Characterization of the p125 Subunit of Human DNA Polymerase δ and Its Deletion Mutants

INTERACTION WITH CYCLIN-DEPENDENT KINASE-CYCLINS

(Received for publication, December 10, 1997)

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The catalytic subunit of human DNA polymerase (pol) δ was overexpressed in an active, soluble form by the use of a baculovirus system in insect cells. The recombinant enzyme was separated from endogenous DNA polymerases by phosphocellulose, Mono Q-Sepharose, and single-stranded DNA-cellulose chromatography. Recombinant DNA pol δ was also purified by immunoaffinity chromatography. The enzymatic properties of the purified catalytic subunit were characterized. The enzyme was active and possessed both DNA polymerase and associated 3′ to 5′ exonuclease activities. NH2-terminal deletion mutants retained polymerase activity, whereas the core and COOH-terminal deletion mutants were devoid of any measurable activities. Coinfection of Sf9 cells with recombinant baculovirus vectors for pol δ and cyclin-dependent kinase (cdk)-cyclins followed by metabolic labeling with 32Pi showed that the recombinant catalytic subunit of pol δ could be hyperphosphorylated by G1 phase-specific cdk-cyclins. When cdk2 was coexpressed with pol δ in Sf9 cells, pol δ was found to coimmunoprecipitate with antibodies against cdk2. Experiments with deletion mutants of pol δ showed that the NH2-terminal region was essential for this interaction. Coimmunoprecipitation and Western blot experiments in Molt 4 cells confirmed the interaction in vivo. Preliminary experiments showed that phosphorylation of the catalytic subunit of pol δ by cdk2-cyclins had little or no effect on the specific activity of the enzyme.

DNA polymerase (pol) δ is the central enzyme in eukaryotic DNA replication (1) and also serves an important role in DNA repair (2). Isolation of the calf thymus (3) and human (4) enzymes has shown that it consists of at least two core subunits of 125 and 50 kDa. The hallmarks of this polymerase are that it has an intrinsic 3′ to 5′ exonuclease activity, distinguishing it from pol α and pol β. The 125-kDa subunit of human pol δ (p125) has been identified as the catalytic subunit (4). Pol δ is a member of a family of DNA polymerases which includes DNA polymerase α, pol ε, the herpesvirus DNA polymerases, and bacteriophage T4 polymerase (5, 6). Examination of the regions of conserved sequence has led to the identification of domains that are potentially required for DNA interaction, deoxynucleotidylate interaction, as well as the 3′ to 5′ exonuclease activity of pol δ (7). In addition, there are several regions in the NH2- and COOH termini which are conserved among human pol δ, yeast pol δ, and yeast and human pol ε (5, 7).

Studies of the replication of SV40 DNA in vitro have led to the identification of a number of accessory proteins, which, together with pol δ, are required for the formation of a replication complex at the replication fork. These include PCNA, which functions as a sliding clamp and enhances the processivity of pol δ, consistent with its role as the leading strand polymerase (8). Although there have been some mutagenesis studies of the yeast pol δ (9), little has been done with human or mammalian pol δ, largely because of the lack of a suitable expression system. To facilitate structure-function studies of pol δ, it is desirable to have an expression system for the production of the recombinant protein. The expression of the human pol δ catalytic subunit has been achieved in mammalian cells using a vaccinia virus vector (10). In this study we report the expression of p125 in Sf9 cells using a baculovirus vector as well as methods for separating the recombinant protein from endogenous DNA polymerases in baculovirus-infected Sf9 cells. Deletion mutants of p125 were also characterized to investigate the domain structure of pol δ. In addition, we have obtained novel evidence that pol δ p125 is phosphorylated by the cyclin-dependent kinase (cdk)-cyclin complexes and also can coimmunoprecipitated with cdk2 when they are coexpressed in Sf9 insect cells. The interaction of pol δ with the cyclins and cdk's was also confirmed by coimmunoprecipitation and Western blot experiments in Molt 4 cells. Preliminary experiments showed that phosphorylation has moderate or little effect on the activity of the catalytic subunit.

EXPERIMENTAL PROCEDURES

Materials—Sf9 cells were purchased from Invitrogen and were maintained at 27 °C in TNM-FH insect medium supplemented with 10% fetal calf serum and 50 μg/ml gentamycin. Cells were propagated both as adherent monolayers and as nonadherent suspension cultures. These cells were used as the hosts for the propagation of wild type Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) and recombinant baculoviruses. Cyclin and cdk recombinant baculovirus were gifts of Dr. Charles Sherr (St. Jude's Hospital, Memphis, TN). BaculoGold™-linearized baculovirus DNA was purchased from Pharmingen. The baculovirus transfer vector P2bac was purchased from Invitrogen. Plasmid pALTER-1 was purchased from Promega.

