in the presence of *sp* PCNA. Purified *sp* RFC migrated in SDS/PAGE as five distinct protein bands, one of 125 kDa and four with apparent molecular masses of 36–40 kDa, more closely resembling the *h* RFC subunit sizes (145 kDa and 36–40 kDa) than those of *sc* RFC (94 kDa and 36–40 kDa) (data not presented). The relationship of the *sp* RFC small subunits to the corresponding subunits of *h* RFC and *sc* RFC, however, remains to be elucidated.

Isolation of *sp* Pol δ. A summary of the purification procedure utilized for the isolation of *sp* pol δ is presented in Table 1. *sp* Pol δ was purified more than 1,000-fold with a 4% yield compared with the polymerase activity detected after phosphocellulose chromatography. Whereas the activity of the pol δ present in relatively crude fractions (the phosphocellulose fraction) was markedly stimulated by PCNA, RFC and pol δ cofractionate until the hydroxylapatite column and, thus, addition of RFC to these fractions does not stimulate pol δ-catalyzed DNA synthesis further. However, following hydroxylapatite fractionation, RFC and pol δ separate and pol δ activity becomes completely dependent on the addition of both PCNA and RFC (Fig. 1). In the poly(dA):oligo(dT) assay, poly(dT) synthesis was only stimulated 2-fold by *E. coli* SSB. In contrast, in reactions in which singly primed single-stranded M13 DNA was used in place of poly(dA):oligo(dT), DNA synthesis was completely dependent on the presence of an SSB (*E. coli*, *h*, or *sp* SSB; data not presented).

Subunit Structure and Composition of *sp* Pol δ. The availability of highly purified *sp* pol δ and antibodies specific for the p125 catalytic subunit, Cdc1, and Cdc27 prompted us to examine whether these subunits are all components of *sp* pol δ. For this purpose, we examined the elution profiles of both *sp* pol δ DNA synthetic activity and the p125, Cdc1, and Cdc27 subunits by Western blot analyses after hydroxylapatite chromatography. As shown in Fig. 2*A*, pol δ eluted between fractions 14 and 24 (0.1 M potassium phosphate), concomitant with the elution of the p125, Cdc1, and Cdc27 subunits as determined by Western blot analyses (Fig. 2 *B–D*, respectively). These three proteins were detected only in fractions containing polymerase activity, indicating their strong association.

Glycerol gradient sedimentation of the *sp* pol δ-enriched hydroxylapatite fractions resulted in the appearance of DNA synthetic activity that peaked at fraction 10, between the catalase and aldolase marker proteins (Fig. 3*A*). Western blot
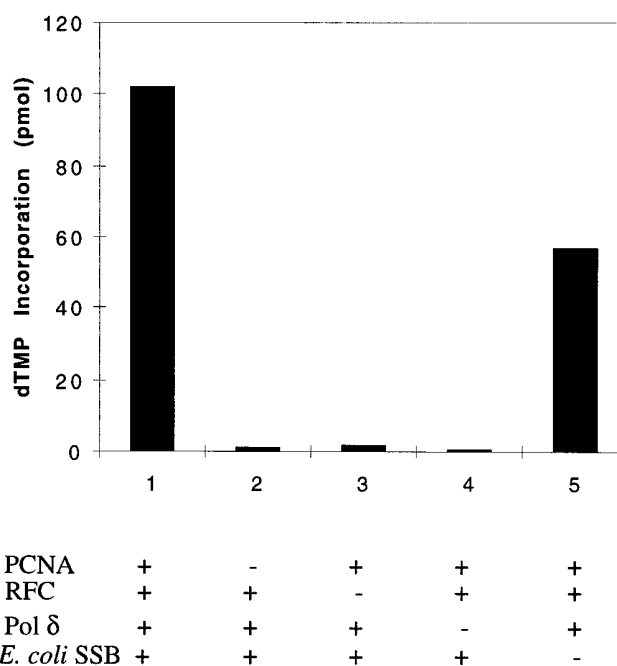

