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| 13. ABSTRACT (Maximum 200)<br><br>Thus far the mechanisms for genetic errors and genomic instability in breast cancer cells have not been fully delineated. Defects in DNA polymerase delta and its accessory proteins could contribute to the molecular etiology of breast cancer. DNA polymerase delta and its accessory proteins are involved in both DNA replication, repair, recombination and transcription. There are linkages between polymerase delta and cell cycle regulation via protein-protein interaction of polymerase processivity factor PCNA with p21, a cyclin dependent kinase inhibitor. We are approaching this study in a multifaceted manner at the protein, message and gene level. It is hoped that the results from these studies will provide a deeper understanding of the linkage between regulation of polymerase delta and its accessory proteins and carcinogenesis in breast cancer. |                                                             |                                                            |                                                                   |  |
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Marietta Lee

PI - Signature

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Date

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## 5. Introduction

There are about 44,000 deaths attributed to breast cancer in 1996 (1). It is reported that 65 % of mammary cancer cells are aneuploid (2). The observed high levels of DNA synthesis and presence of genetic damage in breast tumors suggests that alterations in the DNA replication machinery of these cells may contribute to uncontrolled and error prone DNA synthesis.

We have proposed to examine the role of replication proteins in breast cancer cell lines and tissues in two major areas:

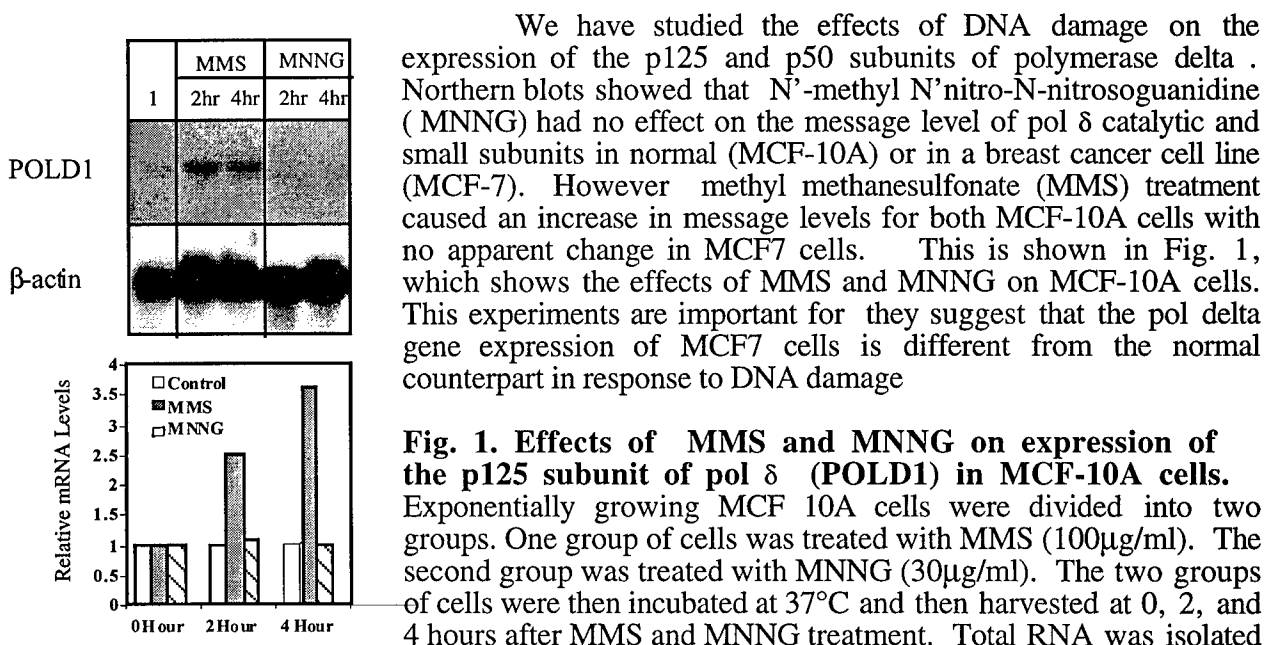
- a) to look for functional changes in DNA replication proteins and for changes in their protein-protein interactions
- b) to directly examine the genes for DNA polymerase delta (pol  $\delta$ ) and PCNA in order to find mutational changes that can provide the basis for a molecular contribution to the cancer state.

Briefly summarized, the goals of the project were to study the pol  $\delta$  system in normal and breast cancer cell lines and tissues. The tasks for the project are: comparisons of the activities of pol  $\delta$ , examination the multi-protein complexes involving pol  $\delta$ , studies of the gene regulation of pol  $\delta$ , assesment of the repair functions of pol  $\delta$ , and a search for mutations in the pol  $\delta$  gene.

In the course of this study, the Principal Investigator moved to the Department of Biochemistry and Molecular Biology, New York Medical College Valhalla, as a tenured professor. The laboratory was physically relocated on October 31<sup>th</sup>, 1997, and fiscal support for this work was placed on hold. The time frame for this Progress Report therefore covers the period September 1 1997 to October 31, 1997. However, it is important to point out that in the interim period the P.I. has successfully relocated her laboratory, and we are well poised to continue our work on this project. .

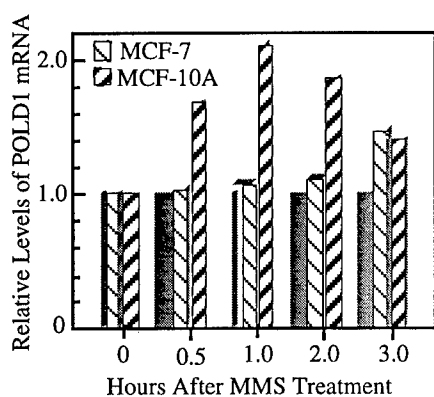
## 6. Body

### Task I. Assay of DNA polymerase and exonuclease activities, protein and mRNA levels.



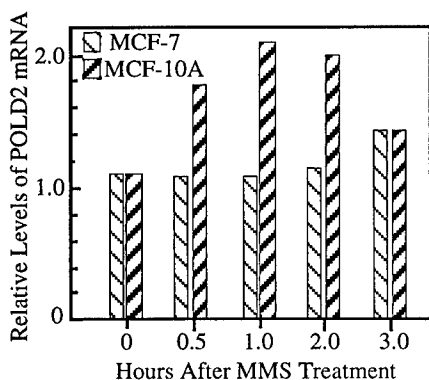
and 20 $\mu$ g of RNA from each time point was used for agarose gel electrophoresis and Northern blotting against probes to POLD1 and  $\beta$ -actin (*upper panel*). Lane 1 shows the control cells. The *lower panel* shows the densitometric estimate of the relative message levels after normalization against  $\beta$ -actin.

Using the methods shown in Fig. 1, the expression of *both* the p125 and p50 subunits of pol  $\delta$  (POLD1 and POLD2 genes, respectively) in response to MMS and MNNG was examined (Figs. 2 and 3). We found that MNNG had little or no effects on expression of POLD1 and POLD2 (not shown). A reproducible effect of MMS was found for both POLD1 and POLD2. The results with these two cell lines show that the expression of the mRNAs for both the p125 and p50 subunits are stimulated on challenge of the cells with the DNA damaging agent, MMS, whereas little change was found for the MCF-7 cancer cell line. These results point to a significant difference, and we plan to examine additional cancer cell lines. The basis for this difference is not known, but could reflect a situation where there is a loss of response of the cells to DNA damage.



**Fig. 2. Effects of MMS treatment on the expression of the p125 mRNA (POLD1) in MCF-7 and MCF-10A cells.**

Conditions were as described for Fig. 1. The diagram shows the relative changes in the levels of the mRNA of the p125 subunit of pol  $\delta$  (POLD1) as a function of time after treatment of MCF-7 and MCF-10A cells with MMS.



**Fig. 3. Effects of MMS treatment on the expression of the p50 mRNA (POLD2) in MCF-7 and MCF-10A cells.**

Conditions were as described for Fig. 2. The diagram shows the relative changes in the levels of mRNA of the p50 subunit of pol  $\delta$  (POLD2) as a function of time after treatment of MCF-7 and MCF-10A cells with MMS. The experiments were performed using the same mRNA samples isolated for the experiment in Fig. 2.

## Task 2. Comparison of DNA replication functions of purified polymerase delta and PCNA of normal and breast cancer cells

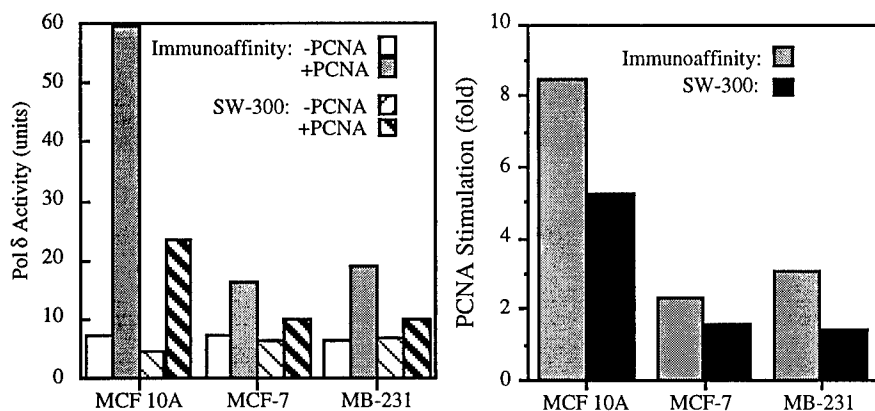
Our paper "Identification of DNA replication and Cell cycle Proteins that Interact with PCNA" was published in Nucleic Acids Research, Vol. 25, 5041-5046, 1997.

#### Task 4. Comparison of DNA replication functions of multi-protein pol $\delta$ complexes of normal and breast cancer cells.

We have initiated experiments with large scale cultures of MCF7, MCF10 A and MDA MB231 cells.

We initiated experiments to examine the behavior of pol  $\delta$  isolated by immunoaffinity chromatography. MCF10A, MCF7, MDA MB231 (normal and cancer breast cell lines) extracts were passed through immunoaffinity columns followed by HPLC gel filtration on S-300 columns. The extracts were assayed for pol  $\delta$  activities in the presence and absence of PCNA. Preliminary data showed that there are differences in total activity as well as PCNA stimulation using purified extracts from MCF-10A (normal) and MCF7, MB-231 (cancer cells). For example using the same amount of cells for each cell line ( $2.5 \times 10^7$  cells), the immunoaffinity purified MCF10 A cells yielded a total pol  $\delta$  activity of about 60 units whereas in the malignant cell lines there were less than 20 units of total activity. When the extracts were passed through a gel filtration column, the total activity of the normal breast cell line was 24 units whereas the malignant breast cancer cell lines the total activities were about 10 units. The results show that PCNA response of the affinity purified pol  $\delta$ , as well as the activity after subsequent gel filtration, differs between the normal cell line (MCF-10A) and the two breast cancer cell lines tested (MCF-7 and MDA MB231). The ability of pol  $\delta$  from normal MCF-10A cell extracts to be stimulated by PCNA was 8 fold and from the malignant extracts were 2 to 3 fold in the immunoaffinity purified extracts. After gel filtration pol  $\delta$  activity from MCF-10A extracts were stimulated about 6 fold and that from MCF-7 and MB231 extracts were less than two fold. These results are very encouraging and we think that the breast cancer cell lines most certainly contain an error prone DNA polymerase as we have proposed in our grant application.