Construction and Screening of Recombinant Baculoviruses—The coding sequence of pol δ which was used in these studies was derived from the cDNA originally isolated by Yang et al. (7). This coding sequence was inserted into the pALTER vector and corrected by site-directed

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mutagenesis so that His-119, Asn-173, and Gly-776 were mutated to Arg-119, Ser-173, and Arg-776 to conform to the genomic sequence (10, 11). The plasmid pALTER-pol δ containing the corrected full-length pol δ coding sequence (3.5 kilobases) was excised from the pALTER plasmid by BamHI/HindIII digestion, gel purified, and inserted into pBacHi/ HindIII at the 5'-end. The construct was then transfected into Sf9 cells with wild type baculovirus DNA according to Ausebel et al. (12). Wild type BaculoGold™-linearized AcMNPV DNA (1 μg), recombinant plasmid DNA (3 μg), cationic liposome solution (25 μl), and 1 ml of Grace’s insect medium containing no supplements were mixed by vortexing for 10–15 s and incubated for 2 h at room temperature before transfection. The transfection mixture was then layered onto Sf9 cells growing on 60-mm plates. After 4 days at 27 °C, the medium was aspirated and analyzed by virus production by plaque assay. The recombinant baculoviruses were identified as occlusion-negative plaques with a dissecting microscope. Because the BaculoGold™-linearized virus DNA contains a lethal deletion and a lacZ gene, the small portion of nonrecombinant virus plaques stained blue on 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside plates, whereas all recombinants produced colorless plaques on these plates. After three rounds of plaque purification, pure recombinant baculoviruses were obtained. Occlusion-negative viral stocks were prepared from the final supernatants, titered, and stored at 4 °C. Deletion mutants of pol δ were constructed as described in Ref. 13.

Induction of Sf9 Cells with Recombinant Baculovirus and Preparation of Cell Extracts—Recombinant viral stocks (0.5 ml) were added to 5-cell pellets (50 ml) of log phase Sf9 cells for 1 h. The inoculum was then removed from the plates, and 8 ml of fresh complete TNM-FH insect medium was added. The infected Sf9 cells were allowed to grow for 2 days at 27 °C and were harvested after 48 h postinfection. Cells were harvested from 80–100-mm plates and collected by centrifugation. The cell pellets were washed twice with ice-cold phosphate-buffered saline, pH 7.4. Subsequent manipulations were carried out at 4 °C. The cells from 80 plates (about 8 × 10^9 cells) were suspended in 5-cell pellet volumes (50 ml) of lysis buffer (40 mM Tris-HCl, pH 7.8, 0.25 mM sucrose, 0.1 mM NaCl, 0.1% Nonidet P-40, 0.1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine-HCl). Cells were disrupted by passage through a French press at 1,000 p.s.i. The lysate was centrifuged at 27,000 × g for 30 min. The supernatant was removed and saved as the soluble extract, and the pellet was suspended in 20 ml of lysis buffer plus 0.5 mM NaCl and sonicated three times for 20 s each at 50 watts on ice. The extract was then centrifuged at 27,000 × g for 30 min, and the supernatant was designated as the high salt-solubilized fraction. Protein concentrations of the first and second extracts were determined using the BioRad protein assay, and the pellet was then homogenized in 0.5–1.0% NP-40 buffer three times. After SDS-PAGE, the separated proteins were transferred to nitrocellulose membranes and probed with antibodies to cdk2, cdk5, or pol δ recombinant baculoviruses (0.5 ml) were added as indicated. The cells were infected at room temperature for 1 h. The recombinant baculovirus was removed with growth medium, and the cells were grown for an additional 2 days at 27 °C before labeling with 32P. Infected Sf9 cells were transfected into a 15-ml tube for 32P labeling. After centrifugation and removal of growth medium, the cells were resuspended in 2 ml of fresh phosphate-free medium containing 200 μCi of 32P (specific activity 3,000 Ci/mmol) and incubated at 37 °C for 2 h. The cells were centrifuged at 3,000 × g for 5 min. The supernatant was removed, and the cells were washed twice with lysis buffer. The cells were then sonicated for 30 s in 40 ml Tris-HCl, pH 7.8, 0.25 mM sucrose, 0.5 mM NaCl, 0.1% Nonidet P-40, 0.1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 10 mM benzamidine-HCl. The crude cell extracts were transferred to microtubes and centrifuged at 15,000 × g for 30 min. About 20 mg of total protein was used for immunoprecipitation in the presence of 20 μg of streptavidin-biotinylated horseradish peroxidase complex. Color development was performed by incubation with 4-chloro-1-naphthol and hydrogen peroxide and terminated with sodium azide.