FIG. 1.   Effects of PCNA, RFC, and *E. coli* SSB on pol δ activity with the poly(dA)·oligo(dT) template. Reaction mixtures (30 μl) contained 40 mM Tris·HCl (pH 7.8), 7 mM MgCl$_2$, 2 mM ATP, 0.5 mM DTT, 166 μg/ml BSA, 50 ng poly(dA)$_{4000}$·oligo(dT)$_{12–18}$ (20:1), and 33 μM [^{3}H]dTTP (300 cpm/pmol). Where indicated, 0.13 unit of RFC, 50 ng PCNA, 0.3 μg *E. coli* SSB, and 0.15 unit of pol δ (hydroxylapatite fraction) were added. Reactions were incubated at 37°C for 60 min, and the incorporation of [^{3}H]dTMP into an acid-insoluble form was measured by liquid scintillation counting.

analyses with antibodies against the p125, Cdc1, and Cdc27 subunits (Fig. 3 *B–D*, respectively) indicated that all three proteins cosedimented at a position coincident with *sp* pol δ activity (as seen following hydroxylapatite fractionation in Fig. 2). As observed after hydroxylapatite chromatography, these three proteins were detected only in fractions containing polymerase activity.

Examination of the glycerol gradient fractions by SDS/PAGE analysis followed by silver staining revealed that pol δ cosedimented as a complex containing five polypeptides with apparent molecular masses of 125, 55, 54, 42, and 22 kDa (Fig. 4). Western blot analysis confirmed that the 125-kDa (Pol3/Cdc6) polypeptide is the large subunit of pol δ, and that the protein bands of 55 and 54 kDa correspond to Cdc1 and Cdc27, respectively (as previously shown; refs. 32 and 42). Based on their amino acid composition, Cdc1 and Cdc27 are 51 and 43 kDa, respectively, suggesting that they migrate more slowly during SDS/PAGE than expected. Western blot analysis of Cdc27 (Figs. 2*D* and 3*D*) revealed the presence of two bands that migrated closely, most likely due to phosphorylation of Cdc27 (S.A.M., unpublished results). The comigration of the p125, Cdc1, and Cdc27 subunits with *sp* pol δ activity was evident in all chromatographic steps (summarized in Table 1) that were carried out with five different extract preparations. The previously unidentified proteins of 42 and 22 kDa were shown to cosediment with *sp* pol δ activity by SDS/PAGE analysis following hydroxylapatite chromatography and glycerol gradient sedimentation. When the two glycerol gradient fractions corresponding to the peak of pol δ activity were pooled, concentrated, and subjected to a second glycerol gradient centrifugation step, all five proteins continued to cosediment with *sp* pol δ activity. These observations suggest that all five protein subunits are associated with *sp* pol δ as a multiprotein complex.
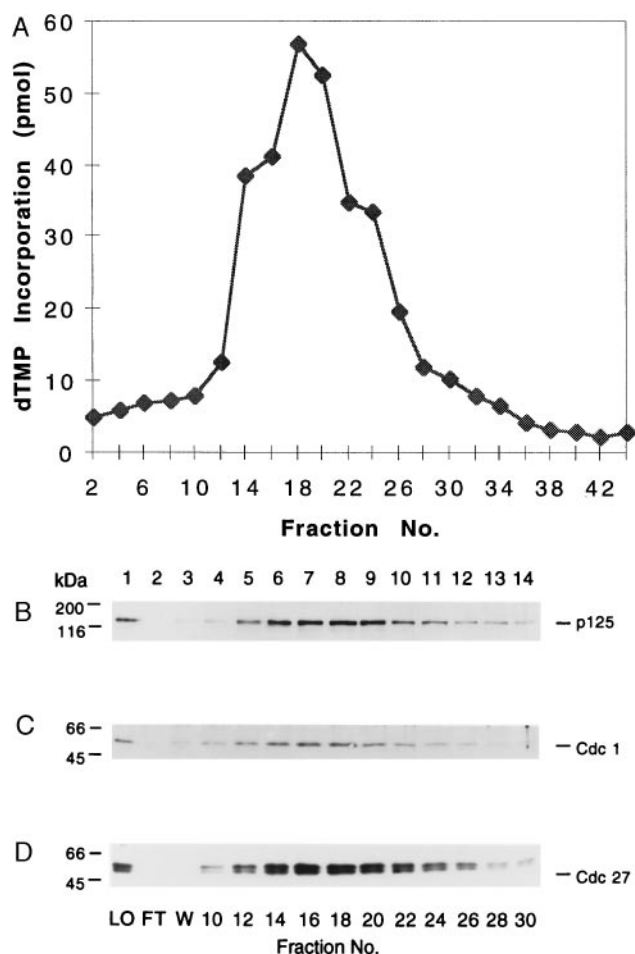
FIG. 2. Elution profile of *sp* pol δ from a hydroxylapatite column. The elution procedure was carried out as described in *Materials and Methods*. (*A*) Elution profile of *sp* pol δ polymerase activity as determined by the poly(dA)·oligo(dT) assay. Aliquots (3.4 μl) of fractions eluting from the hydroxylapatite column were subjected to 10% SDS/PAGE and visualized by Western blotting with polyclonal antibodies against the p125 subunit (*B*), Cdc1 (*C*), and Cdc27 (*D*). LO, load on (single-stranded DNA cellulose fraction); FT, flow through; W, wash.