The basis of this difference will be further analyzed by analysis of the processivity of pol  $\delta$ , and by analysis of the fidelity of the pol  $\delta$  using in vitro assays. We plan to analyze the replication fidelity of the malignant and non-malignant breast cancer cell lines. Dr. Jinyao Mo has set up a fidelity assay (3).

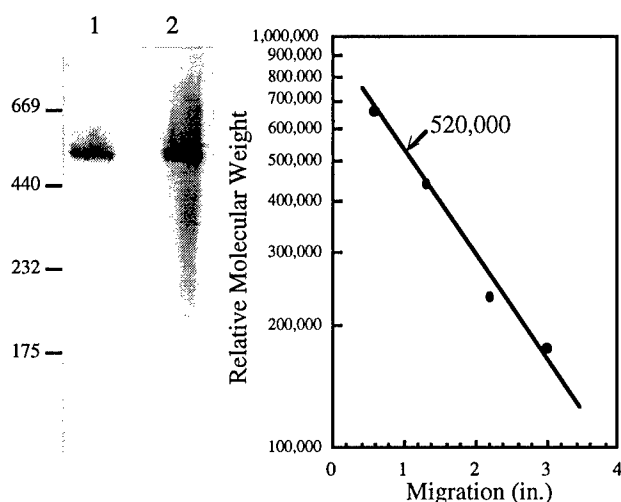


**Fig. 4. PCNA Response of normal and breast cancer cell lines.**

Extracts of MCF10A, MCF7, MDA MB231 cells were subjected to immunoaffinity chromatography. Equal numbers of cells ( $2 \times 10^7$ ) were used. The extracts were then subjected to further purification on SW300 gel filtration columns. The pol  $\delta$  enzyme activities were then assayed on poly(dA)/oligo(dT) in the presence and absence of PCNA (left panel). The right panel shows the fold stimulation of PCNA on the pol  $\delta$  enzymes.

We have also developed a native gel technique (4) in order to analyze the polypeptide composition of the polymerase delta complex. Using this technique the polymerase delta complex is run in a gradient non-denaturing gel in the first dimension and the gel is then soaked in SDS buffer and run in a denaturing condition in the second dimension. This technique will be used to identify the difference in the multiprotein pol delta complex between malignant and non-malignant

breast cells. Our initial results are highly encouraging. Preliminary analyses of calf thymus pol  $\delta$  purified through two initial chromatography steps reveals a single high molecular weight complex ( $M_r$  ca. 520,000) as shown by Western blotting against a pol  $\delta$  antibody (Fig. 5).

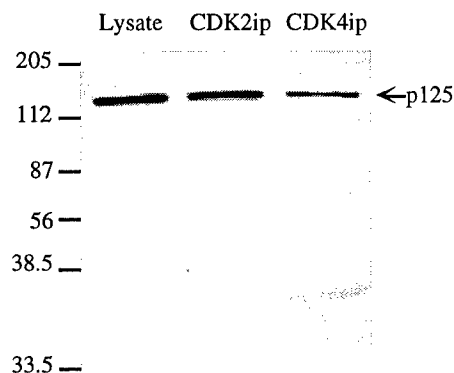


**Fig. 5. Native gel electrophoresis of pol  $\delta$ .**

The samples were run on 5-15% nondenaturing gradient gels at 4°C under conditions where we established that all the protein markers and the pol  $\delta$  band had reached a limiting mobility. In this experiment a crude calf thymus extract which has been subjected to batchwise purification on DEAE-cellulose followed by chromatography on Q-Sepharose (lanes 1 and 2, respectively) were run on non-denaturing gels and analyzed by Western blotting with antibody against pol  $\delta$  p125. The protein standards that were used were thyroglobulin (669,000), ferritin (440,000), catalase (232,000) and *E. coli* MBP- $\beta$ -galactosidase (175,000), in order of size. The

right hand panel shows the correlation of migration of the standards with relative molecular weight.

Our paper "Characterization of the p125 Subunit of Human DNA Polymerase delta and Its Deletion Mutants" was published in *J. Biol. Chem.* 273, 9561-9569 in 1998. This work showed that pol  $\delta$  interacts with cyclin-dependent kinases and cyclins. We have carried out preliminary data using MCF-7 cell lysates and have found that the catalytic subunit of pol  $\delta$  co-immunoprecipitates with Cdk2 and Cdk4. This was performed by immunoprecipitation of cultured cell lysates with antibodies to cdk2 and cdk4 followed by western blot with antibodies to pol  $\delta$  (Fig. 6).



**Fig. 6. Interaction of p125 with cdk2 and cdk4 by co-immunoprecipitation.**

Cell lysates from MCF-7 cells were immunoprecipitated with antibodies against cdk2 and cdk4. The immunoprecipitates were extensively washed with phosphate buffered saline and then subjected to Western blotting with a monoclonal antibody against pol  $\delta$ , and developed using a chemiluminescence stain. The diagram shows the western blot. From right to left, these are the MCF-7 lysate, the immunoprecipitate using the cdk2 antibody, and the immunoprecipitate using the cdk4 antibody. Sizes of the mw standards are shown on the left are in kDa.

We have solicited the help of Diana L. Kuznicki from the Department of Pathology University of Michigan. She is willing to send us matched paraffin and frozen sections of breast cancer samples. We have also synthesized primers which cover the region from amino acid residues 290 to 414. This involves 35 residues encoded by exon 8, the entire 55 amino acid residues encoded by exon 9 and the entire 35 residues of exon 10, the Exo III region, the region covering amino acids 492 to 530, the last 7 residues of exon 12 and the first 32 amino acids encoded by exon 13. We are ready to begin to RT-PCR analyses of the 3' to 5' exonuclease region of pol  $\delta$  in these normal and cancer samples.



## Conclusions

We moved from Miami to New York in Oct 27, 1997. Our grant is still in the process of being transferred to New York. Thus this report represents only progress from Sept 1997 to Oct. 1997. The results we have obtained so far are promising and we expect to make good progress once our full support is resumed. Our laboratory is functioning well and during the past months we have established contacts have made contacts for tissue samples and made primers to further continue the work in full force.

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## Publications

1. Loo, G., Zhang, S.J., Zhang, P., Toomey, N.L., and Lee, M.Y.W.T. (1998) Identification of DNA replication and cell cycle proteins that interact with PCNA. Nucleic Acids Research 25, 5041-5046.
2. Wu, S.M., Zhang, P., Zeng, X.R., Zhang, S.J., Mo, J., Li, B.Q. and Lee, M.Y.W.T. (1998) Characterization of the p125 subunit of Human DNA polymerase delta and its deletion mutants Interaction with cyclin-dependent kinase--cyclins. J. Biol. Chem. 273, 9561-9569

List of personnel receiving pay from this grant

- 1) Marietta Lee, Ph.D
- 2) Peng Zhang, Post doctoral research associate
- 3) Li Liu, graduate assistant
- 4) Jinyao Mo Postdoctoral research associate
- 5) Argentina Leon Student assistant

# Characterization of the p125 Subunit of Human DNA Polymerase $\delta$ 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)  $\delta$  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  $\delta$  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. NH<sub>2</sub>-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  $\delta$  and cyclin-dependent kinase (cdk)-cyclins followed by metabolic labeling with <sup>32</sup>P<sub>i</sub> showed that the recombinant catalytic subunit of pol  $\delta$  could be hyperphosphorylated by G<sub>1</sub> phase-specific cdk-cyclins. When cdk2 was coexpressed with pol  $\delta$  in Sf9 cells, pol  $\delta$  was found to coimmunoprecipitate with antibodies against cdk2. Experiments with deletion mutants of pol  $\delta$  showed that the NH<sub>2</sub>-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  $\delta$  by cdk2-cyclins had little or no effect on the specific activity of the enzyme.

DNA polymerase (pol)<sup>1</sup>  $\delta$  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  $\alpha$  and pol  $\beta$ . The 125-kDa subunit of human pol  $\delta$  (p125) has been identified as the catalytic subunit (4). Pol  $\delta$  is a member of a family of DNA polymerases which includes DNA

polymerase  $\alpha$ , pol  $\epsilon$ , 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, deoxynucleotide interaction, as well as the 3' to 5' exonuclease activity of pol  $\delta$  (7). In addition, there are several regions in the NH<sub>2</sub> and COOH termini which are conserved among human pol  $\delta$ , yeast pol  $\delta$ , and yeast and human pol  $\epsilon$  (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  $\delta$ , 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  $\delta$ , consistent with its role as the leading strand polymerase (8). Although there have been some mutagenesis studies of the yeast pol  $\delta$  (9), little has been done with human or mammalian pol  $\delta$ , largely because of the lack of a suitable expression system. To facilitate structure-function studies of pol  $\delta$ , it is desirable to have an expression system for the production of the recombinant protein. The expression of the human pol  $\delta$  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  $\delta$ . In addition, we have obtained novel evidence that pol  $\delta$  p125 is phosphorylated by the cyclin-dependent kinase (cdk)-cyclin complexes and also can be coimmunoprecipitated with cdk2 when they are coexpressed in Sf9 insect cells. The interaction of pol  $\delta$  with the cyclins and cdk2 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  $\mu$ g/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 baculoviruses 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  $\delta$  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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<sup>1</sup> The abbreviations used are: pol, polymerase; p125, 125-kDa subunit of human pol  $\delta$ ; cdk, cyclin-dependent kinase; AcMNPV, *A. californica* multiple nuclear polyhedrosis virus; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PCNA, proliferating cell nuclear antigen.

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  $\delta$  containing the corrected full-length pol  $\delta$  coding sequence (3.5 kilobases) was excised from the pALTER plasmid by *Bam*HI/*Hind*III digestion, gel purified, and inserted into *Bam*HI/*Hind*III-digested baculovirus transfer vector p2bac. The recombinant p2bac plasmids were cotransfected into Sf9 cells with wild type baculovirus DNA according to Ausubel *et al.* (12). Wild type BaculoGold<sup>TM</sup>-linearized AcMNPV DNA (1  $\mu$ g), recombinant plasmid DNA (3  $\mu$ g), cationic liposome solution (25  $\mu$ l), and 1 ml of Grace's insect medium containing no supplements were mixed by vortexing for 10–15 s and incubated at room temperature for 15 min. 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 for virus production by plaque assay. The recombinant baculoviruses were identified as occlusion-negative plaques with a dissecting microscope. Because the BaculoGold<sup>TM</sup>-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  $\beta$ -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  $\delta$  were constructed as described in Ref. 13.

**Infection of Sf9 Cells with Recombinant Baculovirus and Preparation of Cell Extracts**—Recombinant viral stocks (0.5 ml) were added to a multiplicity of infection between 5 and 10 for the infection 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 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  $8 \times 10^8$  cells) were suspended in 5-cell pellet volumes (50 ml) of lysis buffer (40 mM Tris-HCl, pH 7.8, 0.25 M sucrose, 0.1 M NaCl, 0.1% Nonidet P-40, 0.1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 10 mM benzamidinium-HCl). Cells were disrupted by passage through a French press at 1,000 p.s.i. The lysate was centrifuged at  $27,000 \times g$  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 M NaCl and sonicated three times for 20 s each at 50 watts on ice. The extract was again centrifuged at  $27,000 \times g$  for 30 min, and the supernatant was designated as the high salt-solubilized fraction. Protein concentrations of the first and second extracts were 12 and 9 mg/ml, respectively. The pellet was then dissolved in 1 ml of 8 M urea. The two fractions (low and high salt extracts) were then combined and dialyzed against TGEED buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol).

