The Deoxyribonucleic Acid Polymerases from the Diatom *Cylindrotheca fusiformis*

PARTIAL PURIFICATION AND CHARACTERIZATION OF FOUR DISTINCT ACTIVITIES

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Four extramitochondrial DNA polymerases from the marine photosynthetic diatom *Cylindrotheca fusiformis* were isolated and purified more than 1200-fold by chromatography on DNA–cellulose and DEAE-Sephadex. The enzymes were equally susceptible to inhibition by the thiol-blocking agents *N*-ethylmaleimide and *p*-chloromercuribenzoate, the zinc chelator *o*-phenanthroline, and the nucleic acid interchelators ethidium bromide and acriflavin; they displayed similar pH optima, preferred activated DNA, and had strict dependence on high K⁺ for maximum activity. They were differentiated on the basis of their kinetic parameters, template-primer utilization and salt requirements. The four activities varied with the growth stage of *C. fusiformis*. Activities of polymerases A and D doubled in exponential-phase cells as compared with those in stationary-phase cells, and the increase in polymerase B and chloroplast activity C was 20–40%. The relationship of the diatom polymerases to the complements in other organisms is discussed.

A great deal of research in the past few years has been focused on the DNA polymerases (DNA nucleotidyltransferase, EC 2.7.7.7) and their role in DNA replication. In the prokaryotes, the use of mutants and complementation assays *in vitro* has provided much information on these polymerases and other proteins involved in DNA replication in *Escherichia coli* (Kornberg, 1974; Tait & Smith, 1974; Tait *et al.*, 1974; Lehman & Uyemura, 1976). There have been a few detailed studies to isolate and characterize the DNA polymerases in the protists: two activities have been isolated and extensively studied from the extramitochondrial extracts of *Euglena gracilis* (McLennan & Keir, 1975*a,b*) and yeast (Wintersberger & Wintersberger, 1970; Helfman, 1973; Wintersberger, E., 1974); three activities have been isolated from the macronuclei of *Paramecium* (Tait & Cummings, 1975) but only a single activity has been found in extramitochondrial extracts of the smut fungus *Ustilago maydis* (Banks *et al.*, 1976), and in *Tetrahymena* (Crerar & Pearlman, 1974).

At least three types of extramitochondrial DNA polymerases have been isolated and characterized from mammalian cells, each of which is unique with respect to molecular weight, template-primer specificity, sensitivity to thiol-blocking agents and

cellular localization (see Weissbach, 1975). Nevertheless, little is known about the role of the DNA polymerases in eukaryotes, particularly in mammalian cells where the selection of mutants has proved difficult. An alternative approach to the use of mutants has been to correlate enzyme function with biological processes, e.g., to study the function by estimating the variation of the DNA polymerase activities in tissues actively involved in DNA replication (Chang & Bollum, 1972; Chang *et al.*, 1973). The marine photosynthetic diatom *Cylindrotheca fusiformis* readily lends itself to such an approach.

In this organism DNA replication is uniquely dependent on silicate, and can be controlled by its presence or absence. During normal growth of light–dark synchronized cells in the presence of silicate, the major cellular constituents double during the 12 h–cell-division cycle and initiate DNA replication at 6 h. When light–dark-synchronized cells are deprived of silicate, cellular DNA increases by only 4–10% whereas the rest of the cellular components have increased by at least 75% at the end of the 12 h of deprivation (Darley & Volcani, 1969). If silicate is resupplied, there is an immediate synthesis of DNA coupled with an increase of DNA polymerase activity (Sullivan & Volcani, 1973, 1976). This silicate-dependent DNA replication requires protein synthesis *de novo*, since abrupt inhibition occurs in

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the presence of cycloheximide. Thus *C. fusiformis* appears to be an ideal organism for investigating the regulation of DNA replication and the role of DNA polymerases in this process.

