Evidence That DNA Polymerase δ Isolated by Immunoaffinity Chromatography Exhibits High-Molecular Weight Characteristics and Is Associated with the KIAA0039 Protein and RPA†

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ABSTRACT: DNA polymerase δ, the key enzyme for eukaryotic chromosomal replication, has been well characterized as consisting of a core enzyme of a 125 kDa catalytic subunit and a smaller 50 kDa subunit. However, less is known about the other proteins that may comprise additional subunits or participate in the macromolecular protein complex that is involved in chromosomal DNA replication. In this study, the properties of calf thymus pol δ preparations isolated by immunoaffinity chromatography were investigated. It is demonstrated for the first time using highly purified preparations that the pol δ heterodimer is associated with other polypeptides in high-molecular weight species that range from 260000 to >500000 in size, as determined by FPLC gel filtration. These preparations are associated with polypeptides of ca. 68−70, 34, 32, and 25 kDa. Similar findings were revealed with glycerol gradient ultracentrifugation. The p68 polypeptide was shown to be a PCNA binding protein by overlay methods with biotinylated PCNA. Protein sequencing of the p68, p34, and p25 polypeptide bands revealed sequences that correspond to the hypothetical protein KIAA0039. KIAA0039 displays a small but significant degree of homology to *Schizosaccharomyces pombe* Cdc27, which, like *Saccharomyces cerevisiae* Pol32p, has been described as the third subunit of yeast pol δ. These studies provide evidence that p68 is a subunit of pol δ. In addition, the p68−70 and p32 polypeptides were found to be derived from the 70 and 32 kDa subunits of RPA, respectively.

DNA replication is a vital cellular process in which the basic synthetic reactions are performed by the DNA polymerase enzymes. These enzymes are the central components of larger assemblies of proteins that are required for cellular DNA replication. DNA polymerase δ, the key enzyme for eukaryotic chromosomal replication, has been well characterized as consisting of a core enzyme of a 125 kDa catalytic subunit and a smaller 50 kDa subunit. In *Escherichia coli*, the DNA polymerase III holoenzyme consists of at least 10 different polypeptides (*1*, *2*). Biochemical and genetic studies have enabled the formulation of models in which these form a multiprotein assembly that functions to coordinate both leading and lagging strand DNA synthesis at the replication fork (*2*). This includes a mechanism whereby this complex contains two DNA polymerase molecules linked by a dimerization protein (tau). This model has been proposed for T4 and *E. coli* as a means of concurrent replication of both template strands at the replication fork (*3*−*5*). Similar models have been proposed for eukaryotic systems, although the details of the proteins or their macromolecular assembly are still vague (*6*).

DNA polymerase δ is the main replicative polymerase involved in the duplication of eukaryotic cell chromosomal DNA (*7*−*11*). Despite the central importance of this enzyme, the delineation of its subunit structure to this date is far from complete. Rigorously purified mammalian pol δ has been extensively characterized as a tightly associated heterodimer consisting of a 125 kDa catalytic subunit and a small 50 kDa subunit (*12*−*14*). The pol δ catalytic subunit is highly conserved in eukaryotic cells, e.g., between human and yeast (*15*). In recent work, *Schizosaccharomyces pombe* pol δ has been isolated in a form that contains two additional subunits (*16*), while in *Saccharomyces cerevisiae*, pol δ has been shown to consist of three subunits (*17*). In addition, the recombinant *S. cerevisiae* pol δ heterotrimer can dimerize when analyzed on Superose 6 gel filtration columns (*18*). This third subunit is encoded by the POL32 and Cdc27 genes in *S. cerevisiae* and *S. pombe*, respectively (*17*, *19*), while the fourth subunit, only identified in *S. pombe*, is encoded by the Cdm1 gene (*16*, *19*).

The task of identifying new subunits of mammalian DNA polymerases is made difficult by the low amounts of enzyme in mammalian tissues and the instability of the enzyme (*12*, *13*). Very little is known of the polypeptides that are associated with mammalian pol δ at the level of the demonstration of physical complexes that are more highly organized than the heterodimer. In an effort to facilitate the identification of proteins and enzymes that interact with pol δ and PCNA, we have developed PCNA affinity (*20*) and

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pol δ immunoaffinity chromatography methods (*14*). A pol δ heterodimer consisting of p125 and p50 was readily obtained when immunoaffinity-purified pol δ is subjected to single-stranded DNA cellulose chromatography (*14*). Using a PCNA overlay technique, we have identified another subunit of polymerase δ, p68. A partial protein sequence and a BLAST search identified this polypeptide as KIAA0039 (*21*). In this study, we have investigated the behavior of the immunoaffinity-purified pol δ from calf thymus, and demonstrated that it exhibits the behavior of a higher-order complex with other polypeptides at a high level of purification.

EXPERIMENTAL PROCEDURES

Materials. Single-stranded DNA cellulose and heparin−agarose were obtained from Sigma Chemical Co. Hydrazide Avid gel F was from Unisyn Technologies. Poly(dA)$_{2000}$ was obtained from Midland Certificate Co. Superose 12 columns, a protein biotinylation system, and the ECL chemiluminescence detection reagents were purchased from Amersham-Pharmacia Biotech Inc. Fetal calf thymus glands were obtained from Animal Technologies Inc. The lysis buffer used for homogenization of calf thymus consisted of 50 mM Tris-HCl (pH 7.8), 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.25 M sucrose, and 5% glycerol. TGEED buffer consists of 50 mM Tris-HCl (pH 7.8 or 8.5), 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 5% glycerol. TGEE buffer is the same as TGEED buffer except for the omission of dithiothreitol. KGEED buffer is 20 mM potassium phosphate (pH 7.0), 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 5% glycerol.

Purification of DNA Polymerase δ from Calf Thymus. All steps were carried out at 0−4 °C using the procedures described by Jiang at al. (*14*), unless otherwise indicated. Eight hundred grams of frozen calf thymus tissue in 4 L of lysis buffer was homogenized in a Waring blender. The suspension was centrifuged at 5000 rpm at 4 °C for 1 h. The supernatant was filtered through glass wool.

Batchwise DEAE-Cellulose Adsorption. DE-52 cellulose (1.5 L, Whatman) was equilibrated with TGEED buffer (pH 7.8). The supernatant was mixed with the DE-52 cellulose and stirred for 30 min. The mixture was filtered through a Buchner funnel. The DE-52 cellulose was washed with 10 L of TGEED (pH 7.8), and the pol δ was stripped with 3 L of 20% ammonium sulfate in TGEED (pH 7.8).

