*Proc. Natl. Acad. Sci. USA*
Vol. 87, pp. 6664–6668, September 1990
Biochemistry

# DNA polymerases α, δ, and ε: Three distinct enzymes from HeLa cells

**(mammalian DNA polymerases)**

JUHANI SYVÄOJA†, SUSANNA SUOMENSAARI†, CRAIG NISHIDA‡, JEFFREY S. GOLDSMITH, GLORIA S. J. CHUI, SANJAY JAIN, AND STUART LINN§

Division of Biochemistry and Molecular Biology, Barker Hall, University of California, Berkeley, CA 94720

*Communicated by Michael J. Chamberlin, June 7, 1990 (received for review March 17, 1990)*

ABSTRACT **DNA polymerases α, δ, and ε have been purified and characterized from the same HeLa cell extract in order to determine their relationship by comparing them from the same cell type. The catalytic properties and the primary structures of the large subunits of the DNA polymerases as compared by partial peptide mapping with *N*-chlorosuccinimide are different. Likewise, the small subunit of DNA polymerase ε appears to be distinct from the large subunit of the same polymerase and from the smaller subunits of DNA polymerase α. HeLa DNA polymerase δ is processive only when HeLa proliferating cell nuclear antigen is present, whereas DNA polymerase ε is quite processive in its absence. Inhibitor and activator spectra of DNA polymerases α, δ, and ε also distinguish the three enzymes. These results and immunologic comparisons published elsewhere support the premise that HeLa DNA polymerases α, δ, and ε are distinct enzymes that have common properties with yeast DNA polymerases I, III, and II, respectively.**

Four types of DNA polymerases have been defined in mammalian cells: DNA polymerases α, β, γ, and δ (pol α, β, γ, and δ) (1, 2). An associated exonuclease, low sensitivity to inhibition by 2-[*p*-(*n*-butyl)anilino]dATP (BuAdATP) and $N^2$-[*p*-(*n*-butyl)phenyl]dGTP (BuPdGTP), and high sensitivity to 3′-deoxythymidine 5′-triphosphate (ddTTP), aphidicolin, and *N*-ethylmaleimide characterized pol δ (2). However, two subclasses of pol δ became apparent: DNA pol $\delta_1$ (3, 4), which had low processivity with long templates but high processivity and increased activity with the cell cycle-regulated proliferating cell nuclear antigen (PCNA) (5), and DNA pol $\delta_2$ (6–8), which was highly processive in the absence of PCNA and not activated by it (7, 8). The former was proposed to be involved in DNA replication (9), whereas the latter was implicated in repair (10). Both forms were immunologically and structurally distinct from DNA pol α but possibly showed some similarity to each other (11). To more accurately and methodically define the relationship between these DNA polymerases, we studied all three from the same HeLa cell extract. Based upon differences, we propose that DNA pol α, δ, and ε are indeed three different enzymes that resemble yeast DNA polymerases I, III, and II (pol I, III, and II), respectively.

## MATERIALS AND METHODS

**Materials.** Synthetic deoxypolymers (Midland Certified Reagent Co., Midland, TX) were annealed by incubating poly(dA-dT) or a homopolymer at 1 mM with its complementary oligomer in 10 mM Tris·HCl, pH 7.5/10 mM KCl/0.1 mM EDTA at 68°C for 30 min and then at room temperature for several hours. Unless otherwise indicated, the ratio of polymer-to-oligomer nucleotide residues was 10:1. Activated salmon sperm DNA was prepared as described (12). Unlabeled nucleotides and ddTTP were from Pharmacia and $^3$H-labeled nucleotides were from Amersham. BuPdGTP and carbonyldiphosphonate were gifts of G. E. Wright (University of Massachusetts Medical School). *N*-Chlorosuccinimide was from Sigma. Mouse monoclonal antibody AB151 IgG against human PCNA was from American Biotech, Inc. (Plantation, FL); neutralizing monoclonal antibody SJK 132-20 IgG against human DNA pol α was described by Tanaka *et al.* (13).