Sequence Analysis of p42 and p22. The two previously unidentified protein bands, p42 and p22, that copurified with *sp* pol δ were isolated and subjected to tryptic digestion as described in *Materials and Methods*. The sequences of three distinct tryptic peptides of the isolated 42-kDa band were obtained and are indicated in Fig. 5. No sequences homologous to these peptides were detected using the BLAST SEARCH program; however, a single cosmid, c9G1, was retrieved from the *S. pombe* genomic DNA sequencing project database at the Sanger Center, Cambridge, U.K. (http://www.sanger.ac.uk/projects/s_pombe/), which included sequences encoding all three peptides. These peptides are present within a single ORF of a predicted protein of 42 kDa. The nucleotide sequence and deduced amino acid sequence of p42 are presented in Fig. 5.

Partial peptide sequences generated following tryptic digestion of the 22-kDa subunit were identical to the sequence of the *S. pombe* Cdm1 protein (Fig. 6). The *cdm1*+ gene was initially isolated as a multicopy suppressor of the temperature-sensitive *cdc1*-P13 mutant (32). Thus, p22 (Cdm1) has now been identified in association with the *sp* pol δ multisubunit complex by both genetic and biochemical approaches. The identity of this 22-kDa subunit was confirmed following immunoblot analysis of the *sp* pol δ containing glycerol gradient fractions using antibodies against the Cdm1 protein (Fig. 3*E*). In
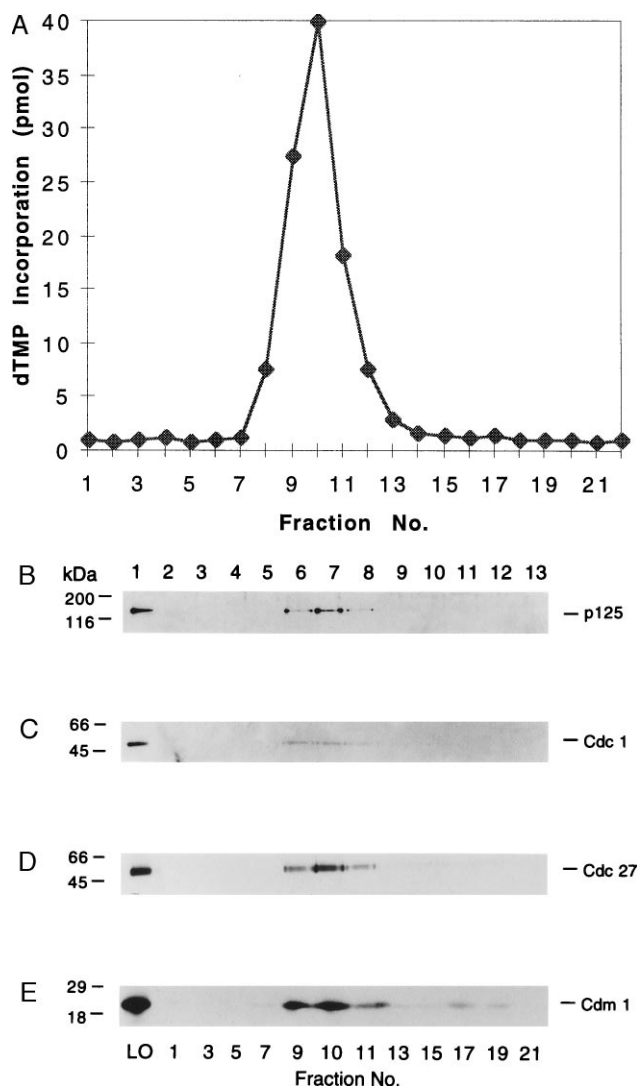
