

## CHARACTERIZATION OF THE HUMAN DNA POLYMERASE $\delta$ CATALYTIC SUBUNIT EXPRESSED BY A RECOMBINANT BACULOVIRUS

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### ABSTRACT

The catalytic subunit of human DNA polymerase (pol)  $\delta$ , p125, was expressed in recombinant baculovirus-infected insect cells, separated from a baculovirus-encoded DNA polymerase, and was purified to homogeneity by affinity trapping with a histidine-octapeptide at the C-terminus of p125 as the ligand. Purified p125 showed DNA polymerase activity resembling conventionally purified calf thymus pol  $\delta$ . However, the two differed in four ways: 1) the specific activity of recombinant p125 was one quarter of the calf thymus pol  $\delta$ ; 2) the recombinant p125 was relatively resistant to aphidicolin; 3) the apparent  $K_m$  for dTTP of the recombinant p125 was estimated at 33  $\mu$ M, 15-fold the value for calf thymus pol  $\delta$ ; and 4) the recombinant p125 was not stimulated by recombinant PCNA, while activity of calf thymus pol  $\delta$  increased 150-fold in response. Furthermore, PCNA did not stimulate either the p125 incubated with p50, a small subunit of pol  $\delta$ , or co-expressed with p50 in insect cells. The full length recombinant p125 migrated slightly faster than pol  $\delta$  from human cell lines, Jurkat or HeLa, upon SDS-polyacrylamide gel electrophoresis, suggesting a post-translational modification. The results indicate that *in vivo* assembly of the fully active complex of pol  $\delta$  requires factors in addition to p125 and p50 subunits, and/or a post-translational modification of p125.

Key Words: DNA polymerase  $\delta$  catalytic subunit, DNA polymerase  $\delta$  small subunit, proliferating cell nuclear antigen, recombinant baculovirus, aphidicolin

Abbreviations: CBB, Coomassie brilliant blue; Cdc, cell division cycle; cdk, cyclin dependent kinase; *E. coli*, *Escherichia coli*; HRPO-, horse radish peroxidase conjugated-; ID50, dose of reagent that inhibits the reaction by 50%; m.o.i., multiplicity of infection; MoMLV, moloney murine leukemia virus; PCNA, proliferating cell nuclear antigen; PMSF, phenylmethylsulfonyl fluoride; pol, DNA polymerases; RFC, replication factor C; RT-PCR, reverse transcribed polymerase chain reaction.

### INTRODUCTION

In eukaryotic cells, DNA polymerases (pol), designated as pol  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\epsilon$  have been identified.<sup>1)</sup> Recently, pol  $\zeta$ , an error-prone DNA polymerase,<sup>2)</sup> and pol  $\eta$ <sup>3)</sup>, an error free enzyme which is the target of Xeroderma pigmentosum variant type were described. In addition, two kinds of DNA polymerase-like enzymes, terminal deoxynucleotidyl transferase<sup>4)</sup> and

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telomerase,<sup>5)</sup> also appear at a particular stage of development or tissues.<sup>6)</sup> Of the different forms, pol  $\alpha$ ,  $\delta$  and  $\epsilon$  are implicated in chromosomal DNA replication in S phase. Pol  $\alpha$ , complexed with primase, works in the initiation of both leading and lagging strands by forming initiator RNA-DNA fragments.<sup>7)</sup> Pol  $\delta$  elongates both strands using primer DNA.<sup>8)</sup> Genetic analysis of yeast showed pol  $\epsilon$  to be essential for DNA replication and survival,<sup>9)</sup> although its catalytic domain of pol  $\epsilon$  is reported to be dispensable for survival.<sup>10)</sup> At present, pol  $\delta$  and  $\epsilon$  are thought to function in the elongation fork of DNA replication, but the exact mechanisms remain to be clarified.

In the present study, we have focused on pol  $\delta$ . By conventional purification, it is isolated as a heterodimer composed of a catalytic subunit of 125 kDa (p125) and a small subunit of 50 kDa polypeptide (p50), for example, from calf thymus,<sup>11)</sup> human placenta<sup>12)</sup> or HeLa cells.<sup>13)</sup> A single polypeptide form of pol  $\delta$  has also been isolated from *Drosophila melanogaster*,<sup>14)</sup> and both single and heterodimeric forms were obtained from mouse cells.<sup>15)</sup> In *Saccharomyces cerevisiae*, a third subunit of pol  $\delta$  was identified.<sup>16)</sup> and in *Schizosaccharomyces pombe*, a number of additional subunits have been found<sup>17)</sup> Recently, a third subunit of mammalian pol  $\delta$ , termed p66, was reported in the homology to the Cdc27 subunit of *Schizosaccharomyces pombe* polymerase  $\delta$ .<sup>18)</sup> Thus, the subunit composition of pol  $\delta$  might be more complex than hitherto thought.

In addition to DNA polymerizing activity, p125 acts as a 3'-5' exonuclease, which contributes to its high fidelity.<sup>19)</sup> Besides its own subunits, pol  $\delta$  requires a number of accessory molecules to exert its full activity. Proliferating cell nuclear antigen (PCNA) forms a doughnut-shaped trimer with a 3.5 nm hole<sup>9)</sup> and works as the sliding clamp that improves processivity.<sup>9)</sup> It also requires replication factor C (RFC) as a clamp loader.<sup>9)</sup>

Recently, cDNAs of both catalytic and small subunits of human pol  $\delta$  were cloned. The former is a 3.5 kb sequence that encodes a polypeptide of 1107 amino acid residues.<sup>20)</sup> The primary structure of human pol  $\delta$  shares conserved regions not only with pol  $\delta$  from yeast,<sup>20)</sup> but also with DNA polymerases of human DNA viruses<sup>20)</sup> and eukaryotic pol  $\alpha$ .<sup>20)</sup> The catalytic subunit of pol  $\delta$  has been expressed using insect cells,<sup>21,22)</sup> *Escherichia coli* (*E. coli*),<sup>23)</sup> and mammalian cells<sup>24)</sup> using recombinant viruses or plasmids. Although active recombinant proteins have been obtained, a number of questions remain unanswered regarding low specific activity and lack of stimulation by PCNA. In the present study, characteristics of recombinant human pol  $\delta$  p125 expressed in insect cells were compared with those of pol  $\delta$  purified from calf thymus.

## MATERIALS AND METHODS

*Materials* - Primers for reverse transcription of mRNA and PCR clonings were synthesized by Amersham Pharmacia Biotech UK Limited (Buckinghamshire, UK). Plasmid pUC18, a GST fusion expression vector pGEX-4T-1, poly(dA-dT), poly(dA)-oligo(dT), dATP, dTTP and [*methy*l-<sup>3</sup>H]dTTP were purchased from the same source. A cDNA library prepared from human placenta was a gift of Medical & Biological Laboratories Co., Ltd. (MBL, Nagoya, Japan). *Taq* DNA polymerase and *LA taq* DNA polymerase were from Takara Shuzo Co. (Kyoto, Japan), *pfu* DNA polymerase from Stratagene (La Jolla, CA, USA) and MoMLV reverse transcriptase from Promega Co. (Madison, WI, USA). Bacmid and transfer vectors, pFastBac 1 and pFastBacHb, were from Gibco BRL Co. (Rockville, MD, USA). The pFastBac 1 was modified to express recombinant protein with an additional 8 histidine-tag at the C-terminus by inserting 5'-GAGCTAGCACATCACCACCATCACCATCATCACTAAGCT-3' between *Xho*I and *Hind*III

sites (pFastBac-c8his). Restriction enzymes were from New England Biolabs (Hertfordshire, UK). Cobalt-Sepharose 6B was from Clontech (Palo Alto, CA, USA) and phosphocellulose from Whatman Co. (Springfield Mill, UK). Grace's insect medium was from Gibco Lifetechnologies Inc. (Rockville, MD, USA). Homogeneously purified calf thymus DNA polymerase  $\delta^{25}$  was a generous gift of Dr. Toshiki Tsurimoto at the Nara Institute of Science and Technology (Nara, Japan).