**SDS/Polyacrylamide Gel Electrophoresis and Immunoblotting.** SDS/polyacrylamide gels (14) were electrophoresed at 20 mA and stained with silver (15). Immunoblotting was as described (8) using a Bio-Rad kit and goat anti-mouse IgG conjugated to alkaline phosphatase.

**DNA Polymerase and Protein Assays.** Assays for pol δ (16) contained 40 mM Hepes·KOH (pH 6.5), 1 mM $MgCl_2$, 10 mM KCl, 2 mM dithiothreitol, 0.03% Triton X-100, 2% glycerol, 80 μg of bovine serum albumin per ml, 50 μM dATP, 50 μM [$^3$H]dTTP (200 cpm/pmol), and 45 μM poly(dA-dT). Incubation was for 30 min at 37°C. Unless otherwise indicated, DNA pol α and ε were assayed as described (8) with activated salmon sperm DNA or poly(dA)·oligo(dT), respectively. Incubation for DNA pol ε was at 30°C during purification and at 37°C thereafter. One unit of polymerase is the amount that catalyzes the incorporation of 1 nmol of total nucleotides per hour. Protein was determined by the Bradford method (17).

## RESULTS

**Purification of DNA pol δ.** Chromatography of a fractionated HeLa cell extract on DEAE-Sephacel resolves pol δ from DNA pol ε and α and from PCNA (Fig. 1). Roughly 1100-fold further purification of pol δ was achieved in subsequent steps (Table 1). The apparent increase in activity after the heparin–agarose and glycerol gradient steps is unexplained. The purification schemes of DNA pol α, δ, and ε and of PCNA from the same extract are schematized in Fig. 2.

**Some Properties of DNA pol δ and ε.** Monoclonal antibody raised against human pol α did not inhibit HeLa pol δ or pol ε while almost completely inhibiting DNA pol α (Fig. 3*A*). PCNA increased the activity of the pol δ some 150-fold when

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Abbreviations: BuAdATP, 2-[*p*-(*n*-butyl)anilino]dATP; BuPdGTP, $N^2$-[*p*-(*n*-butyl)phenyl]dGTP; PCNA, proliferating cell nuclear antigen; pol α, δ, and ε, DNA polymerases α, δ, and ε; pol I, II, and III, DNA polymerases I, II, and III; ddTTP, 3′-deoxythymidine 5′-triphosphate.

†Present address: Department of Biochemistry, University of Oulu, Linnanmaa, SF-90570 Oulu, Finland.
‡Department of Biochemistry, University of California, San Francisco, CA 94143.
§To whom reprint requests should be addressed.