Phenyl−Agarose Hydrophobic Chromatography. The DE-52 cellulose fraction was loaded onto a phenyl−agarose column (bed volume of 500 mL) equilibrated with KGEED buffer [20% ammonium sulfate (pH 7.0)]. The phenyl−agarose column was washed with 1 L of KGEED and eluted with 1 L of TGEED (pH 8.5).

Immunoaffinity Chromatography. The peak fractions from step 2 were pooled and precipitated by addition of 0.32 g/mL ammonium sulfate. The suspension was stirred for 30 min and kept on ice for an additional 30 min, and then centrifuged at 10000*g* for 45 min. The precipitate was resuspended in TGEE buffer, and the conductivity was adjusted to that of TGEE buffer with 80 mM NaCl. The solution was divided into two equal batches, which were individually subjected to immunoaffinity chromatography. The immunoaffinity column (bed volume of 20 mL) was equilibrated with TGEE buffer (pH 7.8). After the sample had been loaded, the column was washed with 60 mL of TGEE buffer containing 0.4 M NaCl. Pol δ was eluted with 30% ethylene glycol and 0.4 M NaCl in TGEE. The peak fractions from two batches of immunoaffinity chromatography were then combined.

Single-Stranded DNA Cellulose Chromatography. The fractions from the immunoaffinity column were combined, and the conductivity was adjusted to that of TGEED buffer containing 50 mM NaCl and loaded onto a ssDNA cellulose column (bed volume of 20 mL). The column was washed with 50 mL of TGEED buffer (pH 7.8). The enzyme was eluted with a 200 mL gradient from 50 to 700 mM NaCl.

Heparin−Agarose Chromatography. The fractions from the ssDNA cellulose column were combined and the conductivities adjusted to that of TGEED buffer containing 50 mM NaCl, and the fractions were loaded onto a heparin−agarose column (bed volume of 2 mL). The column was washed with TGEED buffer (pH 7.8), and the pol δ was eluted with 0.4 M NaCl in TGEED buffer.

FPLC Gel Filtration Chromatography. A preparation obtained after immunoaffinity chromatography was dialyzed against TGEED buffer (pH 7.8) with two changes over a period of 16 h, and then concentrated to 300 μL by centrifugal concentration (5000*g* at 4 °C) using Centricon 30 filters (30 000 MW cutoff, Millipore). The concentrated pol δ was then chromatographed on a FPLC Superose 12 column (HR 10/30, Pharmacia) equilibrated with TGEED (pH 7.8) containing 150 mM NaCl at a flow rate of 0.25 mL/min. A total of 72 fractions of 0.25 mL each were collected.

Glycerol Gradient Ultracentrifugation. Sedimentation analysis was carried out using a Beckman ultracentrifuge with a SW 41 rotor. The buffer was TGEED (pH 7.8) with 150 mM NaCl. The proteins were laid on the top of glycerol gradients in 12 mL tubes and centrifuged at 30 000 rpm for 16 h. After centrifugation, fractions were withdrawn from the bottom of the tubes. The sedimentation velocities were estimated by the use of standard proteins.

SDS−Polyacrylamide Gel Electrophoresis. Fractions were precipitated with 10% trichloroacetic acid, washed with cold 80% acetone, and dissolved in SDS loading buffer. The samples were loaded on a 10% SDS−polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue or silver (Bio-Rad).

Western Blotting. Western blotting was performed using 78F5 and 38B5 pol δ monoclonal antibodies (*14*). Prestained protein standards (Sigma Chemical Co.) were used as molecular weight markers and also to provide for visual confirmation of efficient transfer. Nitrocellulose blots were blocked in 5% w/v nonfat dry milk in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% (v/v) Tween 20 (TBST) for 1 h at room temperature. The blot was then incubated with monoclonal antibodies against pol δ for 12 h at 4 °C. After three 10 min washes in TBST, the blot was incubated with the streptavidin−horseradish peroxidase conjugate diluted in TBST (1:10000) for 1 h at room temperature with constant rocking. The blot was then washed five times with TBST for 20 min each and developed by chemiluminescence (ECL detection system, Amersham-Pharmacia Biotech Inc.).

Nondenaturing Polyacrylamide Gel Electrophoresis. The samples were run on a 5 to 15% gradient gel with a 3.5% stacking gel at 4 °C for a sufficient length of time such that all the protein markers and the pol δ band had reached limiting mobilities. An 18 h period of electrophoresis at 200 V was found to be suitable in preliminary trial experiments. SDS and 2-mercaptoethanol were excluded from the gel. In this experiment, a crude calf thymus extract which has been subjected to batchwise purification on DEAE-cellulose was analyzed. The DEAE sample was concentrated via ammonium sulfate precipitation, and the sample was subsequently desalted on a desalting column. The sample (1 mg of protein) was electrophoresed for 18 h at 4 °C along with the protein standards. Proteins were transferred to a nitrocellulose membrane at 12 V for 12−14 h at 4 °C. The membranes were immunoblotted with 78F5 pol δ p125 monoclonal antibody.

Overlay Blotting with Biotinylated PCNA. Recombinant PCNA was expressed in *E. coli* and purified to near homogeneity as previously described (*22*). PCNA was labeled with biotin by reaction with biotinamidocaproate *N*-hydroxysuccinamide ester (Amersham-Pharmacia Biotech Inc.). The reaction mixtures contained 0.5 mg of PCNA and 0.5 mg/mL biotinamidocaproate *N*-hydroxysuccinamide ester in a total volume of 0.5 mL in 20 mM bicarbonate buffer (pH 8.6). After reaction for 60 min at room temperature, the PCNA was purified on a Sephadex G25 column (bed volume of 5 mL) equilibrated with phosphate-buffered saline containing 1% bovine serum albumin. Samples (1−5 μg of protein) to be overlayed were run onto a 10% SDS−PAGE gel and transferred to nitrocellulose membranes. Prestained protein standards (New England Biolabs) were used as molecular weight markers. The nitrocellulose membrane was blocked with 5% nonfat dry milk in TBST for 45 min at room temperature followed by three washes of TBST for 10 min each. The blot was then incubated with biotinylated PCNA (1 μg/μL) diluted in TBST (1:900) at 4 °C overnight. The blot was washed five times with TBST for 15 min. It was subsequently incubated with the streptavidin−horseradish peroxidase conjugate diluted in TBST (1:5000) for 1 h at room temperature with constant rocking. The blot was then washed five times with TBST for 20 min each and developed by chemiluminescence.