As a first approach to such a study, we undertook a detailed examination of the diatom DNA polymerases. In this and the following paper (Okita & Volcani, 1977), we report the partial purification and the properties of four high-molecular-weight DNA polymerases from the extramitochondrial fraction of *C. fusiformis*. We demonstrate that the activities of all four enzymes depend on the growth stage of the diatom and that their properties are similar to those of the DNA polymerases in other lower eukaryotes.

Experimental

Buffers

The following four buffers were used: I, 20mM-Hepes*/NaOH (pH 7.6 at 25°C)/1 mM-MgCl₂/0.1% bovine serum albumin/0.4M-sucrose; II, 20mM-Hepes/NaOH (pH 7.6)/0.6M-sucrose; III, 20mM-Tris/HCl (pH 7.8)/50 mM-NaCl/1 mM-2-mercaptoethanol/1 mM-Na₂EDTA; IV, 50mM-Tris/HCl (pH 7.3)/100 mM - NaCl / 1 mM - 2 - mercaptoethanol / 1 mM - Na₂EDTA/10% (v/v) glycerol.

Materials

Unlabelled nucleotides, salmon spermatozoan DNA, Triton X-100, phenylmethanesulphonyl fluoride, *o*-phenanthroline, calf thymus DNA, DNAase I, pancreatic RNAase and 2-mercaptoethanol were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; bovine serum albumin was from Calbiochem, San Diego, CA, U.S.A.; [*Me*-³H]dTTP was from Schwarz/Mann, Orangeburg, NY, U.S.A.; cellulose CF II and glass-fibre GF/C filters were from Reeve Angel, Clifton, NJ, U.S.A.; acrylamide and *NN'*-methylenebisacrylamide were obtained from Eastman-Kodak, Rochester, NY, U.S.A.; poly[d(A-T)], poly[d(A)]-oligo[d(T)₁₀] and poly(A)-oligo[d(T)₁₀] were purchased from Collaborative Research, Waltham, MA, U.S.A.; nitrocellulose filters were from Matheson-Higgins, Woburn, MA, U.S.A.; 2,5-diphenyloxazole, 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and Aquasol scintillation 'cocktail' were from New England Nuclear, Boston, MA, U.S.A.; novobiocin was generously given by Merck, Sharp and Dohme Research Laboratory, Rahway, NJ, U.S.A. All other chemicals were A.R. grade.

* Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; DNAase, deoxyribonuclease; RNAase, ribonuclease; SDS, sodium dodecyl sulphate.

Methods

Culture conditions. *C. fusiformis* was grown under forced aeration in an artificial seawater medium (Darley & Volcani, 1969) at 20°C, 17000lx. Cells (2×10⁶–3×10⁶ cells/ml) from the actively growing (exponential-growth) stage were used as starting material for the purification of DNA polymerases. For the growth-stage-dependence studies, cells (4×10⁶ cells/ml) from the late-exponential phase were used, as well as cells taken 72h after growth had ceased (late-stationary phase). Cells were collected with a Sharples centrifuge (Sharples Corp., Philadelphia, PA, U.S.A.).

Preparation of extramitochondrial supernatant. All steps were carried out at 4°C. Cells were washed twice in 1.5 vol. of buffer I, resuspended in 3 vol. of buffer I, and disrupted by passage through an ice-cold pressure cell (Yeda Research and Development Co. Ltd., Rehovot, Israel) at 32.1 MPa (4500 lb/in²). The homogenate was diluted with 2 vol. of buffer II, centrifuged at 3000g_{av.} for 10 min, and the resulting pellet was washed twice with 2 vol. of buffer III. The supernatants were combined with the initial 3000g_{av.} supernatant, and centrifuged at 10000g_{av.} for 10 min to remove the mitochondria. This supernatant fluid was combined with the 3000g_{av.} pellet and solid NaCl was added to a final concentration of 1.5M. The enzymes were extracted from membranes and nucleic acid for at least 30 min with gentle stirring, and then centrifuged in a Spinco ultracentrifuge at 50000g_{av.} for 30 min. The final supernatant was used for further purification of the DNA polymerases, as described in the Results section.

