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Structural relationship between DNA polymerases ε and ε* and their occurence in eukaryotic cells

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ABSTRACT

Monoclonal antibodies raised against the N-terminal half of human DNA polymerase ε bind both to a large >200 kDa form of DNA polymerase ε from HeLa cells and to a small 140 kDa form (DNA polymerase ε*) from calf thymus, while antibody against the C-terminal half binds to DNA polymerase ε but does not bind to DNA polymerase ε*. These results indicate that the two enzymes have common structural motifs in their N-terminal halves, and that DNA polymerase ε* is very likely derived from DNA polymerase ε by removal of its C-terminal half. DNA polymerase ε as well as DNA polymerase ε* was detected in extracts from cells of numerous eukaryotic species from yeast to human. The results indicate that DNA polymerase ε and its tendency to occur in a smaller form, DNA polymerase ε*, are evolutionarily highly conserved and that DNA polymerase ε may occur universally in proliferating eukaryotic cells.

INTRODUCTION

Five types of DNA polymerases, α, β, γ, δ and ε, have been purified from mammalian cells (1). Except for mitochondrial polγ, the cDNAs encoding these enzymes have now been cloned. Polα and polδ are the sole DNA polymerases required for replication of simian virus 40 DNA *in vitro* (2), a model system for mammalian DNA replication. No role has been assigned for polε in this system. There is, however, strong genetic evidence that yeast *Saccharomyces cerevisiae* polε is also required for replication of chromosomal DNA (3). Both human and yeast polε have been implicated in repair of UV-damaged DNA (4,5).

Polε was purified to apparent homogeneity from HeLa cells and it consisted of a >200 kDa catalytic polypeptide and a 55 kDa non-catalytic polypeptide (4,6). A less purified form of polε from calf thymus (7) also contained a >200 kDa polypeptide with polymerase activity (8). The cDNA encoding human polε was recently cloned and the molecular weight of the protein calculated to be 258 kDa (9). A small 140 kDa form of polε, named polε* (10), was first purified close to homogeneity from rabbit bone marrow (11) and then from calf thymus (7,12,13). Earlier studies have suggested that polε* may be derived from polε by proteolysis (8,13). Novel monoclonal antibodies against human polε have now enabled reliable study of the structural relationship between polε and polε* and on their occurence in extracts from different eukaryotic cells from numerous species.

MATERIALS AND METHODS

Preparation of monoclonal antibodies against human polε

When preparing monoclonal antibodies against human polε catalytic polypeptide, the desired regions of cDNA clones (9) were amplified by PCR with primers containing extensions for *Eco*RI restriction sites. The obtained PCR products were purified by Geneclean kit (BIO 101, USA), trimmed with *Eco*RI and ligated into the pGEX1λT vector (Pharmacia, Sweden) containing an open reading frame encoding glutathione S-transferase (GST) in front of the cloning site. The construct was transformed into competent *Escherichia coli* strain DH5α or JM109 (14). The cells were grown in LB/ampicillin medium at 30°C, and expression of the fusion protein was induced by adding IPTG. The cells were collected by centrifugation and lysed by sonication. After removal of the debris by precipitation with Triton X-100, the fusion protein was bound to gluthathione–agarose beads (Pharmacia, Sweden) and the polypeptide representing polε was cleaved from GST by thrombin (Boehringer Mannheim, Germany) as described (15). The released protein was purified by SDS–PAGE. The protein was visualized by staining the gel with potassium chloride and it was eluted out from the gel slice as described (16) and dialyzed against PBS (10 mM potassium phosphate, 150 mM NaCl, pH 7.4). Monoclonal antibodies were produced at DiaBor Inc. (Oulu, Finland), using standard protocols (17). Briefly, five 6-week-old female Balb/c mice were immunized at 3 week intervals with 25 μg antigen/mouse emulsified in Freund's adjuvant (Difco Laboratories, USA). Serum samples were tested after second booster and the mouse showing the best response against the antigen in direct ELISA was selected for fusion. The selected mouse received an intravenous boost of 50 μg of the antigen 3 days before the fusion. Spleen cells from the immunized animal were fused with mouse myeloma cell line P3-X63-Ag8.653 using polyethylene glycol (PEG 4000; Gibco,

