Deoxyribonucleic Acid Polymerases of *Euglena gracilis*

PRIMER-TEMPLATE UTILIZATION OF AND ENZYME ACTIVITIES ASSOCIATED WITH THE TWO DEOXYRIBONUCLEIC ACID POLYMERASES OF HIGH MOLECULAR WEIGHT

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(Received 10 February 1975)

The two high-molecular-weight DNA polymerases from *Euglena gracilis*, pol A (mol. wt. 190000) and pol B (mol. wt. 240000), were differentiated on the basis of associated enzymic activities and primer-template utilization. Neither enzyme had endodeoxyribonuclease activity, but pol B, like pol B of yeast and the corresponding enzyme from *Tetrahymena pyriformis*, exhibited at least one other nuclease activity directed against denatured DNA and the RNA of an RNA–DNA hybrid. These nuclease functions preferred an alkaline pH and Mg^{2+} . Pol B also exhibited nucleoside diphosphokinase activity. Both enzymes were active with 'activated' DNA and poly[d(A-T)] as primer-templates and were sensitive, especially pol B, to inhibition by excess of native or heat-denatured DNA. Pol B also utilized oligo[d(T)]-initiated poly[d(A)] and poly(A) templates under certain conditions, whereas pol A exhibited only slight activity with poly[d(A)]. (U)₆ was not used as a primer by either enzyme.

In addition to their ability to polymerize deoxyribonucleoside 5'-triphosphates in a 5'→3' direction, most bacterial and bacteriophage-induced DNA polymerases (DNA nucleotidyltransferase, EC 2.7.7.7) that have been studied in detail can degrade single-stranded DNA in a 3'→5' direction (Kornberg, 1974). This activity may have a 'proof-reading' function *in vivo* and increase the fidelity of DNA replication by excising mismatched nucleotides from the 3' end of the growing strand before the addition of another nucleotide (Brutlag & Kornberg, 1972; Kössel & Roychoudhury, 1974). The pol I enzymes of *Escherichia coli* (Deutscher & Kornberg, 1969) and *Micrococcus luteus* (Miller & Wells, 1972a,b), and possibly the native molecule of pol I of *Bacillus subtilis* with a molecular weight of 110000 (Setlow *et al.*, 1972a,b; Ganesan *et al.*, 1973), also possess a 5'→3' exonucleolytic activity, which may function in 'nick' translation during DNA replication and excision repair (Kelly *et al.*, 1970; Hamilton *et al.*, 1974) and as a ribonuclease H to excise RNA primers during DNA synthesis (Berkower *et al.*, 1973; Roychoudhury & Kössel, 1973).

Despite the apparent importance of the polymerase-related nucleases to bacterial DNA synthesis, unequivocal evidence for the association of such activities with higher-eukaryotic DNA polymerases has not been obtained. These enzymes are generally labile and difficult to purify in large amounts, and the low nuclease activities occasionally reported may be due to contaminating enzymes. Both endonuclease and exonuclease activities have been reported

to be absent from purified preparations (Craig & Keir, 1974; Loeb, 1974). RNAase* H and RNAase H-like activities have been found in partially purified preparations of KB-cell and BHK-21/C13-cell DNA polymerases (Keller & Crouch, 1972; Cooper *et al.*, 1974), but no molecular association between the activities has been demonstrated. There is better evidence for the presence of an active exodeoxyribonuclease function in highly purified preparations of one of the high-molecular-weight DNA polymerases from yeast, pol B (but not pol A) (Helfman, 1973), and in the enzyme from *T. pyriformis* (Crerar & Pearlman, 1974). This may represent an important difference between DNA polymerases from primitive and higher eukaryotes.

Nucleoside diphosphokinase (ATP–nucleoside diphosphate phosphotransferase, EC 2.7.4.6) may also be an integral part of pol I and pol II of *Esch. coli* and pol I of *M. luteus* (Miller & Wells, 1971) and has also been reported in preparations from KB cells (Sedwick *et al.*, 1972), rat liver nuclei (Haines *et al.*, 1972), Landschütz ascites-tumour cells (Shepherd & Keir, 1966) and *T. pyriformis* (Crerar & Pearlman, 1974). Its occurrence may be widespread.

Utilization of natural and synthetic primer-templates has frequently been used to differentiate between the various species of DNA polymerases, and, broadly speaking, the high-molecular-weight, low-molecular-weight and RNA-dependent activities of eukaryotes can be classified according to

* Abbreviations: RNAase, ribonuclease; DNAase, deoxyribonuclease.

template preference (Craig & Keir, 1974; Loeb, 1974). To help determine the relationship between them and the known classes of prokaryotic and eukaryotic DNA polymerases, we have investigated the template activities and additional enzyme functions associated with the two high-molecular-weight DNA polymerases that we have purified from a bleached derivative of the unicellular green flagellate *Euglena gracilis*. In contrast with higher eukaryotes, this organism contains no 3.4S polymerase (McLennan & Keir, 1975b).