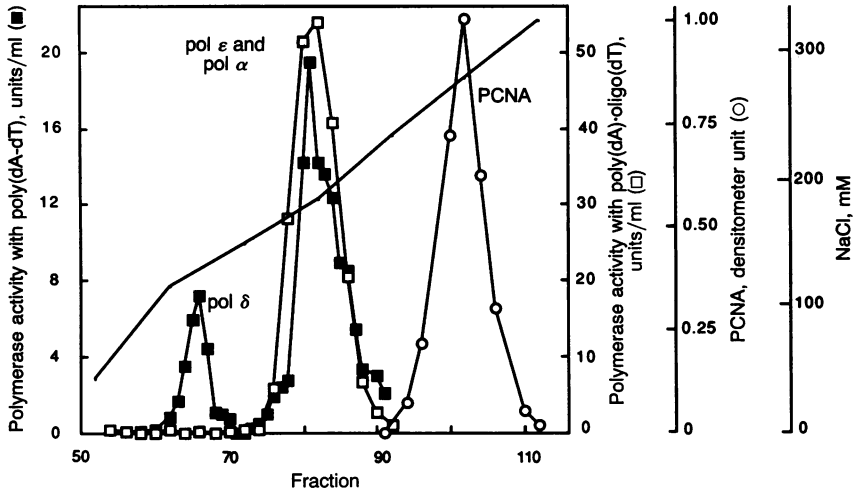


FIG. 1. Chromatography on DEAE-Sephacel. HeLa cells ($6.0 \times 10^{10}$; 84-liter culture) were grown and harvested and the extract was prepared and fractionated with ammonium sulfate as described (10). The material was dialyzed against TDEG (50 mM Tris·HCl, pH 8.0/1 mM dithiothreitol/1 mM EDTA/10% glycerol) and chromatographed on DEAE-Sephacel with a 1120-ml linear gradient from 0 to 500 mM NaCl in TDEG. In the second peak, activity on poly(dA-dT) is due to pol α, whereas that on poly(dA)·oligo(dT) is due to pol ε (8).

poly(dA)·oligo(dT) with long single-stranded areas was used as a primer/template (Fig. 3*B*). By contrast, DNA pol ε was inhibited some 35% by PCNA, as reported previously (8). (The inhibition varies from 20% to 65% depending on the reaction conditions used.) Maximum inhibition is reached at a molecular ratio of roughly 1:1 (PCNA:DNA polymerase), suggesting a possible direct interaction between the two proteins rather than a modification of primer ends that were present in excess compared with PCNA. pol δ required PCNA to act processively, whereas the processivity of pol ε was not significantly altered (Fig. 4).

The only peptides that sedimented with the pol δ activity (at 9.2 S) in the glycerol gradient had molecular masses of 130 and 47 kDa (Fig. 5). Calf thymus pol δ has been reported to have 125- and 48-kDa subunits (9) and yeast pol III has a 125-kDa subunit (19). In some preparations (see Fig. 6*A*) we also find a 124-kDa shadow band of the 130-kDa polypeptide.

In the absence of PCNA, alternating poly(dA-dT) was the preferred primer/template for pol δ (Table 2); poly(rA)·oligo(dT) and duplex DNA did not serve as primer/templates.

**Comparison of Various Inhibitors with DNA Polymerases.** The effects of several compounds on the activities of DNA pol α, δ, and ε were studied (Table 3). Poly(dA)·oligo(dT) and 1 mM $Mg^{2+}$ were used for all assays to make the comparisons. The monoclonal antibody SJK 132-20 also specifically inhibited pol α under these conditions, ruling out cross-

Table 1. Purification of DNA pol δ from HeLa cells

| | Fraction | Volume, ml | Protein, mg | Total activity, units | Specific activity, units/mg |
|---|---|---|---|---|---|
| I | Crude extract | 440 | 4960 | — | — |
| II | 30–50% $(NH_4)_2SO_4$ | 92 | 1651 | — | — |
| III | DEAE-Sephacel | 33.6 | 122 | 176 | 1.4 |
| IV | Phosphocellulose | 44.0 | 5.1 | 36 | 7.0 |
| V | Hydroxylapatite | 7.0 | 0.73 | 32 | 44 |
| VI | Heparin–agarose | 0.6 | 0.11 | 66 | 600 |
| VIIA | Bio-Sil TSK400 (HPLC)* | 4.6 | 0.010† | 21 | 2,100† |
| VIIB | Glycerol gradient* | 2.5 | 0.005† | 78 | 16,000† |

HeLa cells were processed as described in Figs. 1 and 2. The activity of DNA pol δ in fractions I and II could not be assayed specifically.

*Corrected for the fact that only one-third of fraction VI was utilized.

†Estimated.