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USA). Hybrid cells were selected in HAT medium (DMEM, high glucose; Gibco, USA) supplemented with 10% NCTC-135 (Gibco, USA), 10% CPSR serum (Sigma, USA), 10% fetal bovine serum (Bioclear, UK), 5% HECS (Costar, The Netherlands), HAT supplement (Gibco, USA) and penicillin/streptomycin (Gibco, USA). Hybrid cells were screened using direct ELISA against the antigen and those positive were further screened by Western blot. Positive wells were cloned by the limiting dilution method. Medium from positive hybrids was collected and the monoclonal antibody was purified by G-Sepharose affinity chromatography (Pharmacia, Sweden). Monoclonal antibodies 93H3A, 93G1A and 93E24C were raised against polypeptides representing amino acids 1–176, 242–474 and 2095–2257, respectively, of the published sequence (9). The antibodies neutralized neither the polymerase nor exonuclease activity of the enzyme (data not shown).

Direct ELISA

EIA plates with 96 wells (Dynatech, USA) were coated with 100 ng/well of the antigen in PBS overnight at +4°C. Plates were washed (Anthos AutoWash, UK) four times with PBS and blocked with 1% BSA in PBS for 30 min. Samples were incubated for 1 h and after washing with PBS, the bound antibody was detected using the anti-mouse Vector ABC kit (Vector Laboratories, USA) according to the manufacturer's instructions. Plates were read at 405 nm (Anthos 2001, UK) using the MultiCalc program (Pharmacia, Sweden).

Western analysis

Protein samples were separated by SDS–PAGE and blotted onto nitrocellulose filters (Sleicher & Schüll, Germany). After the transfer the filters were washed with TBS (50 mM Tris–HCl, 150 mM NaCl, pH 7.4) and blocked with 5% non-fat milk in TBS for 1 h. Samples were incubated overnight with antibody at 1.5 μg/ml in TBS supplemented with 0.05% Tween-20. The blots were first incubated for 2 h with goat anti-mouse IgG conjugated with alkaline phosphatase (BioRad, USA; 1:3000 dilution in TBS–Tween solution) and then with color developing reagents, 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium in 100 mM Tris–HCl, pH 9.5).

Purification of enzymes and preparation of extracts for Western blots

The extracts for Western blots were prepared as described (13), except that buffers were supplemented with the protease inhibitors phenylmethylsulfonylfluoride and pepstatin A at concentrations of 1 mM and 1 mg/ml, respectively. HeLa polε and calf thymus polε* were purified as described (6,13), except that the hydroxyapatite fraction of the HeLa enzyme was depleted of remaining polα by SJK132-20 antibody (18) immobilized to agarose beads and the last step, glycerol gradient centrifugation, was omitted. HeLa polα and calf thymus polδ were purified according to published protocols (19,20). Human 293 cells and monkey COS cells were cultivated in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum, yeast *Saccharomyces cerevisiae* cells in YPD medium, and *Trichoplusia ni* insect cells in TNM-FK medium (Sigma, USA) supplemented with 10% fetal bovine serum. *Drosophila melanogaster* larvae were kindly donated by Dr Jaakko Lumme (University of Oulu, Finland) and *Xenopus laevis* embryos by Drs Raimo Hissa and Seppo Saarela (University of Oulu, Finland). Calf and chicken tissues were purchased from Animal Technologies Inc. (Tyler, USA).

RESULTS

Monoclonal antibodies against the N-terminal half of polε also recognize polε*

Monoclonal antibodies 93H3A, 93G1A and 93E24C were directed against polypeptides representing amino acid residues 1–176, 242–474 and 2095–2257, respectively, of the published sequence of human polε (9). All these antibodies were specific to >200 kDa polε in the sense that they did not recognize purified HeLa polα or calf thymus polδ on Western analysis (Fig. 1A, data for 93G1A shown). Antibodies 93G1A (Fig. 1B) and 93H3A (Fig. 1C) also recognized the 140 kDa calf thymus polε*, but antibody 93E24C, which was directed against the C-terminus of polε, did not. In Western analysis of partially purified HeLa polε preparation with antibody 93H3A, a smaller form with the same molecular weight as calf thymus polε* appeared (Fig. 1C). When staining of this HeLa polε preparation was prolonged the small form was also recognized by antibody 93G1A, but not by antibody 93E24C, suggesting that this small form probably represented HeLa polε*. Additionally, a weak signal at 120 kDa was occasionally detected in calf thymus polε* preparations with antibody 93G1A (Fig. 1B), but never with antibodies 93H3A or 93E24C. Since 93H1A was raised against a polypeptide representing amino acid residues 1–176 of HeLa polε and 93G1A against a polypeptide representing amino acid residues 242–474, the 120 kDa form was obviously derived from the 140 kDa polε* by removal of a 20 kDa fragment from its N-terminus.

