NONPROTEOLYZED FORM OF DNA-POLYMERASE ε FROM T-CELL SPONTANEOUS LYMPHOMA OF SPRAGUE–DAWLEY INBRED RAT: ISOLATION AND CHARACTERIZATION

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Using a simple isolation procedure and selective assay for the determination of enzyme activity the nonproteolyzed and proteolyzed form of DNA-polymerase ε (pol ε and pol ε*) from the lymphoma of Sprague–Dawley inbred rats were purified. Nonproteolyzed pol ε is composed of two subunits (240 000 and 50 000) with sedimentation coefficient 10.5 S, while the subunit composition of pol ε* was 145 000 and 73 000. Estimated K_m values for dATP and dGTP as well as K_i values for acyclic nucleotide analogs (PMEApp, HPMPApp and PMEDAPpp) in pol ε and pol ε* catalyzed reactions have shown that a proteolysis probably does not affect pol ε binding site for dNTPs. Both enzymes (pol ε and pol ε*) possess 3′-5′-exonuclease activity with different K_m for 3′-OH end of template poly dA-oligo dT_{18} (1.6 μmol/l and 0.36 μmol/l, respectively).

Key words: Pol ε; SD-Lymphoma; Acyclic nucleotide analogs; DNA-Polymerases; PMEA; HPMPA.

Nuclei of eukaryotic cells dispose of four DNA-polymerases (pol α, β, δ and ε; see ref.[1]). DNA-Polymerase ε has been so far reported in two enzyme forms[2,3]: (i) as a protein composed of two polypeptides with molecular weights >200 000 and 50 000 (pol ε) (refs[4–6]), or (ii) as a single polypeptide 140 000 (pol ε*) (refs[6–9]). It was also shown that pol ε* originates in splitting of C-end domain of >200 000 pol ε subunit without any loss of its polymerase and/or 3′-5′-exonuclease activity[10]. Moreover, because only one type of mRNA molecule for the enzyme was confirmed (7.5 kb; ref.[11]) it is very probable, that pol ε* does not originate by an alternative splicing of hnRNA but is proteolytically partly degraded during the posttranslational modification[10] or during a purification procedure. On the other hand, the fact that both enzymes were found in extracts of various cells indicates their possible parallel existence *in vivo* (ref.[10]).

Yeast pol ε is required to indicate replication blocks and UV-damage of DNA. In this response, yeast cells are arrested at S-phase of their cell cycle and induce the transcrip-

tion of genes whose products facilitate DNA repair[12,13]. Disruption in genes for DNA-pol α, δ and ε of *Saccharomyces cerevisiae* is lethal and results in the changes of cell morphology[14] indicative of a deffect in DNA replication. A transcript of pol ε was detected to be 5- to 16-fold more prevalent in proliferating than in quiescent HeLa cells[15]. In contradiction to pol α and δ, pol ε is not required for replication of simian virus SV 40 DNA *in vitro* but may participate in DNA recombination[16–19]. However, sufficient evidence to confirm or exclude the role of DNA-polymerase ε in the replication fork *in vivo* has not yet been presented.

In the present study which is based on our previous experience[20] in isolation of DNA-polymerases α, δ and ε*, we have focused on purification of nonproteolyzed pol ε, its characterization and comparison with pol ε*. For the evaluation of the catalytic properties of both enzymes we employed acyclic nucleotide analogs of the PME- and HPMP-type which were earlier found to be selective substrate/inhibitors of replicative DNA-polymerases[21].

RESULTS AND DISCUSSION

In our previous study[20] we have described reaction conditions under which pol α, δ and ε* can be selectively detected in the eluates from chromatography column. We have also shown that pol α and pol ε* are easily separated on Mono Q column. At an increased ionic strength of the mobile phase (300 mM NaCl) another DNA-polymerase is eluted from the column (Fig. 1); this enzyme is pol ε. The enzyme fraction containing pooled pol ε activity (fraction II, Fig. 1) was efficiently separated from the contaminating pol α on ssDNA cellulose column using a linear concentration gradient of KCl (pol ε ≈ 220 mM KCl; pol α was eluted at 130 mM KCl; Fig. 2). The subsequent affinity chromatography of the prepurified pol ε fraction on Blue Agarose (isocratic elution with 300 mM KCl) followed by centrifugation in glycerol (linear concentration gradient) gave preparation of pol ε which was homogeneous on PAGE (Table I, Fig. 3).