Experimental

Materials

These were as described by McLennan & Keir (1975b). In addition, calf thymus DNA types I and V, dTMP, dTDP, *S*-adenosyl-L-methionine chloride and Triton X-114 were from the Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K., ovalbumin was from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., poly(A)·oligo[d(T)₁₀] (A/T ratio 1:1) was from Boehringer Corp., London W5 2TZ, U.K., and poly[d(A)], d(T)₆, poly(A) and U(pU)₅ were from P-L Biochemicals, Milwaukee, Wis., U.S.A.

Poly(Ali)·oligo[d(T)₁₀] (A/T ratio 23:1), poly[d(A)]·oligo[d(T)₁₀] (A/T ratio 23:1), poly[d(A-T)] and [³H]poly(A)·poly(U) were from Miles Laboratories, Kankakee, Ill., U.S.A., Sarkosyl NL35 was from Ciba-Geigy (U.K.), Manchester, U.K., cellulose F-coated t.l.c. plates were from Anderman and Co., East Molesey, Surrey KY8 0QZ, U.K., and nitrocellulose filters were from Schleicher und Schüll, Dassel, West Germany.

Superhelical [³H]DNA from SV40 virus was generously given by Dr. R. Eason, Department of Biochemistry, University of Glasgow, Glasgow, U.K., and [³H]RNA-DNA and [³H]DNA-RNA hybrids (prepared by the method of Cooper *et al.*, 1974) and *Bacillus subtilis* [³H]DNA were kindly provided by Miss Arja Olivier and Mr. G. C. L. Tait respectively, both of this Department.

Methods

Assay for DNA polymerase. For the determination of synthetic primer-template activities, a modification of the standard assay (McLennan & Keir, 1975b) was used. To ensure maximum recovery of labelled poly- and oligo-nucleotides of relatively low molecular weights, nitrocellulose filters (2.5 cm diam., 0.45 µm pore), were used in place of GF/C discs (Schrier & Wilson, 1973). The assays were incubated at 26°C with 100 mCi/mmol of [*Me*-³H]-dTTP. Since only a few micrograms of primer-templates were used per assay, 0.125 ml of a solution

of native calf thymus DNA was added to each tube after incubation to a final concentration of 25 µg of polynucleotide/assay mixture, followed by 0.25 ml of 10% (w/v) trichloroacetic acid-20 mM-Na₄P₂O₇. Samples were collected and washed as above on nitrocellulose filters except that 0.1 M-HCl replaced the ethanol washes. The filters were then solubilized (30 min at room temperature) in 1 ml of 2-ethoxyethanol and the radioactivity was counted in 10 ml of xylene-Triton X-114 scintillation fluid (Anderson & McClure, 1973). Counting efficiency was 35% in the Intertechnique SL30 liquid-scintillation spectrometer. Blanks obtained by this washing method were of the same order as those obtained with GF/C discs in toluene-based scintillant (50 c.p.m.), and no special washing procedures were found necessary as suggested by Schrier & Wilson (1973).

Assay for endo-DNAase (EC 3.1.4.30). The assay measured the production of open circular DNA and linear pieces of DNA from superhelical [³H]DNA from SV40 virus as determined by alkaline-sucrose-density-gradient sedimentation analysis.

The assay mixture (0.2 ml) contained: approx. 1 µg of superhelical SV40-virus [³H]DNA (6000 c.p.m.); 50 mM-Tris-HCl buffer, pH 8.0; 5 mM-MgCl₂ or -CaCl₂; 5 mM-2-mercaptoethanol; enzyme protein. After incubation for 30 min at 32°C, the samples were chilled on ice, 10 µl of 2 M-NaOH-0.2 M-EDTA was added to each and the resulting solutions were layered on top of linear 5–20% (w/v) alkaline sucrose density gradients, which were prepared as follows. Solutions of 25% (w/v) sucrose and 0.6 M-NaOH-1.4 M-NaCl-2 mM-EDTA were autoclaved at a pressure of 69 kPa (10 lb·in⁻²) for 10 min to destroy contaminating nucleases, and mixed in equal volumes when cool. The pH of the resultant solution was adjusted to 12.0 with saturated NaOH solution and it was then made 0.3% (v/v) with respect to Sarkosyl. Then 4.5 ml portions were dispensed into 5 ml MSE polypropylene centrifuge tubes, which had previously been washed out with ethanol, and were then frozen and thawed three times to produce the gradient (Baxter-Gabbard, 1972). After application of the samples, the gradients were centrifuged at 130 600 *g*_{av} for 2 h at 15°C in the SW50L rotor of a Beckman L-2 ultracentrifuge. About thirty 7-drop fractions were collected by piercing the bottom of the tubes and displacing the gradients upwards with 60% (v/v) glycerol. The fractions were dripped directly into scintillation vials, neutralized with 20 µl of 3 M-HCl and mixed with 0.85 ml of water, and the radioactivity was counted in 10 ml of toluene-Triton scintillator (McLennan & Keir, 1975b).