*Recombinant baculoviruses encoding p125, p50, and PCNA* - cDNAs coding for full length of p125, p50 and PCNA were isolated from the cDNA human placenta library by PCR cloning. Primers were designed based on the reported sequences of human p125,<sup>20</sup> human p50<sup>26</sup> and human PCNA.<sup>27</sup>

The p125 cDNA (nucleotides 30-3,420) was amplified by PCR using a primer set, 5'-TGTTTGAAGCGGGATGGATG-3' and 5'-TATTAATTCTCCCTGGTCCC-3', covering the whole coding sequence. The PCR product, purified with a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), was inserted into the *Sma*I site of pUC18 after blunting. The coding sequence was excised with *Bam*HI and *Eco*RI and inserted into a transfer vector, pFastBac1. After sequencing, this transfer vector (pFastBac-p125) was corrected for the sequence involved in the core domains according to the genomic sequence by Chung *et al.*<sup>31</sup> as described in the RESULTS. Then the corrected pFastBac-p125 was transfected into an *E. coli* strain, DH10bac competent cells (Gibco BRL Co.), containing both bacmid and a helper plasmid. Transposition of a sequence coding p125 to the bacmid occurs during incubation. A single colony from DH10bac containing the recombinant bacmid, designated as bacmid-p125, was cultured in 2 ml of LB medium at 37°C overnight, and the bacmid-p125 DNA was isolated by alkaline miniprep. After the insert size was confirmed by PCR, it was transfected into Sf9 insect cells for the expression of full length of p125. Alternatively, the coding sequence of p125 was introduced into another bacmid to express protein carrying a histidine-octapeptide at the C-terminal. Full length p125 (43-3,363 nucleotides) without a stop codon was amplified by PCR using the corrected pFastBac-p125 as a template and a primer set, 5'-ATGGATGGCAAGCGGCCG-3' and 5'-CCAGGCCTCAGGTCCAGG-3'. Reaction was carried out for 10 cycles with high fidelity *pfu* DNA polymerase. The bacmid-p125-c8his, encoding p125 carrying a histidine-octapeptide at the C-terminal, was constructed with this PCR product.

The cDNA for p50 (55-1,504 nucleotides) was amplified by PCR using a primer set, 5'-ACCAAGCAAGTCAGGAGTGT-3' and 5'-AACCACTTTTTTGGACTCAGGG-3', cloned in pUC18, excised, and then transferred to a transfer vector as described for p125. We used pFastBacHTb for this purpose to make a product carrying a histidine hexamer at the N-terminal. Transposition of the coding region to the bacmid was carried out as described above for p125, and a recombinant bacmid, bacmid-p50-n6his, was obtained

The cDNA for PCNA (66-1,102 nucleotides) was cloned by PCR using a primer set, 5'-AGACTTTCCTCCTTCCCGC-3' and 5'-CCAGATCAGACTTTGGACTTT-3', inserted into pUC18, excised, inserted into another transfer vector, pFastBac 1, to express a full length protein without any histidine-tag. By transposition into a bacmid as above, a recombinant bacmid, bacmid-PCNA, was obtained.

The PCR conditions were: 94°C, 1 min; 58°C, 1 min; 72°C, 3 min; 35 cycles for p125, and 94°C, 1 min; 58°C, 1 min; 72°C; 1 min; 35 cycles for p50 and PCNA, with LA *taq* DNA polymerase. Nucleotide sequences of cloned cDNA were determined using a DNA sequencer, ABI PRISM™ 377 (Applied Biosystem, Inc., Foster City, CA, USA) with appropriate synthetic primers.

*Transfection of Sf9 cells with recombinant bacmid DNA* - The three kinds of bacmid DNA encoding p125, p50, and PCNA were transfected into Sf9 cells according to the supplier's in-

struction manual, in the form of DNA-lipid complex (Gibco BRL Co.). The baculoviruses (designed as Acp125, Acp50, AcPCNA respectively) in culture medium, harvested after 7 days incubation, were infected with Sf9 cells growing in culture flask (75 cm<sup>2</sup>) to amplify the virus titer to 10<sup>8</sup> plaque forming units/ml. Sf9 cells, grown to confluence in culture flasks (75 cm<sup>2</sup>), were infected with baculoviruses at a multiplicity of infection (m.o.i.) of 10. In some experiments, baculovirus harbouring p125 or p50 carrying a histidine tag at the C-terminal or N-terminal (designed as Acp125-c8his and Acp50-n6his) were co-infected into Sf9 cells simultaneously at an m.o.i. of 5. Cells were harvested 48 hours postinfection by centrifugation at 1200 × g, and washed twice with ice-cold Tris-buffered saline (25 mM Tris-HCl, 0.15M NaCl, pH 7.5).

*Purification of recombinant p125 carrying a histidine-tag* - The cells (8 × 10<sup>7</sup>) infected with Acp125-c8his were harvested from 7 flasks, resuspended in 7 ml buffer A (50 mM Tris-HCl, 2 mM EDTA, 10 mM sodium bisulfite, 1 mM phenylmethyl-sulfonyl fluoride (PMSF), 1 mM dithiothreitol, 0.2 M sucrose, pH 7.5) and sonicated on ice three times, each for 10 sec at 50 W. After centrifugation at 25,000 × g for 10 min, the resulting supernatant was loaded onto a phosphocellulose column (1 × 5 cm, P1, Whatman) equilibrated with buffer A. After washing with buffer A, the column was eluted with 0.2 and 0.5 M NaCl in buffer A, successively. All fractions were subjected to Western blotting with an anti-p125 rabbit polyclonal antibody, and the positive fractions were pooled and dialyzed against 2 liters of buffer B (20 mM Tris-HCl, 1 mM PMSF, 0.1 M NaCl, 0.2 M sucrose, pH 8.0) for 2 h at 4°C. The dialyzed fraction, supplemented with 2 mM imidazol, was applied to a cobalt-Sepharose 6B column (1 × 5 cm) equilibrated with buffer B. The column was washed with buffer C (20 mM Tris-HCl, 1 mM PMSF, 0.25 M NaCl, 15 mM imidazol, pH 7.0), and then eluted with buffer D (20 mM Tris-HCl, 0.1 M NaCl, 50 mM imidazol, pH 8.0). Fractions of 2 ml were collected and subjected to SDS-PAGE followed by Western blotting. After dialysis against buffer E (50 mM Tris-HCl, 2 mM 2-mercaptoethanol) containing 50% glycerol, fractions were assayed for DNA polymerase  $\delta$  activity.

*Purification of recombinant p50 carrying a histidine-tag* - The cells (4 × 10<sup>7</sup>) infected with Acp50-n6his were pelleted and lysed, and an extract was made by the same procedures as described for p125. The extract (4 ml) was loaded onto a phosphocellulose column equilibrated with buffer A. Then, pass-through fractions containing expressed p50 were collected, mixed with 2 mM imidazol, and then applied to a cobalt-Sepharose 6B column (1 × 5 cm) equilibrated with buffer B. After washing the column with buffer C, the proteins were eluted with buffer D, and 2 ml fractions were collected. After dialysis against buffer E containing 50% glycerol, fractions were subjected to SDS-PAGE followed by Western blotting using an anti-penta histidine oligopeptide mouse monoclonal antibody (QIAGEN).

*Purification of recombinant PCNA* - Cells (4 × 10<sup>7</sup>) infected with the AcPCNA were collected, lysed, and extracted using the same procedures as described above. The extract (4 ml) was dialyzed against buffer E containing 50 mM NaCl, then loaded onto a FPLC HR 5/5 Mono Q column (Amersham Pharmacia Biotech) equilibrated with buffer E containing 50 mM NaCl. Proteins adsorbed to the column were eluted with a linear gradient (25 ml) of NaCl from 50 to 500 mM in buffer E. Fractions (1 ml) were collected, dialyzed against buffer E containing 50% glycerol, and subjected to SDS-PAGE followed by the Western blotting using an anti-PCNA mouse monoclonal antibody (MBL).