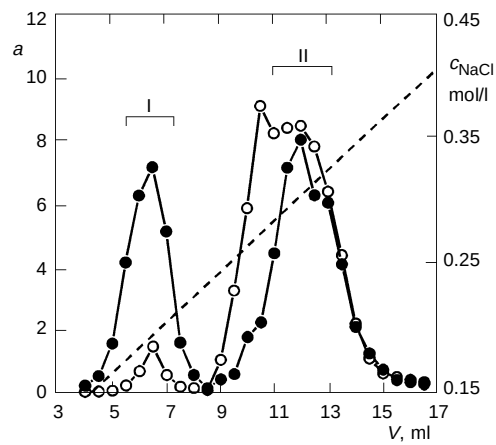

FIG. 1
FPLC of pol ε and ε* on Mono Q column. Enzyme activity of pol α (○), pol ε and ε* (●). NaCl linear concentration gradient (– – – –), DNA-polymerase activity *a* expressed as an incorporation of [^{3}H]dTMP into poly dA-oligo $dT_{12–18}$ template-primer (cpm . 10^{-4}), *V* elution volume, *c* concentration of NaCl, I pooled fractions of pol ε*, II pooled fractions of pol ε

Nearly homogeneous protein of pol ε* was obtained by the modification of the original method[20] where the Heparin Sepharose was replaced by ssDNA cellulose and affinity chromatography on Blue Agarose (for details see Experimental).

Subunit Composition of pol ε and pol ε from SD-Lymphoma*

The pooled fractions with maximum enzyme activity resulting from glycerol gradient centrifugation were concentrated (Centricon-30) and applied on SDS-PAGE (8% gel; Fig. 3). The electrophoretic analysis has shown that pol ε (peak of activity with the sedimentation coefficient 10.5 S) is composed from two polypeptides 240 000 and 50 000. This finding is in a good agreement with so far published data on enzymes isolated

TABLE I
Purification of DNA-polymerase ε from SD-lymphoma

Fraction	Protein, mg	Total activity, U	Specific activity, U/mg
Crude extract	1 300	[a]	[a]
30–50% $(NH_4)_2SO_4$ cut	440	4 764	10.8
DEAE Sephacel	106.5	3 647	34.2
Phosphocellulose	14.7	2 670	181
Mono Q	5.0	1 700	340
ssDNA-cellulose	0.57	772	1 350
Blue Sepharose	0.12	300	2 500
Glycerol gradient[b]	0.03	120	4 000

[a] Not determined. [b] For the last purification step, only an aliquot was used; the presented data refer to the final yield of proteins (see Experimental).

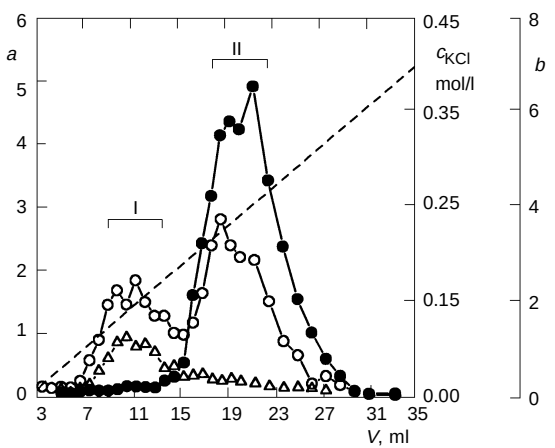

FIG. 2
DNA pol ε on ssDNA-cellulose column. Enzyme activity of pol ε (●), pol α (○), DNA-primase (Δ), KCl linear concentration gradient (– – – –). DNA-polymerase activity *a* expressed as an incorporation of [^{3}H]dTMP into poly dA-oligo $dT_{12–18}$ template-primer (cpm . 10^{-4}), DNA-primase activity *b* expressed as an incorporation of [^{3}H]dGMP into poly dC (cpm . 10^{-3}) in the presence of GTP, *V* elution volume, *c* concentration of KCl, I pooled fractions of pol α, II pooled fractions of pol ε

from other sources[4,5,11,22]: the molecular weight of large subunit of HeLa cells and yeast pol ε estimated from an amino acid composition is 258 000 and 256 000 (ref.[11]). Native enzyme isolated from HeLa cells is composed from two polypeptides >200 000 and 55 000 (refs[4,5]); also the molecular weight of the catalytic subunit of pol ε isolated from *Drosophila melanogaster* analyzed by Western blotting amounts to 250 000 (ref.[22]).

