DNA-POLYMERASES α, δ AND ε FROM T-CELL SPONTANEOUS LYMPHOBLASTIC LEUKEMIA OF SPRAGUE–DAWLEY INBRED RAT: ISOLATION AND CHARACTERIZATION

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Using a single isolation procedure and selective assays for the determination of enzyme activity, highly purified DNA-polymerases α, δ and ε were isolated from the lymphoma of Sprague–Dawley inbred rats. For pol α the subunit composition was 170, 70, 57 and 53 kDa with sedimentation coefficient 8.7 S for the native molecule; pol δ consists of two polypeptides (133 and 46 kDa; sedimentation coefficient 8.2 S), while pol ε is a single polypeptide (140 kDa) and its sedimentation coefficient is 7.0 S. Comparison of the interaction of individual enzymes with known inhibitors and proliferating cell nuclear antigen (PCNA) using the template-primer poly dA-oligo dT_{12-18}, gave the following data: (i) pol α is selectively inhibited by N^2-(*p*-butylphenyl)-2′-deoxyguanosine 5′-triphosphate (BuPdGTP) and stimulated by dimethyl sulfoxide; (ii) all the enzymes are inhibited by *N*-ethylmaleimide and aphidicolin; (iii) PCNA stimulates pol δ approximately 50 times while pol ε is moderately inhibited; (iv) pol α exhibits considerably higher DNA-primase activity with poly dC as template than with poly dT, and negligible 3′-5′-exonuclease activity whereas pol δ and pol ε, which do not exert any DNA-primase activity have approximately the same 3′-5′-exonuclease activity. The ability of individual polymerases to utilize poly dT-oligo dA_{12-18} as a template-primer at different pH values, ionic strengths and Mg^{2+}-concentrations was also investigated. In comparison to poly dA-oligo dT_{12-18} template-primer, pol α has 140% of enzyme activity on poly dT-oligo dA_{12-18} under optimal conditions, whereas the activity of pol ε and pol δ is 4 times and 10 times lower, respectively.

The spontaneous acute T-cell lymphoblastic leukemia in Sprague–Dawley inbred rats has a 17% incidence in animals younger than one year. Since its morphological and immunogenetic characteristics, as well as its clinical course resemble acute lymphoblastic leukemia L2 (refs[1,2]) in children, thus is considered to be a relevant model of human leukemia. The determination of cytostatic effects of newly synthesized drugs in such an in vivo system together with the elucidation of the mechanism of their action at molecular level, is important for a rational approach aimed towards designing compounds for cancer chemotherapy, or may serve as a tool to study the growth and division of

cells. In this context the enzymes involved in the replication of chromosomal DNA are often regarded as suitable targets, especially in investigation concerning the mode of action of nucleoside and nucleotide analogs.

According to the recent knowledge, three DNA-polymerases – α, δ and ε (ref.[3]) – participate in the replication of eukaryotic genome. Their importance for the cell growth has been shown in yeasts[4–6] and their probable function in the replication fork has been demonstrated mainly by extensive studies of the system reconstituting the DNA replication of SV-40 virus in vitro in the presence of human cell extracts[7,8]. While pol α in the complex with DNA-primase initiates the synthesis of both DNA strands during the early stage of replication and forms Okazaki fragments on lagging strand, pol δ forms leading strand and the function of pol ε probably consists in completing lagging strand by filling gaps between the Okazaki fragments[9,10]; moreover, it participates in UV-induced repair synthesis[11] and in DNA recombination[12].

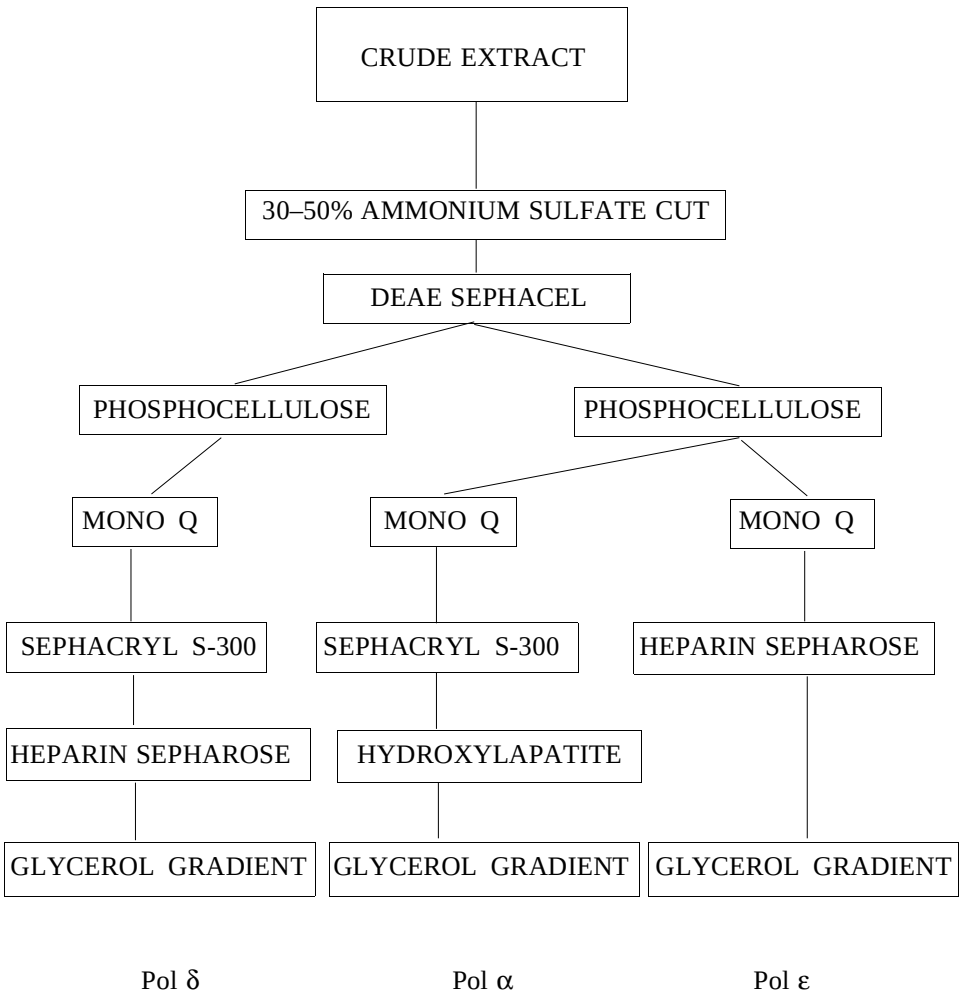
In the present report we describe the isolation of highly purified DNA-polymerases α, δ and ε from rat SD-lymphoma and verification of their identity by comparing their physical, chemical and catalytic properties with the data for the corresponding enzymes isolated from other sources. We expect that a parallel in vivo study of rat lymphoblastic leukemia and of appropriate in vitro system composed of isolated enzymes will elucidate the cytostatic effects of acyclic nucleotide analogs[13–16], and also that it will be useful for design of selective inhibitors of replicative DNA-polymerases as well as of biologically active drugs.