**Phosphocellulose Chromatography**—The dialyzed lysates were loaded onto a phosphocellulose column (5  $\times$  7 cm) equilibrated in TGEED buffer. The column was eluted with a linear gradient of 50–1 M NaCl in TGEED buffer in a total volume of 2 liters. Fractions of 10 ml each were collected and assayed for DNA polymerase activity. Western blots were also performed using 38B5, a monoclonal antibody against the COOH-terminal region of pol  $\delta$  (2, 14).

**HPLC**—The combined fractions from the phosphocellulose column which contained recombinant pol  $\delta$  p125 were dialyzed against TGEED buffer, pH 7.8, passed through an 0.45- $\mu$ m syringe filter, and injected onto a Mono Q HR 5/5 column. The enzyme was eluted with a linear gradient of 0–1 M NaCl for 20 min at 1 ml/min.

**Single-stranded DNA-cellulose Chromatography**—Fractions from the Mono Q column were dialyzed against HEPES buffer (20 mM HEPES, 5 mM MgCl<sub>2</sub>, 10% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.5) and were then loaded onto a single-stranded DNA-cellulose column (0.5  $\times$  6 cm) equilibrated with HEPES buffer. The column was washed with the same buffer, and a gradient of 50–500 mM NaCl in a total volume of 40 ml was applied. Fractions of 1 ml were collected and analyzed by SDS-PAGE, Western blotting, and assays for pol  $\delta$  activity.

**Immunoprecipitation Chromatography**—Monoclonal antibody 78F5 was coupled onto AvidChrom hydrazide (Sigma) as described by Jiang *et al.* (14). The column (1  $\times$  10 cm) was equilibrated with TGEED buffer (50 mM Tris-HCl, 0.1 mM EGTA, 0.5 mM EDTA, 10% glycerol, pH 7.8). The column was washed with the same buffer containing 50 mM NaCl, and pol  $\delta$  was eluted with 0.2 M NaCl in TGEED buffer. Fractions of 1 ml were collected and analyzed as described above.

**DNA Polymerase Assays**—Sparsely 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  $\mu$ g/ml bovine serum albumin, 5% glycerol, 10 mM MgCl<sub>2</sub>, 25 mM HEPES, pH 6.0, 100 cpm/pmol [<sup>3</sup>H]TTP, and 0.2–0.4 unit of pol  $\delta$  in the presence or absence of 0.2  $\mu$ g of PCNA in a total volume of 100  $\mu$ l. Reaction mixtures were incubated for 60 min at 37 °C and were terminated by spotting onto DE81 papers that were then washed four times with 0.3 M ammonium formate, pH 7.8, once with 95% ethanol, and counted as described previously (4).

**Assay for 3' to 5' Exonuclease Activity**—The assay was performed by measuring the release of [<sup>3</sup>H]dTTP from [<sup>3</sup>H]dT<sub>50</sub> as described previously (3). The assay contained 2  $\mu$ M [<sup>3</sup>H]dT<sub>50</sub> (200–300 cpm/pmol), 25 mM HEPES buffer, pH 7.4, 5  $\mu$ g of bovine serum albumin, 5 mM MgCl<sub>2</sub>, and 0.2–0.4 unit of pol  $\delta$  in a total volume of 60  $\mu$ l. Reaction mixtures were incubated for 30 min at 37 °C and were terminated by spotting 20  $\mu$ l onto DE81 filter papers. Filters were washed four times with 0.3 M ammonium formate, pH 7.8, and once with 95% ethanol and counted as described previously (3).

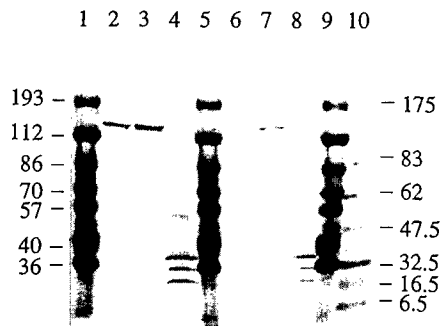
**Western Blot Analysis**—The recombinant proteins expressed in Sf9 cells infected with recombinant baculoviruses were analyzed by Western blotting with pol  $\delta$  monoclonal antibody 38B5 (2, 14). Extracts of Sf9 cells prepared as described above were subjected to SDS-PAGE in 5–15% gradient gels that were then transferred to nitrocellulose membranes. Prestained protein standards (Sigma) were used as molecular weight markers and also to provide visual confirmation of efficient transfer. The nitrocellulose blots were blocked with 5% nonfat dry milk in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, for 1 h at room temperature. The blots were then incubated with monoclonal antibody against pol  $\delta$  for 12 h at 25 °C. After three 10-min washes in 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, the blots were incubated with biotinylated sheep anti-mouse immunoglobulin for 1 h at 27 °C followed by incubation with 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  $\delta$ , Cyclins, and Cdk and <sup>32</sup>P<sub>i</sub> Labeling**—Sf9 cells (10<sup>7</sup>) were grown to exponential stage. Pol  $\delta$ , cyclin, and cdk recombinant baculoviruses (0.5 ml) were added as indicated. The cells were infected at room temperature for 1 h. The recombinant baculoviruses were removed, replaced with growth medium, and the cells were grown for an additional 2 days at 27 °C before labeling with <sup>32</sup>P<sub>i</sub>. Infected Sf9 cells were transferred into a 15-ml tube for <sup>32</sup>P<sub>i</sub> labeling. After centrifugation and removal of growth medium, the cells were resuspended in 2 ml of fresh phosphate-free medium containing 200  $\mu$ Ci of <sup>32</sup>P<sub>i</sub> (specific activity 3,000 Ci/mmol) and incubated at 37 °C for 2 h. The cells were centrifuged at  $3,000 \times g$  for 5 min. The supernatant was removed, and the cells were washed twice with phosphate-buffered saline. The cells were sonicated for 30 s in 40 mM Tris-HCl, pH 7.8, 0.25 M sucrose, 0.5 M NaCl, 0.1% Nonidet P-40, 0.1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 10 mM benzamidinium-HCl. The crude cell extracts were transferred to microtubes and centrifuged at  $15,000 \times g$  for 30 min. About 20 mg of total protein was used for immunoprecipitation in the presence of 20  $\mu$ g of 78F5 pol  $\delta$  monoclonal antibody (2, 14) and 40  $\mu$ l 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  $\mu$ l 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  $\delta$  and Members of the Cyclin and Cdk**—4  $\times$  10<sup>7</sup> exponentially growing Molt 4 cells were prepared and lysed with 300  $\mu$ l of Nonidet P-40 buffer (50 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 150 mM NaCl, and 1% Nonidet P-40). The lysates were precleared with protein A beads (50  $\mu$ l 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  $\mu$ l of fresh protein A beads and incubated at 4 °C for 1 h. Anti-pol  $\delta$  monoclonal antibody (20  $\mu$ g), PCNA monoclonal antibody (20  $\mu$ g), anti-cyclin E and A antibodies (100  $\mu$ l of hybridoma cell supernatant), and anti-cdk2 polyclonal antibody (2  $\mu$ l) 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  $\delta$ .

## RESULTS

**Expression of Pol  $\delta$  p125**—The expression of human pol  $\delta$  in Sf9 cells infected with recombinant baculovirus was analyzed



**FIG. 1. Immunoblot of extracts of Sf9 cells infected with recombinant baculovirus.** Extracts of Sf9 cells infected with recombinant baculoviruses were prepared as described under "Experimental Procedures." The cells were disrupted and extracted in 50 ml of lysis buffer containing 0.1 M NaCl and then with 20 ml of lysis buffer containing 0.5 M NaCl. The pellet was then dissolved in 1 ml of 8 M urea. These three extracts (60 or 30  $\mu$ g of protein/lane) were then analyzed by SDS-PAGE (5–15% acrylamide). Western blotting was performed using monoclonal antibody 38B5 against human pol  $\delta$ . Lanes 1, 5, and 9 are high molecular weight standards as marked; lanes 2–4 are 60  $\mu$ g of the 0.1 M NaCl, 0.5 M NaCl, and 8 M urea extracts, respectively. Lanes 6–8, same as lanes 2–4 but with 30  $\mu$ g of protein/lane; lane 10, low molecular weight protein standards as marked.

by immunoblotting with a pol  $\delta$  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  $\delta$  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  $\delta$  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  $\delta$  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 as 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.

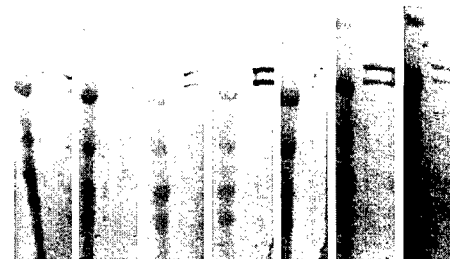
**Purification of Recombinant Pol  $\delta$** —Cells from 80 100-mm plates of Sf9 cells infected with recombinant baculovirus were harvested as described under "Experimental Procedures." A potential complication for the isolation of the recombinant human pol  $\delta$  from Sf9 is the presence of endogenous DNA polymerases (15), which could compromise studies of the enzymatic properties of human recombinant pol  $\delta$ . 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.

12 24 36 48 60



**FIG. 2. Time course of pol  $\delta$  expression in Sf9 cells.** Sf9 cells infected with recombinant virus were harvested at 12, 24, 36, 48, and 60 h after infection. The cells were lysed and extracted as described under "Experimental Procedures." The 0.1 M and 0.5 M NaCl extracts were combined and analyzed for the expression of pol  $\delta$  by SDS-PAGE (20  $\mu$ g/lane) followed by immunoblotting. Lanes are marked according to time of harvest.

N1 N2 N3 N4 N5 C1 C2



**FIG. 3. Immunoblot of crude recombinant pol  $\delta$  extract using peptide-specific antibodies.** Sf9 cells were infected with recombinant baculovirus, and the cell extracts were immunoblotted using polyclonal antibodies against specific peptides derived from the NH<sub>2</sub>- and COOH-terminal regions of the pol  $\delta$  sequence (13). These were as follows: N1 (84–101), N2 (129–149), N3 (244–262), N4 (276–295), N5 (312–331), C1 (1047–1068), and C2 (1069–1090). The figure shows a composite of individual blots, each of which shows two lanes, the left lane being the prestained protein standards and the right lane, the Sf9 cell extracts (20  $\mu$ l, 50  $\mu$ g of protein).