The latter of the enzymes studied (peak of activity with the sedimentation coefficient of 7.0 S) consists of two polypeptides (145 000 and 73 000). The larger subunit is undoubtedly the proteolyzed form of pol ε, *i.e.* pol ε*, while the smaller polypeptide is most probably either an additional product of the proteolytic degradation, or a contaminant. Also in this case, our data are in agreement with the previous findings of other authors[3,4,6,10,20].

Characterization of pol ε from SD-Lymphoma

For the identification of DNA-polymerase ε, we have used the preparation resulting from the Blue Agarose column step of our previously described isolation procedure. The data summarized in Tables II and III, demonstrate that the enzyme is inhibited by aphidicolin or *N*-ethylmaleinimide, but it does not possess any reverse trancriptase activity (template: poly A-oligo dT_{12-18}; data not shown), therefore, the presence of pol β or γ can be clearly excluded[23–25]. The enzyme preparation does not contain any DNA-primase activity (Table III) and does not interact either with the monoclonal antibody antiDNA pol α SJK 237-71 (data not shown) or with BuPdGTP* (ref.[23]). Though the enzyme preparation possesses 3′-5′-exonuclease activity, it is not stimulated with

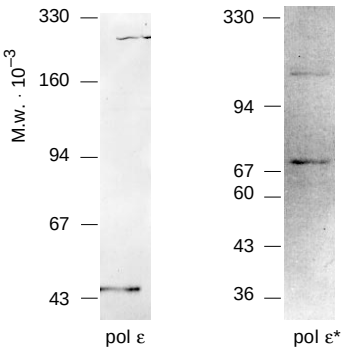

FIG. 3
SDS-PAGE polypeptide pattern of pol ε and pol ε* after glycerol gradient centrifugation. M.w. molecular weight. For details see Experimental

* Abbreviations: BuPdGTP, N^2-(4-butylphenyl)-2′-deoxyguanosine 5′-triphosphate; HPMPApp, (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine diphosphate; PMEApp, 9-[2-(phosphonomethoxy)ethyl]adenine diphosphate; PMEDAPpp, 2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine diphosphate; PCNA, proliferating cell nuclear antigen.

PCNA (Tables II, III). Therefore, both the presence of pol α and δ should also be excluded[23,26–28]. The above properties as well as its subunit composition unambiguously confirm that the enzyme activity corresponds solely to nonproteolyzed pol ε.

*Comparison of Catalytic Activity of pol ε and pol ε**

The values of $^{dATP}K_m$ (template-primer poly dT-oligo $dA_{12–18}$), $^{dGTP}K_m$ (template-primer poly dC-oligo $dG_{12–18}$) and K_i/K_m for acyclic nucleoside phosphonate analogs (HPMPApp, PMEApp and PMEDAPpp) which are selective DNA-pol inhibitors[21] (Table IV) were also estimated for both enzyme preparations. The nearly identical values of these parameters indicate that the proteolysis probably does not affect the binding site of pol ε for dNTPs. On the other hand, different K_m(3′-OH) values of associated 3′-5′-exonuclease towards poly dA-oligo dT_{18} (Fig. 4) has been found (1.6 μmol/l (pol ε) and 0.36 μmol/l (pol ε*), respectively) and non-processive hydrolysis of substrate was observed.

TABLE II
Characterization of DNA-polymerase ε from SD-lymphoma on poly dA-oligo $dT_{12–18}$

Added factor	Concentation	Relative activity[a]
None	–	1.00
PCNA	18 μg/ml	0.64
Aphidicolin	10 μg/ml	0.19
NEM	5 mmol/l	<0.001
BuPdGTP	20 μmol/l	0.92

[a] Relative enzyme activity in the presence of 0.05 U of pol ε per assay.

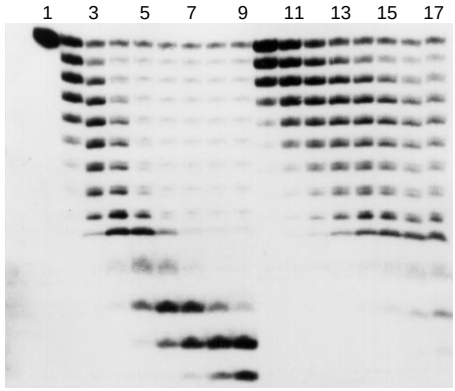

FIG. 4
Processivity of associated 3′-5′-exonuclease of pol ε and pol ε* on dsDNA. Template poly dA-[^{32}P]oligo dT_{18} was incubated with 1 U of pol ε and/or pol ε* and then analyzed on denaturing PAGE gel (start is on the top of gel). Lane 1 control, lanes 2–8 time course of the pol ε* catalyzed reaction, lanes 9–17 time course of the pol ε catalyzed reaction (in both cases time intervals 15 min)