DNA Polymerase Assay. Sparsely primed poly(dA)$_{2000}$/oligo(dT)$_{16}$ was used as the template. The assays contained poly(dA)$_{2000}$/oligo(dT)$_{16}$ (20:1, 0.25 OD$_{260}$ unit/mL), 200 μg/mL BSA, 5% glycerol, 10 mM $MgCl_2$, 25 mM HEPES (pH 6.0), 20 μM [^{3}H]TTP (100 cpm/pmol, 5 μCi/nmol), and 0.2−0.4 unit of pol δ, in the presence or absence of 0.2 μg of PCNA in a total volume of 100 μL. When poly[d(AT)] was used as a template, assays were performed in the absence of PCNA as described by Lee et al. (*13*). The reaction mixtures were incubated for 60 min at 37 °C, and the reactions were terminated by spotting onto DE-81 papers which had been washed four times with 0.3 M ammonium formate (pH 7.8) and once with 95% ethanol and counted as previously described (*13*). One unit of DNA polymerase activity is the amount that catalyzes the incorporation of 1 nmol of dTMP per hour at 37 °C.

Protein Quantitation. Protein was quantified by the Bradford method with BSA as a standard (*23*).

Peptide Sequencing. Sequence analyses were performed by the Harvard University Microchemistry Facility using single microcapillary reverse-phase HPLC nanoelectrospray tandem mass spectrometry on a Finnigan LCQ quadrupole ion trap mass spectrometer.

RESULTS

Immunoaffinity-Purified Pol δ Behaves as a High-Molecular Complex That Is Much Larger Than the Heterodimer. A number of methods have been reported for the rigorous isolation of pol δ from mammalian sources by conventional methods. These generally lead to the isolation of a tightly associated heterodimer of 125 and 50 kDa subunits, although there are reports of the isolation of the free p125 catalytic subunit (*24*, *25*). The amounts recovered are generally very small (tens of micrograms). We reported a more facile procedure that involves batchwise purification of pol δ from calf thymus through DE-52 and phenyl−agarose supports, followed by immunoaffinity chromatography using a monoclonal antibody directed against the p125 catalytic subunit (*14*). This procedure yields about 1 mg of protein, which on SDS−PAGE contains the p125 and p50 polypeptides of pol δ but also a number of other polypeptide bands. However, a persistent association of a ca. 70 kDa band was also noticed. Final purification to the heterodimer was achieved by ssDNA cellulose chromatography (*14*). The specific activity of the preparation was similar to that reported previously (*13*, *14*).

The question which arose was whether any of the polypeptide bands found with the pol δ heterodimer after immunoaffinity chromatography represented replication proteins, i.e., whether the immunoaffinity-purified enzyme contains additional pol δ subunits or other replication proteins that contribute to a higher-order complex. FPLC gel filtration analysis was used to determine if the immunoaffinity-purified enzyme behaved as a higher-molecular weight complex(es) than could be ascribed to a heterodimer. To do this, the immunoaffinity-purified enzyme was concentrated before FPLC analysis. This was done as any higher-order complexes were more likely to be dissociated on dilution. The column fractions were assayed for DNA polymerase δ activity using poly(dA)/oligo(dT) as a template in the absence and presence of PCNA. The fractions were also assayed with poly[d(AT)] alternating copolymer as a template in the absence of PCNA as described previously (*13*). The enzyme activity behaved in a polydisperse manner, but the pol δ activity eluted with a molecular weight higher than that found for the heterodimer (M_r = 175 000), as shown in Figure 1. The major peaks of pol δ activity ranged from a relative molecular weight of 230000 to >500000 as determined from calibration of the column with protein standards. In Figure 1, the identities of the 125 and 50 kDa polypeptides as the subunits of pol δ were confirmed by Western blotting. SDS−PAGE of the peak fractions of the high-molecular weight fractions of immunoaffinity-purified pol δ revealed that the preparations contained the 125 and 50 kDa subunits as major components; i.e., these represent very highly purified preparations. Typical SDS−PAGE profiles for the fractions obtained on FPLC gel filtration of the immunoaffinity-purified enzyme are shown for two separate preparations in Figure 2. In addition to the p125 and p50 polypeptides, we consistently observed the presence of a number of other polypeptides. These included a ca. 70 kDa band, which often appeared as a doublet, and

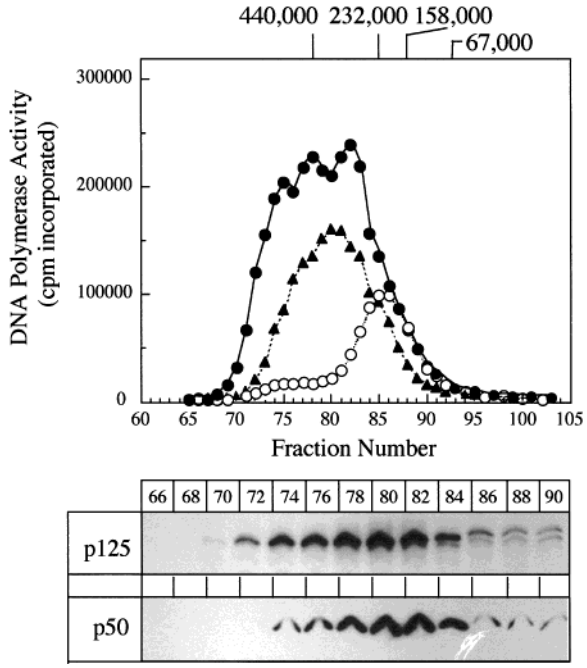

FIGURE 1: FPLC gel filtration analysis of immunoaffinity-purified fetal calf thymus DNA polymerase δ. Calf thymus pol δ was purified by immunoaffinity chromatography and concentrated by centrifugation on Centricon filters as described in Experimental Procedures. The concentrated preparation was run on a Superose 12 HR10/30 column on a Bio-Rad Biologics FPLC system. Fractions of 0.15 mL were collected. The column was calibrated with protein standards (ferritin, catalase, aldolase, and albumin). (Top) Five microliters of each fraction was assayed with poly(dA)/oligo(dT) in the presence (●) and absence (○) of added calf thymus PCNA at 37 °C for 30 min. Activated poly(dAT) was also used as a template (▲) and assayed in the absence of PCNA. (Bottom) Samples of the active fractions were subjected to SDS−PAGE (10% acrylamide) and transferred to nitrocellulose membranes. The membrane was blotted with antibodies against p125 and p50.