*Assays of DNA polymerase activity* - Pol  $\delta$  activity was measured in a reaction mixture (25  $\mu$ l) containing 40 mM HEPES-KOH (pH 6.5), 5 mM MgCl<sub>2</sub>, 2 mM DTT, 0.03% Triton X-100, 2% glycerol, 80  $\mu$ g/ml bovine serum albumin, 50  $\mu$ M dATP, 50  $\mu$ M [<sup>3</sup>H]dTTP (200 cpm/pmol), and 45  $\mu$ M poly(dA-dT) co-polymer or 40  $\mu$ g/ml poly(dA)-oligo(dT)<sub>12-18</sub> (28). After in-

cubation for 60 min at 37°C, acid-insoluble radioactivity was assessed (29). One unit of DNA polymerase catalyzes the incorporation of 1 nmol dNTPs into DNA at 37°C for 60 min under the conditions described above.

*Antibody against the pol  $\delta$  catalytic subunit* - A cDNA encoding the N-terminal region between 43 and 1063 nucleotides for human p125 (20) was cloned by PCR from Raji cell cDNA library, using a primer set, 5'-ATGGATGGCAAGCGGCGG-3' and 5'-CAGCGAGCAGATCTGGATG-3'. The products were cloned in pUC18 after blunting, and the coding sequence was inserted into pGEX-4T-1, which was then introduced into *E. coli* BL21. The GST-fused p125 was expressed in the *E. coli* by induction with 0.1 mM isopropyl- $\beta$ -D-thiogalactoside. Frozen *E. coli* cell pellets were lysed in PBS containing 1% Triton X100, 1 mM PMSF, 1 mM benzamidine, 2  $\mu$ M pepstatin, 5  $\mu$ M leupeptin by sonication for 10 sec, 3 times, at 50 W on ice. A soluble extract was obtained by centrifugation, and loaded on a glutathione-Sepharose column (Amersham Pharmacia Biotech). The column was washed and the adsorbed GST-fused p125 fragment was eluted with 10 mM glutathione in 50 mM Tris-HCl, pH 9.6. After dialysis against PBS, 100  $\mu$ g of the purified p125-GST fusion protein was injected 4 times into Nippon white rabbits intraperitoneally with Freund's incomplete adjuvant every other week. The rabbits were sacrificed 3 days after the last immunization. Rabbit IgG against p125 was affinity-purified using a column of Sepharose CL-4B conjugated with p125 GST fusion protein. The affinity purified anti-p125 was then passed through Sepharose CL-4B conjugated with GST protein to deplete antibodies against GST.

*Western blotting* - Protein samples were boiled for 5 min in a SDS-sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue] and subjected to SDS-PAGE, then electrophoretically transferred to polyvinylidene difluoride (PVDH) membranes at 200 mA at room temperature. After the membranes were immersed in a blocking solution [10 mM Tris-HCl (pH 7.5) 150 mM NaCl, 10% skim milk] at 37°C for 1 h, they were incubated with either an anti-p125 rabbit polyclonal antibody, an anti-penta histidine oligopeptide mouse monoclonal antibody, or an anti-PCNA mouse monoclonal antibody, at room temperature for 1 h. After washing 5 times with washing buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl], the membranes were incubated with horse radish peroxidase-conjugated (HRPO-) goat anti-rabbit IgG (BECKMAN COULTER, Miami, Florida, USA), or HRPO-goat anti-mouse IgG (BECKMAN COULTER), at room temperature for 1h. Expressed polypeptides were detected with the ECL system (Amersham Pharmacia Biotech).

## RESULTS

*Nucleotide sequence of the cDNA for pol  $\delta$  catalytic subunit p125* - The nucleotide sequence of the cDNA for human pol  $\delta$  catalytic subunit, p125, obtained by PCR was determined. As summarized in Table 1, the sequence obtained here differed at several points from those described by other groups.<sup>20,30,31</sup> Among 21 different nucleotides, 8 would result in amino acid changes. Compared with the genomic sequence by Chung *et al.*,<sup>31</sup> our sequence differed at Arg-119, Ser-173, Cys-360, Lys-648, and Ile-736. We corrected our sequence at three sites which are involved in the core region of pol  $\delta$ ,<sup>20</sup> by means of site-directed mutagenesis (GeneEditor<sup>TM</sup>, Promega). The corrections made were: C-1079 to G (Ser to Cys), G-1942 to A (Glu to Lys), and C-2147 to T (Thr to Ile), as shown in Table 1. Corrections were confirmed by sequencing.

*Nucleotide sequences of cDNA for p50 and PCNA* - With the cDNA for p50, there was no difference in nucleotide sequence between our clone and that published.<sup>26</sup> With our PCNA clone, one substitution was found: the guanine, at 350 nucleotides downstream from ATG start codon,

Table 1. Sequences comparison of the p125 catalytic subunit

| Nucleotide position <sup>a</sup> | HSDNAPOLD <sup>b</sup>  |                   | HSDNAPOLC <sup>c</sup> |      | POLD1 <sup>d</sup> |      | pFastBac-p125 <sup>e</sup>     |                               |
|----------------------------------|-------------------------|-------------------|------------------------|------|--------------------|------|--------------------------------|-------------------------------|
|                                  | Nucleotide <sup>h</sup> | A.A. <sup>i</sup> | Nucleotide             | A.A. | Nucleotide         | A.A. | Before correction <sup>f</sup> | After correction <sup>g</sup> |
| 88                               | CGG                     |                   | TGG                    | Trp  | CGG                | Arg  | CGG                            | CGG                           |
| 252                              | CCA                     |                   | CCA                    |      | CCA                |      | CCG                            | CCG                           |
| 324                              | GCG                     |                   | GCG                    |      | GCG                |      | GCA                            | GCA                           |
| 356                              | CAC                     | His               | CGC                    |      | CGC                | Arg  | CAC                            | CAC                           |
| 518                              | AAC                     | Asn               | AGC                    |      | AGC                | Ser  | AAC                            | AAC                           |
| 810                              | GCC                     |                   | GCT                    |      | GCT                |      | GCT                            | GCT                           |
| 1079                             | TGT                     |                   | TGT                    |      | TGT                | Cys  | TCT                            | TGT                           |
| 1173                             | GAT                     |                   | GAC                    |      | GAC                |      | GAT                            | GAT                           |
| 1414                             | TAC                     |                   | CAC                    | His  | TAC                | Try  | TAC                            | TAC                           |
| 1539                             | CTA                     |                   | CTG                    |      | CTG                |      | CTA                            | CTA                           |
| 1548                             | GCT                     |                   | GCC                    |      | GCC                |      | GCC                            | GCC                           |
| 1662                             | AAG                     |                   | AAA                    |      | AAA                |      | AAG                            | AAG                           |
| 1761                             | ATT                     |                   | ATC                    |      | ATC                |      | ATC                            | ATC                           |
| 1860                             | ACA                     |                   | ACG                    |      | ACA                |      | ACG                            | ACG                           |
| 1942                             | AAG                     |                   | AAG                    |      | AAG                | Lys  | GAG                            | aAG                           |
| 1956                             | CGT                     |                   | CGG                    |      | CGG                |      | CGG                            | CGG                           |
| 2147                             | ATC                     |                   | ATC                    |      | ATC                | Ile  | ACC                            | AtC                           |
| 2244                             | AGC                     |                   | AGT                    |      | AGT                |      | AGT                            | AGT                           |
| 2325                             | GGC                     |                   | GGG                    |      | GGG                |      | GGG                            | GGG                           |
| 2326                             | GGG                     | Gly               | CGG                    |      | CGG                | Arg  | CGG                            | CGG                           |
| 2493                             | GCG                     |                   | GCC                    |      | GCC                |      | GCC                            | GCC                           |

- a. The first nucleotide of the ATC start codon is designated +1
- b. The cDNA sequence published by Yang *et al.* (20)
- c. The cDNA sequence published by Chung *et al.* (30)
- d. The genomic sequence published by Chung *et al.* (31)
- e. Our clone (see MATERIALS AND METHODS)
- f. pFastBac-p125, originally cloned in our laboratory.
- g. pFastBac-p125, corrected for the sequence involved in the core domains according to POLD1
- h. Nucleotides different from POLD1 are shown in boldface letters.
- i. Only the encoded amino acids different from POLD1 are shown.
- j. Corrected nucleotides are shown in lower case letters.
- k. Corrected amino acids are shown in boldface letters.