Coinfection of Sf9 Cells with Pol δ, Cyclins, and Cdk2s and 32P, Labeling—Sf9 cells (10^6) were grown to exponential stage. Pol δ, cyclin, and cdk recombinant baculoviruses (0.5 ml) were added as indicated. The cells were infected at room temperature for 1 h. The recombinant baculovirus was removed with growth medium, and the cells were grown for an additional 2 days at 27 °C before labeling with 32P. Infected Sf9 cells were transfected into a 15-ml tube for 32P labeling. After centrifugation and removal of growth medium, the cells were resuspended in 2 ml of fresh phosphate-free medium containing 200 μCi of 32P (specific activity 3,000 Ci/mmol) and incubated at 37 °C for 2 h. The cells were centrifuged at 3,000 × g for 5 min. The supernatant was removed, and the cells were washed twice with lysis buffer. The cells were then sonicated for 30 s in 40 ml Tris-HCl, pH 7.8, 0.25 mM sucrose, 0.5 mM NaCl, 0.1% Nonidet P-40, 0.1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 10 mM benzamidine-HCl. The crude cell extracts were transferred to microtubes and centrifuged at 15,000 × g for 30 min. About 20 mg of total protein was used for immunoprecipitation in the presence of 20 μg of streptavidin-biotinylated horseradish peroxidase complex. Color development was performed by incubation with 4-chloro-1-naphthol and hydrogen peroxide and terminated with sodium azide.

RESULTS

Expression of Pol δ p125—The expression of human pol δ in Sf9 cells infected with recombinant baculovirus was analyzed

DNA Polymerase Assays—Sparingly primed poly(dA)·oligo(dT) was used as the template as described by Lee et al. (3). The standard reaction for the poly(dA)·oligo(dT) assay contained 0.25 optical density units/ml poly(dA)·oligo(dT) (20:1), 200 μg/ml bovine serum albumin, 5% glycerol, 10 mM MgCl₂, 25 mM HEPES, pH 6.0, 100 μm/μmol [3H]TTP, and 0.2–0.4 unit of pol δ in the presence or absence of 0.2 μg of PCNA monomeric antibody (2, 14) and 40 μl of protein A-Sepharose slurry at 4 °C overnight. The Sepharose beads were washed twice with sonication buffer and boiled for 5 min in 50 μl of SDS sample buffer. The proteins released from the beads were then subjected to SDS-PAGE and autoradiography.

Immunoprecipitation and immunoblotting of Molt 4 Cells with Pol δ and cdk2s of the Cyclin and Cdk—4 × 10^7 exponentially growing Molt 4 cells were prepared and lysed with 300 μl of Nonidet P-40 buffer (50 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 150 mM NaCl, and 1% Nonidet P-40). The lysates were precloned with protein A beads (50 μl of a 10% suspension) by rotating at 4 °C for 30 min. The supernatants were removed by centrifugation and transferred to a fresh tube. The antibody used for immunoprecipitation was then added in the presence of 50 μl of fresh protein A beads and incubated at 4 °C for 1 h. Anti-pol δ monoclonal antibody (20 μg), PCNA monoclonal antibody (20 μg), anti-cyclin E and A antibodies (100 μl of hybridoma cell supernatant), and anti-cdk2 polyclonal antibody (2 μl) were used for the experiments. The extracts were then centrifuged and washed with Nonidet P-40 buffer three times. After SDS-PAGE, the separated proteins were transferred to a nitrocellulose membrane and Western blotted with antibodies to cdk2, cdk5, or pol δ.
by immunoblotting with a pol δ monoclonal antibody (38B5; see "Experimental Procedures"). The infected cells were disrupted by passage through a French press in 0.1 M KCl and centrifuged to provide the first extract. The pellet was reextracted by sonication in 0.5 M KCl (second extract). The pellet was then dissolved in 1 ml of 8 M urea. Immunoreactive protein was found to be present in the two salt extracts but not in the urea extract when equal amounts of protein were loaded from each fraction (Fig. 1). These experiments showed that pol δ was expressed as a soluble protein that can be extracted completely by 0.5 M KCl. Immunoblots of the corresponding extracts of Sf9 cells infected with wild type AcMNPV using the same antibody showed the absence of immunoreactive polypeptide (not shown). The time course of pol δ expression was examined by immunoblot analysis of cells taken at intervals after infection with recombinant virus (Fig. 2). For these experiments the 0.1 and 0.5 M KCl extracts were combined. Very little p125 immunoreactivity was observed at 12 h postinfection, and the peak of expression was found to be between 36 and 48 h (Fig. 2).

The recombinant pol δ was immunoblotted using a series of peptide-specific antibodies (Fig. 3) as described by Hao et al. (5). The different peptide-specific antibodies (N1, N2, N3, N4, N5, C1, and C2) recognized the recombinant p125 expressed in the baculovirus system. This experiment provided additional confirmation of the identity of the overexpressed protein. Note that the immunoblots (Fig. 3) for p125 appear as a doublet. As we will show, p125 could be purified to a single polypeptide of 125 kDa, although it was often observed as a doublet. A similar behavior was encountered in the isolation of the calf thymus enzyme. At present the most likely explanations are that this may reflect posttranslational modification of the enzyme by phosphorylation or partial proteolysis.