Assay for DNAase. The assay measured the conversion of [³H]DNA into acid-soluble material. The assay mixture (0.25 ml) contained: approx. 2 nmol (calculated as nucleotide residues) of native or denatured ³H-labelled *B. subtilis* DNA

(≤ 10000 c.p.m.); 40 mM buffer; bivalent cation; 8 mM-2-mercaptoethanol; 10% (v/v) glycerol; 0.02% bovine serum albumin; enzyme protein. The composition and pH of the various buffers and the concentrations of bivalent cations are given in the Results section. After incubation at 32°C for 1 h, the assay mixtures were chilled on ice and 0.1 ml of calf thymus DNA solution (2.5 mg/ml) was added to each, followed by 0.25 ml of 7% (v/v) HClO_4 . After standing for 20 min, the tubes were centrifuged for 45 min at 1000 g_{av} , and the radioactivity of a 0.25 ml portion of each supernatant was counted in 10 ml of toluene-Triton scintillator fluid.

Assay for other nuclease activities. [^3H]RNA-DNA, [^3H]DNA-RNA and [^3H]poly(A)·poly(U) in the native or denatured configurations were replaced in the DNA assay with 40 mM-Tris-HCl buffer, pH 8.0, and 5 mM-MgCl₂ in addition to the other assay components.

Assay for nucleoside diphosphokinase. The assay mixture (0.4 ml) contained: 10 mM-imidazole-HCl buffer, pH 7.2; 10 mM-2-mercaptoethanol; 2 mM-MgCl₂; 0.1 mM-ATP; 20 μM -[$\text{Me-}^3\text{H}$]dTDP (500 mCi/mmol); enzyme protein; it was incubated at 32°C. Samples (0.1 ml) were taken at intervals, added to 10 μl of marker solution (0.4 mM-dTTP, 0.2 mM-dTDP, 0.4 mM-dTMP, 0.1 M-EDTA) and evaporated to complete dryness in a vacuum desiccator. The residues were dissolved in 5 μl of distilled water and applied to a cellulose-coated t.l.c. plate. Ascending chromatography was performed with the solvent butan-1-ol-acetone-acetic acid-aq. 5% (w/v) ammonia-water [9:3:2:2:4 (by vol.)]; (Randerath, 1966)] and the spots corresponding to dTMP, dTDP and dTTP were located with a short-wave u.v. lamp after development, by comparison with reference nucleotides. The cellulose containing each nucleotide was scraped into 1 ml of distilled water and the suspensions were centrifuged for 10 min at 1000 g_{av} . The radioactivity of a 0.8 ml portion of each supernatant was then counted in 10 ml of the toluene-Triton scintillator fluid.

Preparation of primer-templates. These were dissolved in 10 mM-Tris-HCl buffer (pH 7.8)-10 mM-KCl. DNA was denatured by heating a solution of native DNA at 100°C for 10 min followed by rapid cooling in an ice-water mixture. 'Activated' DNA, in which many 3'-hydroxyl groups had been produced by 'nicking' with DNAase I, was prepared as before (McLennan & Keir, 1975b) and by the method of Momparler *et al.* (1973) by annealing single-stranded DNA with the oligodeoxyribonucleotides produced by extensive digestion of native DNA with DNAase. Unless otherwise stated, the former preparation was used.

Immediately before use, solutions (1 mg/ml) of poly(A)·oligo[d(T)₁₀] and poly[d(A)]·oligo[d(T)₁₀] were heated for 15 min at 55°C (Cavalieri *et al.*, 1974)

and allowed to cool to room temperature. Unless this procedure is followed, these primer-templates are used only poorly (Wickremasinghe & Johnston, 1974). Various volumes of solutions (1 mg/ml) of poly[d(A)] and U(pU)₅, and of poly(A) and d(T)₆, were similarly treated together to produce poly-[d(A)]-oligo(U)₆ and poly(A)-oligo[d(T)₆] with various R/Y ratios.

Results

In all these experiments the enzyme preparations used were fractions VIIIA and VIIIB of the purification scheme previously described for the *Euglena* DNA polymerases (McLennan & Keir, 1975b).