RESULTS AND DISCUSSION

The isolation of three replicative DNA-polymerases α, δ and ε from SD-lymphoma in a single isolation procedure followed by the specific combination of purification steps is described in Scheme 1. The reaction conditions for determination of the catalytic activity of these enzymes were chosen so as to enable the best estimation of the individual polymerases in the presence of each other (see Experimental) during the purification steps. Synthetic DNA template-primer, poly dA-oligo $dT_{12\text{-}18}$, has been used; under the assay conditions for pol α, pol ε has about 10% of its enzyme activity and the average activity of pol δ is about 2%. This enzyme is assayed only after the addition of PCNA which strongly stimulates the reaction proportionally to the purity of pol δ. The conditions applied for the estimation of pol ε activity are highly selective (the contribution of the total pol α and δ is maximally 1%). Data on the purification are given in Table I and represent the average of three experiments.

All the three DNA-polymerases were salted out from the crude extract at 30–50% ammonium sulfate saturation. During this step about 70% of the contaminating proteins were removed while the activity of the isolated enzymes was significantly stabilized. The subsequent separation of individual enzymes was reached by combining chromatography on DEAE Sephacel, phosphocellulose P-11 and Mono Q. Pol δ is separated

from the mixture of pol α and pol ε on DEAE Sephacel (Fig. 1) and freed from the residual pol ε on phosphocellulose column (Fig. 2). The contaminating pol α was removed using Mono Q (the "tail" peak of the elution profile; Fig. 3). Detection of pol δ consists in the determination of its increased activity in the presence of PCNA; the stimulation of enzyme activity in "tail" pol α peak is not seen. Independently of that the presence of a negligible amount of pol δ in pol α peak cannot be completely ex-



SCHEME 1

cluded. Subsequent steps given in the Scheme 1 further increased the relative purity of pol δ.

Crude separation of pol α and pol ε was achieved on phosphocellulose (Fig. 4). Pol α was further purified on Mono Q (Fig. 5), where contamination of pol δ was separated and pol ε was purified by chromatography on Mono Q (Fig. 6). Here the elution profile of pol ε displays a massive "front" peak (I) of enzyme activity, distinctly separated

TABLE I
Purification of DNA-polymerases α, δ and ε from SD-lymphoma

Fraction	Protein, mg	Total activity, U	Specific activity U mg^{-1}
Crude extract (I)	1 530	–[a]	–[a]
30–50% $(NH_4)_2SO_4$ cut (II)	476	–[a]	–[a]
	Pol δ		
DEAE Sephacel	102.6	4 500	43.8
Phosphocellulose	22.1	2 070	93.7
Mono Q	4.0	552	138.0
Sephacryl S-300 HR	0.83	262	315.7
Heparin Sepharose	0.071	69	971.8
Glycerol gradient[b]	0.024	39	1 625.0
	Pol α		
DEAE Sephacel	105.6	5 133	48.6
Phosphocellulose	8.2	2 150	262.2
Mono Q	2.2	948	430.9
Sephacryl S-300 HR	0.52	758	1 457.7
Heparin Sepharose	0.22	641	2 913.6
Glycerol gradient[b]	0.075	452	6 027.0
	Pol ε		
DEAE Sephacel	105.6	1 920	18.2
Phosphocellulose	7.5	1 268	169.1
Mono Q	0.62	260	419.3
Heparin Sepharose	0.054	72	1 336.9
Glycerol gradient[b]	0.025	59	2 360.0

[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).

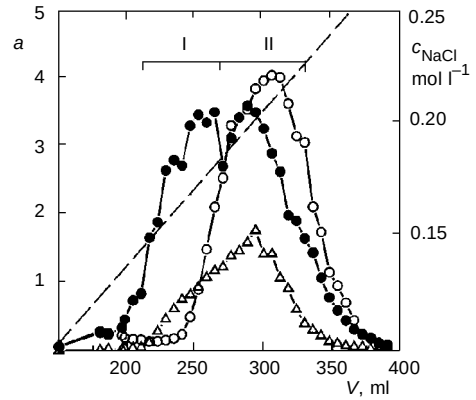


FIG. 1

Partial separation of pol δ on DEAE Sephacel. Enzyme activities of pol α (○), pol δ (●), pol ε (Δ). 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, I pooled fractions of pol δ, II pooled fractions of pol α + ε

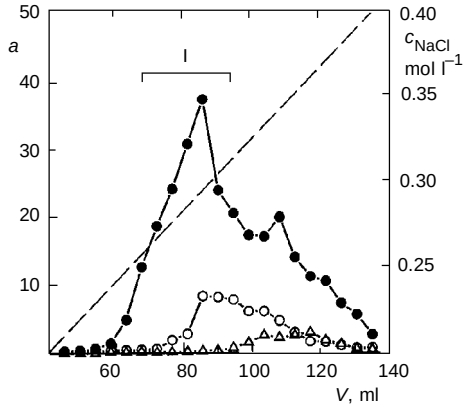


FIG. 2

Chromatography of pol δ on phosphocellulose column. Enzyme activities of pol α (○), pol δ (●), pol ε (Δ). 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, I pooled fractions of pol δ

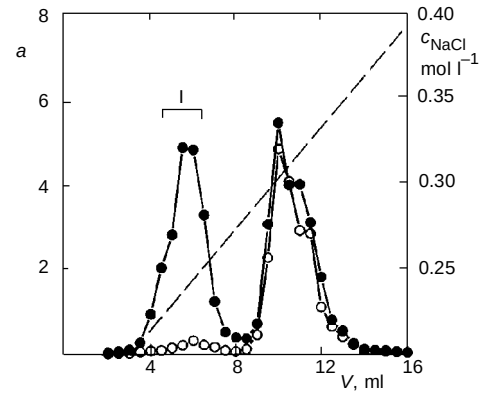


FIG. 3

FPLC of pol δ on Mono Q column. Enzyme activities of pol α (○) (without PCNA), pol δ (●) (with PCNA). 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, I pooled fractions of pol δ