RECOMBINANT HUMAN DNA POLYMERASE  $\delta$ 

Table 2. Purification of recombinant p125

| Fraction            | Total protein <sup>a</sup><br><i>mg</i> | Total activity <sup>b</sup><br><i>units</i> | Specific activity<br><i>units/mg</i> | Yield<br><i>%</i> |
|---------------------|---|---|--------------------------------------|-------------------|
| Crude extract       | 40.0                                    | 2349  | 58.7                                 | 100               |
| Phosphocellulose    | 3.9                                     | 2422  | 621                                  | 103               |
| Cobalt Sepharose 6B | 0.017                                   | 60.2  | 3539                                 | 2.5               |

a. Protein concentrations of each fraction were determined by the Bradford assay with BSA as the standard.  
 b. Activity was determined as described in the "MATERIALS AND METHODS" with poly(dA-dT) as the template. One unit of enzyme incorporates 1 nmol of nucleotide per h at 37°C.

was adenine in the published report,<sup>27)</sup> and this substitution would result in an amino acid change (Lys-117 to Arg). This sequence was not corrected because the amino acid residue is not involved in the interdomain connector loop of PCNA (amino acids 119–133) which participates in direct interactions with pol  $\delta$ .<sup>32)</sup>

*Purification of active p125 catalytic subunit* - Steps in purification of a recombinant, full-length p125 protein carrying a histidine-tag at the C-terminal are summarized in Table 2. The p125, expressed in insect cells, was largely insoluble, and only approximately 1% could be recovered in soluble form after extraction (data not shown). Eluted protein from the cobalt-Sepharose 6B column (MATERIALS AND METHODS) exhibited the expected molecular mass (125 kDa) upon SDS-PAGE (Fig. 1A), and was reactive with an anti-human pol  $\delta$  p125 rabbit polyclonal antibody (Fig. 1D). The protein band was also immunodetected by an anti-penta histidine oligopeptide mouse monoclonal antibody (data not shown). Fractions showed pol  $\delta$  activity in proportion to the protein amounts (Fig. 1G). The purity of recombinant p125 protein was approximately 95% as determined by Coomassie Brilliant Blue (CBB) staining of bands in SDS-PAGE. By this procedure, 17  $\mu$ g of p125 was obtained from  $8 \times 10^7$  Sf9 cells infected with Acp125-c8his (MATERIALS AND METHODS).

Possible contamination with baculovirus DNA polymerase in the purified p125 fraction was examined by a control experiment using Sf9 cells infected with a baculovirus encoding the non-catalytic subunit. The crude extract of cells infected with Acp50-n6his also showed high DNA polymerase activity. This control extract was fractionated by the column chromatography with both phosphocellulose and cobalt-Sepharose 6B, in the same way as for p125-expressing cells, and the corresponding fractions were measured with respect to pol  $\delta$  activity. As shown in Fig. 1G, the eluted fractions from the cobalt-Sepharose 6B column of the control sample did not show any DNA polymerase activity. Thus, the activity of purified p125 fraction is solely due to the expressed pol  $\delta$  and not to any contaminating viral DNA polymerase.

*Purification of p50* - Approximately 10% of recombinant p50 protein carrying histidine-tag became soluble in the extract from the Acp50-n6his infected Sf9 cells, as assessed by CBB staining of bands in SDS-PAGE (data not shown). The p50 protein was purified by means of the cobalt-Sepharose 6B column as described in the MATERIALS AND METHODS. Purified p50 migrated as a 50 kDa protein on the SDS-PAGE, and reacted with an anti-penta histidine oligopeptide mouse monoclonal antibody (Fig. 1, B and E). By this procedure, 75  $\mu$ g of recombinant p50 was obtained from  $4 \times 10^7$  cells infected with the Acp50-n6his. The purity of the final fraction was over 95%.

*Purification of PCNA* - Recombinant PCNA, expressed in the AcPCNA infected Sf9 cells, was more soluble than p125 or p50. Approximately 70% of expressed PCNA was solubilized by the extraction (data not shown). The expressed PCNA was purified by a conventional procedure as described in the MATERIALS AND METHODS and migrated at 35 kDa upon SDS-PAGE as a single band that reacted with an anti-PCNA mouse monoclonal antibody (Fig. 1, C and F).