Endo-DNAase activity

Pol A and pol B were shown to be devoid of Mg²⁺- or Ca²⁺-dependent endo-DNAase activity on a double-stranded DNA substrate by their inability to convert closed circular superhelical SV40-virus DNA into open circles and linear strands, as determined by centrifugation of the DNA through alkaline sucrose density gradients. The enzyme samples used contained ovalbumin as the stabilizing protein (McLennan & Keir, 1975b), since some preparations of bovine serum albumin have slight endonuclease activity. The centrifugation profile of the substrate DNA (Fig. 1a) showed that 85% of the DNA sedimented as the denatured supercoiled form with a sedimentation coefficient of 53S, whereas the remainder sedimented at about 14-16S as single-stranded circles and linear pieces. The proportion of these forms was not significantly increased by incubation of the DNA with 0.56 μg of pol A or 0.67 μg of pol B protein and Mg²⁺ or Ca²⁺. For example, Fig. 1(b) shows the profile of DNA incubated with pol B and Mg²⁺. Incubation with 100 μg of pancreatic DNAase I degraded the DNA to a large extent (Fig. 1c), so the polymerase preparations must have contained considerably less than 0.02 mg of detectable endo-DNAase/100 mg. Crerar & Pearlman (1974) report slight endonuclease activity directed towards single-stranded viral DNA in the purified enzyme from *T. pyriformis*, but neither pol A nor pol B was tested for this.

Exo-DNAase activity

Assays were performed at pH values 4.6, 7.2 and 9.3, these being optimal for the known DNAases of *Euglena gracilis* (Slater *et al.*, 1972), and at pH 8.3. Native and denatured ^3H -labelled DNA substrates were used in the presence of 0.5 mM- or 5 mM-Mg²⁺, -Mn²⁺ or -Ca²⁺. Under these conditions no detectable hydrolysis of DNA was catalysed by pol A.

Fig. 1. *Endo-DNAase* assay by alkaline-sucrose-density-gradient centrifugation

Each assay mixture contained approx. 1 μ g (6000 c.p.m.) of superhelical SV40-virus [3 H]DNA and (a) no enzyme protein, (b) 0.67 μ g of pol B protein or (c) 100 μ g of pancreatic DNAase I. Samples were then centrifuged in alkaline sucrose density gradients as described in the Experimental section.

On the other hand, pol B preparations did exhibit DNAase activity (Table 1); only the assay conditions that supported this activity are shown. There was a distinct preference for a heat-denatured DNA substrate, native DNA being degraded only at neutral pH. Thus it did not correspond well to any of the known DNAases of *Euglena*, but displayed similarities to the pol B nuclease of yeast (Helfman, 1973). The most efficient assay conditions used were pH 8.3 and 5 mM-Mg $^{2+}$. Under these conditions and assuming the degradation to be exonucleolytic, nucleotide residues (or oligonucleotides) were released in an acid-soluble form at a rate of 72 nmol/h

Table 1. *Exo-DNAase* activity of pol B

Each assay mixture contained approx. 2 nmol of DNA substrate (calculated as nucleotide residues) and 0.55 μ g of pol B protein. Buffers used were sodium acetate, pH 4.6, imidazole-HCl, pH 7.2, Tris-HCl, pH 8.3, and glycine-NaOH, pH 9.3 (all at 40 mM).

pH	Nature of DNA substrate	Bivalent cation	Exo-DNAase activity (nmol of nucleotide residues released/h per mg of protein)
7.2	Native	0.5 mM-Mg $^{2+}$	10
7.2	Native	5 mM-Mg $^{2+}$	12
7.2	Native	0.5 mM-Mn $^{2+}$	8
7.2	Native	5 mM-Mn $^{2+}$	7
7.2	Denatured	0.5 mM-Mg $^{2+}$	15
7.2	Denatured	5 mM-Mg $^{2+}$	42
7.2	Denatured	0.5 mM-Mn $^{2+}$	34
7.2	Denatured	5 mM-Mn $^{2+}$	17
7.2	Denatured	0.5 mM-Ca $^{2+}$	12
7.2	Denatured	5 mM-Ca $^{2+}$	12
8.3	Denatured	0.5 mM-Mg $^{2+}$	34
8.3	Denatured	5 mM-Mg $^{2+}$	72
8.3	Denatured	0.5 mM-Mn $^{2+}$	13
8.3	Denatured	5 mM-Mn $^{2+}$	8
9.3	Denatured	0.5 mM-Mg $^{2+}$	21
9.3	Denatured	5 mM-Mg $^{2+}$	36
9.3	Denatured	0.5 mM-Mn $^{2+}$	8
9.3	Denatured	5 mM-Mn $^{2+}$	9

per mg of protein. The DNA polymerase activity of the same sample under optimal assay conditions (pH 7.2, 0.2 mM-Mn $^{2+}$) was 1750 nmol of nucleotide residues incorporated/h per mg of protein, but at pH 8.3 and in the presence of 5 mM-Mg $^{2+}$ it was 155 nmol of nucleotide residues incorporated/h per mg of protein. The turnover number of the 3' $\rightarrow$ 5' exonuclease function of pol I of *Esch. coli* is only 2% of the corresponding polymerase activity (Kornberg, 1974) under 'nuclease-optimal' conditions, so the DNAase activity of pol B is certainly present in amounts sufficient to suggest an association with the polymerase molecule.