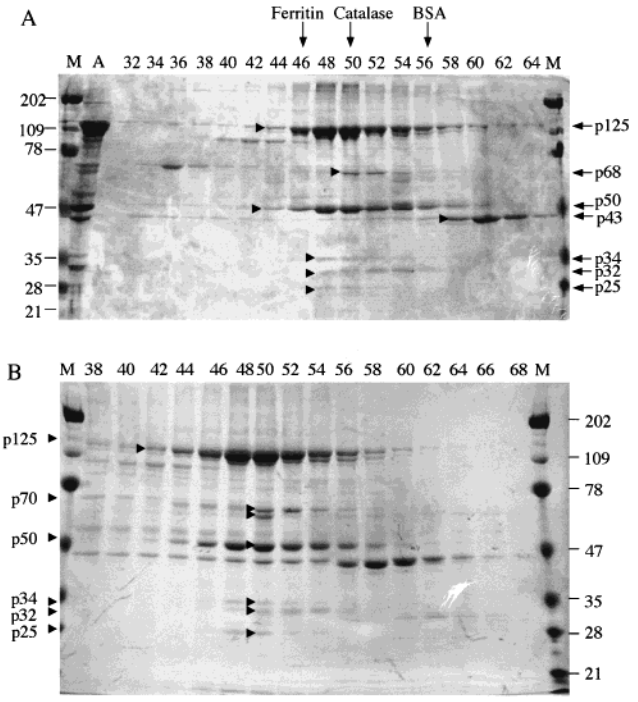

FIGURE 2: SDS−PAGE of immunoaffinity-purified pol δ after FPLC gel filtration. Calf thymus pol δ was purified to the immunoaffinity chromatography step as described for Figure 1, and subjected to FPLC gel filtration using a Superose 12 column. In these experiments, fractions of 0.25 mL were collected. Samples of the active fractions were run on SDS−PAGE and stained with Coomassie Brilliant Blue, and the diagram (A and B) shows the results from two separate representative experiments. Protein standards were as indicated on the left of the diagram and were run in the lanes marked M. A sample of the immunoaffinity-purified enzyme preparation before gel filtration was run in the lane marked A (panel A). The numbers refer to the column fractions which were analyzed. The position of elution of ferritin (MW of 440 000), catalase (232 000), and bovine serum albumin (BSA, 67 000) are shown by the arrows above the gel which indicate the fraction numbers at which these standards eluted.

bands at 43, 34, 32, and 25 kDa. These experiments were repeated for at least 20 preparations, and the appearance of these polypeptides was consistent, although the amounts were variable from preparation to preparation. These bands did not appear in stoichiometric amounts with p125 and p50.

In the following experiments, (a) additional evidence was obtained which shows that the immunoaffinity-purified enzyme behaves as a larger physical entity than can be ascribed to the size of the heterodimer, (b) the identities of the ancillary polypeptides were investigated by protein sequence determination, and (c) the PCNA response of the immunoaffinity-purified enzyme is shown to differ from that of the heterodimer.

Comparison of Physical Properties of the Immunoaffinity-Purified Pol δ with Those of the Heterodimer. The immunoaffinity-purified enzyme was passed through a single-stranded DNA cellulose column and subsequently onto a heparin−agarose column as described in Experimental Procedures, leading to the isolation of a heterodimer of p125 and p50 (not shown) as previously reported (*14*). Calibration of the FPLC gel filtration column on which the immunoaffinity preparations were chromatographed (Figure 2) indicated that the p125 polypeptide (based on SDS−PAGE and protein staining) eluted with a peak between fractions 49 and 50, corresponding to relative molecular weights between 250 000 and 300 000 (not shown). The behavior of the enzyme was not due to aggregation since similar findings were obtained when the amounts of enzyme loaded were reduced 10-fold. Previous studies have shown that the heterodimer behaves as a protein with a relative molecular weight of 173 000 (*13*). This was confirmed with the FPLC gel filtration column used in these studies (not shown). A plot of the Stokes radii of the immunoaffinity-purified enzyme as determined by FPLC chromatography on Superdex 200 gave a value of 57 Å based on the peak fraction containing the p125 band as determined by SDS−PAGE. This is larger than the value of 53 Å that we have previously determined for the heterodimer by conventional purification (*13*).

Similar results were obtained by using glycerol gradient ultracentrifugation (Figure 3); i.e., the immunoaffinity-purified enzyme sedimented with a much higher velocity ($S_{20,w}$ = 9.2) than the heterodimer which under the same experimental conditions migrated as a species of about 7.0 S (not shown). The fractions of pol δ activity obtained after glycerol gradient ultracentifugation were run on SDS−PAGE and stained with silver (Figure 3, inset). It may be noted that p68, p34, p32, and p25 polypeptides also cosedimented with the core enzyme. These experiments demonstrate that the immunoaffinity-purified calf thymus pol δ activity

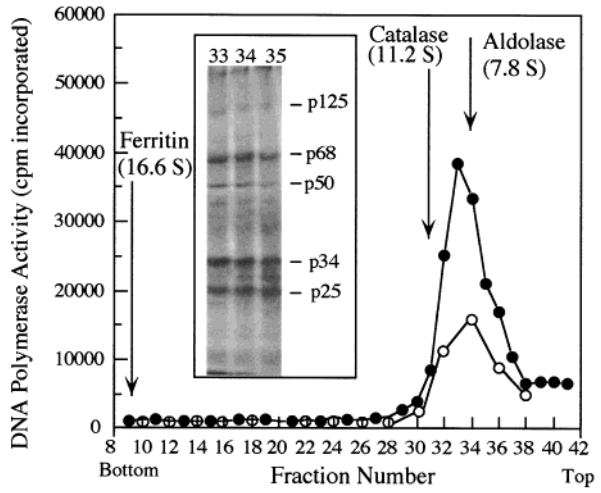

FIGURE 3: Glycerol gradient centrifugation of the immunoaffinity-purified pol δ. Pol δ was purified to the immunoaffinity chromatography step. The peak fractions were concentrated to 150 μL on Centricon filters (Experimental Procedures), laid on the top of a 10 to 45% glycerol gradient (11 mL) containing 150 mM NaCl, 1 mM DTT, and 50 mM Tris-HCl (pH 7.8), and centrifuged for 16 h at 25 000 rpm. After centrifugation, fractions of 0.25 mL each were withdrawn from the bottom of the tubes and assayed for polymerase activity in the presence (●) and absence (○) of PCNA using poly(dA)/oligo(dT) as the template. Migration positions of protein standards (ferritin, catalase, and aldolase) are shown by the arrows. In the inset, the proteins from the peak fractions of activity (fractions 33−35) were concentrated using Centricon 30 filters and loaded onto a 10% SDS−polyacrylamide gel. The gel was visualized by silver staining. The lines show the p125, p68, p50, p34, and p25 polypeptides.