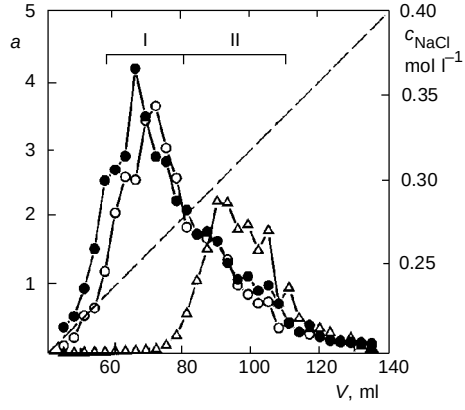


FIG. 4

Partial separation of pol ε on phosphocellulose column. Enzyme activities of pol α (○), pol δ (●), pol ε (Δ). 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, I pooled fractions of pol α and the residual pol δ, II pooled fractions of pol ε

from that of the contaminating pol α, and two smaller peaks in the "tail" part of the elution profile. Further manipulation was performed only with peak I resulting thus in relatively large losses of pol ε activity on Mono Q column (Table I). The existence of two enzyme forms (pol ε and pol ε*) has been reported during the isolation of pol ε from calf thymus[17] and yeast[18] (pol ε* is a partly proteolytically degraded pol ε with the same catalytic properties). A more detailed analysis of the enzyme activities in the "tail" part might lead to similar findings. Like pol δ, also pol α and pol ε after the Mono Q purification step do not exert any other polymerase activity and further purifi-

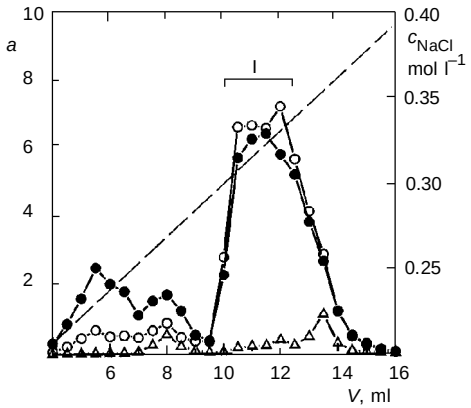


FIG. 5
FPLC of pol α on Mono Q column. Enzyme activities of pol α (❍) (without PCNA), pol δ (●) (with PCNA), pol ε (Δ). NaCl linear concentration gradient (– – –), DNA-polymerase activity *a* expressed as an incorporation of [^{3}H] dTMP into poly dA-oligo dT_{12-18} template-primer (cpm 10^{-4}), *V* elution volume, I pooled fractions of pol α

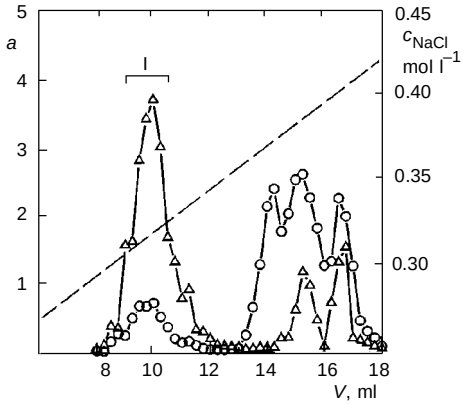


FIG. 6
FPLC of pol ε on Mono Q column. Enzyme activities of pol α (❍), pol ε (Δ). NaCl linear concentration gradient (– – –), DNA-polymerase activity *a* expressed as an incorporation of [^{3}H] dTMP into poly dA-oligo dT_{12-18} template-primer (cpm 10^{-4}), *V* elution volume, I pooled fractions of pol ε

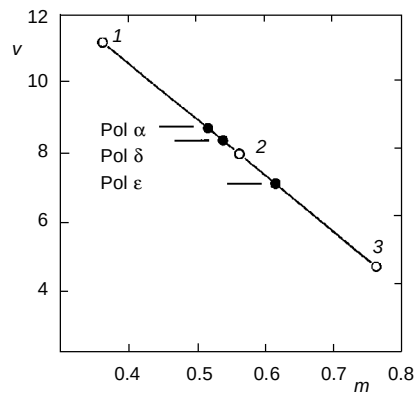


FIG. 7
Sedimentation of pol α, δ and ε in the linear concentration glycerol gradient 15–35% (v/v). *1* Catalase, *2* aldolase, *3* bovine serum albumin, *v* sedimentation velocity (S), *m* relative mobility

cation steps resulted only in their increased specific activity. The final step of purification of each enzyme by centrifugation in the linear concentration gradient of glycerol (with aliquots of individual isolates only) served for the determination of their sedimentation velocity.

Subunit Composition of Pol α, δ and ε from SD-Lymphoma

The sedimentation coefficients of pol α, δ and ε from SD-lymphoma in the presence of 400 mM NaCl were 8.7 S, 8.2 S and 7.0 S, respectively (Fig. 7); catalase (11.2 S), aldolase (7.8 S) and bovine serum albumin (4.6 S) were used as standards. The enzyme subunits of samples from glycerol gradients was determined by SDS-PAGE and the results are given in Fig. 8 (pol α), Fig. 9 (pol δ) and Fig. 10 (pol ε). The fractions of pol α with the highest enzyme activity contain dominant polypeptides of 170, 70, 57

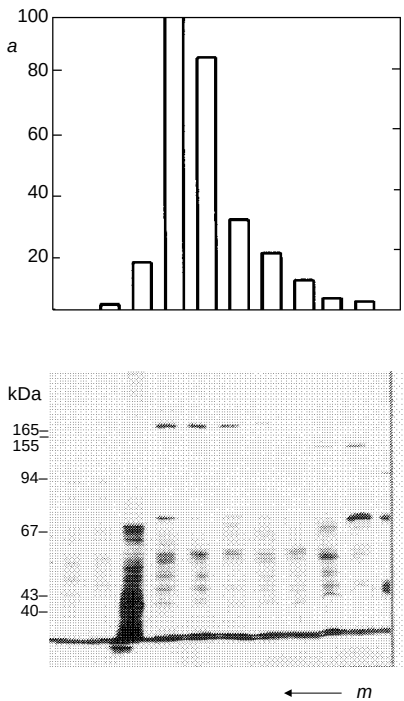


FIG. 8
SDS-PAGE polypeptide pattern of pol α after glycerol gradient centrifugation. *a* Relative DNA-polymerase activity, *m* direction of sedimentation

