Expression and Characterization of the Small Subunit of Human DNA Polymerase δ*

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DNA polymerase δ is a heterodimer consisting of a catalytic subunit of 125 kDa and a small subunit of 50 kDa (p50). We have overexpressed p50 in Escherichia coli and have characterized the recombinant protein. p50 was readily overexpressed using the pET vector as an insoluble protein. A procedure was developed for its purification and renaturation. Examination of the physical-chemical properties of renatured p50 showed that it is a monomeric protein with an apparent molecular weight of 60,000, a Stokes radius of 34 Å, and a sedimentation coefficient of 4.1 S. Its physical properties were indistinguishable from p50 expressed as a soluble protein using the pTACTAC vector. Examination of the effects of recombinant p50 on the activity of DNA polymerase δ showed that p50 is able to slightly stimulate (about 2-fold) the activity of the recombinant 125-kDa catalytic subunit using poly(dA)oligo(dT) as a template in the absence of proliferating cell nuclear antigen. In the presence of proliferating cell nuclear antigen, activity is stimulated about 5-fold. Seven stable hybridoma cell lines were established that produced monoclonal antibodies against p50. One of these antibodies (13D5) inhibited the activity of calf thymus DNA polymerase δ. This antibody, when coupled to a solid support, also was found to provide a method for the immunoadfinity purification of recombinant p50 and of DNA polymerase δ from calf thymus or HeLa extracts. Immunoprecipitation and enzyme-linked immunosorbent assays also confirmed that p50 interacts with the catalytic subunit of DNA polymerase δ.

DNA polymerase (pol) δ is involved in both DNA replication (1) and DNA repair (2) in mammalian cells. Its activity is stimulated by PCNA, a DNA sliding clamp which provides the processivity required for its role in DNA replication (3, 4). In addition to PCNA, a number of other accessory proteins are required for the assembly of a functional replication complex at the leading strand of the replication fork. These include the multi-subunit replication factor C (RF-C) complex and replication protein A (RP-A), a single-stranded DNA-binding protein (5), pol δ isolated from calf thymus (6) and human placenta (7) is a heterodimer of 125- and 50-kDa polypeptides. The catalytic activity had been clearly demonstrated to be associated with the 125-kDa subunit (5) and for pol δ have been reported that contain only the active 125-kDa subunit (8, 9). Goulian et al. (9) isolated two preparations of mouse DNA polymerase δ, one of which consisted of a single polypeptide of 123–125 kDa and was unresponsive to PCNA stimulation. It was suggested that the 50-kDa polypeptide is required for the stimulation of DNA polymerase δ by PCNA (9). However, yeast pol δ catalytic subunit overexpressed in Escherichia coli was stimulated 2.5- to 3-fold (10). Human pol δ catalytic subunit expressed in the vaccinia virus system also showed a slight increase in activity in the presence of PCNA (11). This increase in activity is significantly lower than the 34-fold stimulation of the native human DNA polymerase δ core reported previously in our laboratory (7). In this study we report the expression of human p50 in E. coli and its biochemical characterization.

EXPERIMENTAL PROCEDURES

Expression of p50—The cDNA of p50 was isolated by PCR cloning using primers based on the reported sequence of human p50 (12). The coding sequence was inserted into both the pET21a (Novagen) and pTACTAC vectors (13, 14). PCR amplification was used for the insertion of the coding sequence of human p50 between the NdeI and HindIII sites of the vectors. The primer 5′-CAGAGTGTGGCATATTCCCTTCTGACC-3′ was used for the 5′ end of the coding sequence with an engineered NdeI site (underlined residues) at the initiating methionine codon (bold residues). The antisense primer at the 3′ end (5′-CCACAGGCTTGAGTCCAGGGCC-3′) had a HindIII site (underlined residues) after the termination codon (bold residues). The primers were phosphorylated with T4 polynucleotide kinase before use. The PCR conditions used were 95 °C for 1.5 min, 45 °C for 2 min, and 72 °C for 3 min, for 30 cycles. The product was a single band of about 1.4 kilobases, which was subsequently purified on a Centricon 100 column followed by phenol/chloroform extraction. After digestion with NdeI and HindIII, the PCR product was ligated into the pET vector that had been previously digested with NdeI and HindIII and purified by agarose gel electrophoresis. The construct was then used to transform E. coli DH5α competent cells. The correctness of the inserted DNA was confirmed by DNA sequencing. A single colony from E. coli DH5α cells harboring the pET21a/p50 construct was used to inoculate a 5-ml culture in Terrific medium (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 0.017 M NaHPO4, 0.072 M KH2PO4, 50 μg/ml ampicillin). After overnight growth at 37 °C, the 5-ml culture was used to inoculate a 2-liter culture in LB medium and grown at 37 °C for 4–6 h until the absorbance reached 0.6 at 600 nm. Isopropyl-β-thiogalactoside was then added to a concentration of 0.5 mM, and the culture was grown for an additional 8 h at 37 °C. A similar procedure was used for the insertion of the coding sequence for p50 into the pTACTAC vector (14, 15).