Other nucleolytic activities

Nuclease activity on double-stranded RNA, single-stranded RNA and DNA-RNA hybrids was also examined. The DNA-RNA hybrids were prepared by the transcription of denatured DNA by a BHK-cell DNA-dependent RNA polymerase or by *Esch. coli* RNA polymerase (Cooper *et al.*, 1974). The exact nature of the hybrid products is unknown, so quantitative assessment of the results is rather difficult. It is likely that the DNA is present in great excess over the RNA. From the results given in Table 2, however, it is apparent that the RNA of a [3 H]RNA-DNA hybrid is much more sensitive to hydrolysis by pol B preparations when it is

Table 2. Nuclease activity of pol B on various substrates

Assay mixtures contained 2 μ g of each substrate (≤ 7000 c.p.m.) and were performed as described in the Experimental section at pH 8.0 with 5 mM-Mg²⁺. Each assay mixture contained 0.55 μ g of pol B protein.

Substrate	Conformation	Nuclease activity [% of radioactivity (c.p.m.) rendered acid-soluble in 1 h]
[³ H]RNA-DNA	Native	37.0
[³ H]RNA-DNA	Denatured	0.5
[³ H]DNA-RNA	Native	3.0
[³ H]DNA-RNA	Denatured	3.0
[³ H]Poly(A)·poly(U)	Native	1.0
[³ H]Poly(A)·poly(U)	Denatured	0.8

hybridized to DNA than after it is dissociated from the DNA by heat-denaturation. The amount of DNA that is concomitantly degraded is comparable with that which could be hydrolysed from a denatured DNA substrate, so it is possible that hybridized DNA is not susceptible to nuclease action. Native and denatured RNA can also be degraded to a slight extent. Slight degradation of double- and especially single-stranded RNA by RNAases H has been reported (Berkower *et al.*, 1973; Wyers *et al.*, 1973; Banks, 1974), and this may be a property of some of these enzymes. If one catalytic enzyme site is responsible for all these activities, then the RNAase H-type activity cannot be a true RNAase H, since such enzymes degrade only the RNA moiety of an RNA-DNA hybrid. Further, the RNAase activity demonstrated here was found not to be susceptible to inhibition by *S*-adenosyl-L-methionine up to a concentration of 5 mM. This compound has been used as an inhibitor of RNAase H (Stavrianopoulos & Chargaff, 1973; Cooper *et al.*, 1974). Nevertheless, the RNA of such a hybrid is degraded efficiently, and the pH and bivalent cation optima of this activity were investigated (Fig. 2). An alkaline pH of 8.4 and 5 mM-Mg²⁺ were preferred, conditions that are very similar to those required for exo-DNAase activity. Mn²⁺ at a concentration of 0.5–1 mM was 25% as efficient as Mg²⁺ in the assay. This activity may also be closely related to the polymerase since two different pol B preparations with polymerizing activities of 275 and 350 pmol of dTMP residues incorporated/h per 10 μ l of enzyme solution respectively (a ratio of 0.78) had RNAase H activities that solubilized 45% and 55% (a ratio of 0.82) of the total hybrid radioactivity (c.p.m.)/h per 10 μ l of enzyme solution respectively. It is tempting to suggest that the DNAase and RNAase activities of pol B correspond to the 3'→5' and 5'→3' nuclease functions of the bacterial pol I enzymes respectively. The latter activity might also account for the observed hydro-

Fig. 2. pH and bivalent cation optima of the pol B RNAase H activity

Assays were performed as described in the Experimental section with 2 μ g (≤ 7000 c.p.m.) of [³H]RNA-DNA hybrid and 0.67 μ g of pol B protein per assay. (a) Imidazole-HCl buffer was used over the pH range 7.2–7.6 and Tris-HCl buffer over the pH range 7.8–9.0. (b) Various amounts of Mg²⁺ (●) or Mn²⁺ (○) were added at pH 8.0.

lysis of native DNA, but such a conclusion must await the results of further investigation. Pol A preparations again showed no tendency to hydrolyse any of the substrates.