Purification of Recombinant Pol δ—Cells from 80 100-mm plates of Sf9 cells infected with recombinant baculovirus were harvested as described under "Experimental Procedures." A potential complication for the isolation of the recombinant human pol δ from Sf9 is the presence of endogenous DNA polymerases (15), which could compromise studies of the enzymatic properties of human recombinant pol δ. We have circumvented this by passing the crude extract through a phosphocellulose column ("Experimental Procedures"). When the crude extract was chromatographed on a phosphocellulose column, two peaks of activity were detected using poly(dA)-oligo(dT) as a template. One peak eluted at about 0.4 M NaCl and the second at 0.6–0.7 M NaCl (Fig. 4, center panel). To determine which of the peaks was the overexpressed pol δ, immunoblots were performed using monoclonal antibody 38B5. Only the first peak of activity (fractions 80–120) was immunoblotted; the second peak (fractions 120–160) did not contain immunoreactive protein (Fig. 4, top panel). The second peak also corresponded to the peak of polymerase activity eluted at about 0.7 M KCl when extracts of Sf9 cells infected with wild type AcMNPV baculovirus were chromatographed (Fig. 4, bottom panel). DNA polymerase δ isolated from the calf thymus was reported to elute between 235 and 320 mM KCl (3). The second peak was presumed to be endogenous DNA polymerase in baculovirus-infected Sf9 cells, which has been reported to elute from phosphocellulose at high salt concentrations (15).

The peak fractions that immunoblotted with pol δ antibody were pooled, dialyzed, and chromatographed on a Mono Q HPLC column. The column was eluted with a salt gradient as described under "Experimental Procedures" (Fig. 5). Assay of the fractions revealed a peak of DNA polymerase activity which eluted at about 350 mM NaCl. Calf thymus DNA pol δ elutes at 260 mM KCl under the same conditions (3, 4). The preparation contained a 125-kDa polypeptide that was immunoblotted by antibody 38B5 (Fig. 5, inset). The recombinant p125 was puri-
fied to near homogeneity by passage through a single-stranded DNA-cellulose column ("Experimental Procedures"). The fractions were assayed for DNA polymerase activity using poly(dA)·oligo(dT) as template (center panel). The fractions containing the two peaks of activity (80–170) were immunoblotted using an antibody against pol δ (38B5) as shown in the top panel. BC refers to the extract before chromatography. A cell extract from Sf9 cells infected with the control baculovirus was also chromatographed on phosphocellulose, and the fractions were assayed for DNA polymerase activity as shown in the bottom panel. Immunoblots of the peak fractions failed to show any immunoreactive protein (not shown).
p125 Interaction with Cdk-cyclins

The enzymatic properties of the recombinant pol δ catalytic subunit were compared with those of native calf thymus pol δ, which had been isolated by immunoaffinity chromatography (14), and with the endogenous DNA polymerase activity from SF9 cells infected with wild type AcMNPV (Fig. 8). The latter was the material obtained after phosphocellulose chromatography as in Fig. 4, and was the material obtained after phosphocellulose chromatography (see Fig. 4, bottom panel). DNA polymerase and exonuclease activities were assayed as in panel A. The inset shows the SDS-PAGE of the peak fractions stained for protein and also immunoblotted using an antibody against pol δ.

Characterization of Recombinant p125—The enzymatic properties of the recombinant pol δ catalytic subunit were compared with those of native calf thymus pol δ, which had been isolated by immunoaffinity chromatography (14), and with the endogenous DNA polymerase activity from SF9 cells infected with wild type AcMNPV (Fig. 8). The latter was the partially purified preparation obtained after phosphocellulose chromatography (see Fig. 4, bottom panel). The activities of the recombinant pol δ catalytic subunit were similar to those of native pol δ and the SF9 polymerases in that they were inhibited by aphidicolin (Fig. 8A) and resistant to 2-(p-n-butylandimino)-9-(2-deoxy-β-D-ribofuranosyl)adenine 5'-triphosphate (not shown). A well known characteristic of calf thymus pol δ is its sensitivity to inhibition by N-ethylmaleimide; recombinant pol δ was inhibited in a manner similar to calf thymus pol δ, whereas the SF9 polymerase was significantly more resistant to N-ethylmaleimide (Fig. 8B). The inhibition by low levels of salt is another characteristic of calf thymus pol δ (Fig. 8C). Recombinant p125 differed from the calf thymus enzyme in that it was less sensitive to inhibition. The SF9 DNA polymerase activity was not inhibited but slightly stimulated at 100 mM KCl and was only inhibited at much higher salt concentrations (Fig. 8C). The heat inactivation of the three polymerases was also examined. The enzyme was heated to 45 °C and assayed for polymerase activity at the indicated times. DNA polymerase δ from calf thymus and the p125 subunit displayed a similar behavior when heat-treated and were much less sensitive to

| Purification step | Protein Activity | Specific activity | Recovery |
|------------------|------------------|------------------|---------|
| Cell extract     | 800              | 6,272            | 7.8     | 100   |
| Phosphocellulose | 26               | 936              | 36      | 15    |
| Mono Q HR 5/5    | 2.2              | 616              | 280     | 20    |
| ssDNA cellulose* | 0.11             | 132              | 1,200   | 2     |

ssDNA, single-stranded DNA.

**TABLE I**

Assays was performed using poly(dA)·oligo(dT) template.