Purification and Renaturation of Recombinant p50—The cells from 1 liter of culture harboring the p50-pET21a plasmid were harvested by centrifugation at 3,000 × g, 4 °C for 30 min, and frozen overnight. The frozen cells were suspended in lysis buffer (25 mM Tris-HCl, pH 7.5, 10 mM DTT, 50 mM KCl, 0.5 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM ethylenediaminetetraacetic acid, 1 mg/ml bovine serum albumin, 1 mM NAD, 10 mM 3-mercaptopropanoic acid, 1 mg/ml lysozyme) by vigorous sonication. The suspension was centrifuged at 10,000 × g for 30 min and the supernatant was concentrated by centricon filtration. The concentrate was dialyzed against 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5 mM DTT, 10 mM 2-mercaptoethanol, for 30 min and again by centrifugation. The dialyzed sample was loaded onto a 5-mL Sephacryl S-200 column (Pharmacia) equilibrated with 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5 mM DTT, 10 mM 2-mercaptoethanol, and eluted. Purified DNA polymerase δ was collected, concentrated, and analyzed by SDS-PAGE. The enzyme was further purified by affinity chromatography using 5′-GAGC-3′ (antisense primer at the 3′ end) and 5′-CAGGAGTGTGCAATTTTCCTAGGC-3′ (sense primer at the 5′ end) in the PCR reaction. The PCR product was ligated into the pET vector that had been previously digested with NdeI and HindIII and purified by agarose gel electrophoresis. The construct was then used to transform E. coli DH5α competent cells. The correctness of the inserted DNA was confirmed by DNA sequencing. A single colony from E. coli DH5α cells harboring the pET21a/p50 construct was used to inoculate a 5-ml culture in Terrific medium (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 0.017 M NaH2PO4, 0.072 M KH2PO4, 50 μg/ml ampicillin). After overnight growth at 37 °C, the 5-ml culture was used to inoculate a 2-liter culture in LB medium and grown at 37 °C for 4–6 h until the absorbance reached 0.6 at 600 nm. Isopropyl-β-thiogalactoside was then added to a concentration of 0.5 mM, and the culture was grown for an additional 8 h at 37 °C. A similar procedure was used for the insertion of the coding sequence for p50 into the pTACTAC vector (14, 15).

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The abbreviations used were: pol, polymerase; PCNA, proliferating cell nuclear antigen; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.