DNA polymerase assay. The standard assay mixture (0.12 ml) contained: 50 mM-Tris/HCl, pH 8.0; 100 mM-KCl; 5 mM-MgCl₂; 1 mM-2-mercaptoethanol; 100 μM each of dATP, dCTP and dGTP; 2 mM-ATP; 60 μg of activated salmon spermatozoan DNA; 10 μM-[*Me*-³H]TTP (300 Ci/mol); and enzyme protein. After incubation for 30 min at 30°C the reaction was stopped by the addition of 0.5 ml of chilled 10% (w/v) trichloroacetic acid/10 mM-Na₄P₂O₇. The assay tubes were left on ice for 20 min and their contents collected by suction on glass-fibre filters which were then washed with 3×5 ml washes of 1% trichloroacetic acid/1 mM-Na₄P₂O₇ followed by 2×1 ml washes in ethanol. After air-drying under suction for at least 5 min, the filters were counted for radioactivity in a Beckman LS-250 liquid-scintillation spectrometer with 0.3% (w/v) 5-phenyloxazole and 0.02% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in toluene/Triton X-100 (2:1, v/v) as the scintillator fluid. A unit of DNA polymerase activity is defined as the amount of enzyme that catalyses the incorporation of 1 nmol of TMP into acid-soluble material in 1 h.

With synthetic template primers, the standard

assay method was modified as follows. The assays were incubated at 26°C with 500Ci of [$Me\text{-}^3H$]-dTTP/mol. Because only 5 μ g of template primer was used in each reaction, 0.1ml of a solution of heat-denatured calf thymus (1mg/ml) and bovine serum albumin (1mg/ml) was added to each tube before the addition of ice-cold 10% trichloroacetic acid and 20mM- $Na_4P_2O_7$. The contents of each tube were mixed, and after 15min at 0–2°C, insoluble material was collected on nitrocellulose filters (0.45 μ m pore size). Each filter was washed with 20ml of 10% (w/v) trichloroacetic acid at 4°C and 10ml of chloroform/methanol (1:1, v/v). Each filter was transferred to a glass scintillation vial, left to air-dry for 10min, and then dissolved in 0.5ml of methyl-Cellosolve. Aquasol (10ml) was added and after thorough mixing radioactivity was determined in a Beckman LS-250 liquid-scintillation spectrometer at a counting efficiency of about 35% as determined by the addition of radioactive internal standards.

Column chromatography. DEAE-Sephadex A-50 was swollen and washed in buffer IV. Native DNA-cellulose was prepared by the method of Alberts & Herrick (1971), washed in boiling ethanol, and dried under vacuum before complexing with the native DNA solution.

Other methods. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard, and in fractions V and VI by the method of Warburg & Christian (1941). Activated DNA was prepared by a modification of the method described by Loeb (1969): salmon spermatozoan DNA (2mg/ml) was incubated with pancreatic deoxyribonuclease I (0.04 μ g/ml) at 37°C. Maximum priming activity was reached at 15–20min whereby 2% hydrolysis occurred. SDS/polyacrylamide slab-gel electrophoresis was performed by the methods of Laemmli (1970) and Ames (1974). Details will be discussed by Okita & Volcani (1977). Native polyacrylamide gels were prepared, electrophoresed and analysed for DNA polymerase activity as described by McLennan & Keir (1975a).

Results

Purification of DNA polymerases

All operations were carried out at 4°C.