EXPERIMENTAL

Materials

Acyclic nucleotide analogs PMEA, PMEDAP and HPMPA were prepared according to the described procedures[34,35], their diphosphates were synthetized by the morpholidate method[36]. BuPdGTP was generous gift of Dr G. E. Wright, Department of Pharmacology, University of Massachusetts Medical School, Worcester, MA, U.S.A. Nucleotides (dTTP, dATP, dGTP and ATP), oligoribonucleotides (oligo dA_{12-18}, oligo dT_{12-18}, oligo dG_{12-18}), DEAE Sephacel, FPLC column Mono Q HR 5-5 and MicroSpin G-25 columns were products of Pharmacia P-L Biochemicals (Sweden). ssDNA-Cellulose, Blue Agarose (Reactive Blue 4), poly A, poly dT, poly dC, poly dA, EDTA, urea, bovine serum albumine (BSA) and DTT were purchased from Sigma (Czech Republic). Whatman (Great Britain) supplied Phosphocellulose P-11, glass filters GF/A and DEAE paper discs (DE-81). Glycerol (99%, Riedel–deHaën) was redistilled in vacuum (glass apparatus). Trichloroacetic acid (TCA) was from LOBA (Austria), labelled deoxyribonucleotides [8-^{3}H]dATP (888 GBq/mmol), [methyl-^{3}H]dTTP (1.5 TBq/mmol), [8-^{3}H]dGTP (659 GBq/mmol), [γ-^{32}P]ATP (110 TBq/mmol) and T4 polynucleotide kinase were products of Amersham (Great Britain). PCNA was purified to homogeneity according to the published method[30] from *E. coli* strain BL 1/DE 3 harboring a plasmid encoding the human PCNA cDNA sequence. This bacterial strain was kindly provided by Dr B. Stillman, Cold Spring Harbor Laboratory (Cold Spring Harbor, NY, U.S.A.). Monoclonal antibodies SJK 237-71 IgG against the human DNA pol α were prepared according to ref.[31].

TABLE III
Enzyme activities associated with DNA-polymerase ε from SD-lymfoma

Enzyme activity	Template	Pol ε	Pol ε*
Primase[a]	poly dT	<0.1	<0.1
	poly dC	<0.1	<0.1
3′-5′-Exonuclease K_m, μmol/l	poly dA-oligo dT_{18}	1.6 ± 0.3	0.36 ± 0.07

[a] The results are given in picomoles of nucleotide incorporated (under conditions of primase assay) when the amount of an enzyme corresponding to 0.05 U on poly dA-oligo dT_{12-18} was used.

TABLE IV
Comparison of catalytic properties of pol ε and pol ε*

Enzyme	$^{dGTP}K_m$ μmol/l	$^{dATP}K_m$ μmol/l	$^{analog}K_i/^{dATP}K_m$		
			HPMPApp	PMEApp	PMEDAPpp
Pol ε	16.8	4.2	0.12	0.25	0.23
Pol ε*	14.8	6.1	0.12	0.24	0.22

Template-primers were prepared by annealing of oligodeoxynucleotide to the corresponding polydeoxynucleotide at 60 °C for 5 min with the subsequent cooling to 20 °C in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

Kinetic Experiments

All reaction mixtures contained 200 μg/ml BSA, 1 mM DTT and 10% glycerol; enzyme kinetics were followed at 37 °C in the 25 μl volume; one unit (U) of the DNA-polymerase activity is defined as an amount of enzyme which catalyzes an incorporation of 1 nmol of dTTP into acid-insoluble precipitate after 30 min under the conditions listed below.

Determination of K_m for dATP, K_m for dGTP and K_i for HPMPApp, PMEApp and PMEDAPpp was carried out under conditions described earlier[21].