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Expression of pol δ50

0.25 M sucrose, 1 mM EDTA, 0.1 mM EGTA, 10 mM benzamidine HCl, 0.2 mM phenylmethylsulfonfluoride, 1 mM diithiothreitol (DTT), 0.1% Triton X-100, 0.1 M NaCl) and disrupted using a French press. The suspension was centrifuged at 27,000 × g at 4 °C for 30 min. The pellet was resuspended in 50 ml of lysis buffer containing 0.5 M NaCl, and sonicated. After glycerol precipitation was centrifuged at 27,000 × g for 30 min. The resulting pellet was then sonicated in 10 ml of 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 1 mM EDTA, 1 mM DTT, and 0.5 μl urea to solubilize the pellet and was centrifuged at 12,000 × g at 4 °C for 30 min. The resulting supernatant (5 ml, 60 mg of protein) was loaded onto a Sephacryl S300HR column (2.5 ml) equilibrated with 20 mM Tris-HCl, pH 7.5, 80 mM NaCl, 0.1 mM EDTA, 0.5 mM NaCl, and 2 μl urea. Fractions (4 ml) were collected, and the elution of p50 was monitored by SDS-PAGE and protein staining. Renaturation was performed by HPLC chromatography in a buffer containing 0.25M NaCl instead of 2 M urea. A portion of the peak fraction (0.45 ml) was injected onto a Waters Protein Pak 300SW column equilibrated with 20 mM Tris-HCl, pH 7.5, 5% glycerol, 1 mM EDTA, 0.1 mM EGTA, 0.5 mM NaCl, and 2 mM DTT. Fractions of 0.5 ml were collected and analyzed for p505 by immunoblotting using a monoclonal antibody.

Generation of Monoclonal Antibodies against Recombinant p50 and Preparation of a p50 Immunoaffinity Column—A panel of murine hybrido ma cell lines that produced antibodies against p50 was developed by immunizing three female BALB/c mice with recombinant p50 purified from a S300HR step. The mice were then immunized with 100 μg of p50 emulsified with complete Freund's adjuvant. Two weeks later the mice were given a booster injection of another 100 μg of p50 with incomplete Freund's adjuvant. The booster was repeated at monthly intervals. One of the mice exhibited a high titer after two booster injections and was sacrificed 3 days after the final booster injection. The procedure for fusion of the spleen cells to produce hybridomas was as described by Lee et al. (16). Western blotting of hybridoma cell supernatants was used to screen for positives. SDS-PAGE and Western blotting were performed as described previously (17). The antigen used for screening was the native polypeptide isolated by immunoadfinity chromatography from the calf thymus (17). Positive hybridoma cultures were isolated by limiting dilution three times. Seven stable hybridomas were established. Antibodies were isolated by purification on protein A-Sepharose columns.

Immunoadfinity Purification of Recombinant p50 Expressed in the pTACTAC Vector—Purified antibody 13D5 was coupled onto Avidin-Chrom hydrazide (Sigma) as described by Jiang et al. (17). A cell lysate from 6 liters of an induced culture of E. coli harboring the p50-pTAC plasmid (6 g of protein in 300 ml) was precipitated by the addition of ammonium sulfate to 50% saturation. The pellet was then resuspended in TGEE buffer (40 mM Tris-HCl, pH 7.8, 10% glycerol, 1 mM EDTA, 0.1 mM EGTA), 0.15 M NaCl, and 2 μl urea. The resulting supernatant (5 ml, 60 mg of protein) was loaded onto a Sephacryl S300HR column (2.5 ml) equilibrated with 20 mM Tris-HCl, pH 7.5, 5% glycerol, 1 mM EDTA, 0.1 mM EGTA, 0.5 mM NaCl, and 2 μl urea. Fractions (4 ml) were collected, and the elution of p50 was monitored by SDS-PAGE and protein staining. Renaturation was performed by HPLC chromatography in a buffer containing 0.25M NaCl instead of 2 M urea. A portion of the peak fraction (0.45 ml) was injected onto a Waters Protein Pak 300SW column equilibrated with 20 mM Tris-HCl, pH 7.5, 5% glycerol, 1 mM EDTA, 0.1 mM EGTA, 0.5 mM NaCl, and 2 mM DTT. Fractions of 0.5 ml were collected and analyzed for p505 by immunoblotting using a monoclonal antibody.