Table 1: Molecular Weights of Mammalian DNA Polymerase Preparations

ref	source	Stokes radius (Å)	sedimentation coefficient	molecular mass
Goulian et al. (*24*)	mouse	54	8.0	178
		43	6.3	112
Lee et al. (*13*)	calf thymus	53	7.9	173
this work	calf thymus	57	9.2	215

behaves as a larger complex than the heterodimer. These findings are the first demonstration that highly purified pol δ in which the core enzyme is the principle component behaves as a macromolecular complex. The calculated molecular weights are shown in Table 1, and are larger than those previously reported for the calf thymus (*13*) and mouse (*24*) pol δ preparations.

Sequence Analysis of Polypeptides in the Pol δ Complex. The identities of the polypeptides that are associated with pol δ preparations were investigated by sequence analysis of the bands excised from the preparations shown in Figure 2, i.e., of the fractions obtained on FPLC gel filtration of immunoaffinity-purified pol δ. A list of the protein sequences obtained from polypeptides excised from SDS−PAGE gels from the two preparations (Figure 2) is shown in Table 2. The p125 and p50 bands were also excised, and the peptide sequences that were obtained exactly matched the known sequences of p125 and p50 (Table 2).

We have previously shown that the doublet of proteins of 68−70 kDa that are associated with high-molecular weight form of affinity-purified pol δ are PCNA binding polypeptides (*21*). Sequencing of bands from this region was performed by MS/MS peptide sequencing, yielding sequences that are identical to the KIAA0039 cDNA sequence (*21*). Additional analysis showed that the peptide sequences obtained from the p68/p70 band (Figure 2 and Table 2) could be arranged into two groups, showing that there were two polypeptides that were migrating in this region. Five sequences (Table 2) were exactly identical with the open reading frame encoded by the human cDNA (Genbank entry D26018) for the hypothetical protein KIAA0039 (*26*). The cDNA encoded by KIAA0039 contains an open reading frame of 466 amino acids. The hypothetical KIAA0039 protein has a predicted molecular mass of 51.4 kDa. The extreme C-terminus contains a consensus PCNA binding site, consistent with the results of the PCNA overlay experiments (*21*). The latter observation makes this protein a strong candidate for the human homologue of the "third" subunits of yeast pol δ, which are encoded by the Cdc27 and POL32 genes in *S. pombe* and *S. cerevisiae*, respectively (*16, 17*). The second of the groups of sequences obtained from the p68 polypeptide was found to be a match for the 70 kDa subunit of RPA, the eukaryotic single-stranded DNA binding protein.

The p34 and p25 bands also yielded sequences identical to those in the KIAA0039 sequence. Five peptide sequences obtained from the p34 polypeptide, and one peptide sequence obtained for the p25 polypeptide, were found to be derived from KIAA0039. This indicates that these two bands are proteolytic fragments of the p68 full-length KIAA0039 protein. The association of these KIAA0039 fragments with the high-molecular weight fraction of pol δ strongly suggests that they may be associated with nicked species of KIAA0039 which retained an ability to associate with pol δ. This could also explain failure to find a consistent stoichiometric association of KIAA0039 in these particular experiments.

The p43 polypeptide, which is present in the immunoaffinity-purified preparation but is clearly separated from the heterodimer on gel filtration (see Figure 2), was also sequenced and was identified as actin.

PCNA Overlay of Immunoaffinity-Purified Pol δ. We have shown that biotinylated PCNA can be used to identify PCNA binding proteins, and have used this method to demonstrate that PCNA interacts with pol δ p125 (*21*). The p50 subunit does not bind PCNA by this method (*21*). The results of a typical overlay experiment in which pol δ preparations at different stages of purification were examined are shown in Figure 4A. It is seen that there are a number of PCNA binding polypeptides in the crude extract, and that the prominent ones are p125 and a doublet at around 68 kDa. The partial protein sequence of this 68 kDa band was obtained, and a BLAST search identified this polypeptide as KIAA0039 (*21*). This protein is retained in the preparation up to the immunoaffinity step, and is retained with the p125 band during subsequent FPLC gel filtration on Superose 12 (Figure 4A). Thus, highly purified pol δ preparations are associated with a ca. 68 kDa polypeptide which is the mammalian counterpart of *S. pombe* Cdc27. If the immunoaffinity-purified pol δ enzyme is first chromatographed on ssDNA−cellulose, this polypeptide is removed and only the p125 band can be detected by PCNA overlay (Figure 4B).

Native Gel Electrophoresis. The nature of the complex(es) involving the p125 catalytic subunit of pol δ was investigated by Western blot analyses of partially purified pol δ on

Table 2: Sequence Analysis of Polypeptides in Immunoaffinity-Purified Calf Thymus Pol δ[a]

excised polypeptide band (kDa)	amino acid sequence	residue numbers	identity	Genbank accession no.
125	TEGGEDYTGATVIEPLK	574−590	p125 subunit of pol δ	M80395
	GLLPQILENLLSAR	653−666		
50	YIHPDDELVLEDELQR	126−141	p50 subunit of pol δ	U2109
	QAASVEAVKMLDEIL	269−283		
	YSSMEDHLEILEWTL	355−369		
70	VVILMELEVLK	93−103	RPA 70 kDa subunit	M63488
	LFSLELVDESGEIR	221−234		
	NEQAFEEVFQNANFR	554−568		
68	WLSYTLGVHVNQAK	25−38	KIAA0039 protein	D26018
	QMLYDYVER	39−47		
	DSGPLFNTDYDILK	110−123		
	FSAIQCAAAVPR	132−143		
	GIMGMFASK	191−199		
34	WLSYTLGVHVNQAK	25−38	KIAA0039 protein	D26018
	QMLYDYVERK	39−48		
	AMLKDSGPLFNTDYDILK	106−123		
	DSGPLFNTDYDILK	110−123		
	GIMGMFASK	191−199		
32	IGNVEISQVTIVGIIR	66−81	RPA 32kDa subunit	gi|4506585|
	IDDMTAAPMDVR	94−105		
	KSLVAFK	139−145		
	PRGLNFQDLK	222−231		
	NQLKHMSVSSIK	232−243		
25	DSGPLFNTDYDILK	110−123	KIAA0039 protein	D26018

[a] Protein sequences from polypeptides extracted after SDS−PAGE were obtained as described in Experimental Procedures.