FIG. 3. Glycerol gradient sedimentation of *sp* pol δ. This step was carried out as described in *Materials and Methods*. (*A*) Glycerol gradient fractions were assayed for pol δ polymerase activity using the poly(dA):oligo(dT) assay. Aliquots (10 μl) of glycerol gradient fractions were subjected to Western blot analyses using polyclonal antibodies against the p125 subunit (*B*), Cdc1 (*C*), Cdc27 (*D*), and Cdm1 (*E*). LO, load on (hydroxylapatite fraction). In a separate gradient, sedimented under identical conditions, catalase and aldolase peaked at fractions 5 and 13, respectively.

contrast to the other subunits, Western blot analysis indicated that a small portion of the Cdm1 subunit appeared to dissociate from the complex during glycerol gradient centrifugation. A detailed characterization of *cdm1*+ will be presented elsewhere (S.A.M., N. Reynolds, A. Watt, and P. A. Fantes, unpublished work).

DISCUSSION

The previous demonstration that there is an interaction between the *S. pombe* Cdc1 and Pol3 subunits and between Cdc1 and Cdc27 suggested that these three proteins would be found in a complex. In this study, we have isolated and characterized *S. pombe* DNA polymerase δ and confirmed that Cdc1 and Cdc27 are associated with the catalytic 125-kDa subunit of pol δ and that these three proteins copurify with pol δ activity.

The Pol3 subunit, Cdc1, and Cdc27 have each been shown to be essential for cell cycle progression. *S. pombe* cells carrying temperature-sensitive mutations in the Pol3 subunit undergo
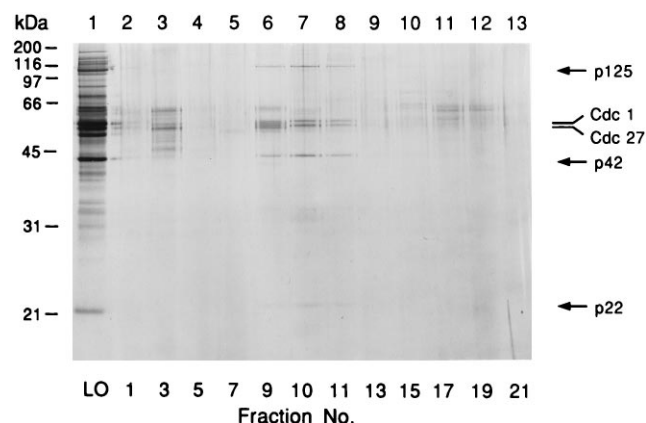
FIG. 4. Polypeptide composition of *sp* pol δ. Aliquots (20 μl) of the glycerol gradient fractions were subjected to 12% SDS/PAGE analysis followed by silver staining. LO, load on.