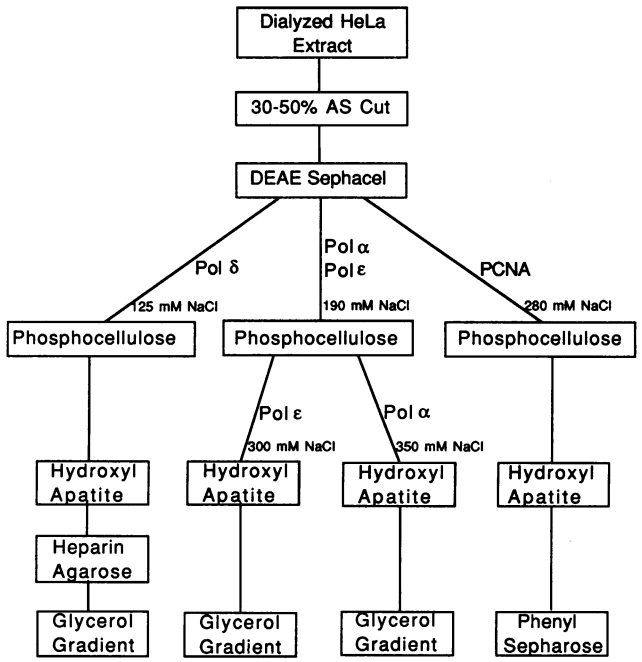


FIG. 2. Scheme for the separation and purification of HeLa DNA pol α, δ, and ε and PCNA. To purify DNA pol δ (Table 1), the material from DEAE-Sephacel (Fig. 1) was applied to phosphocellulose, equilibrated with 100 mM NaCl in TDEGT (defined in Fig. 1), and eluted with a linear gradient from 0 to 600 mM NaCl in TDEGT. Activity eluting at 340 mM NaCl was diluted 1:5 with PDGT (20 mM potassium phosphate, pH 7.5/5 mM dithiothreitol/20% glycerol/0.05% Triton X-100) and chromatographed on hydroxylapatite with a 100-ml gradient from 20 to 600 mM potassium phosphate. Activity eluting around 180 mM potassium phosphate was diluted 1:1 with PDGT and applied to a 0.6-ml heparin–agarose column (18) and eluted with PDGT with potassium phosphate at 100, 200, 300, 400, 500, and 600 mM. Activity eluting with the 200 mM solution was further purified by glycerol gradient centrifugation. DNA pol ε was purified from the DEAE-Sephacel material (Fig. 1) as described (8). DNA pol α, which elutes from the DEAE-Sephacel with DNA pol ε (Fig. 1; ref. 8), was applied to phosphocellulose with the pol ε, and the portion of pol α activity that eluted near 350 mM NaCl and was separated from pol ε was taken and purified as indicated. PCNA was detected by immunoblotting with a monoclonal antibody (8) and quantitating the bands by densitometry. DEAE-Sephacel fractions 97–105 (Fig. 1) were combined and purified as indicated. AS Cut, an ammonium sulfate fractionation as described in ref. 10.

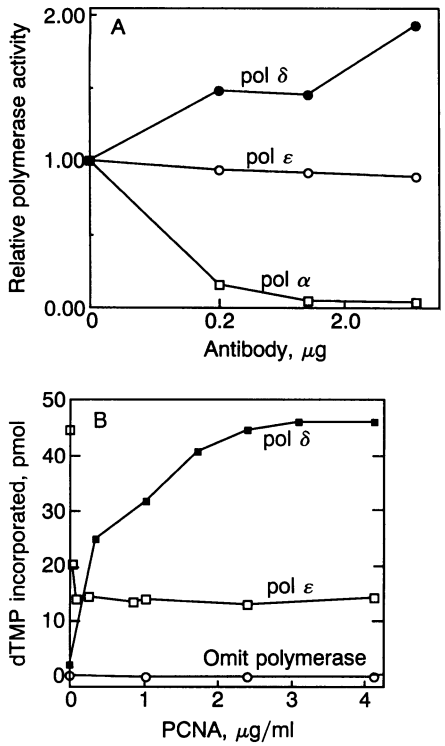


FIG. 3. Effects of pol α IgG and PCNA upon HeLa DNA polymerases. (*A*) Neutralization studies were as described (13) with 0.1 unit of each enzyme at its highest purity. DNase I-activated DNA, poly(dA-dT), or poly(dA)·oligo(dT) was used as the primer/template for DNA pol α, δ, or ε, respectively. A control IgG, P3 (13), did not neutralize any of the polymerases (data not shown). (*B*) Assay conditions for both polymerases were as described for pol δ, using poly(dA)·oligo(dT) as the primer/template (5), except that the polymer ratio of $dT_{16}$ to $dA_{4000-5000}$ was 1:10, and PCNA was added as indicated.