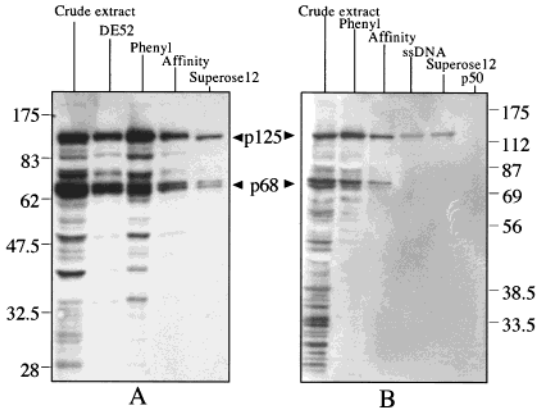

FIGURE 4: PCNA overlay analysis of DNA pol δ at different stages of purification. (A) Samples of a pol δ preparation from the crude extract, DE-52, phenyl−agarose, immunoaffinity, and Superose 12 gel filtration steps were run on SDS−PAGE (10% acrylamide) and transferred to nitrocellulose membranes. The membrane was blotted using biotinylated recombinant PCNA, and visualized using the streptavidin−horseradish peroxidase conjugate and a chemiluminescence method (ECL detection system). (B) Samples of a pol δ preparation at the crude extract, phenyl−Sepharose, immunoaffinity, ssDNA cellulose, and Superose 12 gel filtration steps were subjected to SDS−PAGE and analyzed using biotinylated PCNA as described above. Recombinant p50 (2.5 μg) was also run on this gel, showing that it is not overlayed by PCNA. The positions of the prestained protein standards are marked on the sides. The positions of the p125 and p68 bands are shown by the arrowheads.

nondenaturing gel electrophoresis in gradient gels under conditions where limiting mobility of the proteins was reached. Under these conditions, the migration of proteins or stable protein complexes can be correlated with their relative molecular weights. Our analyses of calf thymus pol δ purified through the initial DE-52 column revealed a single high-molecular weight complex ($M_r \sim 520\,000$) by Western blotting against a pol δ p125 antibody (Figure 5A, lane 1). Similar results were obtained when the material was purified through a subsequent Q-Sepharose column (Figure 5A, lane 2). This monodisperse behavior of the pol δ complex on native gel electrophoresis is striking and argues for the maintenance of a very discrete complex under these conditions. Examination of the purified heterodimeric form of pol δ under the same conditions gave a single band at 175 000 (not shown). Further analysis was performed by excision of the 520 kDa bands obtained from nondenaturing gel electrophoresis. These were subjected to SDS−PAGE, and assessed by PCNA overlay analysis. The results revealed the presence of polypeptide bands at 125 and 68 kDa, and a band at ca. 58 kDa. The latter is not the small subunit of the pol δ (p50) which does not interact with PCNA in the overlay analysis (*21*). This work further confirmed that p68 is strongly associated with high-molecular weight forms of pol δ. The results of these experiments support those shown above for the presence of a PCNA binding protein of ca. 68 kDa which is associated with the pol δ heterodimer in a high-molecular weight complex. In addition, these experiments indicate the possible existence of yet another novel PCNA binding protein of about 58 kDa that is associated with the complex, although we cannot rule out the possibility that it is a proteolytic product of the 125 kDa catalytic subunit.

Sensitivity to PCNA. When the response of the immunoaffinity- and gel filtration-purified enzyme to PCNA was compared to that of the heterodimer, it was observed that the former consistently exhibited a higher response than the latter. To eliminate the possibility that this was due to

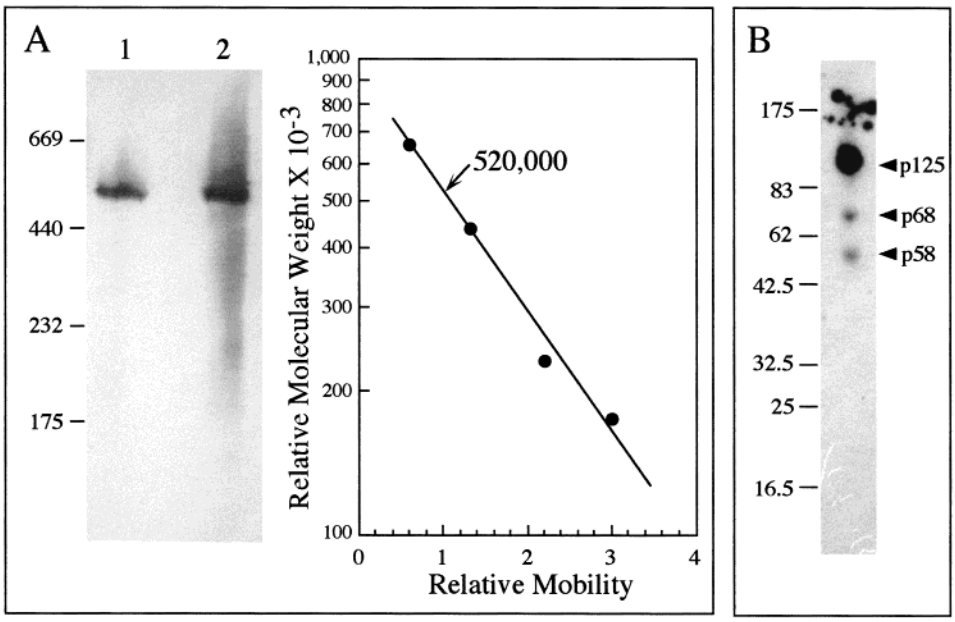

FIGURE 5: Native gel electrophoresis of pol δ. (A) Nondenaturing gel electrophoresis of pol δ. Calf thymus pol δ that was purified through the DE-52 step (lane 1) and then further purified on a Q-Sepharose column (lane 2) was subjected to electrophoresis on a gradient gel (5 to 15% acrylamide) under nondenaturing conditions and electrophoresed until the marker proteins had reached a limiting mobility (Experimental Procedures). The proteins were then transferred to nitrocellulose membranes which were then Western blotted with an antibody against the p125 subunit of pol δ. The diagram on the right shows the estimation of the size of the pol δ complex. Protein standards were used to estimate the size of the complex containing the p125 subunit of pol δ. These were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa). The arrow shows the migration position and estimated size of the protein complex containing the pol δ p125 subunit. (B) Two-dimensional PCNA overlay analysis. The gel slice from the first dimension corresponding to the 520 kDa band was cut with reference to the Western blot, immersed in SDS−PAGE sample buffer without shaking for 2 h at 37 °C, and then secured on top of the stacking gel of a 5−15% SDS−polyacrylamide gradient gel with 0.5% agarose in SDS sample buffer without glycerol. The proteins were transferred onto a nitrocellulose membrane, and the PCNA overlay was performed as described in Figure 4. The positions of the p125, p68, and p58 bands are marked by arrows.