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  $\delta$ , 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  $\delta$  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  $\delta$  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  $\delta$  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-

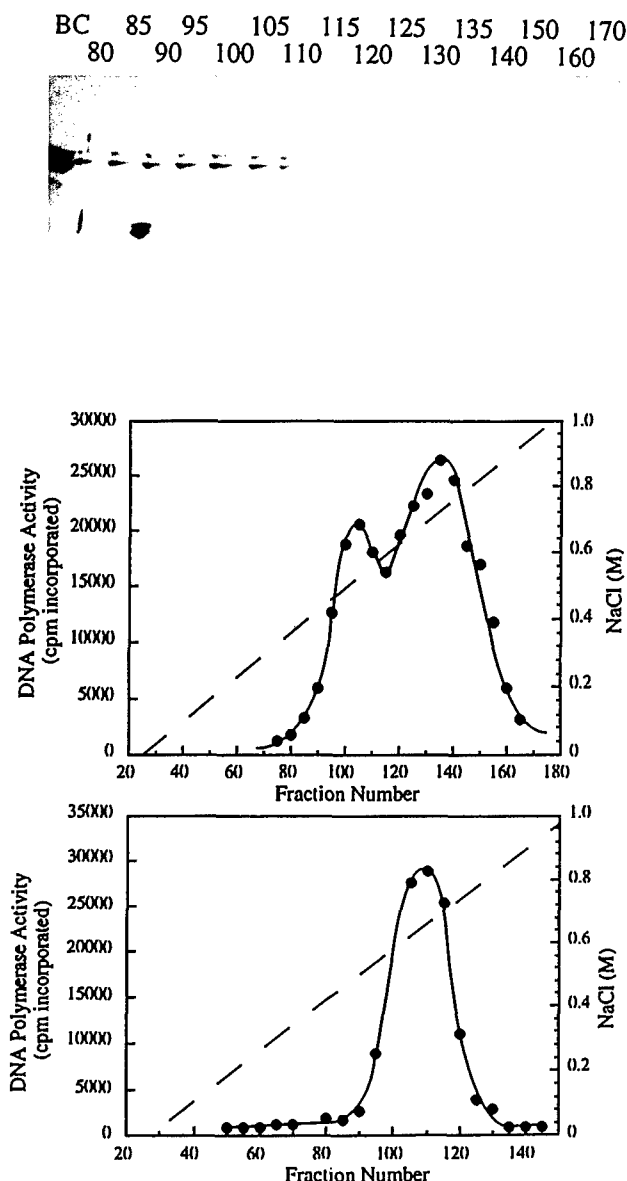


FIG. 4. **Phosphocellulose chromatography of Sf9 cell extracts infected with recombinant baculovirus.** A cell extract from Sf9 cells infected with recombinant baculovirus was chromatographed on phosphocellulose as described under "Experimental Procedures." The fractions were assayed for DNA polymerase 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  $\delta$  (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).

fied to near homogeneity by passage through a single-stranded DNA-cellulose column ("Experimental Procedures"). DNA polymerase activity and exonuclease activities were assayed and found to coelute (Fig. 6). The enzyme was found to be nearly homogeneous as shown by Coomassie Blue staining of SDS-PAGE of the peak fraction (Fig. 6, *inset*).

**Immunoaffinity Purification of Recombinant Pol  $\delta$** —We have shown previously that calf thymus pol  $\delta$  can be isolated by immunoaffinity chromatography using monoclonal antibody 78F5 coupled to AvidChrom hydrazide (14). Crude Sf9 cell extracts were chromatographed on a pol  $\delta$  immunoaffinity column ("Experimental Procedures"). The column was washed

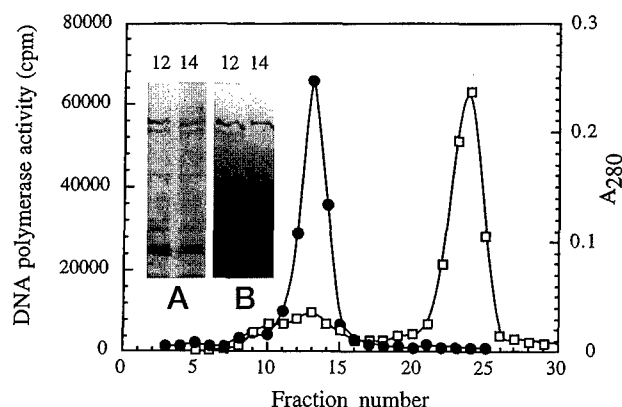


FIG. 5. **Mono Q chromatography of recombinant pol  $\delta$ .** The peak fractions from the phosphocellulose chromatography step were combined and subjected to HPLC on a Mono Q 5/5 column (see "Experimental Procedures"). The enzyme was eluted with a linear gradient of 0–1 M NaCl in 20 min at 1 ml/min. The fractions were assayed for DNA polymerase activity (*closed circles*). The elution of protein is shown by the absorbance at 280 nm (*squares*). The *inset* shows the SDS-PAGE of fractions 12 and 14, which were stained for protein (*left panel*) and immunoblotted using a monoclonal antibody against pol  $\delta$  (*right panel*).

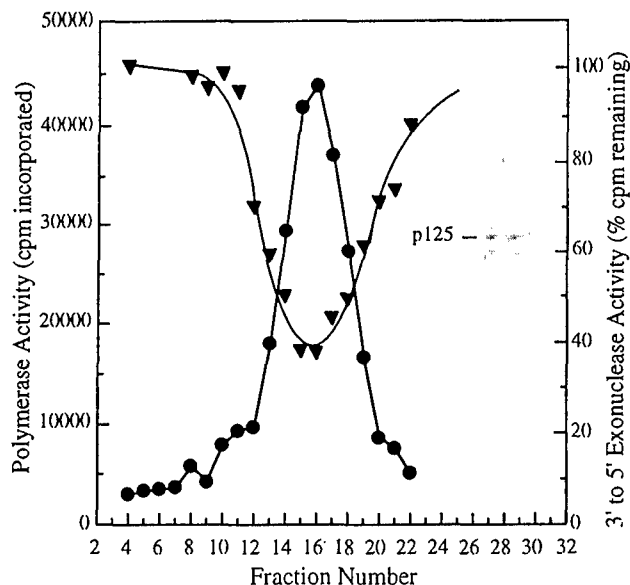
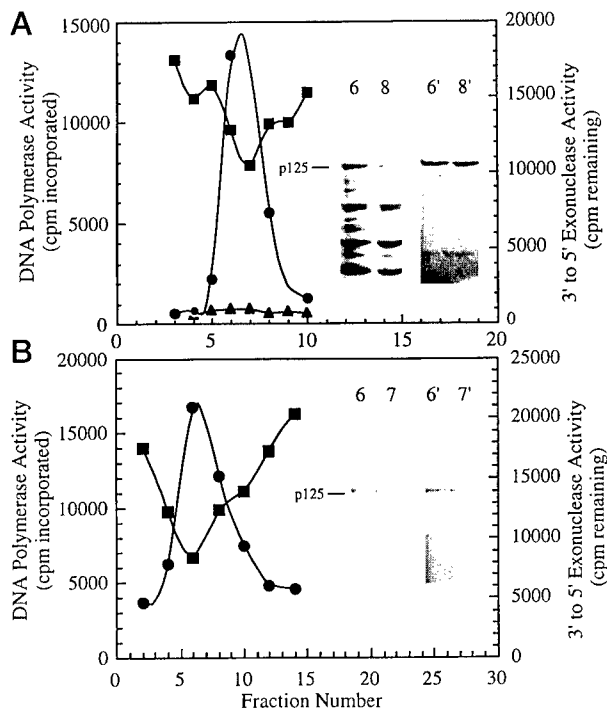


FIG. 6. **Single-stranded DNA-cellulose chromatography.** The fractions from the peak of the Mono Q column which immunoblotted with the pol  $\delta$  antibody were combined, dialyzed against buffer, and loaded onto a single-stranded DNA-cellulose column as described under "Experimental Procedures." Fractions of 1 ml were collected and assayed for DNA polymerase activity (*circles*) and for exonuclease activity (*inverted triangles*). The *inset* shows the SDS-PAGE of fraction 16, which was stained for protein.

with buffer containing 50 mM NaCl, and pol  $\delta$  was eluted by 0.2 M NaCl as shown by analysis for DNA polymerase and exonuclease activities (Fig. 7A) and Western blotting (Fig. 7A, *inset*). The enzyme obtained was still impure (Fig. 7A, *inset*) as determined by SDS-PAGE gels stained for protein. Sf9 cells infected with wild type virus were also passed through this immunoaffinity column, and no detectable DNA polymerase activity was recovered (Fig. 7A). This demonstrated that DNA polymerase activities from the Sf9 cells infected with wild type virus did not bind to the column. Note that the overexpressed p125 catalytic subunit could be eluted from the immunoaffinity column by simply using 0.2 M KCl, whereas calf thymus DNA pol  $\delta$  holoenzyme is eluted at 0.4 M NaCl and 30% ethylene glycol (14). The peak fractions were combined and rechromatographed on the same column. This allowed for the isolation of



**FIG. 7. Immunoaffinity chromatography of recombinant pol  $\delta$ .** Panel A, an extract from cells infected with recombinant baculovirus was chromatographed on a pol  $\delta$  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  $\delta$  (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  $\delta$ .

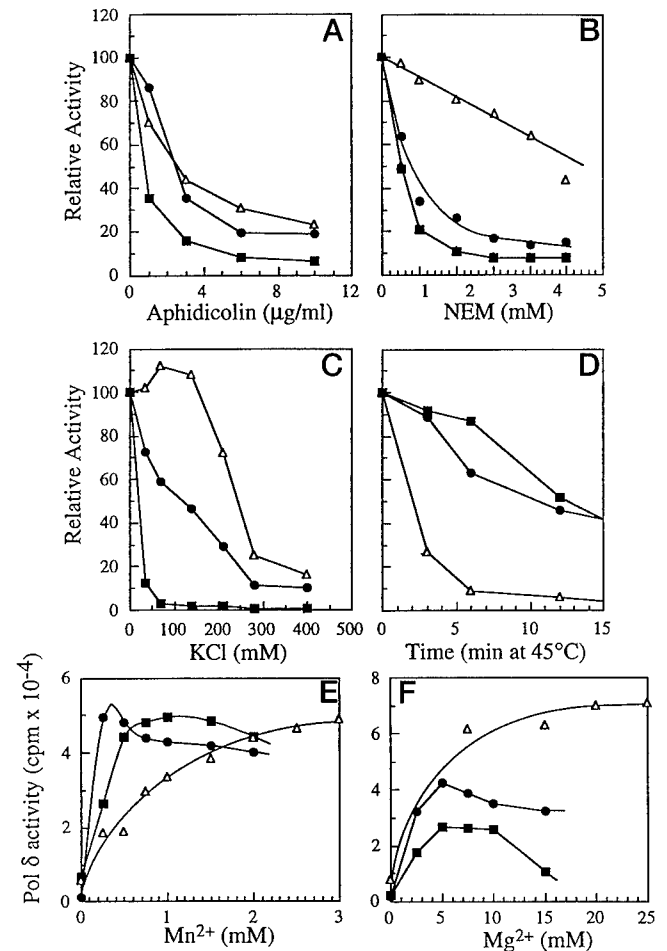
the recombinant p125 in a nearly homogeneous form (Fig. 7B). Starting with 800 mg of total protein in the crude extract, about 0.11 mg of nearly homogeneous protein was recovered, presenting a purification of 153-fold and a final specific activity of 1,200 units/mg of protein using poly(dA)-oligo(dT) as a template (Table I).

**Characterization of Recombinant p125**—The enzymatic properties of the recombinant pol  $\delta$  catalytic subunit were compared with those of native calf thymus pol  $\delta$ , 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  $\delta$  catalytic subunit were similar to those of native pol  $\delta$  and the Sf9 polymerases in that they were inhibited by aphidicolin (Fig. 8A) and resistant to 2-(*p*-n-butylamino)-9-(2-deoxy- $\beta$ -D-ribofuranosyl)adenine 5'-triphosphate (not shown). A well known characteristic of calf thymus pol  $\delta$  is its sensitivity to inhibition by *N*-ethylmaleimide; recombinant pol  $\delta$  was inhibited in a manner similar to calf thymus pol  $\delta$ , 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  $\delta$  (Fig. 8C). Recombinant p125 differed from the calf thymus pol  $\delta$  in that it was less sensitive to inhibition. The Sf9 DNA polymerase ac-

**TABLE I**  
Purification of recombinant DNA pol  $\delta$  p125  
Assays was performed using poly(dA) · oligo(dT) template.

| Purification step            | Protein<br>mg | Activity<br>units | Specific activity<br>units/mg | Recovery<br>% |
|------------------------------|---------------|-------------------|-------------------------------|---------------|
| 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 <sup>a</sup> | 0.11          | 132               | 1,200                         | 2             |

ssDNA, single-stranded DNA.