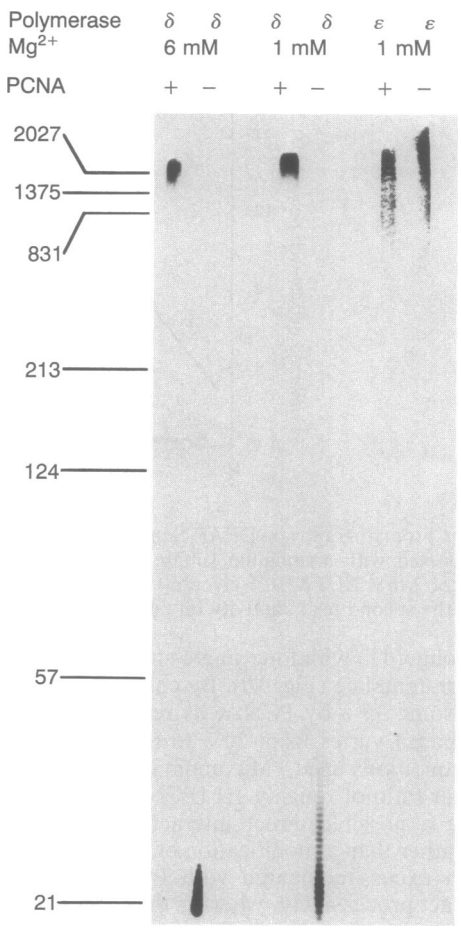


FIG. 4. Processivities of DNA pol δ and ε. Reaction conditions were those used for assays, except that the final concentration of $(dA)_{4000-5000}\cdot(dT)_{16}$ was 300 μM, only one oligo $dT_{16}$ was annealed per poly $dA_{4000-5000}$, and [$^3$H]dTTP was replaced with [$^{32}$P]dTTP (10,000 cpm/pmol). After 5 min at 37°C, samples were taken to determine nucleotide incorporated; then the remaining material was immediately extracted with phenol/chloroform, concentrated with 1-butanol, dialyzed, evaporated to dryness, and resuspended in TBE (89 mM Tris·HCl/89 mM boric acid/4 mM EDTA) containing 50% formamide and 0.1% bromphenol blue. After heating for 3 min at 100°C, samples were subjected to electrophoresis at 1600 V in a prerun 8% polyacrylamide gel prepared in 8 M urea in TBE. Approximately the same amount of radioactivity was added to each pair of lanes. Reactions with 6 mM $MgCl_2$ and pol δ had incorporated 0.114 and 0.28 nucleotide per primer terminus, respectively, in the presence or absence of PCNA. With 1 mM $MgCl_2$, these values were 0.112 and 1.42. Two $MgCl_2$ concentrations were utilized so that pol δ could be compared with observations in the literature at 6 mM and observations with pol ε at 1 mM. Ten-fold more pol δ was utilized in the absence of PCNA. pol ε reactions had incorporated 0.45 and 0.59 nucleotide per primer terminus in the presence and absence of PCNA, respectively. $^{32}$P-labeled markers (indicated in base pairs) were *Hae* III digests of pBR328 and *Eco*RI digests of λ DNA.

contamination. As expected, all three enzymes were sensitive to aphidicolin, and pol α was more sensitive to inhibition by BuPdGTP than were DNA pol δ and ε. However, the relative sensitivities of these enzymes to ddTTP were different from those reported under different conditions. DNA pol α was reported to be much more resistant to ddTTP than pol ε ($\delta_{II}$) (20), whereas in this case, the sensitivities were in the same range. DNA pol δ was resistant even to 5 mM ddTTP.

As reported for pol δ from human placenta (21), dimethyl sulfoxide activated HeLa pol δ. However, contrary to reports for calf thymus pol α, HeLa pol α was also activated by dimethyl sulfoxide. By contrast, pol ε was inhibited as reported previously (8).