**FIG. 7. Immunoaffinity chromatography of recombinant pol δ.** Panel A, an extract from cells infected with recombinant baculovirus was chromatographed on a pol δ immunoaffinity column as described under "Experimental Procedures." The column was eluted with 50 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, and 200 mM NaCl. Fractions of 1 ml were collected. The fractions were assayed for DNA polymerase activity (solid circles) and for 3' to 5' exonuclease activity (solid squares). The inset shows the SDS-PAGE of fractions 6 and 8 stained for protein with Coomassie Blue. The same fractions were immunoblotted using an antibody against pol δ (lanes 6' and 8'). An extract from cells infected with control baculovirus was also chromatographed on the same column and assayed for DNA polymerase activity (solid triangles). Panel B, the active fractions from the first immunoaffinity chromatography (panel A) were pooled, dialyzed against the equilibration buffer, and rechromatographed on the same column. DNA polymerase and exonuclease activities were assayed as in panel A. The inset shows the SDS-PAGE of the peak fractions stained for protein and also immunoblotted using an antibody against pol δ.

**FIG. 8. Characterization of recombinant pol δ: comparison with native calf thymus pol δ and endogenous DNA polymerases in baculovirus-infected SF9 cells.** Effects of different compounds and conditions were assayed using poly(dA)·oligo(dT) as a template. Assay conditions were as described under "Experimental Procedures" for the DNA polymerase activities of recombinant pol δ (closed circles), native calf thymus pol δ (closed squares), and endogenous DNA polymerase from wild type baculovirus overexpressed in SF9 cells (open triangles). PCNA was added in the assays for calf thymus pol δ. The endogenous DNA polymerase from wild type baculovirus overexpressed in SF9 cells was the material obtained after phosphocellulose chromatography as in Fig. 4, bottom panel. Panel A, effect of aphidicolin; panel B, effect of N-ethylmaleimide; panel C, effect of KCl; panel D, effect of heat treatment at 45 °C for varying amounts of time; panels E and F, effects of Mn²⁺ and Mg²⁺, respectively, on the DNA polymerase activity of recombinant pol δ.
heat than the Sf9 polymerase (Fig. 8D).

Recombinant pol δ was stimulated by Mn²⁺ in a manner similar to that already known for calf thymus pol δ. Optimal activation was observed between 0.3 and 0.5 mM Mn²⁺, whereas optimal activity of the Sf9 polymerase was obtained at about 3 mM Mn²⁺ (Fig. 5E). Maximal activation of both calf thymus and recombinant pol δ by Mg²⁺ was reached at about 5 mM, whereas the Sf9 polymerase activity was stimulated maximally at 20 mM Mg²⁺ (Fig. 8F). These experiments showed that the properties of the recombinant p125 subunit were quite consistent with those of the calf thymus native enzyme.

Deletion Mutagenesis of p125—Extensive compilation and alignment of DNA polymerase sequences from a broad phylogenetic spectrum, i.e. from both prokaryotes and eukaryotes, have shown that these fall into two major protein families (16, 17). DNA pol δ belongs to the α-like or B family of DNA polymerases (16). A distinguishing feature of this family is the presence of a conserved core region containing six distinct conserved regions, I–VI, which are thought to contain the catalytic domain for polymerase activity. Unlike pol α, the NH₂-terminal regions of pol δ possess several regions (N1–N5) that are conserved in the Epstein-Barr virus and herpesvirus DNA polymerases (5).

Deletion mutants of the full-length human pol δ (1,107 residues) were constructed. These were p97, in which the N1 and N2 regions of the NH₂-terminus (2–249) were deleted; p109, in which N3, N4, and part of the N5 region including the ExoI domain (186–321) were deleted; p82, in which regions IV, A, B, II, VI, and III (336–715) were deleted; and p94, in which regions C, V, CT-1, CT-2, CT-3, and ZnF1 (778–1,047) were deleted (7). These were purified to near homogeneity by phosphocellulose, Mono Q, and single-stranded DNA-cellulose chromatography as described above. SDS-PAGE of the mutants (Fig. 9) showed that these had the expected molecular weights. Assays for enzyme activity showed that only p109 (Δ186–321) and p97 (Δ2–249) retained DNA polymerase activity. The p82 and p94 mutants had negligible activities (Table II). This is expected as most of the core region involved in deoxynucleotide interaction was deleted in p82, whereas most of the COOH-terminal domain responsible for DNA interaction was deleted in p94 (Fig. 9).