**FIG. 8. Characterization of recombinant pol  $\delta$ : comparison with native calf thymus pol  $\delta$  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  $\delta$  (closed circles), native calf thymus pol  $\delta$  (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  $\delta$ . 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  $Mn^{2+}$  and  $Mg^{2+}$ , respectively, on the DNA polymerase activity of recombinant pol  $\delta$ .

tivity 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  $\delta$  from calf thymus and the p125 subunit displayed a similar behavior when heat-treated and were much less sensitive to

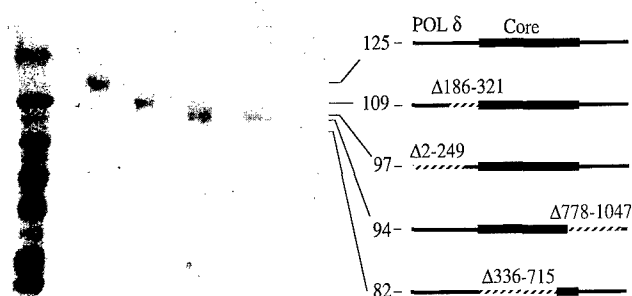


FIG. 9. Expression of deletion mutants of pol  $\delta$  p125. Deletion mutants were constructed as described in Ref. 13. These mutants were purified to homogeneity by phosphocellulose, Mono Q, and single-stranded DNA-cellulose chromatography. The protein staining of the purified mutants after SDS-PAGE are shown. The map of the deletions is shown on the right.

heat than the Sf9 polymerase (Fig. 8D).

Recombinant pol  $\delta$  was stimulated by  $Mn^{2+}$  in a manner similar to that already known for calf thymus pol  $\delta$ . Optimal activation was observed between 0.3 and 0.5 mM  $Mn^{2+}$ , whereas optimal activity of the Sf9 polymerase was obtained at about 3 mM  $Mn^{2+}$  (Fig. 8E). Maximal activation of both calf thymus and recombinant pol  $\delta$  by  $Mg^{2+}$  was reached at about 5 mM, whereas the Sf9 polymerase activity was stimulated maximally at 20 mM  $Mg^{2+}$  (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  $\delta$  belongs to the  $\alpha$ -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  $\alpha$ , the  $NH_2$ -terminal regions of pol  $\delta$  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  $\delta$  (1,107 residues) were constructed. These were p97, in which the N1 and N2 regions of the  $NH_2$  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 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 ( $\Delta$ 186–321) and p97 ( $\Delta$ 2–249) 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  $\delta$  by Cyclin-dependent Protein Kinases**—Sf9 cells were coinfecting with recombinant viruses harboring pol  $\delta$  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  $^{32}P_i$  for 2 h at 37 °C in low phosphate medium, sonicated, and analyzed by immunopre-

TABLE II  
Relative specific activities of recombinant p125 and its deletion mutants

Enzymes were purified to near homogeneity as described under "Experimental Procedures" and assayed for DNA polymerase activity using poly(dA) · oligo(dT) as template.

| Enzyme            | Protein <sup>a</sup> | Specific activity | Relative specific activity |
|-------------------|----------------------|-------------------|----------------------------|
|                   | mg/ml                | units/mg          |                            |
| p125              | 0.0168               | 1,270             | 100                        |
| $\Delta$ 186–321  | 0.0178               | 1,290             | 102                        |
| $\Delta$ 2–249    | 0.0150               | 896               | 71                         |
| $\Delta$ 336–715  | 0.0195               | <0.01             | <0.01                      |
| $\Delta$ 778–1047 | 0.0187               | <0.01             | <0.01                      |

<sup>a</sup> Concentration of protein in the final preparation was assayed using Coomassie Blue.

cipitation using a mixture of pol  $\delta$  monoclonal antibodies followed by SDS-PAGE and autoradiography as described previously (13). The results (Fig. 10) showed that pol  $\delta$  was hyperphosphorylated when it was coexpressed with the  $G_1$  phase-specific cdk-cyclins, cdk4-cyclin D3 or cdk2-cyclin E. The relative intensity of phosphorylation when pol  $\delta$  was coexpressed with these cdk-cyclins was about 10-fold greater than when pol  $\delta$  was expressed on its own. The relative phosphorylation of pol  $\delta$  after coinfection with the S or  $G_2/M$ -specific cdc2-cyclins (cdc2-cyclin A or cdc2-cyclin B1) was about 20% of that of the  $G_1/S$ -specific cdk-cyclins. Cdk2-cyclin A and cdk4-cyclin D2 gave phosphorylation intensities that were similar to the control values obtained when pol  $\delta$  was expressed alone. The relative intensity of cdk4-cyclin D1 coinfecting with pol  $\delta$  was lower than that of pol  $\delta$  alone. Our results indicate that pol  $\delta$  is phosphorylated by cdk4-cyclin D3 and cdk2-cyclin E and is a likely substrate of these  $G_1/S$ -specific cdk-cyclins.

**Activity of Phosphorylated and Unphosphorylated Forms of Pol  $\delta$** —The effects of coexpression of p125 with cdk2-cyclin E, cdk2-cyclin A, and cdk4-cyclin D3 on the activity of pol  $\delta$  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  $\delta$  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.

**Coimmunoprecipitation of Cdk2 and Pol  $\delta$** —It was found that pol  $\delta$  could be coimmunoprecipitated 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  $\delta$  (not shown). The interaction of pol  $\delta$  with cdk2 was investigated further by examination of the coimmunoprecipitation of deletion mutants of pol  $\delta$  with cdk2. The results (Fig. 11) showed that all of the deletion mutants tested were coimmunoprecipitated with the exception of the mutant in which the  $NH_2$  terminus (residues 2–249) were deleted. These results demonstrate that there is likely a direct interaction between cdk2 and pol  $\delta$ , although the possibility that this interaction is mediated by a third protein cannot be discounted.

**Coimmunoprecipitation of Pol  $\delta$  with Members of the Cdk-Cyclins**—The coimmunoprecipitation of pol  $\delta$  with cdk2 could also be observed in cultured Molt 4 cell extracts when cell extracts were immunoprecipitated with pol  $\delta$  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  $\delta$  antibody also showed that cdk2 was coimmunoprecipitated with pol  $\delta$  (Fig. 12, last lane). When cyclin E was used as the precipitating antibody, the coimmunoprecipitation of pol  $\delta$  was observed. The coimmunoprecipitation of cdk2 and cdk5 by PCNA antibody was also observed under the same experimental conditions (Fig. 12).



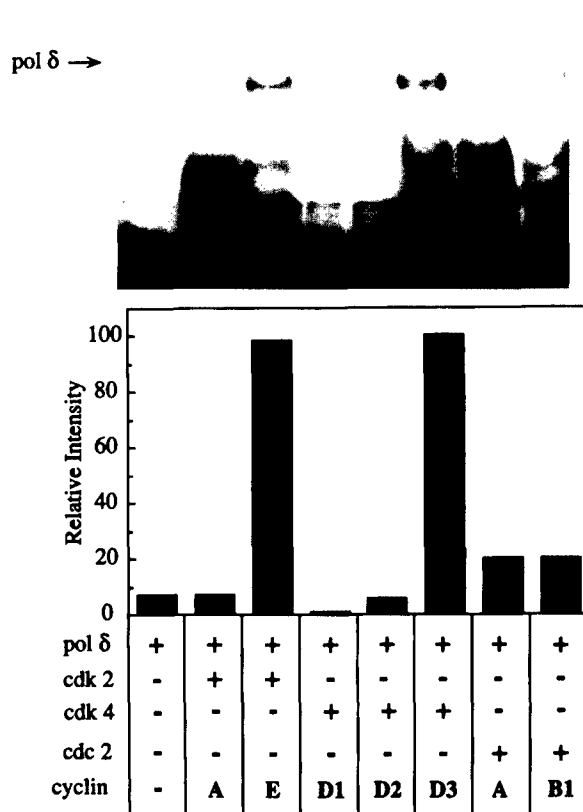


FIG. 10. *In vivo* phosphorylation of recombinant pol  $\delta$  in Sf9 insect cells. The indicated cdk-cyclins and pol  $\delta$  were coexpressed in Sf9 cells by coinfection as described under "Experimental Procedures." The cells were labeled metabolically with  $^{32}\text{P}_i$ , and the cell lysates were immunoprecipitated with 20  $\mu\text{g}$  of pol  $\delta$  monoclonal antibody and 40  $\mu\text{l}$  of protein A-Sepharose slurry. The immunoprecipitates were subjected to SDS-PAGE and then autoradiographed (upper panel). Relative intensities of the pol  $\delta$  p125 polypeptide were determined by densitometry.

TABLE III

Specific activities of p125 coinfecting with different combinations of cdk-cyclins

Lysates obtained from equal amounts of coinfecting cells were precipitated with 50% ammonium sulfate. The precipitates were dissolved in TGEED buffer containing 150 mM KCl, and equal volumes (0.5 ml) of each were loaded onto a Superose 6 HPLC gel filtration column (see "Experimental Procedures"). The results show the protein concentration and pol  $\delta$  activities of the peak fractions. The presence of the cdk-cyclins in the eluates was confirmed by immunoblot (not shown).

| Cotransformant | Protein concentration<br>mg/ml | Specific activity<br>units/mg |
|----------------|--------------------------------|-------------------------------|
| 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  $\delta$  interacts with cdk2 and a cyclin *in vivo* and point to the existence of macromolecular complexes between pol  $\delta$  and the cdk-cyclins.

## DISCUSSION

The studies reported here show that the catalytic subunit of DNA pol  $\delta$  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  $\delta$  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

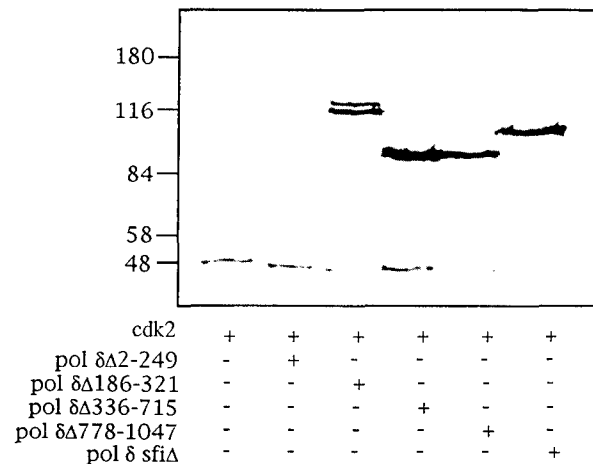


FIG. 11. Analysis of the ability of the deletion mutants of pol  $\delta$  to bind to cdk2. Sf9 cells (about  $10^7$ ) were coinfecting with pol  $\delta$  deletion mutants and cdk2 recombinant baculoviruses as indicated. The levels of expression of these mutants were similar as determined by immunoblotting 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  $\text{NH}_2$ - and  $\text{COOH}$ -terminal pol  $\delta$  monoclonal antibodies.