Carbonyldiphosphonate, a tripolyphosphonate analog, was reported to inhibit a form of calf thymus pol δ but not pol α (22). When tested with the three HeLa enzymes, the compound was remarkably specific for pol δ. At 15 μM, only 7% of pol δ activity remained, whereas pol α was essentially unaffected and pol ε was inhibited by only 20%. At 100 μM, pol δ was essentially completely inactivated, whereas pol α and ε retained roughly half their activity. Hence, this compound, like dimethyl sulfoxide, easily distinguishes pol δ from pol ε. Hopefully, the data in Table 3 will clear up some conflicts in the literature arising because of confusion between pol δ and pol ε and provide means for distinguishing the three large polymerases.

**Comparison of the Primary Subunit Structures of DNA Polymerases by Partial Peptide Mapping with *N*-Chlorosuccinimide.** To study the structural relation of the DNA polymerases, the high molecular mass subunits were subjected to partial peptide mapping with *N*-chlorosuccinimide (Fig. 6). The maps of the 130-kDa (133 kDa on this gel) and 124-kDa peptides associated with the pol δ preparation are clearly related to one another but different from those of the 180-kDa peptide of pol α and the 215-kDa peptide of pol ε. Therefore, there is no indication that the large subunits of the three enzymes are related by proteolysis or other posttranslational modifications.

The four subunits of pol α (noted with molecular masses of 180, 73, 59, and 49 kDa) are known to be distinct from one another (24). The 55-kDa subunit of pol ε is clearly different from each of these and from the large pol ε subunit, although we cannot rule out some similarity between the 55-kDa

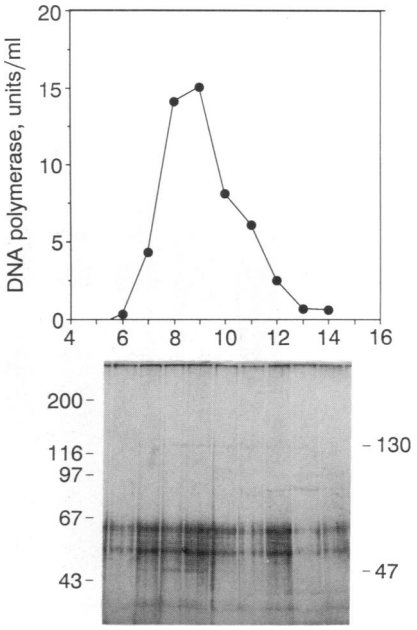


FIG. 5. Glycerol gradient sedimentation of HeLa cell DNA pol δ. The heparin–agarose fraction was applied to and sedimented through a glycerol gradient as described (8) and then samples were electrophoresed on an SDS/polyacrylamide gel that was then stained with silver. The bands between 55 and 63 kDa and near the gel bottom were present in all lanes and are artifacts of the staining procedure. They are distinguishable by color on the original gel and fractions 8–10 had only 130- and 47-kDa protein bands. Molecular mass markers (indicated in kDa) were myosin, β-galactosidase, phosphorylase b, bovine serum albumin, and ovalbumin.

subunit of pol ε and the 73-kDa subunit of pol α. There was insufficient 47-kDa pol δ subunit for these studies.

## DISCUSSION

PCNA activation, primer/template preferences, antigenic properties, subunit structure, and peptide maps indicate that pol δ from HeLa cells is unique and resembles that reported from calf thymus (3, 4, 9). Its catalytic properties are clearly different from those of DNA pol ε (or $\delta_2$), which we have characterized from HeLa cells (8, 10) and others have studied from calf thymus (6, 7, 19, 25). The purification of DNA pol δ and ε simultaneously from the same HeLa extract and the differences in the partial peptide maps of their large subunits support the premise that the enzyme proteins are different in spite of the weak cross-reactivity with a polyclonal antibody and possible common motifs in peptide maps found earlier between HeLa DNA pol ε and calf thymus DNA pol δ (11).