differences in assay conditions, a systematic comparison was made of the PCNA responses of (a) the recombinant human p125 catalytic subunit, (b) a p125 mutant in which the N-terminus containing the PCNA binding region was deleted (*10*), (c) the recombinant heterodimer produced by coexpression in Sf9 cells, (d) the calf thymus heterodimer isolated as described previously by immunoaffinity and ssDNA cellulose chromatography (*14*), and (e) the immunoaffinity-purified calf thymus pol δ preparation. Different time points and concentrations of PCNA were used to optimize the assay conditions, and the same preparation of human PCNA was used for all the assays. The results (Figure 6) show that the p125 catalytic subunit has a small but detectable response to PCNA, which is eliminated by deletion of the N-terminus. The recombinant human p125/p50 heterodimer and the calf thymus heterodimer isolated to near homogeneity were activated by PCNA, to comparable extents, about 12−16-fold, in contrast to the immunoaffinity-purified enzyme, which was activated by nearly 40-fold. This suggested that components required for the full response to PCNA were removed from the immunoaffinity-purified pol δ during its purification to the heterodimer.

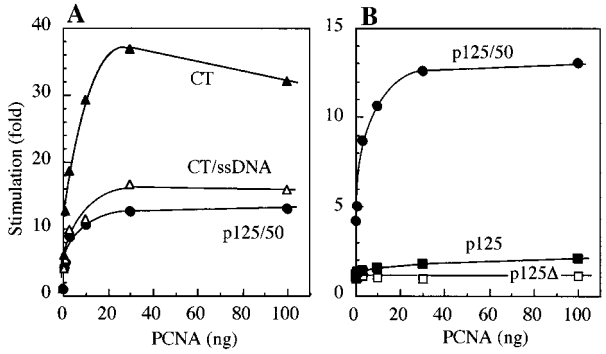

FIGURE 6: PCNA sensitivity of pol δ after immunoaffinity and ssDNA cellulose chromatography. Pol δ activity was determined using poly(dA)/oligo(dT) as the template−primer complex, and results are shown as the fold stimulation of activity in the presence of varying amounts (nanograms) of PCNA per 50 μL assay. Linearities of the assays were determined by time course measurements (not shown). (A) The black triangles show the response of immunoaffinity-purified calf thymus pol δ (CT). The white triangles show the same preparation after subsequent purification to a homogeneous heterodimer on ssDNA cellulose and heparin−agarose chromatography. The black circles show the PCNA response of recombinant human pol δ p125/p50 obtained by coexpression of the two subunits in Sf9 cells. (B) The response of the recombinant human pol δ p125/p50 heterodimer is compared with that of the recombinant p125 subunit (■) and its deletion mutant (p125Δ) in which the 186 N-terminal amino acids were deleted (□). All recombinant human pol δ proteins were expressed in Sf9 cells and purified as previously described (*40*). In these assays, the same preparation of purified calf thymus PCNA was used rather than recombinant PCNA, and all assays were conducted in the same experiment.

DISCUSSION

DNA polymerase δ is now well recognized as the key DNA polymerase in eukaryotic DNA replication, and the p125 and p50 subunits are well conserved between mammals and yeast (*15*, *27*). There are questions as to the complete polypeptide composition of pol δ, as well as that of other

associated replication proteins that together with pol δ form the multiprotein assembly that functions in chromosomal replication. In addition, evidence which shows that pol δ is also involved in DNA repair processes (*28*, *29*) indicates that the pol δ holoenzyme may associate dynamically with different proteins to form more than one type of higher-order assembly. As noted in the introductory section, extensive investigation in prokaryotes of *E. coli* DNA polymerase III has led to the identification of a number of polypeptides which have been studied in various subassemblies. The investigation of the identity and functions of replication proteins in mammalian systems is difficult because of the small amounts of material available, and because these protein complexes are likely to be dissociated upon most conventional methods of isolation, including gel filtration. In this study, we have taken advantage of a method for the immunoaffinity purification of pol δ from calf thymus, which yields relatively large amounts of enzyme, sufficient for both a proteomics approach and the possibility that the methods might be gentle enough to allow isolation of total or partial assemblies of the pol δ heterodimer associated with its natural protein partners.

Investigation of the behavior of the immunoaffinity-purified pol δ revealed that it can be shown to exist in protein complexes that are much larger (>250000) than the heterodimer. Moreover, these complexes represent assemblies that are highly purified and contain the heterodimer as the primary polypeptide component. Our studies indicate that the immunoaffinity purification does in fact lead to isolation of the heterodimer with at least some of its associated proteins. For the preliminary purification steps, we have used batchwise, rather than gradient elution, methods to minimize separation of associated proteins, and used the DEAE-cellulose and phenyl−Sepharose supports originally devised for the purification of pol δ (*13*). We have also found that ssDNA cellulose, as well as heparin−agarose chromatography, which are efficient for the isolation of the heterodimer, may do so by removing other associated proteins.

Comparison of the gel filtration behavior of the heterodimeric and the immunoaffinity-purified pol δ revealed a shift in the native molecular weight of 175 000 of the pol δ core to more than 500 000. As observed by Maki et al. (*4*), gel filtration of diluted pol III* leads to its dissociation from 800 kDa to fractions corresponding to 530, 480, and 380 kDa in size. The studies reported here provide the first evidence for high-molecular weight forms of mammalian pol δ in a highly purified state.