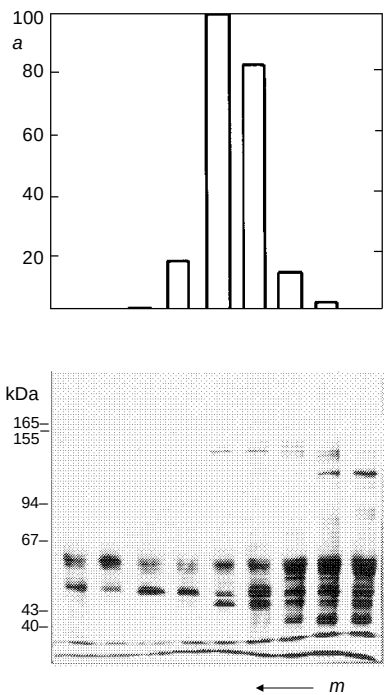


FIG. 9
SDS-PAGE polypeptide pattern of pol δ after glycerol gradient centrifugation. *a* Relative DNA-polymerase activity, *m* direction of sedimentation

and 53 kDa, and small amounts of high molecular weight subunit (about 200 kDa) and three other polypeptides (48, 45 and 42 kDa). Pol δ enzyme activity corresponds to the occurrence of 133 and 46 kDa subunits; additional two bands (62 and 50 kDa) have been observed also in the fractions without enzyme activity. Similar, though less clear situation, is observed with pol ε. In the fractions with highest enzyme activity we distinguished a few very weak bands in the region of 140 kDa which are evidently responsible for the catalytic activity of the sample.

The published data on sedimentation coefficients of these enzymes differ not only in relation to their source but also with respect to the concentration and composition of salts in the sedimentation medium. Although the subunit structure of DNA-polymerases somewhat varies according to their source, purity and/or extent of proteolysis, the individual enzymes exhibit characteristic features.

The sedimentation constants of highly homogenous preparations of pol α are between 8–10 S and the enzymes are composed from four subunits. Pol α from mouse leukemic cells L1210 has sedimentation coefficient of 8.9 S (at 200 mM KCl) and the polypeptides 180, 70, 56 and 47 kDa (ref.[19]); subunits from *Drosophila melanogaster* pol α are 182, 73, 60 and 50 kDa (ref.[20]). The sedimentation coefficient for immuno-

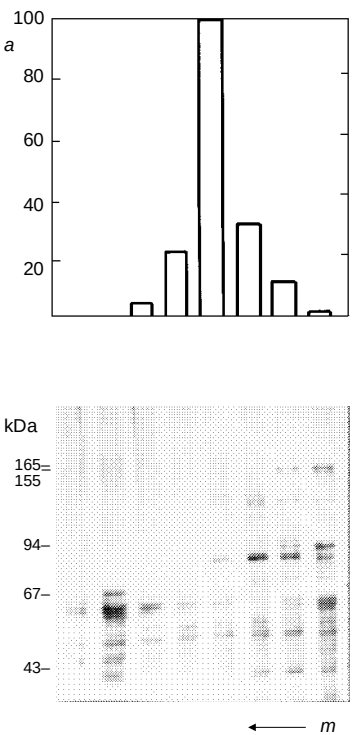


FIG. 10
SDS-PAGE polypeptide pattern of pol ε after glycerol gradient centrifugation. *a* Relative DNA-polymerase activity, *m* direction of sedimentation

affinity purified pol α from calf thymus is 9 S (500 mM KCl) and the subunit composition 180, 155, 148, 73, 59 and 48 kDa; however, the 155 and 148 kDa subunits are partially degraded forms of the 180 kDa polypeptide[21]. Another sample from the same source had 9.3 S (200 mM KCl) with 185, 160, 68, 55 and 48 kDa polypeptides (ref.[22]). Pol α from rat SD-lymphoma is not completely homogeneous, nevertheless, the occurrence of 170 and 70 kDa polypeptides is associated with polymerase activity. The identity of five smaller subunits (42–57 kDa) two of which are responsible for the DNA-primase activity of enzyme complex[23] is not obvious.

Both the subunit masses and sedimentation velocity of pol δ from SD-lymphoma agrees with the published data. Pol δ is conceived as heterodimer; the subunit compositions and sedimentation coefficients were: 125 and 48 kDa, 7.9 S (500 mM KCl) for calf thymus[24], 125 and 50 kDa, 8.0 S (200 mM KCl) for L1210 mouse leukemic cell enzyme[25], 130 and 47 kDa, 9.2 S for enzyme from HeLa cells[26] and 125, 123 and 48 kDa, 8.2 S (500 mM KCl) for enzyme from rabbit bone marrow[27].

The catalytic activity of pol ε from rat SD-lymphoma is obviously due to 140 kDa polypeptides while the contribution of smaller subunits is not fully understood. The enzyme resembles its counterpart from calf thymus[28] which has 140, 125 and 40 kDa subunits and surprisingly low sedimentation coefficient value of 4 S (40 mM KCl, 20 mM KH_2PO_4–K_2HPO_4). Human placenta pol ε contains 167 kDa catalytic subunit and series of polypeptides in the region of 50–80 kDa, sedimentation coefficient 7.8 S (30 mM KCl) (ref.[29]), and the enzyme from rabbit bone marrow has a single 122 kDa subunit, 6.7 S (300 mM KCl) (ref.[30]). Completely different structure has been found in pol ε from HeLa cells: 215 and 55 kDa subunits and considerably different sedimentation coefficient depending on ionic strength of potassium phosphate (11.3 S at 130 mM and 6.8 S at 550 mM) (ref.[31]). This structural heterogeneity is most probably due to the existence of the region in the molecule of pol ε that is highly sensitive to proteolysis; thus its tryptic cleavage results in two polypeptide fragments, (122 and 136 kDa) having 2–3 fold higher enzyme activity in vitro[32]. On the other hand, two forms – pol ε and pol ε* – isolated from calf thymus contained the large 210 and 145 kDa catalytic subunits, respectively[17]. Pol ε* is not formed merely as a contaminant during the isolation procedure – it probably has an important function in vivo[17]. We cannot exclude that pol ε from rat SD-lymphoma described in this paper might be the proteolysed form of the original enzyme which could occur in one of the both fractions with pol ε activity separated on Mono Q (Fig. 6). This possibility is currently under investigation.