Table 2. Primer/template preferences of DNA pol δ in the absence of PCNA

| Primer/template | Relative activity |
|---|---|
| Poly(dA-dT) | 1.00 |
| Poly(dA)·oligo(dT) | 0.12 |
| Poly(dT)·oligo(dA) | 0.03 |
| Poly(dC)·oligo(dG) | 0.13 |
| Poly(rA)·oligo(dT) | <0.01 |
| DNase-activated salmon sperm DNA | 0.03 |
| Heat-denatured salmon sperm DNA | 0.03 |
| Native salmon sperm DNA | <0.01 |

Except for primer/templates and the required dNTPs at a concentration of 50 μM with $^3$H label in an appropriate nucleotide, the standard reaction conditions for DNA pol δ were used. One-tenth unit of enzyme was used per assay.

Table 3. Comparison of effects of inhibitors upon HeLa DNA polymerases

| Compound added | Relative DNA polymerase activity | | |
|---|---|---|---|
| | α | ε | δ |
| None | 1.00 | 1.00 | 1.00 |
| SJK 132-20 IgG (3.4 μg) | 0.02 | 0.97 | 1.60 |
| Aphidicolin (10 μg/ml) | 0.15 | 0.32 | 0.05 |
| ddTTP (μM) | | | |
| 50 | 0.97 | 0.96 | 1.02 |
| 500 | 0.50 | 0.25 | 1.32 |
| Dimethyl sulfoxide (10%) | 1.83 | 0.28 | 2.60 |
| BuPdGTP (10 μM) | 0.22 | 0.89 | 0.69 |
| Carbonyldiphosphonate (μM) | | | |
| 15 | 0.95 | 0.80 | 0.07 |
| 100 | 0.58 | 0.49 | <0.03 |

Except for the added factors and the use of poly(dA)·oligo(dT) and 1 mM $MgCl_2$ for all three DNA polymerases, reaction conditions were as described in the text. Neutralization with SJK 132-20 antibody was as described (13) and was included in the event that the unusual primer/template would unmask contaminating pol δ or pol ε in the pol α. One-tenth unit of each enzyme was used. For reactions with carbonyldiphosphonate, Tris·HCl was replaced with Hepes·KOH for pol α. DNA pol δ was inhibited equally by this compound at pH values of 6.5 and 7.5.

This work also points out similarities between mammalian DNA pol δ and pol ε and yeast DNA pol III (26) and pol II (27), respectively, which are known to be encoded by different genes (28). Like pol δ, pol III prefers poly(dA-dT) primer/template, has low processivity with poly(dA)·oligo(dT), and has processivity and activity increased by either yeast or mammalian PCNA (29). The subunit composition of pol III (19, 26, 30) also appears at this time to resemble that of HeLa pol δ.

Yeast pol II, conversely, is not especially affected by PCNA, is highly processive with poly(dA)·oligo(dT) (31, 32), and shows salt effects with activated DNA that resemble those of pol ε of mammalian cells (29, 30). The recent finding of a 200-kDa form of pol II in yeast, pol II* (33), and similar inhibitor responses and exonuclease activities also supports the similarity. Finally, a high degree of similarity, including primary structure of the catalytic subunit, has been reported between human DNA pol α (34) and yeast DNA pol I (31).

The inhibitor/activator spectra of DNA pol α, δ, and ε are clearly distinct. Variances from previous reports may reflect different reaction conditions, primer/template, and/or enzyme purity. In addition, previous preparations may have contained mixtures of pol ε and δ that were then unknown to be distinct. In this regard, previous inhibitor studies aimed at estimating the roles of the different DNA polymerases in replicative or repair synthesis of DNA ought to be reevaluated in some cases.

Since actively growing HeLa cells were utilized in these studies, we would expect the DNA polymerases involved in DNA replication to be abundant. Paradoxically, although thought to be involved in DNA replication, the amount of DNA pol δ in terms of activity and protein is only around 10% that of DNA pol ε, the enzyme implicated in DNA repair. This difference could reflect loss of pol δ during the purification, or, alternatively, the highly processive pol ε may be involved in replication.