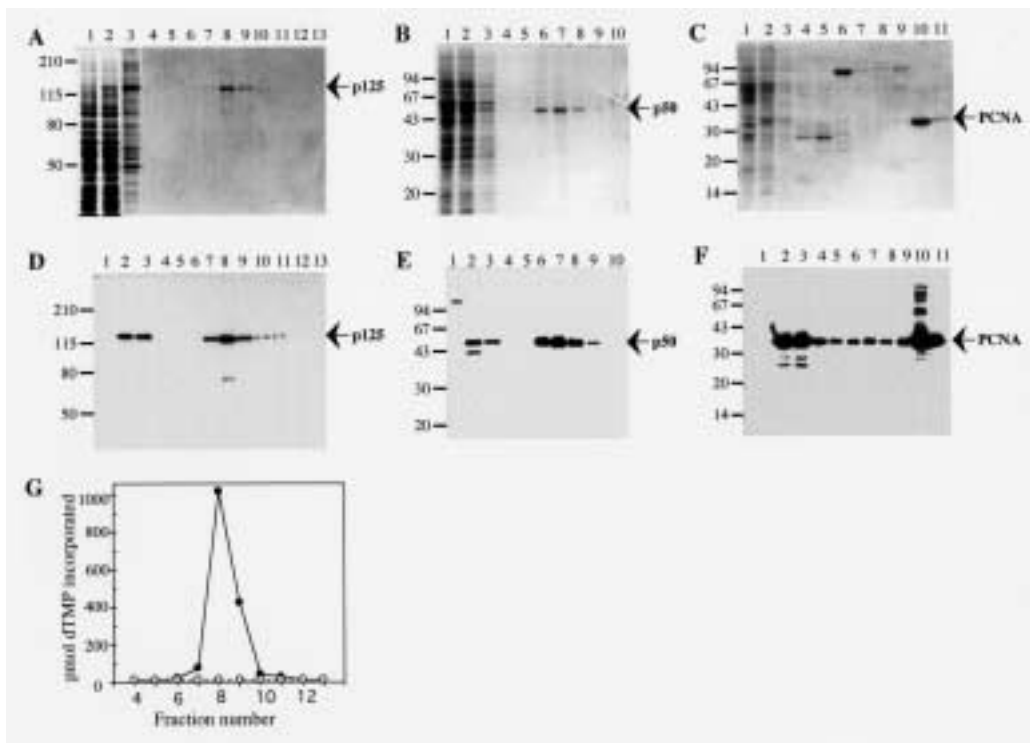


Fig. 1. Purification of recombinant proteins expressed in baculovirus infected Sf9 cells. Sf9 cells were infected with baculoviruses encoding p125, p50, and PCNA, and the overexpressed proteins were purified as described in the MATERIALS AND METHODS. SDS-PAGE patterns at the purification steps of p125 catalytic subunit (panels A and D), p50 small subunit (panels B and E) and PCNA (panels C and F) are shown. Protein bands were detected by CBB staining (panels A, B, C) and immunoblotting using anti-p125 (panel D), anti-histidine-tag (panel E), and anti-PCNA (panel F) antibodies. Sizes (kDa) of molecular weight standards (Bio Rad, Hercules, CA, USA) are indicated on the left in the figure. Lane 1 in panels A and D: crude extract from Sf9 cells infected with Acp50-n6his as negative controls; lane 1 in panels B, C, E, and F: crude extract from Sf9 cells infected with Acp125-c8his. Lane 2 in panels A and D: crude extract from Sf9 cells infected with Acp50-n6his. Lane 2 in panels B and E: crude extract from Sf9 cells infected with Acp125-c8his. Lane 2 in panels C and F: crude extract from AcPCNA infected Sf9 cells. Lane 3 in all panels, phosphocellulose fractions. Lanes 4 to 13, in panels A and D: fractions 1 to 10 eluted from cobalt Sepharose 6B. Lanes 4 to 10 in panels B and E: fractions 1-7 eluted from cobalt-Sepharose 6B. Lanes 4 to 11 in panels C and F: fractions 32 to 39 eluted from Mono Q. Panel G: pol  $\delta$  activity of p125 fractions in panel A (solid circles). As control, Acp50 infected Sf9 cells were processed in the same way, and the fractions from cobalt-Sepharose 6B column were assayed (open circles).

Approximately 300  $\mu\text{g}$  of PCNA was recovered from  $4 \times 10^7$  cells infected with the AcPCNA. The purity of the final preparation was over 95%.

*Activity of recombinant p125* - The specific activity of the purified recombinant p125 protein was 3,500 units/mg protein (Table 2), lower than that of calf thymus pol  $\delta$  purified by a conventional method (15,000 units/mg protein, in the presence of PCNA). While the recombinant p125 alone showed activity, the conventionally purified calf thymus pol  $\delta$  was almost inactive in the absence of PCNA. The recombinant PCNA greatly stimulated the calf pol  $\delta$  (163-fold), but failed to stimulate the recombinant p125 (Table 3).



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Table 3. Effects of p50 and PCNA on activity of recombinant p125

| Cofactors added                   | Conventionally purified pol $\delta$ | Recombinant p125 | Recombinant p125 coexpressed with p50 |                          |
|-----------------------------------|--------------------------------------|------------------|---------------------------------------|--------------------------|
|                                   |                                      |                  | P1 pass through <sup>a</sup>          | P1 absorbed <sup>b</sup> |
| None                              | 1.00 <sup>c</sup>                    | 1.00             | 1.00                                  | 1.00                     |
| PCNA (1.2 $\mu$ g/assay)          | 163.58                               | 1.03             | 1.05                                  | 0.92                     |
| p50 (1.2 $\mu$ g/assay)           | nd <sup>d</sup>                      | 1.08             | nd                                    | nd                       |
| PCNA+p50 (0.6 $\mu$ g each/assay) | nd                                   | 0.99             | nd                                    | nd                       |

a. p50 fraction (see Fig. 3)

b. p125 fraction (see Fig. 3)

c. Activities are expressed relative to the value without cofactors, corresponding to 1.00.

d. Not determined.

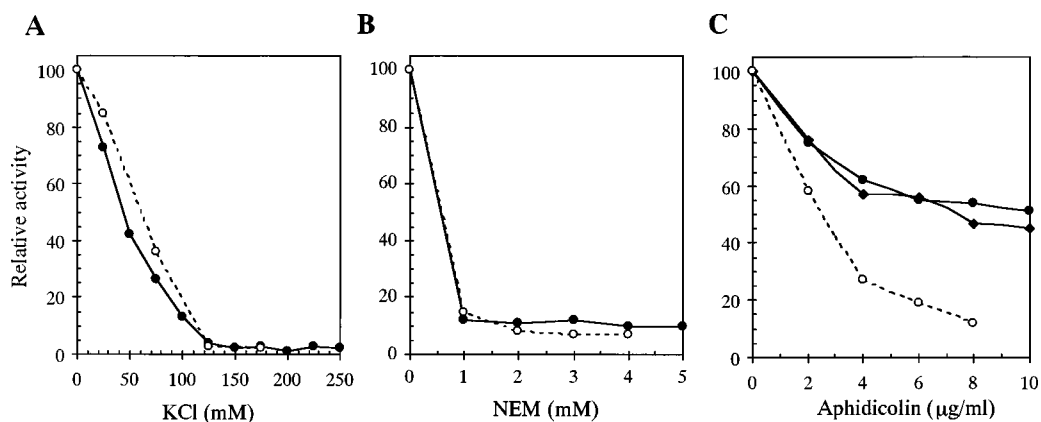


Fig. 2. Effects of inhibitors on the activity of recombinant p125 and calf thymus pol  $\delta$ . Recombinant p125 (solid circles) and calf thymus pol  $\delta$  (open circles) were assayed in the presence of KCl (panel A), NEM (panel B) and aphidicolin (panel C) at the concentrations indicated. The calf thymus pol  $\delta$  was assayed in the presence of PCNA (0.6  $\mu$ g/assay). In panel C, the recombinant p125 was assayed in the presence of PCNA (0.6  $\mu$ g/assay) and p50 (0.6  $\mu$ g/assay) (solid diamonds).

*Effects of inhibitors* - The effects of various inhibitors on the recombinant p125 were studied in comparison with the conventionally purified calf thymus pol  $\delta$ . A high concentration of KCl inhibited the reactions of both in the same manner (Fig. 2A). Both were also strongly inhibited by an SH-blocking reagent, 1 mM N-ethylmaleimide (Fig. 2B). On the other hand, a difference was observed in sensitivity to aphidicolin, a potent inhibitor of replicative DNA polymerases, pol  $\alpha$ ,  $\delta$ , and  $\epsilon$ .<sup>33</sup> The ID<sub>50</sub> with the conventionally purified pol  $\delta$  was 2.5  $\mu$ g/ml, against 10  $\mu$ g/ml (4 times) with the recombinant p125. The aphidicolin resistance of p125 was not affected by the addition of p50 and PCNA to the assay system (Fig. 2C).

*Effects of small subunit p50 on activity of p125* - Since the recombinant p125 was active in the absence of the small subunit p50, the purified p50 was mixed with p125 to see whether there would be further stimulation. As shown in Table 3, the p50 did not affect the activity of p125. Furthermore, overnight incubation of p125 with p50 at 0°C did not enhance the activity (data not shown).

*Effects of PCNA* - Unlike the conventionally purified pol  $\delta$ , the recombinant p125 was not stimulated by PCNA. The simultaneous addition of both p50 and PCNA also showed no stimulation of the activity (Table 3). In contrast, the activity of calf thymus pol  $\delta$  was stimulated 163-fold by the same recombinant PCNA at the concentration of 1.2  $\mu\text{g}/\text{assay}$  (Table 3). In an effort to reconstruct the PCNA-dependent stimulation of recombinant pol  $\delta$ , Sf 9 cells were co-infected with both Acp125-c8his and Acp50-n6his, in the expectation that large and small subunits would form a heterodimer *in vivo*. It has been reported that the co-infection of these two results in a heterodimer that is stimulated by PCNA.<sup>34</sup> From co-infected Sf9 cells, we purified p125 (p125 fraction) and p50 (p50 fraction) by procedures involving phosphocellulose and the cobalt-Sepharose 6B columns described above. It was found that the expressed p50 did not form a complex with p125 sufficient to persist during the purification. The p50 was separated from p125 almost completely at the step of phosphocellulose column, with p125 being adsorbed, while p50 passed through (Fig. 3). Although trace amounts of p50 or p125 were detected by Western blotting in the p125 or the p50 fraction, respectively, after the cobalt-Sepharose 6B column chromatography (data not shown), the molar ratio between p125 and p50 was far from the stoichiometry of the heterodimer (Fig. 3). These fractions thus obtained were also refractory to the effects of PCNA (Table 3).