Nucleoside diphosphokinase activity

In the presence of dATP, dGTP and dCTP, pol B was found to incorporate label from [³H]dTDP into acid-insoluble material at about 12% of the rate obtained with dTTP (McLennan & Keir, 1975b), suggesting the presence of a nucleoside diphosphokinase activity in fraction VIIIB. This was confirmed by measuring the production of [³H]dTTP from [³H]dTDP in the presence of ATP (no primer-template present). The substrate and product were separated by t.l.c. on cellulose. No label was found in dTMP, thus demonstrating that the reaction measured was not of the form:

but that ATP had served as the phosphoryl donor. The conversion followed a linear time-course up to about 30 min, the reaction slowing down thereafter (Fig. 3). The specific activity of the diphosphokinase was 8.4 nmol of [^3H]dTDP phosphorylated/30 min per mg of protein, whereas the specific activity of the polymerase function under the similar conditions of the standard assay (2 mM- Mg^{2+}) (McLennan & Keir, 1975b) was 123 nmol of [^3H]dTTP residues incorporated/30 min per mg of protein. Thus dTDP could be phosphorylated at only 7% of the rate required to attain the maximum rate of DNA synthesis. dATP appeared to substitute for ATP with an efficiency of about 65% after a 30 min incubation. Optimal assay conditions were not sought, so a higher rate of diphosphokinase activity may be feasible.

Since Kornberg (1974) has reported that nucleoside diphosphokinase activity may not be present in homogeneous preparations of pol I of *Esch. coli*, the activity that we have observed in the present work may be associated with one of the contaminants in fraction VIII B (McLennan & Keir, 1975b), but its presence in other eukaryotic DNA polymerase fractions, especially the highly purified enzyme from *T. pyriformis* (Crerar & Pearlman, 1974), may indicate otherwise. Pol A was not examined for kinase activity.

Utilization of natural and synthetic primer-templates

Natural primer-templates. It should be stressed that in terms of assay composition and enzyme/template ratios the conditions under which the primer-templates were tested may not have been optimal for each one. However, under all conditions used,

'activated' DNA, i.e. native DNA that had been 'nicked' with pancreatic DNAase I, was the best natural primer-template tested for both enzymes (Table 3). The activity demonstrated with commercial native salmon testis type III was probably due to the presence of 'nicks' in the DNA. Calf thymus type I DNA, which contains fewer 'nicks', promoted very little activity with either

Fig. 3. Nucleoside diphosphokinase activity of pol B as a function of time

Pol B (2.2 μg of protein) was assayed in 0.4 ml as described in the Experimental section with ATP as the phosphoryl donor. Portions (0.1 ml, containing 0.55 μg of protein) were analysed at time-intervals for the presence of [^3H]dTTP by t.l.c.

Table 3. Activities of pol A and pol B with natural DNA primer-templates

Assays were at 32°C in the presence of either 2 mM- Mg^{2+} or 0.2 mM- Mn^{2+} and with 5 μg of primer-template and 0.35 μg of pol A or 0.67 μg of pol B protein per assay. The results are expressed as percentages of the activity produced by 5 μg of an 'activated'-DNA primer-template in the presence of 2 mM- Mg^{2+} , this being 38 and 122 nmol of dTMP residues incorporated/h per mg of protein for pol A and pol B respectively. All primer-templates were prepared as described in the Experimental section.

Primer-template	Polymerase activity (% of 'activated'-DNA control)			
	Pol A		Pol B	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
'Activated' salmon testis DNA	100	160	100	155
'Activated' salmon testis DNA + 20 μg of native salmon testis DNA	92	74	27	71
'Activated' salmon testis DNA + 20 μg of denatured salmon testis DNA	32	35	0	0
Native salmon testis DNA	19	21	17	13
Denatured salmon testis DNA	7	14	7	9
'Momparker' activated DNA	9	14	13	13
Native calf thymus DNA type I	0.5	1.0	0.5	0
None	0	0	0	0

Table 4. Activities of *pol A* and *pol B* with synthetic primer-templates

Assays were at 26°C with 100mCi/mmol of [³H]dTTP in the presence of either 2mM-Mg²⁺ or 0.2mM-Mn²⁺ and with 5 µg of each primer-template and 0.7 µg of *pol A* or 1.34 µg of *pol B* protein per assay. The results are expressed as percentages of the activities produced by 5 µg of an 'activated'-DNA primer-template in the presence of 2mM-Mg²⁺, this being 22 and 71 nmol of nucleotide residues incorporated/h per mg of protein for *pol A* and *pol B* respectively. The A+T content of salmon testis DNA was taken as 58% (Hashizume & Sasaki, 1968).