cell cycle arrest in late S phase at the restricted temperature (43–45). Cells with temperature-sensitive mutations in either Cdc1 or Cdc27 undergo cell cycle arrest at the restricted temperature with a 2C DNA content and become hypersensitive to hydroxyurea at the permissive temperature. Cells harboring Cdc1 or Cdc27 mutations are highly elongated, contain a single nucleus, and do not enter mitosis. This cell
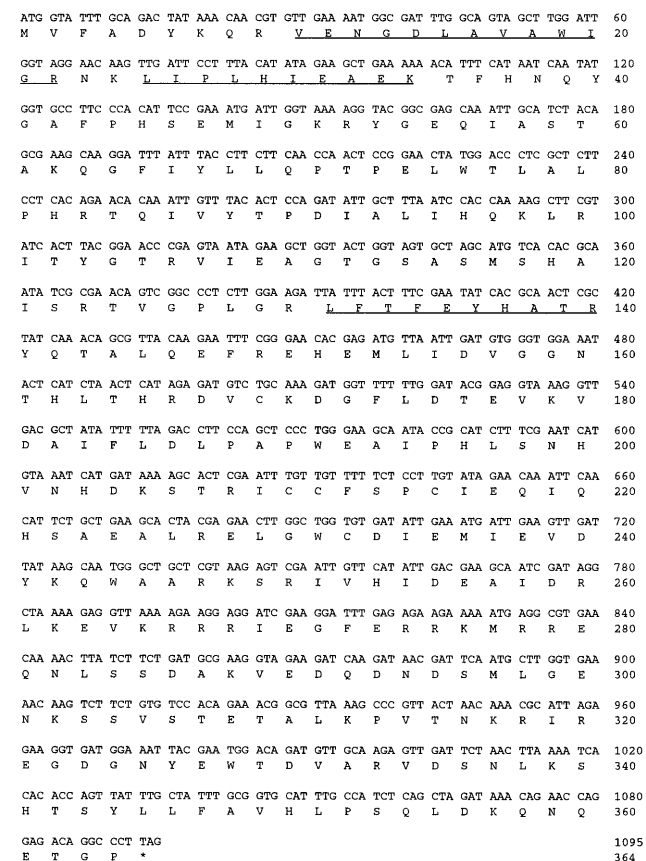

FIG. 5. Nucleotide and amino acid sequences of the *sp* pol δ p42 subunit. The three peptide sequences determined by chemical sequencing of tryptic peptides derived from the isolated 42-kDa subunit are underlined. These sequences were identical to the DNA sequence of the cosmid c9G1 retrieved from the *S. pombe* database (available from the Sanger Center, Cambridge, U.K.). The calculated molecular mass from the deduced amino acid sequence of the p42 subunit is 41,737 daltons, in keeping with the 42-kDa size observed following SDS/PAGE analysis.

```
                                                          **
MKKRTTQAKKSGQNTNIRDVFPHVVRSNSSQSHIGKK VSS    40
 *
EQSPTPDVTITTKTLDER IKEDDELSKEVE EATNQIMAER    80
 *    **              *** ***
ISEPIHCENI TKVEFILHHF DTTARYGPYLGMTRMQRWKR   120
                  **
AKNFNLNPPETVGKILMLEEADEENRKK ESLFYDLQTIP G   160
```
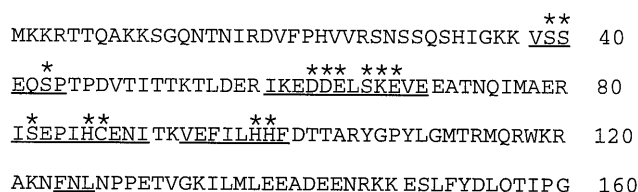
FIG. 6. The complete amino acid sequence of the 22-kDa subunit deduced from clones of the *cdm1* gene that rescued temperature-sensitive *cdc1* mutants. The partial peptide sequences determined by chemical analysis of the 22-kDa subunit isolated as a part of the *sp* pol δ holoenzyme are underlined. Several of the peptides included sites at which no amino acid assignment could be made, and these are indicated with an asterisk above that position.