Characterization of Pol α, δ and ε from SD-Lymphoma

The identification of DNA-polymerases α, δ and ε isolated from rat SD-lymphoma was carried out using their specific inhibitors, on the basis of their interaction with PCNA on template-primer poly dA-oligo $dT_{12\text{-}18}$ under reaction conditions used for the isolation, and by determination of DNA-primase and 3′-5′-exonuclease activities (see Experimental).

The enzyme preparations used were obtained by standard purification procedure except for the last glycerol gradient step (Scheme 1). The results are summarized in Tables II and III. The enzymes do not contain pol β or γ; it follows from the fact that they are inhibited by *N*-ethylmaleimide (NEM) and aphidicolin, and no reverse transcriptase activity using template-primer poly A-oligo dT_{12-18} under optimum conditions for pol β (ref.[33]) and pol γ (ref.[34]) has been found (data not shown).

In analogy to the enzymes isolated from other sources[35], pol α from rat SD-lymphoma differs from pol δ and pol ε by considerably higher sensitivity to N^2-(*p*-butylphenyl)-2′-deoxyguanosine 5′-triphosphate (BuPdGTP) as well as by the presence of DNA-primase activity which prefers poly dC template to poly dT (ref.[36]). Similar properties of pol δ and pol ε from SD-lymphoma to those of the enzymes from other sources[37] have been observed; the prevailing characteristic of pol δ is its minimal catalytic activity

TABLE II
Characterization of DNA-polymerases from SD-lymphoma on poly dA-oligo dT_{12-18}

Added factor	Concentration	Pol α[a]	Pol δ[a]	Pol ε[a]
None	–	1.00	0.02	1.00
PCNA	18 μg ml^{-1}	0.98	1.00[b]	0.72
COMDP	50 μmol l^{-1}	0.72	0.25	0.35
Aphidicolin	10 μg ml^{-1}	0.30	0.05	0.15
NEM	5 mmol l^{-1}	<0.01	<0.01	<0.01
BuPdGTP	20 μmol l^{-1}	0.20	0.89	1.03
DMSO	10% (v/v)	2.80	0.48	0.83

[a] Relative enzyme activity in the presence of 0.1 U per assay (see Experimental for details). [b] Relative enzyme activity of pol δ-PCNA was taken as 1.00.

TABLE III
Enzyme activities associated with DNA-polymerases from SD-lymphoma

Activity	Template	Pol α	Pol δ	Pol ε
Primase[a]	poly dT	2.0	0.4	<0.1
	poly dC	51.0	<0.1	<0.1
3′,5′-Exonuclease[a]	poly dA-oligo dT_{12-18}-oligo [^{3}H]$dC_{\approx 15}$	1.0	49.0	32.0

[a] Results are given in picomoles of nucleotide incorporated (at conditions of primase assay) or removed (at conditions of 3′-5′-exonuclease assay) when the amount of an enzyme corresponding to 0.1 U on poly dA-oligo dT_{12-18} was used (see Experimental for details).

with poly dA-oligo dT_{12-18} template-primer in the absence of PCNA. On the other hand, with this template-primer pol ε is very active and it is weakly inhibited by PCNA. In contrast to pol α both pol δ and pol ε possess 3′-5′-exonuclease activity (Table III). They are also nearly equally inhibited by carbonyldiphosphonate (COMDP) while pol α is 2–3 fold less sensitive to this inhibitor.

Carbonyldiphosphonate was regarded as a selective inhibitor of pol ε; the value of the inhibition constant (K_i) for this enzyme from calf thymus is 20 fold lower than for pol α (ref.[38]); however, in enzymes from HeLa cells it exerted a certain selectivity towards pol δ (ref. [26]): at the concentration of 15 μmol l^{-1} this compound inhibits pol δ by 93%, pol ε only by 20% while it is almost inactive against pol α (ref.[26]). The recent study concerning inhibition of calf thymus enzymes demonstrated that the selectivity of COMDP is low: IC_{50} values for pol δ and pol ε are similar (4.4 μmol l^{-1} and 6.7 μmol l^{-1}) and IC_{50} for pol α is only 4–6 fold higher (26.2 μmol l^{-1}) (ref.[39]).

The presence of 10% dimethyl sulfoxide (DMSO) results in a considerable stimulation of pol α, inhibition of pol δ and only weak inhibition of pol ε from SD-lymphoma. In the presence of 10% DMSO, the activity of pol δ from calf thymus is not affected[40] but the activity of the enzyme from HeLa cells is considerably stimulated[26]. Under the same conditions pol ε from HeLa cells and calf thymus are strongly inhibited whereas human placenta pol ε is stimulated[29], so it may be concluded that the effect of DMSO is rather unspecific and might depend on the purity of the sample and on the assay conditions used for the determination of enzyme activity.

Enzyme Activity of Pol α, δ and ε from SD-Lymphoma on Synthetic Homopolymeric Template-Primers

For comparison, the reaction catalyzed by all three polymerases on template-primers poly dA-oligo dT_{12-18} and poly dT-oligo dA_{12-18} as examined; the reaction conditions were optimized with respect to pH (6.5–8), concentration of $MgCl_2$ (0–15 mmol l^{-1}) and KCl (0–100 mmol l^{-1}) (see Table IV). Poly dA-oligo dT_{12-18} is the most frequently used template-primer for the enzyme activity determination of eukaryotic DNA-polymerases. The reaction conditions for individual polymerases are not characterized by sharp optima (data not given). A considerable influence of the ionic strength of KCl on the activity of both pol α and pol ε which depends on the type of buffer used in the assay was observed (Fig. 11). In Tris-HCl buffer both enzymes display the highest activity in the absence of KCl, whereas in Hepes(K^+) buffer pol ε activity is, in contrast to pol α, strongly stimulated by 75 mM KCl (this fact was taken into consideration when the selective medium for pol ε was prepared).