*Enzyme kinetics* - Parameters of enzyme kinetics were assessed using the recombinant p125, and were compared with those of calf thymus pol  $\delta$  (Table 4). Km values for the template-primer, poly(dA)-oligo(dT), were found to be identical, *i.e.*, 3.6  $\mu\text{g}/\text{ml}$ , corresponding to 7.2  $\mu\text{M}$  in deoxynucleotides (Table 4). In contrast, the Kms for the substrate, dTTP, differed markedly, the recombinant p125 having a value of 33  $\mu\text{M}$ , which was 15 times higher than that obtained with the calf thymus pol  $\delta$  (2.2  $\mu\text{M}$ ). This was not affected by the addition of p50

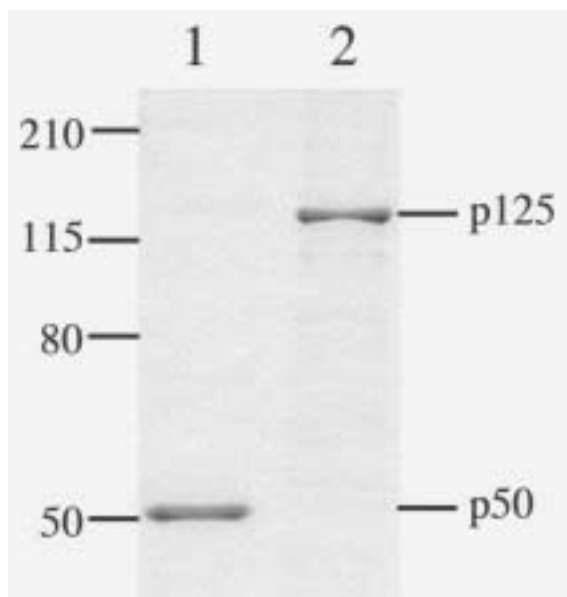


Fig. 3. Purification of recombinant proteins co-expressed in Acp125-c8his and Acp50-n6his coinfecting Sf9 cells. Resolved samples with SDS PAGE were detected by Coomassie Brilliant Blue staining. Lane 1, purified proteins from the pass through fraction from phosphocellulose (p50 fraction). Lane 2, purified proteins from the fraction eluted with 500 mM NaCl (p125 fraction).

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and PCNA (Table 4).

*Electrophoretic behavior of recombinant p125* - A crude extract was prepared from the Acp125 infected Sf9 cells, which express a full length of p125 without any fusion partner, and subjected to PAGE in parallel with that prepared from human Jurkat cells, followed by Western blotting with an anti-p125 antibody. As shown in Fig. 2, the pol  $\delta$  catalytic subunit in Jurkat cells migrated as a doublet bands, the major band at the position corresponding to 125 kDa, and the minor one, at 120 kDa. The recombinant p125 protein that was expressed in Sf9 cells migrated at positions corresponding to the 120 kDa, the minor band of Jurkat cell pol  $\delta$  catalytic subunit. The difference in the electrophoretic mobility between the recombinant p125 and the naturally occurring pol  $\delta$  was also seen in exactly the same manner with Hela cell pol  $\delta$  (data not shown). These results suggest that p125 is post-translationally modified in mammalian cells.

Table 4.  $K_m$  values of recombinant p125 (mM)

| Substrate or Template | Conventionally purified pol $\delta^a$ | Recombinant p125            |                                |
|-----------------------|--|-----------------------------|--------------------------------|
|                       |  | with cofactors <sup>b</sup> | without cofactors <sup>c</sup> |
| dTTP                  | 2.2                                    | 33.0                        | 33.0                           |
| Poly(dA)-Oligo(dT)    | 7.2                                    | nd <sup>d</sup>             | 7.2                            |

a. Activity was measured in the presence of recombinant PCNA (0.6  $\mu$ g/assay).

b. Activity was measured in the presence of recombinant PCNA and recombinant p50 (0.6  $\mu$ g each/assay).

c. Activity was measured in the absence of recombinant PCNA and recombinant p50.

d. Not determined.

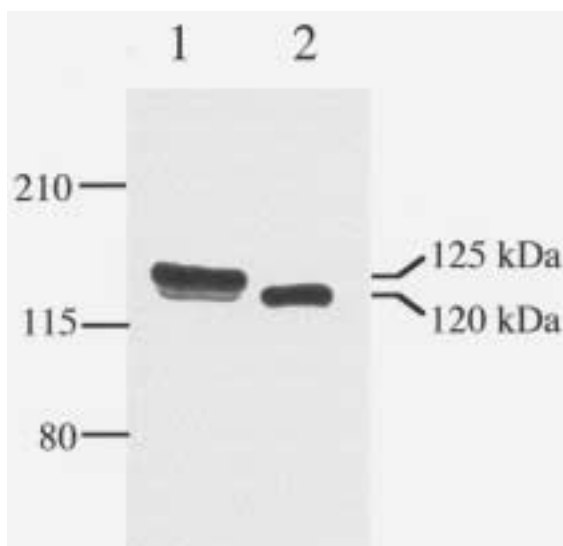


Fig. 4 Electrophoretic mobility of the recombinant p125. Crude extract from Jurkat cells (lane 1), Acp125 infected Sf9 cells (lane 2) were subjected to PAGE and stained with an anti-p125 antibody as described in the MATERIALS AND MEHTODS. Sizes (kDa) of molecular weight standards (Bio Rad, Hercules, CA, USA) are indicated on the left in the figure.

## DISCUSSION

We have studied the functions of DNA polymerases from the viewpoints of regulation by interaction with nuclear proteins,<sup>35-38)</sup> inhibition<sup>28,39,40)</sup> and fidelity of replication.<sup>41-43)</sup> In the present study, we expressed the catalytic subunit of pol  $\delta$  p125, in an insect cell line Sf9 in large quantities. The p125 protein was expressed efficiently in Sf9, but only a relatively small proportion could be solubilized by extraction and purified by means of affinity column chromatography. Efforts to renature larger amounts of insoluble p125 to an active form were not successful. The baculovirus-insect cell expression system also presents difficulty regarding purification of expressed enzymes such as DNA polymerase, since the infected cells contain a number of enzymes and proteins required for viral reproduction including viral DNA polymerase. The latter must be separated from the expressed recombinant p125. For this purpose, the histidine-tag affinity purification method was successfully applied here, and the purified p125 showed DNA polymerase activity when assayed in the pol  $\delta$  reaction mixture. However, in comparison with the pol  $\delta$  purified from calf thymus, it demonstrated a lower specific activity, resistance to inhibition by aphidicolin, a much higher  $K_m$  value for dTTP, and a lack of response to PCNA. It has been reported that the specific activity of recombinant p125 expressed in insect cells is 5 or 14% of that of the native enzyme.<sup>21,22)</sup> The p125 expressed in either insect cells<sup>21)</sup> or *E. coli*.<sup>23)</sup> similarly lacked the typical response to PCNA. The higher  $K_m$  value for dTTP is also consistent with the results of Zhou *et al.*<sup>21)</sup>

Aphidicolin is known to inhibit pol  $\alpha$ ,  $\delta$  and  $\epsilon$ <sup>33)</sup> and the herpes simplex virus DNA polymerase,<sup>44)</sup> by competing with dNTP.<sup>45)</sup> Although the chemical structure of aphidicolin differs from that of dNTP, it binds to the enzyme at a site close to the location of dNTP binding.<sup>46)</sup> The 15 times higher  $k_m$  value for dTTP of the recombinant p125 (Table 4) may mean that post-translational modification of the enzyme protein in insect cells differs from that of the natural enzyme in mammalian cells. In vivo, pol  $\delta$  exists as a heterodimer composed of p125 and p50, and this may affect the conformation of the catalytic subunit, p125. Misfolding might be expected to decrease its affinity for substrates, causing a higher  $K_m$  for dTTP and lower inhibition by aphidicolin.