In addition to the two DNA polymerases, δ and ε, that have been compared in this report, two mammalian DNA pol δ-like preparations, one from rabbit bone marrow (32, 35) and the other from human placenta (16), have been reported but not characterized extensively enough to categorize them as more δ- or ε-like. Hopefully the classification of these enzymes will soon occur.

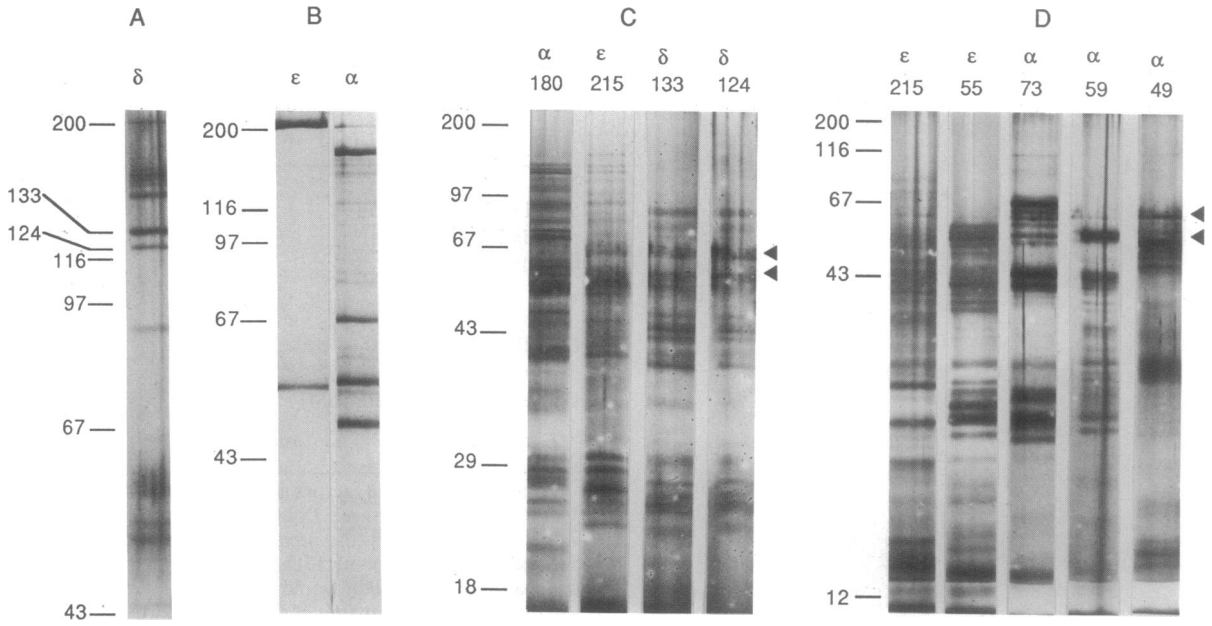


FIG. 6. Partial peptide mapping of DNA pol α, δ, and ε with *N*-chlorosuccinimide. Silver-stained SDS/polyacrylamide gel patterns of unhydrolyzed DNA pol δ TSK-400 fraction (*A*) and DNA pol α and ε preparations (*B*) and partial *N*-chlorosuccinimide peptide maps of the various subunits (*C* and *D*) are shown. For hydrolysis, samples of 300–600 ng of each peptide were obtained from SDS/polyacrylamide gels after staining with 0.05% Coomassie brilliant blue. The peptides were cleaved with *N*-chlorosuccinimide inside gel slices and electrophoresed as described (23), except that 2 M mercaptoethanol was replaced with 200 mM dithiothreitol in the equilibration solution and a 0.5-mm gel with 10% polyacrylamide was used. After electrophoresis, the gels were stained with silver. Molecular mass markers (indicated in kDa) included those in Fig. 5 plus carbonic anhydrase, β-lactoglobulin, and cytochrome *c*. Arrowheads indicate silver stain artifact bands, which were abnormally colored and present in control lanes lacking sample.


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