With poly dT-oligo dA_{12-18} as a template-primer the eukaryotic DNA-polymerases exerted zero or minimum activity[26,31,40,41]. We have observed that the catalytic activity of pol α and ε in the presence of this template-primer is highly dependent on concentration of Mg^{2+}-ions; in both cases very sharp optima are obtained at an unusually low

TABLE IV
Activity optima of pol α, δ and ε on synthetic homopolymeric template-primers

Template-primer	Pol	Buffer[a]	pH	$MgCl_2$, mmol l^{-1}	KCl, mmol l^{-1}
$(dA)_n$. dT_{12-18}	α	Tris-HCl	7.5	5.0	0.0
	δ-PCNA[b]	Hepes(K^+)	7.0	8.0	20.0
	ε	Hepes(K^+)	7.5	5.0	75.0
$(dT)_n$. dA_{12-18}	α	Tris-HCl	7.5	0.2	40.0
	δ-PCNA[b]	Hepes(K^+)	7.0	8.0	40.0
	ε	Hepes(K^+)	7.5	0.2	75.0

[a] 20 mM Tris-HCl and 40 mM Hepes(K^+); [b] PCNA 18 μg ml^{-1}.

TABLE V
Activity of DNA-polymerases α, δ and ε on synthetic template-primers under the optimized conditions *a*

Template-primer	DNA-Pol	Specific activity[b], U mg^{-1}
$(dA)_n$. dT_{12-18}	α	6 150
	δ-PCNA	1 055
	ε	1 510
$(dT)_n$. dA_{12-18}	α	8 650
	δ-PCNA	102
	ε	345

[a] See Table IV. [b] Values correspond to the enzyme purity before glycerol gradient centrifugation (see Scheme 1).

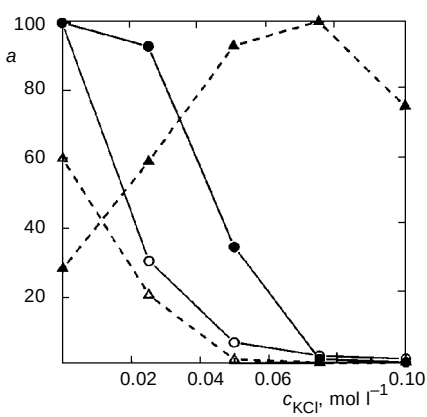


FIG. 11
Effect of different buffers and of ionic strength on catalytic activity of pol α and pol ε on poly dA-oligo dT_{12-18} template-primer. Pol α, Tris-HCl pH 7.5 (○); pol α, Hepes(K^+) pH 7.5 (Δ); pol ε, Tris-HCl pH 7.5 (●); pol ε, Hepes(K^+) pH 7.5 (▲); *a* relative DNA-polymerase activity, *c* concentration (mol l^{-1})

concentration (0.2 mmol l^{-1}). It is worth mentioning that pol δ in the complex with PCNA is active on this template-primer at usual range of $MgCl_2$ concentration (optimum 8 mmol l^{-1}). The influence of PCNA on catalytic activity and processivity of pol α, pol δ and pol ε with poly dT-oligo dA_{12-18} is similar to that observed with poly dA-oligo dT_{12-18}; the presence of PCNA is indispensable for pol δ but it does not influence pol α and pol ε. The quantitative comparison of all three polymerase activities from SD-lymphoma on both template-primers under optimum conditions is given in Table V. Whereas poly dT-oligo dA_{12-18} is less efficient template-primer for pol δ and pol ε (7% and 15% activity, respectively, to compare with poly dA-oligo dT_{12-18}), the opposite is true for pol α (140% of activity to compare with poly dA-oligo dT_{12-18}). In spite of the fact that the extent of incorporation of dAMP even at optimum conditions for pol ε and especially for pol δ is relatively low, it is highly significant. Thus poly dT-oligo dA_{12-18} could serve as a template-primer useful for the studies in vitro, e.g. for the comparison of inhibitory effects of adenine nucleotide analogs on replicative DNA-polymerases.

EXPERIMENTAL

Materials

All reagents were purchased from Sigma unless otherwise noted. Nucleotides (ATP, GTP, dATP, dGTP, and dTTP), oligodeoxynucleotides (oligo dA_{12-18}, oligo dT_{12-18}), terminal deoxynucleotidyl transferase (calf thymus), DEAE Sephacel, Heparin Sepharose CL-6B, Mono Q HR 5/5 FPLC column and Sephacryl S-300 HR were products of Pharmacia P-L Biochemicals. Phosphocellulose (P-11), glass microfibre filters (GF/A) and ion exchange papers (DE 81) were from Whatman. Glycerol (99%) was obtained from Riedel-de Haen and redistilled in a glass apparatus. Trichloroacetic acid (TCA) was from LOBA. Radiolabeled nucleotides [8-^{3}H]dATP (888 GBq $mmol^{-1}$), [8-^{3}H]dGTP (481 GBq $mmol^{-1}$), [methyl-^{3}H]-dTTP (1.5 TBq $mmol^{-1}$), [5-^{3}H]dCTP (625 GBq $mmol^{-1}$) and [α-^{32}P]dATP (15 TBq $mmol^{-1}$) were purchased from Amersham. N^2-(*p*-Butylphenyl)deoxyguanosine 5′-triphosphate (BuPdGTP) and sodium α-oxomethylenediphosphonate (carbonyldiphosphonate, COMDP) were generous gifts from Dr G. E. Wright, Department of Pharmacology, University of Massachusetts Medical School, Worcester, MA. Proliferating cell nuclear antigen (PCNA) was purified to homogeneity according to the method reported by Fien and Stillman[42] from *E. coli* strain BL 21/DE 3 harboring a plasmid encoding the human PCNA cDNA sequence[43]. The bacterial strain was kindly provided by Dr B. Stillman, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Hydroxylapatite was prepared according to published procedure[44].

Template-primers (poly dA-oligo dT_{12-18} and poly dT-oligo dA_{12-18}) were prepared by annealing of oligodeoxynucleotide to the corresponding polydeoxynucleotide (at 60 °C for 5 min with the subsequent cooling at room temperature; a base ratio 1 : 10) in 10 mM Tris-HCl, 1 mM EDTA pH 8.0. The template to determine 3′-5′-exonuclease activity, poly dA-oligo dT_{12-18}-oligo[^{3}H]-$dC_{\approx 15}$ was prepared by terminal deoxynucleotidyl transferase[28].

SD-Tumors

Spontaneous acute lymphoblastic leukemia[1] (designated SD4) arose in Sprague–Dawley 8 month old female inbred rat. Enlarged lymph nodes taken from animals suffering from leukemia (10^6 cells in

phosphate-buffered saline) were inoculated s.c. to syngenic SD animals and lymphoma tissues subcutaneously growing (SD-tumors) within 7 week were used for enzyme purification.