cycle arrest is dependent on the *rad/hus* checkpoint pathway as demonstrated in *cdc1 rad/hus* or *cdc27 rad/hus* double-mutant cells, which fail to arrest prior to mitosis. Furthermore, Cdc1 or Cdc27 inactivation induces synthesis of the large transcript of *suc22* (the small subunit of ribonucleotide reductase), which is associated with inhibition of DNA replication and DNA damage in *S. pombe* (32). Together, these results demonstrate that Pol3, Cdc1, and Cdc27 play important roles in replication, repair, and cell cycle progression (43–46).

The identification of two additional *sp* pol δ subunits of 42 and 22 kDa was based on their copurification with the p125, Cdc1, and Cdc27 subunits and with PCNA-dependent polymerase activity. Sequence data obtained from tryptic digests of the p42 and p22 protein bands indicated that they were not proteolyzed fragments of the p125, Cdc1, and/or Cdc27. The p42 subunit possesses a weak but significant sequence similarity with the budding yeast nuclear protein GCD14, which is of unknown function (47). The 22-kDa subunit was found to be identical to the Cdm1 protein, which was previously isolated by its ability to rescue cells harboring a temperature-sensitive *cdc1* mutation (32). The significance of this interaction, as well as the role of the p22 and p42 subunits in governing the activity of *sp* pol δ, awaits further genetic and reconstitution studies.

Pol δ purified from mammalian cells has been shown to be a heterodimer of 125 and 48 kDa with no apparent Cdc27, p42, or Cdm1 homologues present. There are several possible explanations for this. Cdc27, p42, and Cdm1 may be unique to yeast, exhibit a lower affinity for the mammalian 125- and 48-kDa subunits, and thus separate from the heterodimer during purification or may only be required for the repair or checkpoint functions of pol δ and be dispensable for replication. Alternatively, exponentially growing fission yeast cells are predominantly in the G₂ phase, and the association of p42, Cdc27, and Cdm1 with p125 and Cdc1 may be cell-cycle-dependent. Further studies with mammalian pol δ should distinguish between these possibilities.

The purified recombinant human p125-kDa subunit has catalytic and 3′ to 5′ exonuclease activities *in vitro*, suggesting that the 48-kDa subunit is not required for these activities. Recent studies have shown that polymerase activity of the human p125 subunit is not stimulated by PCNA (48); however, coexpression and subsequent purification of the p125- and 48-kDa subunits (homologous to Pol3 and Cdc1) yield a complex whose activity is stimulated by PCNA (28), suggesting that the 48-kDa subunit is responsible for mediating any interaction between pol δ and PCNA.

It is interesting to note that as with pol δ there is a discrepancy in the subunit structure of pol ε from human or *S. cerevisiae* origins. Pol ε derived from human cells has been shown to include only two subunits of 215 and 55 kDa (49, 50), whereas *sc* pol ε contains subunits of >200, 80, 34, 30, and 29 kDa (5, 51, 52). Genes encoding the largest three of these *sc* subunits have been cloned, and those encoding the 200- and 80-kDa subunits have been shown to be essential. A single

nonessential gene encodes both the 30- and 34-kDa subunits, suggesting that they may represent posttranslationally modified products of this gene (52).

The pol δ holoenzyme reconstituted from combinations of *h* and *sp* PCNA, RFC, and pol δ efficiently supported poly(dT) synthesis with a poly(dA):oligo(dT) template but showed variable efficiency in catalyzing nucleotide incorporation into a singly primed M13 DNA template. This difference was most evident in reactions containing *sp* pol δ, *h* PCNA, and *sp* RFC or *h* RFC. Other distinctions included a more marked salt sensitivity of reactions containing *sp* pol δ compared with those containing *h* pol δ (data not presented). Whether these effects are due to differences in the subunit structure and/or stability of *sp* pol δ and *h* pol δ is presently unclear.