RESULTS

Expression of the p50 Subunit in E. coli—The cDNA for the p50 subunit was cloned by PCR amplification from a human liver cDNA library using primers based on the reported cDNA sequence of the human p50 subunit (12). The cDNA clone consisted of a 1519-nucleotide sequence with an open reading frame of 469 amino acid residues encoding the identical amino acid sequence as reported by Zhang et al. (12). The p50 protein was readily overexpressed in E. coli when the pET21a vector was used (see “Experimental Procedures”). As has been commonly observed for a number of overexpressed proteins, the recombinant p50 protein was expressed as an insoluble form in inclusion bodies. The p50 was extracted from the inclusion bodies using 6 M urea, and approximately 60 mg of protein could be obtained from a 1-liter cell culture. The major protein component of the extract was p50, as determined by SDS-PAGE and immunoblotting (not shown). The solubilized protein was then purified by gel filtration in the presence of 2 M urea on a Sephacryl S300HR column. During this process the p50 protein remained in solution in 2 M urea. A portion of the peak fraction was then subjected to HPLC gel permeation chromatography in the presence of 0.25M NaCl to remove the urea. This process was found to allow the apparent renaturation of the p50, as it now behaved as a soluble protein as shown by the HPLC elution (Fig. 1A). The presence of the p50 protein was determined by immunoblotting using 13D5, a p50 monoclonal antibody (see “Experimental Procedures”). It is seen that most of the protein is in the included fraction of the column with a small amount eluting in the void volume.

Effect of p50 on Pol δ Activity—To test whether this solubilized p50 material had any effect on the p125 catalytic subunit of pol δ, samples of the fractions were added to a standard assay for pol δ activity in the presence of PCNA as purified recombinant p125 obtained by expression in S99 cells. The results show that the renatured p50 was able to stimulate pol δ activity to a roughly 2.5-fold level that this stimulatory effect is nearly identical to that observed with the p125 protein (Fig. 1B). To further establish that this was a functional effect of p50 on pol δ activity, samples of the peak fraction from the HPLC run (fraction 30, Fig. 1A) were used to determine whether this stimulation was concentration-dependent. The results (Fig. 1C) showed that a response could be seen in the range of 0.1–1 μg of p50/assay. The renatured recombinant p50 did not have any
The presence of 0.5 M of p50 was found to be 4.1 S by glycerol gradient ultracentrifugation (Fig. 2). The elution of p50 was monitored by Western blotting of the fractions using a monoclonal antibody to p50. The immunoblot of the peak fractions is shown in the inset. Panel B, glycerol gradient ultracentrifugation. Proteins were ultracentrifuged in sample volumes of 200 μl on glycerol gradients from 10 to 30% in TGEE buffer, 0.5 M NaCl and 1 mM DTT in a total volume of 5 ml. The samples were ultracentrifuged in a Beckman SW50.1 rotor at 105,000 × g, 4°C, for 17 h. Fractions (200 μl) were collected from the top of the tube. SDS-PAGE and Western blots were performed for determination of the elution of p50. Standards used were catalase, 11.3 S; aldolase, 7.3 S; BSA, 4.4 S; carbonic anhydrase, 3.9 S; and cytochrome c, 1.7 S.

Expression of p50 as a Soluble Protein—Because of concerns that the renatured p50 might not represent a native folded protein, we expressed p50 in the pTACTAC vector, which has been shown to allow expression of several proteins in a soluble form when induced at room temperature rather than at 37 °C (13–15). The results showed that this vector allowed the expression of p50 in a soluble state, although the levels were much lower than in the pET21a vector. The soluble p50 expressed using the pTACTAC vector therefore required a much more extensive purification than the material from the pET vector, where the inclusion bodies were largely composed of p50 itself. This problem was solved by the use of an immunopurification column using a monoclonal antibody against p50, 13D5 (see below). The cell lysate from 6 liters of culture was partially purified by 50% ammonium sulfate precipitation and passed through a monoclonal anti-p50 column as described under “Experimental Procedures.” The yield of soluble protein was 0.26 mg starting from 6 liters of cells and provided a near homogeneous preparation of p50 (Fig. 3). The p50 expressed using the pET21a and pTACTAC vectors and that of p50 in the calf thymus pol δ core were all immunoblotted by monoclonal antibody 13D5, which provided additional confirmation of the identity of the recombinant proteins (Fig. 4).