Evidence for the Phosphorylation of Pol δ by Cyclin-dependent Protein Kinases—Sf9 cells were coinfected with recombinant viruses harboring pol δ and different pairs of recombinant baculoviruses harboring cdk-cyclins. The cdk-cyclin pairs were cdk2-cyclin A, cdk2-cyclin E, cdk4-cyclin D1, cdk4-cyclin D2, cdk4-cyclin D3, cdc2-cyclin A, and cdc2-cyclin B1. After 48 h of infection, the cells were labeled with ³²P, for 2 h at 37 °C in low phosphate medium, sonicated, and analyzed by immunoprecipitation using a mixture of pol δ monoclonal antibodies followed by SDS-PAGE and autoradiography as described previously (13). The results (Fig. 10) showed that pol δ was hyperphosphorylated when it was coexpressed with the G₁ phase-specific cdk-cyclins, cdk4-cyclin D3 or cdk2-cyclin E. The relative intensity of phosphorylation when pol δ was coexpressed with these cdk-cyclins was about 10-fold greater than when pol δ was expressed on its own. The relative phosphorylation of pol δ after coinfection with the S or G₂/M-specific cdc2-cyclins (cdc2-cyclin A or cdc2-cyclin B1) was about 20% of that of the G₁/S-specific cdk-cyclins. Cdc2-cyclin A and cdc4-cyclin D2 gave phosphorylation intensities that were similar to the control values obtained when pol δ was expressed alone. The relative intensity of cdk4-cyclin D1 coinfected with pol δ was lower than that of pol δ alone. Our results indicate that pol δ is phosphorylated by cdk4-cyclin D3 and cdk2-cyclin E and is a likely substrate of these G₁/S-specific cdk-cyclins.

Activity of Phosphorylated and Unphosphorylated Forms of Pol δ—The effects of coexpression of p125 with cdk2-cyclin E, cdk2-cyclin A, and cdk4-cyclin D3 on the activity of pol δ were assessed by examination of the activities in the lysates after gel filtration on an HPLC column (Table III). There were no striking effects on the specific activities of the pol δ catalytic subunit assayed using poly(dA)·oligo(dT) as a template (Table III). Immunoblots for the cdk-cyclins in the fractions confirmed that these were also present in the fractions.

Coinmunoprecipitation of Cdk2 and Pol δ—It was found that pol δ could be coinmunoprecipitated with cdk2 from Sf9 cell extracts when they were coexpressed in experiments in which the extracts were immunoprecipitated with antibody against cdk2 and immunoblotted with antibody against pol δ (not shown). The interaction of pol δ with cdk2 was investigated further by examination of the coinmunoprecipitation of deletion mutants of pol δ with cdk2. The results (Fig. 11) showed that all of the deletion mutants tested were coinmunoprecipitated with the exception of the mutant in which the NH₂-terminus (residues 2–249) were deleted. These results demonstrate that there is likely a direct interaction between cdk2 and pol δ, although the possibility that this interaction is mediated by a third protein cannot be discounted.

Coinmunoprecipitation of Pol δ with Members of the Cdk-Cyclins—The coinmunoprecipitation of pol δ with cdk2 could also be observed in cultured Molt 4 cell extracts when cell extracts were immunoprecipitated with pol δ antibody and Western blotted with antibody to cdk2 (Fig. 12, first lane). The reciprocal experiment using cdk2 as the precipitating antibody followed by immunoblotting with pol δ antibody also showed that cdk2 was coinmunoprecipitated with pol δ (Fig. 12, last lane). When cyclin E was used as the precipitating antibody, the coinmunoprecipitation of pol δ was observed. The coinmunoprecipitation of cdk2 and cdk5 by PCNA antibody was also observed under the same experimental conditions (Fig. 12).
These experiments show that pol δ interacts with cdk2 and a cyclin in vivo and point to the existence of macromolecular complexes between pol δ and the cdk-cyclins.

**DISCUSSION**

The studies reported here show that the catalytic subunit of DNA pol δ can be expressed in Sf9 cells in an active form and can be isolated by a conventional purification protocol or by an immunoaffinity chromatography procedure. Isolation of the recombinant protein was aided by the use of antibodies against pol δ which did not cross-react with the endogenous DNA polymerase in baculovirus-infected Sf9 cells. We took advantage of an immunoaffinity chromatography procedure to purify the recombinant pol δ in a facile manner and to ascertain that it was separated from any endogenous DNA polymerases. The properties of the overexpressed p125 catalytic subunit were compared with those of the native enzyme. Assays of the enzyme activity using poly(dA)·oligo(dT) as a template showed that the specific activities of the preparations were only about 1,200 units/mg (Table I) compared with about 25,000 units/mg protein for the calf thymus holoenzyme (14). This difference is likely the result of the lack of, or of a greatly attenuated response to PCNA by the free catalytic subunit. Other studies of pol δ preparations containing only the catalytic subunit have suggested that it is not PCNA-responsive (18, 19), whereas our previous studies of recombinant pol δ expressed in vaccinia virus have indicated a weak response (2–3-fold stimulation). The baculovirus-expressed pol δ shows little or no response to PCNA, whereas the response is restored by the presence of the p50 subunit (20–22). In other aspects, the enzymatic behavior of the recombinant p125 is very similar to that of the holoenzyme.

**FIG. 10.** In vivo phosphorylation of recombinant pol δ in Sf9 insect cells. The indicated cdk-cyclins and pol δ were coexpressed in Sf9 cells by coinfection as described under “Experimental Procedures.” The cells were labeled metabolically with [32P]Pi, and the cell lysates were immunoprecipitated with 20 μg of pol δ monoclonal antibody and 40 μl of protein A-Sepharose slurry. The immunoprecipitates were subjected to SDS-PAGE and then autoradiographed (upper panel). Relative intensities of the pol δ p125 polypeptide were determined by densitometry.