A lack of response to PCNA could be due to lack of the small subunit, p50, as reported by Zhou *et al.*<sup>34)</sup> In our present study, however, stimulation by PCNA was not apparent either with addition of p50 and overnight incubation, or the co-expression of baculoviruses coding for the two proteins (Table 3). Analysis of enzyme samples after incubation or co-expression did not show any evidence of formation of stable complexes between p125 and p50 (Fig. 3), and this might be a prerequisite for PCNA responsiveness. Additional factors may be required for the formation of a mature pol  $\delta$  protein complex. According to Zuo *et al.*,<sup>17)</sup> the pol  $\delta$  complex of *Saccharomyces pombe* is composed of 5 subunits, p125 (catalytic) and p55 (Cdc1, corresponding to p50 of human pol  $\delta$ ), p54 (Cdc27), p42 (homologous to budding yeast GCD14), and p22 (Cdm1, which can rescue *cdc1*<sup>18)</sup>). Recently, a subunit of mammalian pol  $\delta$ , termed p66, was found, having homology with the Cdc27 subunit of *Schizosaccharomyces pombe* pol  $\delta$ .<sup>18)</sup> Some factors which are missing in the recombinant pol  $\delta$ -expression system may be required for the assembly of pol  $\delta$  complex, though mammalian counterparts of p42 or Cdm1 have yet to be identified.

Another possible reason for different characters is a post-transcriptional modification like protein phosphorylation. The catalytic subunit of pol  $\delta$  from human Jurkat cells showed doublet protein bands on PAGE, and the recombinant p125 migrated to the same position as the fast-migrating minor band. The Acp125 encodes a full-length p125 protein, from the first ATG to the stop codon. Histidine-tag affinity purification ensures the intact C-terminal. Since the prod-

uct is a homogeneous peptide in size, partial cleavage of the N-terminal is also unlikely. It has been reported that pol  $\delta$  p125 is phosphorylated *in vivo* by cyclin E/cdk2 and cyclin D3/cdk4,<sup>22)</sup> and that this is maximal around the G1/S transition. With the human p125, there are 6 potential target sites for cyclin/cdk which have (S/T)P motifs, at Ser-207, Ser-788, Thr-83, Thr-150, Thr-238, and Thr-640. Phosphorylation of p125 at these sites might cause slower migration of pol  $\delta$  from mammalian cells, similar to the case of the phosphorylated retinoblastoma protein.<sup>47)</sup>

In conclusion, the assembly of pol  $\delta$  complexes in cells may be regulated by multiple factors, and function may depend on post-translational modification. The proper assembly may be essential for a functional pol  $\delta$  that is stimulated by PCNA and sensitive to aphidicolin $\delta$

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### REFERENCES

- 1) Burgers, P.M.J., Bambara, R.A., Cambell, J.L., Chang, L.M.S., Douney, K.M., Hubsher, U., Lee, M.Y.W.T., Linn, S.M., So, A.G. and Spadari, S.: Revised nomenclature for eukaryotic DNA polymerases. *Eur. J. Biochem.*, 191, 617–618 (1990).
- 2) Nelson, J.R., Lawrence, C.W. and Hinkle, D.C.: Thymidine-thymidine dimer bypass by yeast DNA polymerase  $\zeta$ . *Science*, 272, 1646–1649 (1996).
- 3) Matsutani, C., Kusumoto, R., Yamada, A., Dohmae, N., Yokoi, M., Araki, M., Iwai, S., Takio, K. and Hanaoka, F.: The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase  $\eta$ . *Nature*, 399, 700–704 (1999).
- 4) Nakamura, H., Tanabe, K., Yoshida, S. and Morita, T.: Terminal deoxynucleotidyl-transferase of 60,000 daltons from mouse, rat, and calf thymus. Purification by immunoabsorbent chromatography and comparison of peptide structures. *J. Biol. Chem.*, 256, 8745–8751 (1981).
- 5) Greider, C.W. and Blackburn, E.H.: A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. *Nature*, 337, 331–337 (1989).
- 6) Gleider, C.W., Collins, K. and Autexier, C.: DNA telomerases in *DNA Replication in Eukaryotic Cells* (DePamphilis, M.L. ed), pp. 619–638 (1996), Cold Spring Harbor Laboratory Press, New York.
- 7) Salas, M., Miller, J.T., Leis, J. and DePamphilis, M.L.: Mechanisms for Priming DNA Synthesis in *DNA Replication in Eukaryotic Cells* (DePamphilis, M.L. ed), pp. 131–176 (1996), Cold Spring Harbor Laboratory Press, New York.
- 8) Wang, T., S.-F.: Cellular DNA Polymerases in *DNA Replication in Eukaryotic Cells* (DePamphilis, M.L. ed), pp. 461–493 (1996), Cold Spring Harbor Laboratory Press, New York.
- 9) Tania, A.B. and Stephen, P.B.: Polymerases and the replisome: Machines within machines. *Cell*, 92, 295–305 (1998).
- 10) Kesti, T., Flick, K., Keranen, S., Syvaaja, J.E. and Wittenberg, C.: DNA polymerase  $\epsilon$  catalytic domains are dispensable for DNA replication, DNA repair, and cell viability. *Mol. Cell*, 3, 679–685 (1999).
- 11) Lee, M.Y.W.T., Tan, C.K., Downey, K. M. and So., A.G.: Further studies on calf thymus DNA polymerase  $\delta$  purified to homogeneity by a new procedure. *Biochemistry*, 23, 1906–1913 (1984).
- 12) Lee, M.Y.W.T., Jiang, Y., Zhang, S.J. and Toomy, N.L.: Characterization of human DNA polymerase  $\delta$  and its immunochemical relationships with DNA polymerases  $\alpha$  and  $\epsilon$ . *J. Biol. Chem.*, 266, 2423–2429 (1991).
- 13) Syvaaja, J., Suomensaaari, S., Nishida, C., Goldsmith, J.S., Chui, G.S.J. and Linn, S.: DNA polymerases  $\alpha$ ,  $\beta$ ,  $\delta$ : Three distinct enzymes from HeLa cells. *Proc. Natl. Acad. Sci. USA*, 87, 6664–6668 (1990).
- 14) Chiang, C.S., Mitsis, P.G. and Lehman, I.R.: DNA polymerase  $\delta$  from embryos of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA*, 90, 9105–1909 (1993).