1. Johnson, A. L., Barker, D. G. & Johnston, L. H. (1986) *Curr. Genet.* **22,** 107–112.
2. Boulet, A., Simon, M., Faye, G., Bauer, G. A. & Burgers, P. M. J. (1989) *EMBO. J.* **8,** 1849–1854.
3. Stillman, B. (1994) *Cell* **78,** 725–728.
4. Bauer, G. A., Heller, H. M. & Burgers, P. M. J. (1988) *J. Biol. Chem.* **263,** 917–924.
5. Morrison, A., Araki, H., Clark, A. B., Hamatake, R. K. & Sugino, A. (1990) *Cell* **62,** 1143–1151.
6. Wang, T. S.-F. (1996) in *DNA Replication in Eukaryotic Cells*, ed. DePamphilis, M. (Cold Spring Harbor Lab. Press, Cold Spring Harbor, NY), pp. 461–493.
7. Matsumoto, T., Eki, T. & Hurwitz, J. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 9712–9716.
8. Wang, T. S.-F. (1991) *Annu. Rev. Biochem.* **60,** 513–552.
9. Sugino, A. (1995) *Trends Biochem. Sci.* **20,** 319–323.
10. Lee, S.-H., Kwong, A. D., Ishimi, Y. & Hurwitz, J. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 4877–4881.
11. Tsurimoto, T. & Stillman, B. (1989) *EMBO J.* **8,** 3883–3889.
12. Waga, S. & Stillman, B. (1994) *Nature (London)* **369,** 207–212.
13. Lee, S.-H. & Hurwitz, J. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 5672–5676.
14. Tsurimoto, T. & Stillman, B. (1991) *J. Biol. Chem.* **266,** 1961–1968.
15. Burgers, P. M. & Yoder, B. L. (1993) *J. Biol. Chem.* **266,** 1961–1968.
16. Gogol, E. P., Young, M. C., Kubasek, W. L., Jarvis, T. C. & von Hippel, P. H. (1992) *J. Mol. Biol.* **224,** 395–412.
17. Kong, X.-P., Onrust, R., O'Donnell, M. & Kuriyan, J. (1992) *Cell* **69,** 425–437.
18. Krishna, T. S. R., Kong, X.-P., Gary, S., Burgers, P. & Kuriyan, J. (1994) *Cell* **79,** 1233–1244.
19. Lee, S.-H., Pan, Z.-Q., Kwong, A. D., Burgers, P. M. J. & Hurwitz, J. (1991) *J. Biol. Chem.* **266,** 22707–22717.
20. Burgers, P. M. J. (1991) *J. Biol. Chem.* **266,** 22698–22706.
21. Hurwitz, J., Dean, F. B., Kwong, A. D. & Lee S.-H. (1990) *J. Biol. Chem.* **265,** 18043–18046.
22. Kuriyan, J. & O'Donnell, M. (1993) *J. Mol. Biol.* **234,** 915–925.
23. Herendeen, R. H. & Kelly, T. J. (1996) *Cell* **84,** 5–8.
24. Byrnes, J. J., Downey, K. M., Black, V. C. & So, A. G. (1976) *Biochemistry* **15,** 2817–2823.
25. Chung, D. W., Zhang, J., Tan, C.-K., Davie, E. W., So, A. G. & Downey, K. M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 11197–11201.
26. Yang, C. L., Chang, L.-S., Zhang, P., Hao, H., Zhu, L., Toomey, N. L. & Lee, M. Y. W. T. (1992) *Nucleic Acids Res.* **20,** 735–745.
27. Zhang, P., Frugulhetti, I., Jiang, Y., Holt, G., Condit, R. C. & Lee, M. Y. W. T. (1995) *J. Biol. Chem.* **270,** 7993–7998.