**TABLE III**

| Cotransformant | Protein concentration | Specific activity |
|----------------|----------------------|------------------|
| None           | 0.33                 | 34               |
| Cdk2           | 0.36                 | 48               |
| Cdk2-cyclin A  | 0.36                 | 61               |
| Cdk2-cyclin E  | 0.79                 | 38               |
| Cdk4-cyclin D3 | 0.3                  | 34               |

These experiments show that pol δ interacts with cdk2 and a cyclin in vivo and point to the existence of macromolecular complexes between pol δ and the cdk-cyclins.

**FIG. 11.** Analysis of the ability of the deletion mutants of pol δ to bind to cdk2. Sf9 cells (about 10⁷) were coinfected with pol δ deletion mutants and cdk2 recombinant baculoviruses as indicated. The levels of expression of these mutants were similar as determined by immunoblotting of the Sf9 cell lysates. About 10 mg of total protein from each cell lysate was used for immunoprecipitation with cdk2 polyclonal antibody and SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane and immunoblotted with a mixture of NH₂- and COOH-terminal pol δ monoclonal antibodies.

**FIG. 12.** Coimmunoprecipitation of pol δ with members of the cdk-cyclin system. Molt 4 cells were lysed by sonication. About 10 mg of total protein was immunoprecipitated with the first antibody (Im. Ab) plus protein A-Sepharose and then Western blotted with a second antibody (WB Ab). The common band in the last three lanes is an artifact (IgG heavy chain).
activity. Deletions in the core region (amino acids 336–715) and the deletion of regions C and V in the core as well as most of the COOH-terminal region including the zinc finger motifs (778–1047) had no assayable activity (Table II). This is consistent with numerous other studies that indicate that the core region of this family of polymerases is involved in the binding of the incoming dNTP substrate (23, 24) and contains the catalytic center for DNA polymerase activity. The retention of enzymatic activity by the NH2-terminal deletion mutants is consistent with the existence of a domain structure in which the NH2-terminal region does not function in catalysis. That this is likely is also consistent with the structure of T4 polymerase, which contains most of the conserved core but only part of the NH2-terminal region that includes a motif required for the exonuclease activity (5).

The present studies provide the first evidence that the catalytic subunit of pol δ is itself a substrate for cyclin-dependent protein kinases and that this is specific for the G1, cdk-cyclins because other cdk-cyclin combinations were less effective in phosphorylating pol δ when they were coexpressed in Sf19 insect cells. Although the in vivo kinase activity of cdk-cyclin overexpressed in Sf19 insect cells may not reflect actual cellular events in the mammalian cell cycle, the involvement of G1 phase cdk-cyclins is consistent with our previous observations that pol δ is phosphorylated in vivo during the cell cycle and is maximal near the G1/S transition (25). The primary structure of pol δ shows a number of potential phosphorylation sites for the cdks, including six sites possessing the (S/T/P) motif: serines 207 and 788 and threonines 83, 150, 238, and 640 (25). It is well known that in mammalian cells the key regulators of the transition from G1 to S phase of the cell cycle include the G1 cyclins-three D type cyclins (D1, D2, D3) and cyclin E (26). Cyclin E expression is periodic, peaks at the G1/S transition, and regulates S phase commitment together with its catalytic subunit cdk2. Unlike cyclin E, expression of D type cyclins is cell lineage-specific and highly mitogen-dependent, rising on growth factor stimulation and declining rapidly on growth factor withdrawal (27, 28). The current model for G1 cdk-cyclin functions is that cyclin D binds directly to the tumor suppressor gene product pRb, targeting cdk4 to its substrate, and resulting in phosphorylation of pRb during middle to late G1 phase. This reverses the growth-suppressive effects of pRb by releasing the transcriptional factor E2F from its inhibitory constraint; the untethered E2F factor is then able to activate a series of genes required for DNA replication (26). The G1, cdk-cyclins are also thought to phosphorylate other key substrates resident at the DNA replication origin to trigger the actual onset of DNA replication once cells pass the restriction point (29, 30). Pol δ is the central enzyme in eukaryotic DNA replication and is tethered to DNA by a direct interaction with the PCNA clamp, which converts pol δ from a distributive into a highly processive enzyme for DNA synthesis (31, 32). Thus, the finding that pol δ is a substrate for the G1 cyclin-cyclins is of significance as it provides a potential linkage for the cell cycle control of DNA synthesis. However, our studies do not reveal any major effects of phosphorylation on the activity of the p125 catalytic subunit, and only small increases (<2-fold) were observed after coexpression with cdk-cyclins (Table III). Pol α-primase has also been shown to be phosphorylated, and phosphorylation does not or only moderately changes its enzymatic properties (33–35). However, the ability of pol α-primase to initiate SV40 DNA replication in vitro was found to be inhibited markedly after phosphorylation by cyclin A-dependent kinases (36).