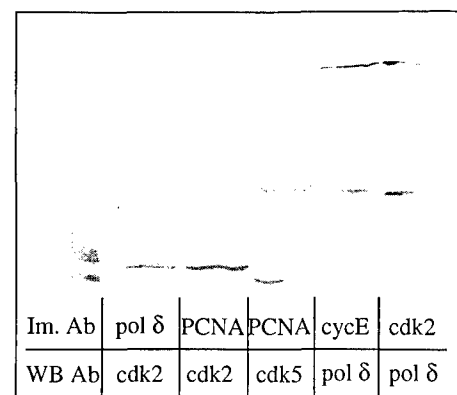


FIG. 12. Coimmunoprecipitation of pol  $\delta$  with members of the cdk-cyclin system. Molt 4 cells were lysed by sonication. About 10 mg of total protein was immunoprecipitated with the first antibody (Im. Ab) plus protein A-Sepharose and then Western blotted with a second antibody (WB Ab). The common band in the last three lanes is an artifact (IgG heavy chain).

the recombinant pol  $\delta$  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  $\delta$  preparations containing only the catalytic subunit have suggested that it is not PCNA-responsive (18, 19), whereas our previous studies of recombinant pol  $\delta$  expressed in vaccinia virus have indicated a weak response (2–3-fold stimulation). The baculovirus-expressed pol  $\delta$  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.

Studies of deletion mutants show that deletions (amino acids 2–249 or 186–321) in the  $\text{NH}_2$  terminus retain polymerase



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 NH<sub>2</sub>-terminal deletion mutants is consistent with the existence of a domain structure in which the NH<sub>2</sub>-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 NH<sub>2</sub>-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  $\delta$  is itself a substrate for cyclin-dependent protein kinases and that this is specific for the G<sub>1</sub> cdk-cyclins because other cdk-cyclin combinations were less effective in phosphorylating pol  $\delta$  when they were coexpressed in Sf9 insect cells. Although the *in vivo* kinase activity of cdk-cyclin overexpressed in Sf9 insect cells may not reflect actual cellular events in the mammalian cell cycle, the involvement of G<sub>1</sub> phase cdk-cyclins is consistent with our previous observations that pol  $\delta$  is phosphorylated *in vivo* during the cell cycle and is maximal near the G<sub>1</sub>/S transition (25). The primary structure of pol  $\delta$  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 G<sub>1</sub> to S phase of the cell cycle include the G<sub>1</sub> cyclins—three D type cyclins (D1, D2, D3) and cyclin E (26). Cyclin E expression is periodic, peaks at the G<sub>1</sub>/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 G<sub>1</sub> 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 G<sub>1</sub> phase. This reverses the growth-suppressive effects of pRb by releasing 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 G<sub>1</sub> 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  $\delta$  is the central enzyme in eukaryotic DNA replication and is tethered to DNA by a direct interaction with the PCNA clamp, which converts pol  $\delta$  from a distributive into a highly processive enzyme for DNA synthesis (31, 32). Thus, the finding that pol  $\delta$  is a substrate for the G<sub>1</sub> cdk-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  $\alpha$ -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  $\alpha$ -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  $\delta$  showed that the tertiary structure of pol  $\delta$  is not required for this interaction and that the binding region is

located in the NH<sub>2</sub>-terminal 249 residues of pol  $\delta$ . The NH<sub>2</sub>-terminus of yeast and mammalian pol  $\delta$  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  $\delta$  (5). The binding site of pol  $\delta$  for PCNA has been mapped to the N2 region (13). The data presented also provide the first evidence for complexes that involve pol  $\delta$  and the cdk-cyclins. The targeting of the cdks to a substrate has some precedence since the G<sub>1</sub> 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  $\delta$ . The present findings show that the interaction of pol  $\delta$  with cdk2 and cdk4 needs to be investigated further, in addition to the issue of the cellular role of phosphorylation of pol  $\delta$  by the cdk-cyclins.

There are many levels at which phosphorylation could affect pol  $\delta$  function other than the simple modulation of enzyme activity in a simple assay. This is apparent because physiologically pol  $\delta$  is part of a holoenzyme and part of an extended multiprotein complex. Current findings that p21, a potent inhibitor of G<sub>1</sub> cdks, and pol  $\delta$  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  $\delta$  is a substrate for the cyclin-dependent protein kinases. This was shown by the coexpression of baculovirus vectors for pol  $\delta$  with several different cdk-cyclin combinations in Sf9 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  $\delta$ , possibly triggering its phosphorylation at different sites or times of the cell cycle. Coimmunoprecipitation of pol  $\delta$  deletion mutants with cdk2 also established the site of interaction (Fig. 11). Although the regulation of pol  $\delta$  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  $\delta$  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  $\delta$  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  $\delta$  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  $\delta$  and for that matter other replication proteins. The putative kinase consensus sequences in pol  $\delta$  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  $\delta$ .

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# Identification of DNA replication and cell cycle proteins that interact with PCNA

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## ABSTRACT

The identity of DNA replication proteins and cell cycle regulatory proteins which can be found in complexes involving PCNA were investigated by the use of PCNA immobilized on Sepharose 4B. A column containing bovine serum albumin (BSA) bound to Sepharose was used as a control. Fetal calf thymus extracts were chromatographed on PCNA-Sepharose and BSA-Sepharose. The columns were washed and then eluted with 0.5 M KCl. The salt eluates were examined for the presence of both DNA replication proteins (Pol  $\alpha$ ,  $\delta$ ,  $\epsilon$ , PCNA, RFC, RFA, DNA ligase I, NDH II, Topo I and Topo II) and cell cycle proteins (Cyclins A, B1, D1, D2, D3, E, CDK2, CDK4, CDK5 and p21) by western blotting with specific antibodies. The DNA replication proteins which bound to PCNA-Sepharose included DNA polymerase  $\delta$  and  $\epsilon$ , PCNA, the 37 and 40 kDa subunits of RFC, the 70 kDa subunit of RPA, NDH II and topoisomerase I. No evidence for the binding of DNA polymerase  $\alpha$ , DNA ligase I or topoisomerase II was obtained. Of the cell cycle proteins investigated, CDK2, CDK4 and CDK5 were bound. This study presents strong evidence that PCNA is a component of protein complexes containing DNA replication, repair and cell cycle regulatory proteins.

## INTRODUCTION

The discovery of a stimulating factor for DNA polymerase  $\delta$  (1,2) that eventually led to its identification as proliferating cell nuclear antigen (PCNA) (3) stimulated major advances in our understanding of DNA synthesis at the replication fork. PCNA functions as a sliding clamp which endows pol  $\delta$  with a high degree of processivity (4). Studies of *in vitro* SV40 DNA replication have now led to a fuller understanding of the protein machinery required for the formation of a functional mammalian DNA replication fork in which DNA polymerase  $\delta$  (pol  $\delta$ ) and PCNA play a central role. The current model is one in which replication factor C (RFC, also known as activator-1), a complex of five subunits, first binds to the primer-template terminus and loads the PCNA onto the 3' hydroxyl end of the primer strand of the DNA primer-template in an ATP-dependent process. Following the formation of a RFC/PCNA complex, pol  $\delta$  is then

recruited to assemble an elongation complex that catalyzes DNA synthesis in the presence of deoxynucleotide triphosphates (5-9). Replication protein A (RPA), a ssDNA binding protein, is involved in both initiation and elongation, as it stimulates pol  $\delta$  activity in the presence of RFC and PCNA (6,10). A DNA helicase activity is essential to the replication machinery and serves mainly to unwind replication origins during the initiation phase of DNA replication and to separate parental DNA strands during the elongation phase. A helicase which is highly associated with pol  $\delta$  has been isolated (11). Recently, six human helicases have been purified to near homogeneity (12). Furthermore, a nuclear DNA helicase II (NDH II) has also been purified (13). Like the large T antigen of SV40, it was found to unwind both DNA and RNA. Molecular cloning of NDH II revealed a high homology to human RNA helicase A (14). Pol  $\alpha$ /primase is primarily involved in the synthesis of RNA primers plus short stretches of DNA primers on the lagging strand, while the actual elongation of the primers is performed by pol  $\delta$  in a process requiring polymerase 'switching' (15). A topoisomerase activity is also required, and studies using the SV40 system showed that either topoisomerase I or topoisomerase II is capable of removing positive supercoils ahead of the replication fork (16,17). The model of the protein assembly at the replication fork now resembles that of the well defined prokaryotic systems, requiring the presence of two pol  $\delta$  molecules (18).

A major area of research which is currently emerging is the exploration of the biochemical and genetic mechanisms by which cell cycle regulation of DNA synthesis is achieved. There have been rapid advances in delineating the existence of cell cycle proteins: these include the cyclins A and B, a family of G1 cyclins (E, D cyclins) and a family of cyclin dependent kinases (CDKs) (19,20). There is now evidence for the cell cycle control of mammalian DNA replication by the cyclin-CDK system (21). A number of studies point to the existence of protein-protein interactions of DNA synthesis proteins with cell cycle dependent protein kinases or cyclins, as well as the phosphorylation of DNA synthesis proteins by CDKs. DNA polymerase  $\alpha$  is phosphorylated in a cell cycle specific manner and is a substrate for p34<sup>cdc2</sup> (22,23). The RPA complex purified from HeLa cells or Manta cells is also phosphorylated in a cell cycle dependent manner by one or more members of cyclin/CDK2 family, and its phosphorylation has been shown to stimulate the initiation of SV40 DNA synthesis *in vitro* (24,25). Recent studies by Pan *et al.* (26) showed that both CDK2/cyclin A and DNA-dependent protein

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kinase phosphorylate the 34 kDa subunit of RPA. However, phosphorylated and unphosphorylated forms of RPA were equally active in SV40 DNA replication and nucleotide excision repair (26). Using immunoprecipitation and western blot experiments, Xiong *et al.* (27,28) showed combinatorial interactions of D type cyclins, cyclin-dependent kinases with PCNA and with p21. p21, also known as WAF1, CIP1 or Sdi1, is an inhibitor of the CDKs that control the initiation of the S phase of the cell cycle and DNA replication. The N-terminal region of p21 contains the CDK inhibitory domain whereas the C-terminal region contains a PCNA binding domain that leads to the inhibition of DNA synthesis (29).

In addition, both pol  $\delta$  and PCNA have been shown to be required for DNA repair (30). Thus, PCNA, through its interactions with elements of both the DNA replication apparatus and the cell cycle regulatory system, has emerged as an important locus for protein-protein interactions that may provide communication between DNA replication, DNA repair and cell cycle control. Definition of the number and nature of these protein-protein interactions will therefore be important. In this study, immobilized recombinant PCNA is used as a means for the isolation of proteins that bind to PCNA.

## MATERIALS AND METHODS

### Immunoblotting

After electrophoresis in 5–15% gradient gels, proteins were transferred to nitrocellulose membranes. Prestained protein standards (Sigma Chemical Co.) were used as molecular weight markers and provided visual confirmation of efficient transfer. The nitrocellulose blots were incubated with 3% BSA in phosphate-buffered saline as a blocking agent. The blot was then incubated with the primary monoclonal antibody at a final concentration of 5  $\mu$ g/ml or with a polyclonal antibody at ~1:500 dilution for 12 h at 25°C. After washing, the blot was incubated with biotinylated sheep anti-mouse immunoglobulin, followed by streptavidin-biotinylated peroxidase preformed complex. When polyclonal antibodies were used, the second antibody was anti-rabbit IgG biotinylated species-specific whole antibody instead of anti-mouse IgG. Color development was performed by incubation with 4-chloro-1-naphthol and hydrogen peroxide and was terminated with sodium azide.