DNA-Polymerase Assays

During a purification procedure the reaction mixtures for estimation of pol α, δ and ε activities contained in final volume of 25 μl: poly dA-oligo dT_{12-18} (20 μg ml^{-1}), 5 mM $MgCl_2$, 1 mM DTT, bovine serum albumin (BSA; 200 μg ml^{-1}), 10% (v/v) glycerol and 20 μM [methyl-^{3}H]-dTTP (0.2 TBq $mmol^{-1}$). The activities of pol α and δ were then followed in the presence of 40 mM Hepes(K^+), pH 7.0; the pol δ reaction mixture contained PCNA (*E. coli*; 18 μg ml^{-1}) and the pol ε activity was screened in 40 mM Hepes(K^+), pH 7.5 with 75 mM KCl. The reactions were carried out at 37 °C for 15 min and stopped by spotting an appropriate aliquot onto GF/A glass paper discs (Whatman) which were then immersed into cold 5% TCA containing 20 mM $Na_4P_2O_7$. 10 H_2O. Discs were three times extensively washed with the same solution and then with an excess of 96% ethanol, dried and TCA-insoluble radioactivity was counted in a toluene-based scintillation fluid. One enzyme unit (U) is defined as the amount of enzyme that incorporates 1 nmol of dTTP into acid-insoluble precipitate after 15 min at 37 °C under the conditions listed above.

DNA-Primase Assay

Activity of DNA-primase was followed in the reaction mixture (25 μl) which contained: poly dC and/or poly dT (20 μg ml^{-1}), 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM DTT, BSA (200 μg ml^{-1}), 10% (v/v) glycerol, 1 mM GTP and/or ATP, 20 μM [^{3}H]-dGTP and/or [^{3}H]-dATP (0.4 TBq $mmol^{-1}$) and an appropriate aliquot of the enzyme preparation to be tested. After the incubation for 15 min at 37 °C, TCA-insoluble counts were determined using GF/A filters as was described above.

3′-5′-Exonuclease Assay

DNA-Exonuclease activity was tested in the reaction mixture (25 μl) which contained: poly dA-oligo dT_{12-18}-oligo [^{3}H]-$dC_{\approx 15}$ (10 TBq $mmol^{-1}$ of 3′-OH termini), 20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, BSA (200 μg ml^{-1}), 10% (v/v) glycerol and an appropriate aliquot of the enzyme tested. The reaction mixture was incubated for 15 min at 37 °C and transferred onto a strip (2 cm^2) of DE-81 paper (Whatman). The strips were washed in 0.5 M ammonium formate (3 × 100 ml) and then with an excess of 96% ethanol, dried and the bound radioactivity was determined in a toluene-based scintillation fluid.

Protein Assay

A concentration of proteins was determined by the method of Bradford[45] with bovine serum albumin as the standard.

Buffers

Buffer A: 10 mM Hepes(K^+), pH 7.7, 75 mM sucrose, 0.25% dextran (m.w. ≈ 500 000), 4 mM $MgCl_2$, 0.25 mM EGTA, 0.5 mM DTT, 5 mM benzamidine, pepstatin A (1 μg ml^{-1}), leupeptin (1 μg ml^{-1}), 0.2 mM phenylmethylsulfonyl fluoride (PMSF).

Buffer B: 80 mM Hepes(K^+), pH 7.7, 600 mM sucrose, 2% dextran (m.w. ≈ 500 000), 32 mM $MgCl_2$, 2 mM EGTA, 933 mM KCl, 4 mM DTT, 5 mM benzamidine, pepstatin A (1 μg ml^{-1}), leupeptin (1 μg ml^{-1}), 0.2 mM PMSF.

Buffer C: 50 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM EDTA, 10% (v/v) glycerol, 5 mM benzamidine, 0.2 mM PMSF.
Buffer D1: 20 mM potassium phosphate, pH 7.5, 5 mM DTT, 20% (v/v) glycerol, 0.05 Triton X-100, 5 mM benzamidine, 0.2 mM PMSF.
Buffer D2: 400 mM potassium phosphate, pH 7.5, 5 mM DTT, 20% (v/v) glycerol, 0.05% Triton X-100, 5 mM benzamidine, 0.2 mM PMSF.
Buffer E: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 20% (v/v) glycerol, 0.05 Triton X-100, 5 mM benzamidine, 0.2 mM PMSF.
Buffer F: 50 mM Tris-HCl, pH 7.5, 400 mM NaCl, 1 mM EDTA, 5 mM DTT, 0.05% Triton X-100.
Buffer TBE: 90 mM Tris-boric acid, pH 8.3, 2 mM EDTA.
Storage buffer: 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 5 mM DTT, 0.05% Triton X-100, 30% (v/v) glycerol.

Purification Procedure

Crude extract. SD-tumors (100 g) frozen at –70 °C were sliced, ground with the aid of sieve to small pieces (1 mm diameter), suspended in 200 ml of buffer A, homogenized in a Dounce tissue grinder (Wheaton; five strokes of pestle B and 30 strokes of pestle A) and then stirred for 30 min at 0 °C. The resulting homogenate was diluted with 150 ml of buffer B and once more stirred for 30 min at 0 °C. The extract was centrifuged 15 min at 30 000 g and then 60 min at 105 000 g. The supernatant was dialyzed against buffer C (12 h, 2 × 5 l) and clarified by centrifugation (30 000 g, 15 min; fraction I).

Ammonium sulfate (30–50%) cut. Proteins salted out from fraction I between 30 to 50% saturation by ammonium sulfate were solubilized in buffer C and then dialyzed against the same solution (6 h, 2 × 2 l; fraction II).

Chromatography on DEAE Sephacel. The fraction II (78 ml) obtained by ammonium sulfate precipitation was applied onto the DEAE Sephacel column (3 × 8.7 cm) equilibrated in buffer C. The column was washed with five volumes of buffer C first and then proteins with activity of pol α, δ and ε were eluted using linear concentration gradient 0–0.35 mM NaCl in buffer C (488 ml); active fractions which correspond to pol δ ($V_e \approx 250$ ml) and pol α + ε ($V_e \approx 306$ ml) were pooled.