The abilities of the p50 expressed in the pTACTAC vector as a soluble protein and of the renatured p50 isolated after expression in the pET vector to affect pol δ activity were compared (Fig. 5). The two proteins were essentially indistinguishable in their behavior. These results show that the procedure used for renaturation of the insoluble p50 results in a protein that is functionally similar to the protein expressed in a soluble state. The recombinant pol δ p125 activity was stimulated about 2-fold by the addition of p50. The combined effect of p50 and PCNA provided about a 5-fold stimulation of the free p125 activity when assayed using poly(dA)–oligo(dT) as a template in the stimulation over a control fraction where no p50 was added. Panel C, activity was assayed using poly(dA)–oligo(dT) as a template in the presence of 0.5 μg of PCNA and a constant amount of recombinant pol δ p125 (see “Experimental Procedures”). The results are plotted as stimulation over a control fraction where no p50 was added. Panel C, pol δ activity was assayed using poly(dA)–oligo(dT) as a template in the presence of 0.5 μg of recombinant PCNA and a constant amount of recombinant pol δ p125. The amounts of p50 used per assay were 0, 0.2, 0.4, 0.6, 0.8, and 1 μg, respectively. The results are plotted as stimulation over a control assay where no p50 was added.

Physicochemical Characterization of Recombinant p50—The p50 obtained after HPLC gel permeation chromatography was characterized to determine whether it behaved as a globular protein. The apparent molecular mass was determined to be ∼60 kDa by HPLC gel permeation chromatography. A Stokes radius of 34 Å was obtained when the data were plotted against the Stokes radii of the protein standards (Fig. 2A). This can be compared with the Stokes radius of bovine serum albumin, a globular protein, which is 36 Å. The sedimentation coefficient of p50 was found to be 4.1 S by glycerol gradient ultracentrifugation (Fig. 2B). These data confirm that the renatured p50 behaves as a monomeric protein and has physicochemical properties consistent with it having refolded into a native globular state. The isoelectric point of p50 was determined in acrylamide gels to be 5.3 (not shown).
activity. Because of concerns that the small effects of the p50 on the pol δ activity were nonspecific, recombinant p50 was heat-denatured. This heat-treated p50 and BSA were used as negative controls (Fig. 6). Examination of their effects as a function of added protein and time showed that the heat-denatured protein had no significant effects when compared with untreated p50 and that the increase in activity is not due to a stabilization of the p125 subunit by the addition of protein to the solution (Fig. 6).

Interaction of the Pol δ p125 and p50 Subunits—The physical interaction of p50 with pol δ p125 was demonstrated by an ELISA procedure (Fig. 7). When the plates were coated with p50 and the response was tested by the addition of p125 and a p125 antibody, a concentration dependence of absorbance could be detected. Similar results were obtained from the reciprocal experiment, in which p125 was bound to the ELISA plates and probed with p50 and a p50 antibody (Fig. 7). These results establish that recombinant p50 does interact with p125.

Generation of Monoclonal Antibodies to p50—Monoclonal antibodies to p50 were generated (see "Experimental Procedures"). The p50 protein obtained by extraction from inclusion bodies and purified by gel filtration in 2 M urea was used as the antigen, and hybridomas were screened by the ability of their cell supernatants to immunoprecipitate the p50 subunit of calf thymus pol δ isolated by immunofinity chromatography (17). Seven stable hybridoma cell lines were selected and established, all of which produced monoclonal antibodies that strongly immunoblotted p50. The monoclonal antibodies were typed as to their immunoglobulin class by the Ouchterlony double-diffusion technique (20). Three antibodies were of the IgG1 class (12B6, 16A8, and 17D2), two were of the IgG2b class (13D3 and 13D5), one was an IgG2a (13D8), and one was IgG3 (13E6). All of the monoclonal antibodies were able to co-immunoprecipitate the p125 subunit of pol δ from HeLa cell extracts. This is illustrated in Fig. 8, in which HeLa extracts were immunoprecipitated with 13D5 (p50) monoclonal antibody and then immunoblotted with a pol δ monoclonal antibody (38B5). The effects of these monoclonal antibodies on the activity of pol δ core purified from calf thymus by immunofinity chromatography was examined. Only one antibody, 13D5, was found to be inhibitory (Fig. 9). This inhibitory antibody did not inhibit the 3'-5'-exonuclease activity of pol δ isolated from the calf thymus (not shown). None of the antibodies inhibited the polymerase activity of the recombinant pol δ p125 overexpressed in the Sf9 cells.