DNA-3′-5′-exonuclease activity was determined in the reaction mixture which contained: 40 mM Hepes(K^+) pH 7.5, 5.0 mM $MgCl_2$, 75 mM KCl and the required concentration of poly dA-oligo dT_{18}. The corresponding substrate oligo dT_{18} (500 pmol) was labelled with ^{32}P (40 μM [γ-^{32}P]ATP, 9.0 TBq/mmol; 30 U T4 polynucleotide-kinase at 37 °C, 25 μl reaction volume) according to the method recommended by Amersham (Tested User Friendly™, T4 Polynucleotide Kinase 5′ End Labelling Protocol, USB™). After enzyme denaturation (75 °C, 25 min), the excess of non-reacted monomer was separated on a Micro-Spin G-25 column, the resulting oligomer was annealed with poly dA in the molar ratio 1 : 10 and subsequently diluted with unlabelled poly dA-oligo dT_{18} to the required concentration. The 3′-5′-exonuclease activity of pol ε and pol ε* on poly dA-[^{32}P]oligo dT_{18} was determined after inactivation of samples by an addition of equal volume of 98% formamide containing 10 mM EDTA, 0.2% Bromophenol Blue and 0.2% Xylene Cyanole FF. The samples were then incubated at 60 °C (5 min), cooled to 4 °C and applied on 20% polyacrylamide gel containing 89 mM Tris-boric acid (pH 8.3), 2 mM EDTA and 7 M urea. After electrophoresis, the gel was dried and quantitatively evaluated on Phosphorimager SF (Molecular Dynamics, Great Britain). The processivity of pol ε and pol ε* 3′-5′-exonuclease was followed in 15-min time intervals (0.4 μM poly dA-[^{32}P]oligo dT_{18}, 1 U of enzyme).

Kinetic constants (K_m and K_i) were determined from the both Lineweaver–Burk and Dixon plots and data were evaluated with the non-linear regression method.

A selective method for distinguishing of pol α, δ, ε and ε* by estimating DNA-polymerase, DNA-primase and DNA-3′-5′-exonuclease activity was described in detail earlier[20]; the selective detection of pol ε was performed under the same conditions as pol ε*.

Purification of pol ε

Buffers. Buffer F: 50 mM Tris-HCl, pH 7.5, 400 mM NaCl, 1 mM EDTA, 5 mM DTT, 0.05% Triton X-100, 10% glycerol. Buffer G: 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 20% glycerol, 5 mM benzamidine, 0.2 mM PMSF. Storage buffer: 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 5 mM DTT, 0.05% Triton X-100, 30% glycerol.

SD-tumors (60 g wet weight) were processed according to the published purification scheme[20]. After the homogenization and salting out steps, the fractions with an activity of pol ε were successively chromatographed on DEAE Sephacel, P-11 and Mono Q HR 5-5 columns[20].

After the elution from Mono Q HR 5-5 column a portion with the larger elution volume ($V_e \approx 12$ ml, fraction II) was diluted with the buffer G (14.6 ml) to conductivity of 50 mM KCl and applied onto ssDNA-cellulose column (1.5 × 1.0 cm) equilibrated with 50 mM KCl in buffer G. The column was then washed with the same buffer (5 column volumes) and the pol ε activity was eluted by linear concentration gradient of KCl (40 ml; 50–400 mM in buffer G). The collected fractions containing the highest enzyme activity ($V_e \approx 19$ ml, 6.3 ml) were diluted with the buffer G (1 : 1) and applied onto

Blue Agarose column (1.6 × 0.4 cm) equilibrated in 100 mM KCl containing buffer G. The affinity column was washed with 100 mM KCl in buffer G (5 column volumes) and the elution was continued with 300 mM KCl in buffer G. The resulting peak of pol ε activity was pooled and divided into two parts. One part was dialyzed against storage buffer[20] and stored at –70 °C for kinetic measurements. The other part was dialyzed against 10% glycerol in the buffer F and then used for sedimentation analysis in the linear concentration gradient of glycerol (15–35%) (ref.[20]). The peak of the enzyme (10.5 S) was concentrated (Centricon-30, JA 20.1, 5 000 rpm) and applied on 8% PAGE.

Purification of pol ε*

Pol ε* was isolated by the pocedure described by Kramata *et al.*[20] with the following modifications: the pooled fractions after the elution from Mono Q HR 5-5 column ($V_e \approx 7$ ml, fraction I) were diluted with the buffer G to the conductivity of 50 mM KCl and applied onto ssDNA-cellulose column (1.6 × 0.4 cm) equilibrated with 50 mM KCl in the buffer G. The proteolyzed form of polymerase ε (ε*) was then processed in the same way as pol ε. The affinity purification on Heparin Sepharose used in the original method[21] was omitted.

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