The results presented indicate that polε* shares common epitopes with the N-terminal half of polε but not with the C-terminus, and that 140 kDa polε* is likely to be derived from 258 kDa polε by proteolysis or by splicing so that about 120 kDa of the C-terminal half of polε has been removed.

Polε is present in extracts from fetal calf thymus

Purification of polε-like polymerase activity from calf thymus to homogeneity or close to homogeneity has repeatedly led to polε* preparation (12,13). Western analysis with the mixture of antibodies 93G1A and 93H3A revealed that a >200 kDa antigen was present and the predominant form of the enzyme, in the extracts from fetal calf thymus (Fig. 2). Although the specificity of the antibodies was not high enough to definitely identify polε* from crude extracts, it is likely to be present at 140 kDa as shown (Fig. 2). These results indicate that polε is present and abundant in calf thymus tissue, even though purification of the enzyme from calf thymus has previously led to polε*.

Polε is present in most proliferating eukaryotic cells

To study the occurence of polε and polε*, we examined extracts from proliferating cells of numerous eukaryotic species. A doublet band of >200 kDa antigens was detected by the mixture of antibodies 93G1A and 93H3A in extracts prepared from cultivated human 293 cells and monkey COS cells (Fig. 3). Both antigens were also recognized by the two antibodies separately (data not shown). Since the antibody 93H3A was raised against

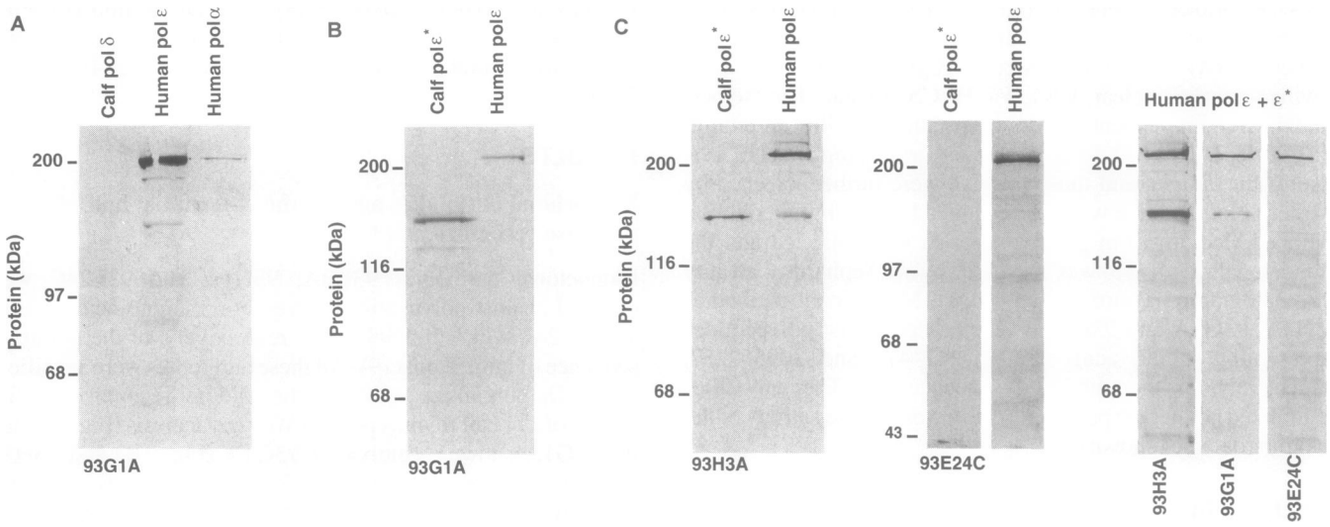

Figure 1. Western analysis of purified polα, δ, ε and ε* with antibodies against different regions of human polε. The enzymes were purified as described in Materials and Methods. 100 ng of each enzyme, given in the quantity of the catalytic polypeptide, was separated on 6% SDS–polyacrylamide gels, transferred onto nitrocellulose, and stained with the indicated monoclonal antibody. Antibodies 93H3A, 93G1A and 93E24C were raised against polypeptides representing amino acid residues 1–176 (N-terminus), 242–474 and 2095–2256 (C-terminus), respectively, of the human polε. (**A**) Specificity of the antibody 93G1A for polε. A band at >200 kDa in the polα lane represents contaminating polε. Standard 15 min color developer treatment of blots was prolonged to 5 h. (**B**) Recognition of calf thymus polε* by the antibody 93G1A. (**C**) Analysis of calf thymus and HeLa polε* and HeLa polε by the three antibodies. With the exception of the left panel, color developer treatment was prolonged to 5 h.