Monoclonal antibody 13D5 was found to be suitable for the preparation of an immunofinity support for the isolation of p50. The purified protein was coupled to AvidChrom hydrazide as described by Jiang et al. (17). The column was useful for the isolation of recombinant p50 expressed as a soluble protein in E. coli (see above). This column also allowed the isolation of pol δ from calf thymus extracts (Fig. 10).

DISCUSSION

Mammalian DNA polymerase δ is a heterodimer consisting of a catalytic subunit of 125 kDa and a small subunit of 50 kDa.
The pol δ catalytic subunit of pol δ is required for PCNA sensitivity of the catalytic subunit. Procedures. Radish peroxidase-conjugated anti-mouse IgG (see "Experimental Procedures"). Binding of p125 to p50 was then monitored by ELISA assays using a monoclonal antibody against p125 (7B7) and a horseradish peroxidase-conjugated anti-mouse IgG (see "Experimental Procedures"). lane 1, immunoprecipitate obtained with control monoclonal antibody 12B1 (non-reactive with p50). Lane 4, immunoprecipitate using antibody 78F5 against pol δ p125. Lane 5, no antibody used. Lane S, protein standards.

(6, 7, 17). Reports of pol δ preparations consisting of the free pol δ catalytic subunits from rabbit reticulocytes (8), mouse (9), and Drosophila (21) supported the possibility that the presence of p50 is required for PCNA sensitivity of the catalytic subunit. The pol δ catalytic subunits of both yeast (10) and mammalian p125 (11) have been overexpressed and exhibit only a slight response to PCNA. Highly purified human placental pol δ preparations purified by conventional means are stimulated at least 10–20-fold and may reach above 30-fold depending on the assay conditions (7, 11). In the studies reported here, we find that the p125 subunit of pol δ overexpressed in baculovirus is stimulated by PCNA less than 2-fold. Thus, our findings are consistent with other reports on the behavior of the isolated p125 subunit in regard to either a lack of, or a low level of, sensitivity to PCNA. However, in the presence of p50 pol δ activity is stimulated about 5-fold by PCNA suggesting that the 50-kDa subunit is required for the stimulation of DNA polymerase δ by PCNA.

The effects of p50 on recombinant p125 are small, and the failure to induce a strong PCNA sensitivity suggests that the reconstitution of a native enzyme was not achieved. Nevertheless, there clearly is an interaction between the two recombinant proteins based on the functional effects of p50 on p125, and the demonstration of a protein-protein interaction via the ELISA system. Several reasons for the failure to observe a high level of PCNA responsiveness are possible. First, either one of the recombinant proteins could be improperly folded or lacking in a required post-translational modification. Second, the proper folding of both proteins may require their co-expression or may require the action of specific chaperone proteins. Third, the formation of the core enzyme may require the presence of other subunits. With regard to the first issue, we have characterized recombinant p50 to confirm that it behaves as a globular protein based on its physicochemical properties and by comparison of its functional effects on pol δ p125 with that of a soluble form of p50 isolated by expression in the pTACTAC. 

Expression of pol δ p50

FIG. 7. Demonstration of the interaction of p50 with the p125 subunit of pol δ by ELISA. Left panel, p50 was immobilized on ELISA plates, and varying amounts of p125 protein were added as indicated. Binding of p125 to p50 was then monitored by ELISA assays using a monoclonal antibody against p125 (7B7) and a horseradish peroxidase-conjugated anti-mouse IgG (see "Experimental Procedures"). Right panel, recombinant p125 (kDa) was immobilized on ELISA plates, and varying amounts of recombinant p50 were added as indicated to the right (in kDa). Binding of p50 to p125 was then monitored by ELISA assays using a monoclonal antibody against p50 (17D2) and a horseradish peroxidase-conjugated anti-mouse IgG (see "Experimental Procedures").