Purification of DNA-polymerase δ. A column of phosphocellulose (2 × 5 cm, 16 ml) was equilibrated in buffer C containing 0.1 mM NaCl. The dialyzed pol δ fraction from DEAE Sephacel (54 ml) was applied onto the column, and elution was continued with five column volumes of equilibration buffer followed by ten column volumes of NaCl linear concentration gradient (0.1–0.5 mol l^{-1} in buffer C). Fractions that exerted pol δ activity ($V_e \approx 81.5$ ml) were pooled, diluted with buffer C to the final NaCl concentration of 0.15 mol l^{-1} (total volume 48 ml) and applied onto Mono Q column (equilibrated with 0.15 M NaCl in buffer C). The elution continued with five volumes of equilibration buffer followed by linear concentration gradient of NaCl (twenty column volumes; 0.15–0.45 mol l^{-1}) in buffer C. Pol δ activity was eluted ($V_e \approx 5.25$ ml), pooled and applied onto Sephacryl S-300 HR column (2 × 83 cm, V_0 93.5 ml; equilibrated in buffer D1 containing 0.2 M NaCl). Fractions with the highest enzyme activity ($V_e \approx 122$ ml) were pooled, diluted with buffer D1 (1 : 1) and applied onto Heparin Sepharose CL-6B column (0.8 × 4 cm, equilibrated in buffer D1). An affinity column was then washed with 5 ml of buffer D1, 5 ml of buffer E containing 0.1 M NaCl and by a linear concentration gradient of NaCl (0.1–0.6 mol l^{-1}) in buffer E. The peak of pol δ activity was pooled and divided into two parts. One part was dialyzed against 10% (v/v) glycerol in buffer F and then used for glycerol gradient centrifugation, second one was dialyzed against storage buffer and stored at –70 °C. The sedimentation analysis (15–35% glycerol (v/v) in buffer F) was carried out for 36 h at 40 000 s^{-1}

(2 °C, Beckman SW 41 Ti rotor). Pol δ sedimentation velocity was compared with catalase, aldolase and bovine serum albumin (Pharmacia LKB; 11.2 S, 7.8 S and 4.6 S).

Purification of DNA-polymerase α. A peak of the pol α + ε activity from DEAE Sephacel column was diluted with buffer C to the final concentration of 0.1 M NaCl (total volume 104 ml) and applied onto a phosphocellulose column (2 × 5 cm) equilibrated in buffer C containing 0.1 M NaCl. The column was washed with 80 ml of equilibration buffer and subsequently linear concentration gradient of NaCl (160 ml, 0.1–0.5 mol l^{-1}) in buffer C was used for pol α and pol ε separation. Fractions with pol α acitivity ($V_e \approx 65$ ml) were pooled, diluted with buffer C to the final concentration of 0.15 M NaCl and transferred onto column of Mono Q (equilibrated with 0.15 M NaCl in buffer C). After a chromatography on Mono Q column at the same conditions as was decribed above pol α ($V_e \approx 11.25$ ml) was pooled, gel-filtered on Sephacryl S-300 HR column (see above; $V_e \approx 119$ ml) and then the enzyme was applied on a hydroxylapatite column (0.8 × 1.0 cm, equilibrated in buffer D1); the column was washed with 2.5 ml of buffer D1 and a linear gradient to buffer D2 (25 ml) was applied. After the hydroxylapatite column part of the enzyme was stored in the storage buffer (–70 °C) and the rest was analyzed by the glycerol gradient centrifugation (see above).

Purification of DNA-polymerase ε. The collected activity of pol ε from phosphocellulose, ($V_e \approx 85$ ml) was treated by the same way (Mono Q, $V_e \approx 10$ ml) as pol α; pooled fractions were diluted with buffer E (1 : 1) and adsorbed on an affinity column (Heparin Sepharose, 0.8 × 4 cm, see above) and then processed in the same way as pol δ and α.

Denaturing Gel Electrophoresis of Proteins

SDS-PAGE system according Laemmli[46] (8% polyacrylamide) was used for the analysis of purified DNA-polymerases. Samples were concentrated by TCA-precipitation[47], heated (95 °C, 3 min) in 12.5 mM Tris-HCl, pH 6.8, 1.0% SDS, 5% (v/v) glycerol and 10 mM DTT. *E. coli* RNA-polymerase (165, 155, 90 and 40 kDa) and Pharmacia P-L Biochemicals LMW and HMW calibration kits were used as protein standards. The gels were stained with silver[48].

Polyacrylamide Gel Electrophoresis of ^{32}P Products

Products of DNA polymerase reactions on poly dT-oligo dA_{12-18} template were analyzed on denaturing polyacrylamide gels[49]. Enzymes were incubated under the optimized conditions: pol α in 20 mM Tris-HCl, pH 7.4, 0.2 mM $MgCl_2$, 40 mM KCl; pol δ in 40 mM Hepes(K^+) pH 7.0, 5.0 mM $MgCl_2$, 40 mM KCl and pol ε in 40 mM Hepes(K^+) pH 7.0, 0.2 mM $MgCl_2$, 75 mM KCl. Moreover, all reaction mixtures (total volume 25 μl) contained 20 μg ml^{-1} poly dT-oligo dA_{12-18} (10 : 1), 200 μg ml^{-1} BSA, 1 mM DTT, 10% (v/v) glycerol and 20 μM [α-^{32}P] dATP (74 GBq $mmol^{-1}$). PCNA when added was present in concentration of 18 μg ml^{-1}. After 10, 30, or 60 min at 37 °C the reaction was terminated by addition of EDTA (pH 8.0) to 20 mmol l^{-1}. The mixture was then treated with one volume of phenol–chloroform–isoamylalcohol (25 : 24 : 1), centrifuged and DNA was precipitated (in the presence of 0.3 M sodium acetate, pH 5.2) from the aqueous phase by two volumes of ethanol, washed with 70% ethanol and then dissolved in 98% deionized formamide, containing 10 mM EDTA, 0.2% bromophenol blue and 0.2% xylene cyanole FF. The solubilized samples were heated at 90 °C for 5 min, cooled in an ice bath and loaded onto polyacrylamide gel (8%) containing TBE buffer and 7 M urea. Denatured [5′-^{32}P] ΦX-174-DNA Hae III digest was used as a marker. After the electrophoresis (1 600 V, 3h) gels were fixed on glass plates in 10% methanol (v/v) and 10% acetic acid (v/v) for 10 min, rinsed with water, dried and autoradiographed.

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