Examination of the interaction of cdk2 with the deletion mutants of pol δ showed that the tertiary structure of pol δ is not required for this interaction and that the binding region is located in the NH2-terminal 249 residues of pol δ. The NH2-terminus of yeast and mammalian pol δ harbors several highly conserved regions (N1–N5) that are also present in herpes and Epstein-Barr viral polymerases (5). These conserved regions are likely protein-protein interaction sites for pol δ (5). The binding site of pol δ for PCNA has been mapped to the N2 region (13). The data presented also provide the first evidence for complexes that include pol δ and the cdk-cyclins. The targeting of the cdks to a substrate has some precedence since the G1, cdk-cyclins are known to form complexes with pRb. The obvious question is whether this has any functional physiological significance in relation to the phosphorylation or regulation of pol δ. The present findings show that the interaction of pol δ with cdk2 and cdk4 needs to be investigated further, in addition to the issue of the cellular role of phosphorylation of pol δ by the cdk-cyclins.

There are many levels at which phosphorylation could affect pol δ function other than the simple modulation of enzyme activity in a simple assay. This is apparent because physiologically pol δ is part of a holoenzyme and part of an extended multiprotein complex. Current findings that p21, a potent inhibitor of G1, cdks, and pol δ compete for the same sites in the interdomain connector loop of PCNA (37, 38) add even more complexity to these questions. Xiong et al. (39, 40) observed that PCNA is in a quaternary complex that includes cyclin D, cyclin-dependent kinases (cdk2, cdk4, cdk5), and p21. No phosphorylation of PCNA and p21 was detected, suggesting that neither of them is the primary substrate of phosphorylation. Thus, there are many possible permutations and speculations possible as to how regulatory systems could emerge from this melange of potential complexes. We have obtained preliminary evidence that pol δ is a substrate for the cyclin-dependent protein kinases. This was shown by the coexpression of baculovirus vectors for pol δ with several different cdk-cyclin combinations in Sf19 cells (Fig. 10) and coimmunoprecipitation Western blot studies in Molt 4 cells (Fig. 12). These results suggest that more than one cyclin might regulate pol δ, possibly triggering its phosphorylation at different sites or times of the cell cycle. Coimmunoprecipitation of pol δ deletion mutants with cdk2 also established the site of interaction (Fig. 11). Although the regulation of pol δ by protein phosphorylation has yet to be demonstrated firmly, this possibility provides a potential mechanism that might provide for the temporal regulation of DNA synthesis in concert with the cell cycle. Although the present evidence indicates that the phosphorylation status of the catalytic subunit of DNA polymerase δ may have no significant effect on its activity, the question of whether phosphorylation has any physiological relevance in affecting or regulating the biological function of polymerase δ still needs to be answered. A role of phosphorylation or binding of the kinase in affecting the properties of the polymerase in vivo in modulating the function of pol δ in DNA replication or repair cannot be excluded. In this regard, note that significant difference was observed when replication protein A is phosphorylated in SV40 DNA replication (41–43) and nucleotide excision repair systems (42). Further studies are needed to answer the question of the regulatory consequences of phosphorylation of pol δ and for that matter other replication proteins. The putative kinase consensus sequences in pol δ also show that it could be a substrate for DNA-dependent protein kinase. The latter kinase phosphorylates serine or threonine residues that are followed or preceded by glutamine residues (S/T-Q or Q-(S/T). It remains to be determined whether other kinases, e.g., DNA-dependent protein kinase, are also involved in the phosphorylation of the catalytic subunit of pol δ.
REFERENCES

1. Waga, S., and Stillman, B. (1994) Nature 369, 207–212
2. Zeng, X.-R., Jiang, Y., Zhang, S.-J., Hao, H., and Lee, M. Y. W. T. (1994) J. Biol. Chem. 269, 13748–13751
3. Lee, M. Y. W. T., Tan, C.-K., Downey, K. M., and So, A. G. (1984) Biochemistry 23, 1900–1913
4. Lee, M. Y. W. T., Jiang, Y., Zhang, S.-J., and Toomey, N. L. (1991) J. Biol. Chem. 266, 2423–2429
5. Hao, H., Jiang, Y., Zhang, S.-J., Zhang, P., Zeng, X.-R., and Lee, M. Y. W. T. (1992) Chromosoma (Berl.) 101, 121–127
6. Wang, T.-S.-F. (1991) Annu. Rev. Biochem. 60, 513–552
7. Yang, C.-L., Chang, L.-S., and Lee, M. Y. W. T. (1992) Nucleic Acids Res. 20, 735–745
8. Krishna, T. S. R., Kong, X.-P., Pan, Z.-Q., and Lee, J. (1994) J. Biol. Chem. 269, 24027–24033
9. Pan, Z.-Q., and Hurwitz, J. (1996) J. Biol. Chem. 271, 505–514
10. Pan, Z.-Q., and Hurwitz, J. (1996) J. Biol. Chem. 271, 505–514
11. Pan, Z.-Q., and Hurwitz, J. (1996) J. Biol. Chem. 271, 24203–24208