Primer-template (R/Y ratios in parentheses)	Polymerase activity (% of 'activated'-DNA control)			
	Pol A		Pol B	
	Mg ²⁺	Mn ²⁺	Mg ²⁺	Mn ²⁺
'Activated' salmon testis DNA	100	164	100	144
Poly[d(A-T)]	11	104	10	71
Poly[d(A)]·oligo[d(T) ₁₀] (23:1)	1.5	1.5	25	27
Poly[d(A)]·oligo[d(T) ₁₀] + oligo[d(T) ₆] (1:1)	0	0	7.5	9
Poly[d(A)]·oligo(U ₆) (23:1)	0	0	<1	<1
Poly[d(A)]·oligo(U ₆) (1:1)	0	0	<1	<1
Poly(A)·oligo[d(T) ₆] (23:1)	0	0	0	0
Poly(A)·oligo[d(T) ₁₀] (23:1)	0	0	0	0
Poly(A)·oligo[d(T) ₆] (1:1)	0	0	0	14
Poly(A)·oligo[d(T) ₁₀] (1:1)	0	0	0	31
Poly[d(A)]	<1	<1	<1	<1
Oligo[d(T) ₆]	0	<1	0	<1
Oligo(U) ₆	0	0	0	0

enzyme. Heat-denatured DNA was also a relatively poor primer-template, and, when present in excess over 'activated' DNA in an assay, it strongly inhibited the 'activated'-DNA-dependent reaction of *pol A* and completely abolished that catalysed by *pol B*. *Pol II* of *B. subtilis* (Gass & Cozzarelli, 1973) and L-cell DNA polymerase (Lindsay *et al.*, 1970) are also inhibited by single-stranded DNA. The inhibition is presumably due to the binding of the enzymes to the DNA, thus decreasing the number of initiation events at 3'-hydroxyl termini, and the relative degrees of inhibition observed with each enzyme agree with their known affinities for DNA-cellulose (McLennan & Keir, 1975b). *Pol B* also appeared to be more sensitive to inhibition by native DNA under similar conditions.

DNA that had been 'activated' by the method of Momparler *et al.* (1973) was not a good primer-template for either polymerase. Such a preparation probably contains single-stranded regions longer than those produced by the method of Loeb (1969). The activity observed is comparable with that obtained with heat-denatured DNA, suggesting that the enzymes are unable to synthesize long stretches of DNA.

Synthetic primer-templates. The assay mixtures involving synthetic primer-templates were incubated at 26°C to increase the stability of the primer-template-enzyme complex (Chang *et al.*, 1972), and the results are expressed in terms of the total amount of deoxyribonucleoside 5'-monophosphate

residues incorporated in each case. In the presence of Mn²⁺, poly[d(A-T)] was the most efficient of the synthetic primer-templates tested for both enzymes, although still not as effective as 'activated' DNA in terms of total nucleotide residues incorporated (Table 4). *Pol A* used poly[d(A)]·oligo[d(T)₁₀] (A/T ratio 23:1) poorly, but *pol B* was 17-18-fold more efficient with such a primer-template. Addition of d(T)₆ primers (A/T ratio 1:1) to this reaction abolished nucleotide incorporation by *pol A* and inhibited the *pol B* reaction by about 70%. Substitution of the d(T)₁₀ primers by (U)₆ (A/U ratio 23:1 or 1:1) led to almost total loss of activity with *pol B*, and *pol A* was completely inactive. Crerar & Pearlman (1974) also found that the enzyme from *T. pyriformis* was unable to catalyse RNA-primed DNA synthesis *in vitro*. No poly(A)-directed activity could be assigned to *pol A* either, and, with d(T)₆ and d(T)₁₀ primers in the A/T ratio 23:1, *pol B* also appeared to lack this activity. However, by increasing the A/T ratio of the poly(A)·oligo[d(T)₆] to 1:1, initiation of poly[d(T)] synthesis was achieved in the presence of Mn²⁺ (but not Mg²⁺), and if poly(A)·oligo[d(T)₁₀] (A/T ratio 1:1) was used in the assay an even greater extent of dTMP incorporation was attained. Thus *pol B* can utilize a polyribonucleotide template if sufficient oligodeoxyribonucleotide primers of the correct length are present, whereas, apart from slight activity with poly[d(A)]·oligo[d(T)₁₀], *pol A* utilized none of the synthetic oligodeoxyribonucleotide-primed homopolymer templates tested.