In other studies, we have found a number of replication proteins bound to PCNA Sepharose (*20*). As with other highly complex protein assemblies, e.g., *E. coli* DNA polymerase or RNA polymerase, conventional methods of isolation seldom yield complete stoichiometric complexes. Examination of the polypeptide compositions of the immunoaffinity-purified material revealed that while the p125 and p50 polypeptides were the major constituents, the preparation contained a number of other polypeptides which remain associated with the heterodimer during gel filtration or glycerol gradient ultracentrifugation. Clearly, some of these may represent impurities, while others may represent bona fide components of a mammalian replication complex. In this study, microsequencing was used to identify some of the associated polypeptides. The significant findings are the identification of KIAA0039 and its proteolytically derived peptides, as well as the 70 and 32 kDa subunits of RPA, as components that are associated with the high-molecular weight form of pol δ. During the course of this study, Hughes et al. (*30*) also identified KIAA0039 as a PCNA binding protein by using PCNA affinity chromatography, and have proposed that this is the third subunit of mammalian DNA polymerase δ, on the basis of the similarity of the sequence with that of Cdc27, and the association of the protein with pol δ on glycerol gradient ultracentrifugation. In their studies, they noted that recombinant KIAA0039 protein exhibited an anomalous migration on SDS−PAGE as a protein of 66 kDa, while its calculated molecular mass is 51.4 kDa. This is consistent with our observations on the behavior of the protein. Two reasons were put forth by Hughes et al. (*30*) to explain the previous failure to detect p66 in purified pol δ preparations. One was that this subunit is refractory to certain silver-staining reagents, and the other was that p66 is not absolutely required for polymerase activity and may have been lost during the lengthy purification procedures. The studies presented here provide stronger evidence that the 68 kDa polypeptide is a likely subunit of pol δ. The presence of the proteolytic products of p68 indicates that the protein is susceptible to nicking, and provides a reasonable explanation for the variability of its appearance in highly purified pol δ preparations. In previous work in this laboratory, p68 was found to be a persistent "impurity" that is associated with the immunoaffinity-purified enzyme and is only removed by a combination of ssDNA cellulose and heparin−agarose chromatography (*14*). The native gel electrophoresis experiments described in this work provide additional evidence for the association of p68 with pol δ. Pairwise alignments using the Clustal W 1.8 program show that there is only 15−16% of sequence identity between Pol32p and Cdc27, Pol32p and p68, and Cdc27 and p68. However, evaluation of the significance of the alignment score for p68 and Cdc27 using the PRSS program (http://www.expasy.ch/tools) provided a score of 0.4; i.e., the alignment score would be attained by chance against the randomly shuffled Cdc27 sequence only 0.4 time in 100 attempts. This indicates that the degree of similarity between these two proteins is significant. Interestingly, all three sequences possess a PCNA binding motif at their C-termini. The finding that p68 is a PCNA binding protein may be responsible for the observations that the immunoaffinity-purified enzyme shows a greater PCNA response than the p125/p50 heterodimer.

If p68 were to associate with the pol δ heterodimer in a stoichiometric fashion, the expected molecular mass of the complex would be 226 kDa; given that the KIAA0039 protein behaves on SDS−PAGE with an anomalous molecular mass of 68 kDa, an upper limit for a relative molecular weight for the complex of 243 000 can be projected. This is at the lower limit of the size range (from 250000 to >500000) that was found for the behavior of the immunoaffinity-purified enzyme on gel filtration, and much smaller than the size of pol δ found on nondenaturing gel electrophoresis (ca. 520 000). The explanations for this could be that there are additional subunits or associated proteins in the complex, or that the trimeric species is capable of dimerization. The current experimental information cannot distinguish between these possibilities. However, it is relevant

that in *S. cerevisiae*, the cognate third subunit encoded by POL32 has been shown to be able to form a hexameric protein with the two classical subunits of pol δ, so that it has been suggested to be a dimerization factor (*17*, *18*). This is not inconsistent with the behavior of pol δ that was observed here, since a hexameric complex would have a molecular weight of about 490 000, but more rigorous studies using reconstituted subunits will be needed to establish this. Gerik et al. (*17*) reported no other subunits in the most purified preparation of pol δ in the yeast *S. cerevisiae* besides p125, p58, and p55. In *S. pombe*, one additional polypeptide was identified as a potential subunit of pol δ that is encoded by the Cdm1 gene (*16*).

The issue of whether there is a subunit of eukaryotic pol δ that confers the property of dimerization is of some significance, since there is evidence that pol δ functions in both the leading and lagging strand synthesis (*6*, *29*). This would suggest that the mechanism for concerted DNA synthesis that depends on a dimerization factor that couples two DNA polymerase enzymes as in the *E. coli* system is conserved in eukaryotic systems (*6*). In *E. coli*, the tau protein serves to maintain a dimeric DNA polymerase, while the *S. cerevisiae* heterotrimer has been shown to dimerize, with the implication that the third subunit may serve a parallel function (*2*, *29*). At the present time, we cannot eliminate the possibility that the larger forms of pol δ activity that we observe may be due to the presence of a dimerization factor.

The second significant finding that was made was the presence of two of the three subunits of RPA in the immunoaffinity-purified pol δ preparations. The finding that RPA, or HSSB (human single-stranded DNA binding protein), is also present in the high-molecular weight form of pol δ after FPLC gel filtration suggests that there may be an interaction of RPA with the replication complex. RPA is an abundant multimeric protein consisting of three subunits (p70, p34, and p14) which is essential for DNA replication and is also involved in DNA repair and recombination (*31*). It binds tightly to single-stranded DNA and affects the activity of other replication proteins, e.g., T antigen, DNA polymerases α (*32*) and δ (*33*), p53 (*34*), and transcriptional initiators, e.g., GAL4 and VP16 (*35*). These findings are consistent with previous studies in which the presence of RPA could be detected by Western blotting of pol δ preparations isolated by PCNA affinity chromatography (*20*). While the presence of RPA in highly purified pol δ complexes may be fortuitous, there are a number of studies which indicate the likelihood that RPA itself may interact with the DNA polymerases in the replication complex. Mutation of the zinc finger domain of RPA has been shown to eliminate DNA replication activity (*36*). Genetic evidence in yeast indicates that the p70 subunit interacts with both pol α and pol δ (*37*). In the complete SV40 DNA replication system, neither prokaryotic, yeast, nor viral SSBs (single-stranded DNA binding proteins) can replace human RPA, thus suggesting that it may participate in specific protein−protein interactions with other replication proteins (*38*). More recently, Yuzhakov et al. (*39*) demonstrated a direct interaction between the pol δ heterodimer and RPA. The p70 subunit of RPA bound to the pol δ heterodimer, but not the p34−p14 subcomplex of RPA. RFC, the clamp loader for PCNA, also binds to pol δ. The binding of pol δ and RPA was found to compete for binding to RFC. In studies of the interactions of these proteins, Yukazhov et al. (*39*) have proposed that RPA forms an important touchpoint for the assembly of the pol δ replication complex. Our findings that RPA is present in a purified high-molecular weight complex of pol δ provide further evidence that the interaction of RPA and pol δ is a significant one.

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