Antibodies used were as follows: polyclonal antibodies against the p145, p40, p37 and p38 subunits of RFC (Dr J.Hurwitz, Memorial Sloan Kettering Cancer Center, NY); polyclonal antibodies against the p70 and p11 subunits of RPA and monoclonal antibody against the p34 subunit of RPA (Dr S.H.Lee, St. Jude's Children's Hospital, Memphis, TN); polyclonal antibodies to human topoisomerase I and II (ToPoGen Inc.); PCNA monoclonal antibody mAB19F4 (American Biotech. Inc., Plantation, FL); p21 monoclonal antibody (Santa Cruz); monoclonal antibody against DNA polymerase  $\epsilon$  (Dr J.E.Syvaaja, University of Oulu, Finland); monoclonal antibody against polymerase  $\alpha$  (American Type Culture Collection); DNA ligase I polyclonal antibody (Dr A.Tomkinson, University of Texas Health Science Center at San Antonio); rabbit antiserum to mouse cyclins D1, D2 and D3 (Dr C.J.Sherr, St. Jude's Children's Hospital, Memphis, TN); monoclonal antibodies to cyclins A, B1 and E and to both CDK2 and CDK5 (Dr E.Lee, Massachusetts General Hospital, Boston); polyclonal antibody to CDK4 (Dr

S.Hanks, Vanderbilt University, TN); monoclonal antibody to NDH II (Dr F.Grosse, Heinrich-Pette Institute for Experimental Virology and Immunology, Germany).

### Preparation of PCNA and BSA affinity columns

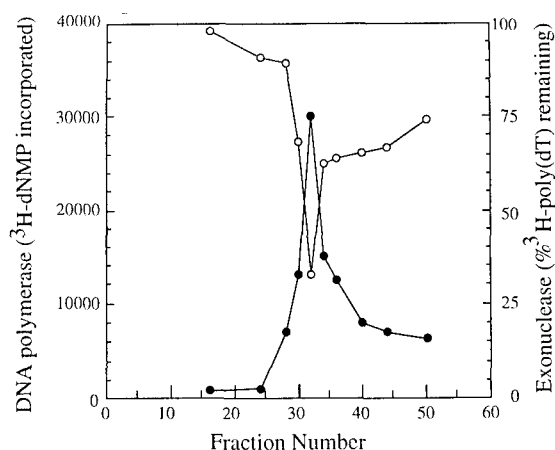
Recombinant PCNA was overexpressed in *E.coli* and purified to homogeneity as previously described (31). Activated CH-Sepharose, which allows coupling to a six carbon spacer arm, was obtained from Pharmacia LKB Biotech. Purified recombinant PCNA (20 mg in 50 ml) was dialyzed against 2 l of 0.1 M NaHCO<sub>3</sub>/0.5 M NaCl, pH 8.0 (four changes at 6–8 h intervals). Activated CH-Sepharose 4B (2 g) was suspended in 30 ml of cold 1 mM HCl. The gel was washed in a column with 400 ml of cold 1 mM HCl followed by 200 ml of 0.1 M NaHCO<sub>3</sub>/0.5 M NaCl, pH 8.0. The coupling reaction was performed by addition of PCNA (20 mg, 50 ml in 0.1 M NaHCO<sub>3</sub>/0.5 M NaCl, pH 8.0) to the washed gel. The suspension was rotated end over end for 18 h at 4°C. The protein content of the supernatant was checked at intervals by absorbance at 280 nm to monitor the progress of the reaction. After an overnight reaction it was estimated that ~2 mg PCNA was bound/ml of gel. The suspension was centrifuged and the supernatant discarded. The gel was then suspended in 50 ml of 1 M ethanolamine, pH 9.0 for 18 h to block unreacted groups. The gel was washed with 200 ml of 1 M NaCl–0.1 M sodium acetate, pH 6.0 followed by 200 ml of 1 M NaCl–0.1 M Tris–HCl, pH 8.0 and 200 ml of 0.5 M NaCl–0.1 M Tris–HCl, pH 8.0. The gel was equilibrated with TGEED buffer (50 mM Tris–HCl, pH 7.8, 10% glycerol, 0.5 M EDTA, 0.1 mM EGTA and 1 mM dithiothreitol). A control column in which bovine serum albumin (BSA) was substituted for PCNA was prepared by the same procedure. In this case ~8.4 mg of BSA were coupled to 5 ml of activated CH-Sepharose 4B. All operations were performed at 4°C.

### Preparation of calf thymus extracts

Frozen fetal calf thymus tissue (10 g) was used to prepare 50 ml of tissue extract. The tissue was homogenized in a blender with 50 ml of lysis buffer (50 mM Tris–HCl, pH 7.8, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM MgCl<sub>2</sub>, 0.25 M sucrose, 10% glycerol, 10 mM KCl, 0.1 mg/ml soybean trypsin inhibitor, 1 mM benzamidine, 0.1 mg/ml bacitracin, 1 mg/ml pepstatin A, 1 mg/ml leupeptin, 0.2 mM phenylmethyl sulfonyl fluoride and 10 mM sodium bisulfite). The homogenate was centrifuged for 1 h at 15 000 g. The extract was then centrifuged at 100 000 g for 1 h. All operations were performed at 4°C.

### Affinity chromatography

Affinity chromatography was performed by mixing 50 ml of calf thymus extract with the PCNA-Sepharose (5 ml) and rotating the suspension end over end for 2 h. The gel was then packed into a column and washed with 100 ml of 50 mM KCl in TGEED buffer and eluted with 0.5 M KCl in TGEED buffer. Fractions of 0.3 ml were collected. Control experiments in which immobilized BSA was used as the column support was performed in parallel.



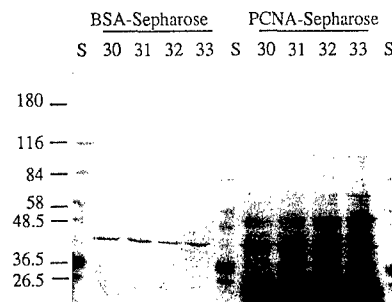
**Figure 1.** Affinity chromatography of calf thymus extract on PCNA-Sepharose. Crude calf thymus extract (50 ml) was rotated end over end with 5 ml of PCNA-Sepharose for 2 h. The gel was then packed onto a column, washed with 100 ml of 50 mM KCl TGEED buffer and stripped with 0.5 M KCl in TGEED (Materials and Methods). Fractions (0.3 ml) were collected and assayed for polymerase activity using poly dA/oligo dT as a template in the presence of PCNA (closed circles) and for exonuclease activity using [ $^3\text{H}$ ]dT<sub>50</sub> (open circles) as previously described (1).

## RESULTS

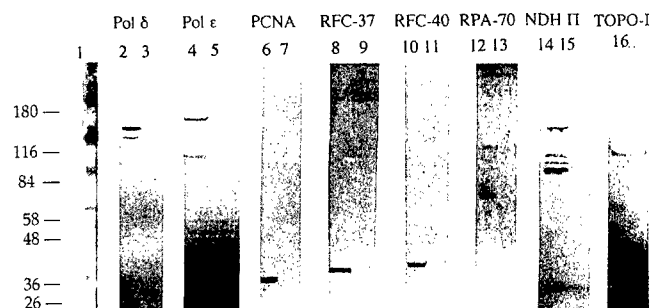
### Affinity purification of DNA replication complex on PCNA-Sepharose

Calf thymus extracts were chromatographed on PCNA-Sepharose as described in Materials and Methods. The eluted fractions were assayed for DNA polymerase  $\delta$  activity using poly dA/oligo dT as a template and for exonuclease activity using [ $^3\text{H}$ ]dT<sub>50</sub>. Preliminary experiments established that pol  $\delta$  was bound to the column and was eluted at ~250 mM KCl when a KCl gradient was applied (not shown). A standard protocol was then used in which the bound material was eluted with 0.5 M KCl (Materials and Methods). No activity was detected in the flow through fractions, and both DNA polymerase and 3'→5' exonuclease activities eluted together and were only detected in the eluate from the PCNA column (Fig. 1). (No activity was bound to a control BSA-Sepharose column when tested with a calf thymus extract.) The SDS-PAGE profile of polypeptides bound to PCNA-Sepharose and eluted with 0.5 M KCl is shown in Figure 2. A number of protein bands ranging from 18 to 210 kDa were present in the eluate from the PCNA-Sepharose column. The gels shown in Figure 2 were deliberately overloaded to show the presence of all bound polypeptides. Comparison with calf thymus extracts chromatographed on a control BSA column showed that there were several bands with two prominent polypeptides (110 and 43 kDa) that also adhered to the BSA column. Experiments were also performed in the presence of 1 mM ATP, since the interaction of PCNA with RFC is ATP dependent (6,7). However, the compositions of the polypeptides that were eluted were the same in the presence or absence of ATP (not shown).

The 0.5 M KCl eluate from the PCNA column was systematically tested for the presence of other replication proteins by western blotting. Representative blots are shown in Figure 3 from a number



**Figure 2.** SDS-gel electrophoresis of proteins bound to PCNA and BSA affinity columns. Fractions 30–33 from the BSA-control column and the PCNA column were subjected to SDS-PAGE and stained for protein. From left to right are fractions 30–33 from the BSA control column, followed by fractions 30–33 from the PCNA column. The latter fractions correspond to the peak of pol  $\delta$  activity. S: pre-stained protein standards (Sigma Chem. Co.,  $\alpha$ -2-macroglobulin, 180 kDa;  $\beta$ -galactosidase, 116 kDa; fructose 6-phosphate kinase, 84 kDa; pyruvate kinase, 58 kDa; fumarase, 48 kDa; lactate dehydrogenase, 36 kDa; triosephosphate isomerase, 26 kDa).



**Figure 3.** Immunoblots against DNA replication proteins bound to the PCNA affinity column. Lane 1: molecular weight markers with weights in kDa as indicated. Lanes 2, 4, 6, 8, 10, 12, 14 and 16 each illustrate fraction 32 from the PCNA column western blotted against pol  $\delta$ , pol  $\epsilon$ , PCNA, RFC-37, RFC-40, RPA-70, NDH II and topoisomerase I antibodies, respectively. Lanes 3, 5, 7, 9, 11, 13 and 15 depict fraction 32 eluted from the BSA column and blotted against the same antibodies. Immunoblots were performed as described in Materials and Methods.

of individual experiments. The catalytic polypeptides of pol  $\delta$  and  $\epsilon$  were found to be present by western blot analysis using specific antibodies to pol  $\delta$  (Fig. 3, lane 2) and pol  $\epsilon$  (Fig. 3, lane 4). The pol  $\delta$  antibody immunoblotted a band of 125 kDa, and the pol  $\epsilon$  antibody detected a band of 145 kDa, in agreement with the previously reported molecular mass of pol  $\epsilon$  isolated from calf thymus extracts (32,33). DNA polymerase  $\alpha$  was not detected in the eluates by western blotting. PCNA itself was detected in the eluate as a 31 kDa band (Fig. 3, lane 6). This could be attributed either to stripping from the column, given that PCNA is trimeric, or due to an interaction of calf thymus PCNA subunits with immobilized PCNA. Antibodies against the individual 145, 40, 37 and 38 kDa subunits of RFC revealed positive results only for RFC-37 and RFC-40 (Fig. 3, lanes 8 and 10). Western blotting was also performed using antibodies to the 70, 34 and 11 kDa subunits of RPA. A positive blot was obtained for the 70 kDa subunit (Fig. 3, lane 12). These results indicate that both RFC and RPA are bound to the PCNA column.