FIG. 8. Immunoprecipitation of HeLa cell extract using p50 monoclonal antibodies. HeLa cell extracts were immunoprecipitated as described under "Experimental Procedures" and then subjected to SDS-PAGE and immunoblotted with a monoclonal antibody against pol δ p125 (78F5). Lane 1, immunoprecipitate obtained with monoclonal antibody 13D5 against p50. Lane 2, control with no antibody used. Lane 3, immunoprecipitate using control monoclonal antibody 12B1 (non-reactive with p50). Lane 4, immunoprecipitate using antibody 78F5 against pol δ p125. Lane 5, no antibody used. Lane S, protein standards.

FIG. 9. Inhibition of pol δ activity by p50 monoclonal antibodies. Immunoadfinity-purified calf thymus pol δ core-purified as described by Jiang et al. (17) was incubated with increasing amounts of purified p50 monoclonal antibodies. After 4 h, 10 μl of the mixture was assayed for DNA polymerase activity using poly(dA)·oligo(dT) as a template in the presence of 500 ng of recombinant PCNA. Data are shown as % inhibition of a control with no added antibody: 13D5 (solid squares), 12B6 (solid inverted triangles), 16A8 (crosses), 17D2 (open squares), 13D8 (solid circles), 13D3 (solid triangles), and 13E6 (solid diamonds).

FIG. 10. Immunoadfinity purification of pol δ using immobilized antibody 13D5. The p50 monoclonal antibody, 13D5, was immobilized on AvidChrom hydrazide support (see "Experimental Procedures"). A partially purified calf thymus preparation (50 ml) after the phenyl-agarose chromatography step (17) was loaded onto a 5-ml immunoaffinity column. The column was washed with 50 ml of TGEE buffer containing 0.1 M NaCl. The column was eluted with TGEE buffer containing 0.5 M NaCl, and fractions were then collected and assayed for pol δ activity (solid circles). Inset, immunoblots of fractions 3, 4, and 5 with a mixture of monoclonal antibodies against p50 (13D6) and p125 (78F5).
yielded genes encoding the small subunit of pol δ. Screens for genes required for cell cycle progression have revealed mutations that lead to defective DNA synthesis and/or repair as required for cell cycle progression (23). It was demonstrated that p50 overexpression is able to rescue mutants of cdc27+ and that the gene product of cdc27+ interacts with the Cdc1 protein.

It seems unlikely that p50 fulfills a simple structural role in maintaining pol δ function, based on studies of yeast genes that encode p50 homologues. A BLAST search of the p50 protein sequence against the GenBank™ data base showed that it has significant identity with a Saccharomyces cerevisiae gene, HYS2, which encodes a protein of 487 residues corresponding to a molecular mass of 55 kDa (22). Alignment of the protein sequences shows that there is a 34% identity between human p50 and the Hys2 protein so that the latter is a likely candidate for the yeast counterpart of the mammalian p50 subunit of pol δ. Genetic analysis of the HYS2 gene (22) showed that it is essential for DNA replication in S. cerevisiae. Additionally, HYS2 mutants display supersensitivity to hydroxyurea, increased levels of mitotic chromosome loss, and recombination.

More recently, MacNeill et al. (23) cloned a Schizosaccharomyces pombe gene, cdc1+1, which displays significant sequence identity (34%) to both the p50 subunit of pol δ and to HYS2. A physical interaction of the Cdc1 protein with the yeast pol δ protein was demonstrated by in vitro co-immunoprecipitation. Thus, genetic studies have pointed to an important requirement for the pol δ small subunit in yeast, since cdc1 and HYS2 mutations lead to defective DNA synthesis and/or repair as well as hypersensitivity to hydroxyurea and methyl methane sulfoxinate. Cellular recognition of these defects in replicated DNA leads to the abnormalities in cell cycle progression.

The findings in both budding and fission yeast that genetic screens for genes required for cell cycle progression have yielded genes encoding the small subunit of pol δ are highly indicative of an important function for this subunit. The p50 subunit could be critical in providing for the interaction of pol δ with other replication proteins, or it may be that regulation of pol δ may be mediated through p50. This function may also involve the interaction of the small subunit of pol δ with another protein which is encoded by cdc27+, another gene that is required for cell cycle progression (23).