28. Zhou, J.-Q., He, H., Tan, C.-K., Downey, K. M. & So, A. G. (1997) *Nucleic Acids Res.* **25,** 1094–1099.
29. Sugimoto, K., Sakamoto, Y., Takahashi, O. & Matsumoto, K. (1995) *Nucleic Acids Res.* **23,** 3493–3500.
30. Pignede, G., Bouvier, D., Recondo, A.-M. & Baldacci, G. (1991) *J. Mol. Biol.* **222,** 209–218.
31. Park, H., Francesconi, S. & Wang, T. S.-F. (1993) *Mol. Biol. Cell* **4,** 145–157.
32. MacNeill, S. A., Moreno, S., Reynolds, N., Nurse, P. & Fantes, P. A. (1996) *EMBO J.* **15,** 4613–4628.
33. Iino, Y. & Yamamoto, M. (1997) *Mol. Gen. Genet.* **254,** 93–97.
34. Arroyo, M. P., Downey, K. M., So. A. G. & Wang, T. S.-F. (1996) *J. Biol. Chem.* **271,** 15971–15980.
35. Gerik, K. J., Gary, S. L. & Burgers, P. M. J. (1997) *J. Biol. Chem.* **272,** 1256–1262.
36. Tempst, P., Link, A. J., Riviere, L. R., Fleming, M. & Elicone, C. (1990) *Electrophoresis* **11,** 537–553.
37. Lui, M., Tempst, P. & Erdjument-Bromage, H. (1996) *Anal. Biochem.* **241,** 156–166.
38. Elicone, C., Lui, M., Geromanos, S., Erdjument-Bromage, H. & Tempst, P. (1994) *J. Chromatogr.* **676,** 121–137.
39. Erdjument-Bromage, H., Lui, M., Sabatini, D. M., Snyder, S. H. & Tempst, P. (1994) *Protein Sci.* **3,** 2435–2446.
40. Geromanos, S., Casteel, P., Elicone, C., Powell, M. & Tempst, P. (1994) *Techniques in Protein Chemistry V*, ed. Crabb, J. W. (Academic, San Diego), pp. 143–150.
41. Waseem, N. H., Labib, K., Nurse, P. & Lane, D. P. (1992) *EMBO J.* **11,** 5111–5120.
42. Hughes, D. A., MacNeill, S. A. & Fantes, P. A. (1992) *Mol. Gen. Genet.* **231,** 401–410.
43. Francesconi, S., Park, H. & Wang, T. S.-F. (1993) *Nucleic Acids Res.* **21,** 3821–3828.
44. Francesconi, S., DeRecondo, A. M. & Baldacci, G. (1995) *Mol. Gen. Genet.* **246,** 561–569.
45. Uchiyawa, M., Galli, I., Griffiths, D. J. F. & Wang, T. S.-F. (1997) *Mol. Cell. Biol.* **17,** 3103–3115.
46. Congley, M. J., Pierce, A. J. & Modrich, P (1997) *J. Biol. Chem.* **272,** 10917–10921.
47. Cziepluch, C., Kordes, E., Pujol, A. & Jauniaux, J. C. (1996) *Yeast* **12,** 1471–1474.
48. Zhou, J. Q., Tan, C.-K., So, A. G. & Downey, K. M. (1996) *J. Biol. Chem.* **271,** 29740–29745.
49. Syvaoja, J. & Linn, S. (1989) *J. Biol. Chem.* **264,** 2489–2497.
50. Kesti, T., Fanti, H. & Syvaoja, J. (1993) *J. Biol. Chem.* **268,** 10238–10245.
51. Hamatake, R. K., Hasegana, H., Clark, A. B., Bebenek, K., Kunkel, T. A. & Sugino, A. (1990) *J. Biol. Chem.* **265,** 4072–4083.
52. Araki, H., Hamatake, R. K., Johnston, L. H. & Sugino, A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 4601–4605.