The nuclear DNA helicase II (NDH II) enzyme was readily detected in the 0.5 M KCl eluate by immunoblotting as a 130 kDa band and three other bands of lower molecular weight ranging from 100 to 84 kDa (Fig. 3, lane 14). The lower molecular weight bands are likely to be proteolytic products. It has been reported that limited tryptic digestion of recombinant NDH II produced active helicases with molecular masses of 130 and 100 kDa (14). The presence of topoisomerase I and II, and ligase I was also tested for by immunoblotting. Only topoisomerase I was detected in the 0.5 M KCl eluate as a 100 kDa band (Table 1 and Fig. 3, lane 16). Examination of the fractions eluted from the BSA column by western blot yielded negative results for all of the above.

It is shown in this report that, in addition to pol  $\delta$ , pol  $\epsilon$ , RFC, RPA, PCNA, nuclear DNA helicase II (NDH II) and topoisomerase I are also present in the 0.5 M salt eluate from the PCNA column (Table 1). This collective elution of various constituents of the DNA replication machinery provides direct evidence for strong interactions between these proteins that directly or indirectly involve PCNA.

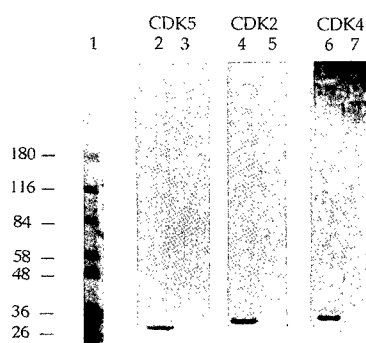
**Table 1.** Proteins which bind to immobilized PCNA as determined by western blotting of column eluates

| Protein        | PCNA column | BSA column | Protein   | PCNA column | BSA column |
|----------------|-------------|------------|-----------|-------------|------------|
| Pol $\alpha$   | —           | —          | CDK2      | +           | —          |
| Pol $\delta$   | +           | —          | CDK4      | +           | —          |
| Pol $\epsilon$ | +           | —          | CDK5      | +           | —          |
| PCNA           | +           | —          | Cyclin A  | —           | —          |
| RFC-37         | +           | —          | Cyclin B1 | —           | —          |
| RFC-38         | —           | —          | Cyclin D1 | —           | —          |
| RFC-40         | +           | —          | Cyclin D2 | —           | —          |
| RFC-145        | —           | —          | Cyclin D3 | —           | —          |
| RPA-11         | —           | —          | Cyclin E  | —           | —          |
| RPA-34         | —           | —          | p21       | —           | —          |
| RPA-70         | +           | —          |           |             |            |
| Ligase I       | —           | —          |           |             |            |
| NDH II         | +           | —          |           |             |            |
| Topo I         | +           | —          |           |             |            |
| Topo II        | —           | —          |           |             |            |

Presence or absence in column eluates as determined by western blotting is shown as + or — respectively.

### Binding of cell cycle regulatory proteins to PCNA-Sepharose

The PCNA-Sepharose column fractions containing peak pol  $\delta$  activity were also tested for the presence of proteins involved in cell cycle regulation. Some principal components were detected through a series of western blots. Positive blots were obtained for cyclin dependent kinase 2 (CDK2), CDK4 and CDK5 (Fig. 4). Neither cyclins nor p21 were detected in the eluates (Table 1).



**Figure 4.** Immunoblots of cell cycle regulatory proteins eluting from the PCNA column. Lane 1: prestained protein markers with weights in kDa as shown. Lanes 2, 4 and 6: cell cycle regulatory proteins, CDK5 (31 kDa), CDK2 (33 kDa) and CDK4 (34 kDa), were bound by and eluted from the PCNA column. Shown also are the corresponding western blots of fraction 32 (lanes 3, 5 and 7) from the control BSA column where no CDK proteins were detected.

### DISCUSSION

Recombinant PCNA was immobilized on Sepharose 4B and was systematically used to investigate the binding of replication and cell cycle proteins from fetal calf thymus extracts by affinity chromatography. As expected, tests for the binding of pol  $\delta$  both by activity and by western blotting confirmed that it was bound. In addition, examination of the protein bands present in the peak of the bound fractions showed the presence of multiple polypeptide components. Some of these may represent adventitious binding although it may be noted that only a few bands with two prominent polypeptides of 110 and 43 kDa were observed in the eluates from the same fractions in the BSA control column. In addition to pol  $\delta$ , several other replication proteins were bound to the affinity column. Specifically, the binding of PCNA, pol  $\epsilon$ , RFC, RPA, NDH II and topoisomerase I was detected. The binding of pol  $\delta$  and RFC was anticipated, since it is known that these proteins interact with PCNA (6). The collective elution of PCNA, pol  $\delta$ , RFC and RPA was striking, in view of the fact that these are all components of the proposed replication complex involved in leading and lagging strand DNA synthesis (16,34).

The interaction of PCNA with pol  $\epsilon$  is still controversial. Lee *et al.* (7) reported that RPA, RFC and PCNA could overcome the salt inhibition of DNA polymerase  $\epsilon$ . Chui and Linn (35) observed strong inhibition of DNA polymerase  $\epsilon$  by salt and found that this inhibition could not be completely overcome by RFC, RPA and PCNA which had little, if any, effect on the processivity of DNA polymerase  $\epsilon$ . A major significance of these present findings is that they reveal a definite interaction, either direct or indirect, between pol  $\epsilon$  and PCNA, thus linking pol  $\epsilon$  to the replication fork. Navas *et al.* (36) have identified the DUN2 gene of *Saccharomyces cerevisiae* as DNA polymerase  $\epsilon$  (Pol2). Mutations in the DUN2 gene displayed properties that suggest that pol  $\epsilon$  has a role as a sensor of replication blocks and some forms of DNA damage, thus linking the DNA replication machinery to the S phase checkpoint (36). However, in *Schizosaccharomyces pombe* it was demonstrated that *cdc 20<sup>+</sup>* encodes the catalytic subunit of pol  $\epsilon$  and the gene product is required for chromosomal replication but not for the S phase checkpoint (G.D'Urso, personal communication). A pol  $\epsilon$  holoenzyme

consisting of pol  $\epsilon$ , PCNA, RPA and RFC may function on the lagging strand of the replication fork (37). This could provide a mechanism for proofreading in the lagging strand because pol  $\epsilon$ , unlike pol  $\alpha$  and similar to pol  $\delta$ , has a 3'→5' exonuclease activity (38,39). Zlotkin *et al.* (40), using UV crosslinking of nascent cellular DNA and immunoprecipitation, showed that DNA polymerase  $\epsilon$  is essential in cellular nuclear DNA replication. Studies of *S.pombe cdc 20*<sup>+</sup> mutants showed that pol  $\epsilon$  plays an important role in the elongation of nascent DNA chains, suggesting that pol  $\epsilon$  participates in the switch from primer extension by pol  $\alpha$  primase to leading strand synthesis (G.D'Urso, personal communication).

The presence of topoisomerase I in the eluates from PCNA-Sepharose is interesting, as it functions to relieve positive superhelicity during replication (41). The positive immunoblots for nuclear DNA helicase II (NDH II) (Fig. 3) in the peak eluates reveals the possibility of a complex involving the helicase enzyme. This is interesting in view of the fact that there may be differences between viral and host chromosomal DNA replication, so that there may be limitations of the *in vitro* SV40 replication system as a model system. Recently, a human nuclear protein that interacts with the constitutive transport element (CTE) of simian retrovirus was identified as RNA helicase A (42). The latter has a high degree of similarity to NDH II which also has RNA helicase activity (14). RNA helicase A was found to be concentrated in the nucleus in normal cells (42). It was also identified as an inherent shuttling protein that interacts with CTE *in vitro* and associates with CTE in its trafficking from the nucleus to the cytoplasm *in vivo* (42). Whether the presence of NDH II is physiologically relevant in the DNA replication complex purified from the PCNA-Sepharose affinity column is still an open question.

These findings are in concert with, and support other studies which have led to the partial purification of macromolecular complexes using conventional protein purification methods (43–45). Immobilized T4 bacteriophage gene 32 protein has been successfully used to characterize and isolate the interacting components of the T4 replication complex (46). The existence of a physical assembly of a mammalian replication complex, the 'replisome' has been inferred from studies of the prokaryotic system, and by consideration of the requirement for processive and uninterrupted DNA synthesis during replication. Evidence for the existence of such complexes is still fragmentary, and is based on the isolation of partially purified protein fractions by conventional methods that contain a number of replication proteins that can functionally replicate viral DNA (18,34). The advantage of an affinity chromatography approach over conventional methods is that it is rapid and is based on protein-protein interactions. This greatly lessens concerns that these complexes may be artifacts of the isolation methods. The current studies using affinity chromatography demonstrates the existence of a system of protein-protein interactions involving the replication proteins that could provide the molecular basis for the formation of a replication complex.

Since PCNA has been reported to bind to the cyclins (27,28), the binding of the cyclins and associated cyclin dependent kinases to the PCNA-Sepharose was also tested. Blots for the cyclin dependent kinases were positive in the case of CDK2, CDK4 and CDK5, while tests for associated cyclins were negative. This is surprising, since both CDKs and cyclins have been reported to associate in quaternary complexes with PCNA and p21 (27,28). It may be that tissue levels of the cell cycle proteins in calf thymus

were too low for detection (28); also, expression of p21 occurs as a result of DNA damage (47). In general, a failure to observe any given protein in our experiments does not preclude its involvement in a replication complex, since the experiments are dependent on the sensitivity of the antibodies, the strength of the association and the stability of the given protein-protein interaction during purification. Recent studies have described several intermolecular interactions between cell cycle proteins and the replication proteins that may be of mechanistic significance in the cell cycle regulation of DNA replication. These include the demonstration that cyclin A or cyclin E-CDK complexes can trigger initiation of DNA synthesis (48), and that cyclin A is required for *in vitro* DNA replication (49). The phosphorylation of replication proteins by cyclin/CDKs has been demonstrated in the case of HSSB-p34 (50). In the latter case, cyclinA/CDK2 but not cyclinE/cdk2 was shown to phosphorylate HSSB. This critical observation indicates that targeting of the CDK2 to HSSB is necessary for phosphorylation to occur. Pol  $\delta$  was reported to be phosphorylated *in vivo* (51). Recently, pol  $\delta$  was found to be phosphorylated by cyclin/CDKs (Zeng and Lee, unpublished observations).

These studies which show the binding of both replication and cell cycle proteins to PCNA provides additional support for a central role of PCNA in the linkage of the processes of DNA replication and cell cycle regulation via protein-protein interactions. In addition, these findings demonstrate the existence of protein-protein interactions between DNA replication proteins and cell cycle regulatory proteins. This interaction of the cyclin-CDK cell cycle regulatory proteins with polymerases and elements of the DNA replication system could be important in understanding the cell cycle control of DNA replication. The findings that PCNA exhibits interaction with multiple protein partners suggest that it may have an important role in the formation of macromolecular complexes involved in DNA replication and its cell cycle control. For this reason, immobilized PCNA may be a particularly useful tool for the isolation of these complexes, a view that is supported by the studies reported. A molecular basis for the multiple protein partners with which PCNA interacts is now emerging, in the form of the recent identification of a short peptide consensus sequence which is found in several PCNA binding proteins including p21 (52), Fen1 (53) and cdc27 (54). There remain major questions as to the number and nature of the interacting protein partners of PCNA, and the mechanisms of how these interactions provide the necessary functional and regulatory outcomes in DNA replication and repair.

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