- 15) Goulian, M., Hermann, S.M., Sackett, J.W. and Grimm, S.L.: Two forms of DNA polymerase  $\delta$  from mouse cells. *J. Biol. Chem.*, 265, 16402–16411 (1990).
- 16) Geric K.J., Li X., Pautz A. and Burgers, P.M.: Characterization of the two small subunits of *Saccharomyces cerevisiae* DNA polymerase  $\delta$ . *J. Biol. Chem.*, 273, 19747–19755 (1998).
- 17) Zuo, S., Gibbs, E., Kelman, Z. and Wang, T.S., O'Donnell, M., MacNeill, S.A. and Hurwitz, J.: DNA polymerase  $\delta$  isolated from *Schizosaccharomyces pombe* contains five subunits. *Proc. Natl. Acad. Sci. USA*, 94, 11244–11249 (1997).
- 18) Hughes, P., Tratner, I., Ducoux, M., Piard, K. and Baldacci, G.: Isolation and identification of the third subunit of mammalian DNA polymerase  $\delta$  by PCNA-affinity chromatography of mouse FM3A cell extracts. *Nucleic Acids Res.*, 27, 2108–2114 (1999).
- 19) Roberts, J.D. and Kunkel, T.A.: Fidelity of DNA replication. *in* DNA Replication in Eukaryotic Cells, ed. M. DePamphilis, pp. 217–248 (1996) Cold Spring Harbor Press.
- 20) Yang, C.-L., Chang, L.-S., Zang, P., Hao, H., Zhu, L., Toomey, N.L. and Lee, M.Y.W.T.: Molecular cloning of a cDNA for the catalytic subunit of human DNA polymerase  $\delta$ . *Nucleic Acids Res.*, 20, 735–745 (1992).
- 21) Zhou, J.-Q., Tan, C.-K., So, A.G. and Downey, K.M.: Purification and characterization of the catalytic subunit of human DNA polymerase  $\delta$  expressed in baculovirus-infected insect cells. *J. Biol. Chem.*, 271, 29740–29745 (1996).
- 22) Wu, S.-M., Zhang, P., Zeng, X., R., Zhang, S.J., Mo, J., Li, B.Q. and Lee, M.Y.W.T.: Characterization of the p125 subunit of human DNA polymerase  $\delta$  and its deletion mutants. *J. Biol. Chem.*, 273, 9561–9569 (1998).
- 23) Hindes, R. and Hubscher, U.: Production of active mouse DNA polymerase  $\delta$  in bacteria. *Gene (Amst)*, 158, 241–246 (1995).
- 24) Zhang, P., Frugulhetti, I., Jiang, Y., Holt, G.L., Condit, R.C., and Lee, M.Y.W. : Expression of the catalytic subunit of human DNA polymerase  $\delta$  in mammalian cells using a vaccinia virus vector system. *J. Biol. Chem.*, 270, 7993–7998 (1995).
- 25) Tsurimato, T., and Stillman, B.: Multiple replication factors augment DNA synthesis by the two eukaryotic DNA polymerases,  $\alpha$  and  $\delta$ . *EMBO J.*, 8, 3883–3889 (1989).
- 26) Zhang, J., Tan, C.K., McMullen, B., Downey, K.M., and So, A.G. : Cloning of the cDNAs for the small subunits of bovine and human DNA polymerase  $\delta$  and chromosomal location of the human gene (POLD2). *Genomics*, 29, 179–186 (1995).
- 27) Almendral, J.M., Huebsch, D., Blundell, P.A., MacDonald-Bravo, H. and Bravo, R.: Cloning and sequence of the human nuclear protein cyclin: Homology with DNA binding proteins. *Proc. Natl. Acad. Sci. USA*, 84, 1575–1579 (1987).
- 28) Shoji-Kawaguchi, M., Izuta, S., Tamiya-Koizumi, K., Suzuki, M. and Yoshida, S.: Selective inhibition of DNA polymerase  $\epsilon$  by phosphatidylinositol. *J. Biochem.*, 117, 1095–1099 (1995).
- 29) Yoshida, S., Kondo, T. and Ando, T.: Multiple molecular species of cytoplasmic DNA polymerase from calf thymus. *Biochim. Biophys. Acta*, 353, 463–474 (1974).
- 30) Chung, D.W., Zhang, J., Tan, C.K., Davie, E.W., So, A.G. and Downey, K.M.: Primary structure of the catalytic subunit of human DNA polymerase  $\delta$  and chromosomal location of the gene. *Proc. Natl. Acad. Sci. USA*, 88, 11197–11201 (1991).
- 31) Chang, L.-S., Zhao, L., Zhu, L., Chen, M.L. and Lee, M.Y.W.T.: Structure of the gene for the catalytic subunit of human DNA polymerase  $\delta$  (POLD1). *Genomics*, 28, 411–419 (1995).
- 32) Zang, P., Sun, Y., Hsu, H., Zhang, L., Zhang, Y. and Lee, M.Y.W.T.: The interdomain connector loop of human PCNA is involved in a direct interaction with human polymerase  $\delta$ . *J. Biol. Chem.*, 273, 713–719 (1998).
- 33) Starrat, A.N. and Loschiavo, S.R.: The production of aphidicolin by *Nigrospora sphaerica*. *Can. J. Microbiol.*, 20, 416–417 (1974).
- 34) Zhou, J.-Q., He, H., Tan, C.-K., Downey, K.M. and So, A.G.: The small subunit is required for functional interaction of DNA polymerase  $\delta$  with the proliferating cell nuclear antigen. *Nucleic Acids Res.*, 25, 1094–1099 (1997).
- 35) Savoysky, E., Suzuki, M., Simbulan, C., Tamai, K., Ohuchi, T., Akiyama, T. and Yoshida, S.: Immunopurified Rb protein inhibits SV40 T antigen-dependent stimulation of DNA polymerase  $\alpha$ . *Oncogene*, 8, 319–325 (1993).
- 36) Simbulan, C.M.G., Suzuki, M., Izuta, S., Sakurai, T., Savoysky, E., Kojima, K., Miyahara, K., Shizuta, Y. and Yoshida, S.: Poly(ADP-ribose) polymerase stimulates DNA polymerase  $\alpha$  by physical association. *J. Biol. Chem.*, 268, 93–99 (1993).
- 37) Suzuki, M., Izuta, S. and Yoshida, S.: DNA polymerase  $\delta$  overcomes an error-prone pause site in the pres-

- ence of replication protein-A. *J. Biol. Chem.*, 269, 10225–10228 (1994).
- 38) Takemura, M., Kitagawa, T., Izuta, S., Wasa, J., Takai, A., Akiyama, T. and Yoshida, S.: Phosphorylated retinoblastoma protein stimulates DNA polymerase  $\alpha$ . *Oncogene*, 15, 2483–2492 (1997).
  - 39) Simbulan, C.M.G., Taki, T., Tamiya-Koizumi, K., Suzuki, M., Savoysky, E., Shoji, M. and Yoshida, S.: Sulfate- and sialic acid-containing glycolipids inhibit DNA polymerase  $\alpha$  activity. *Biochim. Biophys. Acta*, 1205, 68–74 (1994).
  - 40) Ogawa, A., Murate, T., Izuta, S., Takemura, M., Furuta, K., Kobayashi, J., Kamiya, T., Nimura, Y. and Yoshida, S.: Sulfated glycolipid from archaebacterium inhibits eukaryotic DNA polymerase  $\alpha$ ,  $\beta$ , and retroviral reverse transcriptase and affects methyl methanesulfonate cytotoxicity. *Int. J. Cancer*, 76, 512–518 (1998).
  - 41) Izuta, S., Roberts, J.D. and Kunkel, T.A.: Replication error rates for G-dGTP, T-dGTP, and A-dGTP mispairs and evidence for differential proofreading by leading and lagging strand DNA replication complexes in human cells. *J. Biol. Chem.*, 270, 2595–2600 (1995).
  - 42) Suzuki, M., Banskín, D., Hood, L. and Loeb, L.A.: Random mutagenesis of *Thermus aquaticus* DNA polymerase I : Concordance of immutable sites in vivo with the crystal structure. *Proc. Natl. Acad. Sci. USA*, 93, 9670–9675 (1996).
  - 43) Suzuki, M., Avicola, A.K., Hood, L. and Loeb, L.A.: Low fidelity mutants in the O-helix of *Thermus aquaticus* DNA polymerase I. *J. Biol. Chem.*, 272, 11228–11235 (1997).
  - 44) Nishiyama, Y., Suzuki, S., Yamauchi, M., Maeno, K. and Yoshida, S.: Characterization of aphidicolin resistant mutant of herpes simplex type 2 which induces altered viral DNA polymerase. *Virology*, 135, 87–96 (1984).
  - 45) Tsurumi, T., Maeno, K. and Nishijima, Y.: A single base change within the DNA polymerase locus of herpes simplex virus type 2 can confer resistance to aphidicolin. *J. Virol.*, 61, 388–394 (1987)
  - 46) Chang C.C., Boezi, J.A., Warren, S.T., Sabourin, C.L., Liu, P.K., Glatzer, L. and Trosko, J.E.: Isolation and characterization of a UV-sensitive hypermutable aphidicolin-resistant Chinese hamster cell line. *Somat. Cell Mol. Genet.*, 7, 235–253 (1981)
  - 47) Kitagawa, M., Higashi, H., Jung, H.-K., Suzuki-Takahashi, I., Ikeda, M., Tamai, K., Kato, J., Segawa, K., Yoshida, E., Nishimura, S. and Taya, Y.: The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. *EMBO J.*, 10, 7060–7069 (1996).