Crerar & Pearlman (1974) found a similar inhibition of the poly[d(A)]-directed reaction by excess of primers with the enzyme from *T. pyriformis*. They showed the optimal A/T ratio for the poly[d(A)] template to be 10:1, whereas, it was 1:1 for the poly(A) template. Their enzyme, like pol B, exhibited no poly(A)-directed activity with Mg^{2+} . One apparent difference between these two polymerases is the 16-fold preference for Mg^{2+} shown by the *T. pyriformis* enzyme when assayed with apoly [d(A)]·oligo-[d(T)₁₀] primer-template. Pol B showed no bivalent cation preference, but this result is perhaps not surprising in view of the Mn^{2+} preference of pol B in the 'activated'-DNA-dependent reaction (McLennan & Keir, 1975b). Further, Crerar & Pearlman (1971) reported that their *T. pyriformis* enzyme did not use an 'activated' DNA primer-template. This is a property of pol II and pol III of *Esch. coli* and of the T-bacteriophage DNA polymerases (Kornberg, 1974) and is clearly not true of pol A or pol B. Apart from slight activity with poly[d(A)]·oligo[d(T)₁₀] pol A utilized none of the synthetic oligodeoxyribonucleotide-primed homopolymer templates under the assay conditions used.

Finally, both enzyme preparations catalysed a small but reproducible amount of terminal addition activity in the presence of Mn^{2+} and a d(T)₆ primer. (U)₆ could not function as a primer, but poly[d(A)] appeared to be able to do so in the presence of Mg^{2+} or Mn^{2+} . This activity was not investigated further.

Discussion

We have demonstrated that pol A and pol B can be differentiated on the basis of associated nucleolytic activities and by their utilization of primer-templates. Possible models for the association of a nuclease with pol B but not pol A have already been discussed (McLennan & Keir, 1975a).

With their overall preference for 'activated' DNA and the dependence of the poly(A)-directed reaction of pol B on Mn^{2+} , these enzymes behave like DNA-dependent DNA polymerases and are unlike the cellular poly(A)-dependent DNA polymerases (Spadari & Weissbach, 1974), which prefer a poly(A) template to 'activated' DNA, or the viral reverse transcriptases (Goodman & Spiegelman, 1971), which utilize poly(A) in the presence of Mg^{2+} .

The higher-eukaryotic 6-8S species (but not the 3.4S) can initiate synthesis on a DNA template with an oligoribonucleotide primer (Chang & Bollum, 1972a), but poly[d(A)]·oligo(U)₆ (A/U ratio 23:1 or 1:1) promoted only a very limited amount of synthesis with pol B and none at all with pol A. It is possible that the hexaribonucleotide primer was not long enough to form a stable primer-template enzyme complex under these con-

ditions or that, with pol B, the RNAase H activity rapidly removed all the primers although assay conditions would have been suboptimal for this activity. Alternatively, pol A and pol B may be unable to use RNA primers *in vitro*.

An absolute requirement for a poly[d(A)] template over a poly(A) template, especially with Mg^{2+} , is also, in general, a property of the eukaryotic 6-8S species (Chang & Bollum, 1972b; de Recondo *et al.*, 1973; Brun *et al.*, 1974), although in some cases it may copy an RNA template (Smith & Gallo, 1972). The ability of the 3.4S enzyme to copy a poly(A) template is more variable (Craig & Keir, 1974), but many of them do use such a template (Brun *et al.*, 1974; Wickremasinghe & Johnston, 1974). Thus it is very difficult to ascertain functional similarities between pol A and pol B and the 6-8S and 3.4S species on the basis of primer-template utilization. However, in view of the variability of such results from different systems and considering the properties of the 'activated'-DNA-dependent reactions catalysed by pol A and pol B (McLennan & Keir, 1975b), we suggest that these enzymes are both of the 6-8S type. Without further characterization of the separated enzymes of the mammalian 6-8S species (Holmes *et al.*, 1974; Yoshida *et al.*, 1974) it is difficult to say how closely pol A and pol B might correspond to two such enzymic forms. Possible structures for pol A and pol B that would account for their properties have been discussed elsewhere (McLennan & Keir, 1975a).

The *Euglena* enzymes are strikingly similar in their properties to the corresponding pol A and pol B from the yeast *Saccharomyces cerevisiae*, although they exhibit even more common characteristics than the yeast enzymes. The DNA polymerase from *T. pyriformis* also has many properties in common with pol B, and the presence of a nuclease in all three pol B-type preparations may indicate a closer relationship in evolutionary terms to the bacterial DNA polymerases. We therefore suggest that the DNA polymerase complement of eukaryotic protists represents something of an intermediate between that of prokaryotes and higher eukaryotes. The lack of a 3.4S enzyme in such organisms supports such a conclusion. In order to determine the exact roles of these enzymes in the processes of DNA repair and DNA replication, the study of conditional lethal mutants is essential.

We thank the Science Research Council for the award of a studentship to A. G. McL., and the Medical Research Council for support.

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