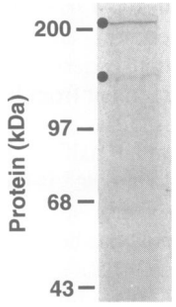

Figure 2. Polε and polε* in extracts from fetal calf thymus. The extract for Western analysis was prepared as described in Materials and Methods. 100 µg of protein was loaded and separated on a 6% SDS–polyacrylamide gel, transfered onto nitrocellulose and stained with a mixture of antibodies 93H3A and 93G1A that were raised against polypeptides representing amino acid residues 1–176 and 242–474, respectively, of human polε. For clarity the signals likely to represent polε (>200 kDa) and polε* (140 kDa) are shown by spots.

a polypeptide containing only 176 N-terminal amino acids, the apparent size difference between the two antigens is caused by proteolysis of the C-terminus or by a post-translational modification. The large >200 kDa antigen, likely to represent polε, was also detected by at least one out of the two antibodies in extracts from *D.melanogaster* larvae, cultivated *T.ni* insect cells and yeast *S.cerevisiae*. No clear signal from an antigen of the size of polε was detected in extracts prepared from *X.laevis* embryos and chicken thymus, but these extracts contained antigens in the size range 140–160 kDa that are likely to represent polε*. The only samples from which no clear signal of the size of polε* was obtained were the insect cells from *T.ni* and *D.melanogaster.*

DISCUSSION

Previous comparisons of HeLa polε and calf thymus polε* revealed similarities in catalytic properties. Furthermore, comparison by peptide mapping suggested that the two enzymes have some structural similarity (13). The three monoclonal antibodies against human polε allow a more reliable structural comparison between the two enzymes. Polε and polε* contain common epitopes that are recognized by both of the two monoclonal antibodies raised against N-terminal parts of polε. A monoclonal antibody against the C-terminus of polε does not recognize polε*, suggesting that polε* is a truncated form of polε that has lost its C-terminal half. So far, we have detected only one message with a length of 7.5 kb (9), suggesting that neither alternative splicing of RNA nor the existence of two genes encoding highly homologous proteins are likely reasons for the existence of two polε forms in extracts. Polε* is thus very likely derived by proteolysis from polε, or by splicing at the protein level.

One or both of polε and polε* were present in detectable quantities in extracts from a variety of proliferating eukaryotic cells. This distribution could not be affected by general protease inhibitors (data not shown), suggesting that polε* may be present *in vivo*. Both polε and polε* have also been purified from *S.cerevisiae* extracts (21). Deletion of a part of the yeast gene showed that the N-terminal half of the yeast polε, which is probably structurally close to polε*, is sufficient for replication and survival of cells (22). Polε has been implicated in repair of UV damage in human fibroblasts (4) and found to be a component of a recombination complex that was able to repair deletions and

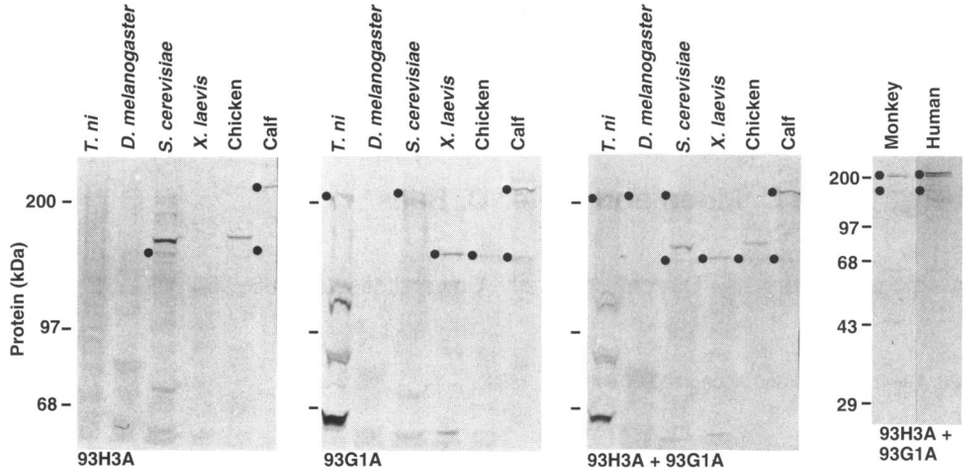

Figure 3. Evolutionary conservation of polε. Extracts for Western analysis were prepared as described in Materials and Methods. 50 µg of protein was loaded and separated on 6 or 7.5% SDS–polyacrylamide gels, transfered onto nitrocellulose filters and stained with antibodies 93H3A and/or 93G1A as indicated. *Trichoplusia ni* sample was from cultivated insect cells, the *D.melanogaster* sample from larvae, the *X.laevis* sample from embryos and samples from chicken and calf were from thymus. Monkey and human samples were from cultivated COS and 293 cells, respectively. For clarity the signals likely to represent polε (>200 kDa) and ε* (140 kDa) are shown by spots.

double-strand breaks (23). It is not known whether polε* can replace polε in these processes.

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REFERENCES

1 Wang, T.S.-F. (1991) *Annu. Rev. Biochem.* **60**, 513–552.
2 Waga, S. and Stillman, B. (1994) *Nature* **369**, 207–212.
3 Morrison, A. and Sugino, A. (1993) *Prog. Nucleic Acid Res. Mol. Biol.* **46**, 93–120.
4 Nishida, C., Reinhard, P. and Linn, S. (1988) *J. Biol. Chem.* **263**, 501–510.
5 Wang, Z., Wu, X. and Friedberg, E.C. (1993) *Mol. Cell. Biol.* **13**, 1051–1058.
6 Syväoja, J.E. and Linn, S. (1989) *J. Biol. Chem.* **264**, 2489–2497.
7 Crute, J.J., Wahl, A.F. and Bambara, R.A. (1986) *Biochemistry* **25**, 26–36.
8 Siegal, G., Turchi, J.J., Jessee, C.B., Mallaber, L.M., Bambara, R.A. and Myers, T.W. (1992) *J. Biol. Chem.* **267**, 3991–3999.
9 Kesti, T., Frantti, H. and Syväoja, J.E. (1993) *J. Biol. Chem.* **268**, 10238–10245.
10 Bambara, R.A. and Jessee, C.B. (1991) *Biochim. Biophys. Acta* **1088**, 11–24.
11 Goscin, A. and Byrnes, J.J. (1982) *Biochemistry* **21**, 2513–2518.
12 Focher, F., Spadari, S., Ginelli, B., Hottiger, M., Grassmann, M. and Hübscher, U. (1988) *Nucleic Acids Res.* **14**, 6279–6295.
13 Kesti, T. and Syväoja. J.E. (1991) *J. Biol Chem.* **266**, 6336–6341.
14 Inoue, H., Nojima, H. and Okayama, H. (1990) *Gene* **96**, 23–28.
15 Smith, D.B. and Johnson, K.S. (1988) *Gene* **67**, 31–40.
16 Prussak, C.E., Almazan, M.T. and Tseng, B.Y. (1989) *Anal. Biochem.* **178**, 233–238.
17 Oi, V.T. and Herzenberger, L.A. (1980) Immunoglobulin-producing hybrid cell lines. In Mishell, B.B. and Shiigi, S.M. (eds), *Selected Methods in Cellular Immunology*, pp. 351–372. San Francisco: Freeman.
18 Tanaka, S., Hu, S.-Z., Wang, T.S.F. and Korn, D. (1982) *J. Biol. Chem.* **257**, 8386–8390.
19 Syväoja, J., Suomensaari, S., Nishida, C., Goldsmith, J.S., Chui, G., Jain, S. and Linn,S. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6664–6668.
20 Weiser, T., Gassmann, M., Thömmes, P., Ferrari, E., Hafkemeyer, P. and Hübscher, U. (1991) *J. Biol. Chem.* **266**, 10420–10428.
21 Hamatake, R.K., Hasegawa, H., Clark, A.B., Bebenek, K., Kunkel, T. and Sugino, A. (1990) *J. Biol. Chem.* **265**, 4072–4083.
22 Morrison, A., Araki, H., Clark, A.B., Hamatake, R.K. and Sugino, A. (1990) *Cell* **62**, 1143–1151.
23 Jessberger, R., Podust, V., Hübscher, U. and Berg, P. (1993) *J. Biol. Chem.* **268**, 15070–15079.