Poly(ethylene glycol) extraction and $(NH_4)_2SO_4$ precipitation. The extramitochondrial supernatant, derived from the disruption of 50g (wet wt.) of exponentially growing cells, was treated with poly(ethylene glycol) 6000 at a final concentration of 10% (w/v). The extract was gently stirred for 30min and then centrifuged at 20000g_{av.} for 10min and dialysed overnight against two 50 vol. changes of buffer III. Solid $(NH_4)_2SO_4$ was slowly added and

gently stirred for an additional 30min after which the mixture was poured into a separatory funnel and the two phases were left to separate overnight. The lower aqueous phase was collected and $(NH_4)_2SO_4$ added to 70% saturation. The precipitate was collected by centrifugation at 25000g_{av.} for 30min. The upper poly(ethylene glycol) phase was discarded, since it contained less than 1% of the total DNA priming activity.

DNA-cellulose. The $(NH_4)_2SO_4$ precipitate was resuspended in buffer III at a protein concentration of 5mg/ml and dialysed for at least 4h against two 50 vol. changes of buffer III. $MgCl_2$, $CaCl_2$ and phenylmethanesulphonyl fluoride were added to final concentrations of 10mM, 2mM and 0.1mM respectively; 50 μ g of DNAase I/ml and 5 μ g of pancreatic RNAase/ml were then added. After incubation at room temperature (24°C) for 30min, the mixture was chilled in an ice bath. Bivalent cations were removed by overnight dialysis against buffer IV and the mixture was layered on a column (3cm $\times$ 1cm) of native DNA-cellulose which was washed with buffer IV until the A_{280} of the eluate reached zero. Buffer IV containing 0.8M-KCl was added at a flow rate of 20ml/h, and 5ml fractions were collected. All DNA polymerase activity was eluted at this ionic strength (Fig. 1).

DEAE-Sephadex A-50. The active fractions from the DNA-cellulose column were collected, dialysed overnight against 500 vol. of buffer IV, and then loaded on to a column (15cm $\times$ 1cm) of DEAE-Sephadex A-50. A 200ml linear gradient of 0.10–0.40M-NaCl was applied at a flow rate of 15ml/h and 2ml fractions were collected. Four distinct DNA polymerase activities were detected, and were designated pol A, pol B, pol C and pol D, in the order of

Fig. 1. Affinity chromatography of fraction IV on DNA-cellulose

DNA polymerase was assayed as described in the text; 5ml fractions were collected. $\square$, A_{280} ; $\bullet$, DNA polymerase activity; the arrow indicates the point at which washing with buffer was terminated and elution with 0.8M-NaCl in buffer III initiated.

Fig. 2. Column chromatography of fraction V on DEAE-Sephadex A-50

Gradient-elution chromatography was performed as described in the text; 2 ml fractions were collected. Tubes 20–24 were combined for pol A, 27–29 for pol B, 32–34 for pol C, and 37–40 for pol D; ●, DNA polymerase; —, NaCl gradient.

Fig. 3. Rechromatography of fraction VI: pol A (a), pol B (b) and pol C (c) on DEAE-Sephadex A-50. Pooled samples from fraction VI were rapidly dialysed and resolved by a second DEAE-Sephadex column. Gradient elution was performed as described in Fig. 2. ●, DNA polymerase; —, NaCl gradient.

elution from the column (Fig. 2). To ensure that each activity was unique, peak fractions from each were collected, dialysed against 100 vol. of buffer IV for 2 h, and rechromatographed on individual columns (4 cm $\times$ 1 cm) of DEAE-Sephadex A-50 at a flow rate of 10 ml/h, by using a 50 ml linear gradient of 0.13–0.30 M-KCl. Separation of the remaining activity was achieved in each case, except for pol D which was

completely labile on this column. Normally there was less than 5% contamination between the DNA polymerase activity in question and the neighbouring polymerase activities (Fig. 3). However, peak fractions of pols A, B and C yielded only 10–26% of the original activity layered on to the column. The reason for this extreme lability is not known. Addition of bovine serum albumin either to the sample before separation by ion-exchange chromatography or to the tubes used in the collection of the gradient fractions did not significantly improve stability. Because only a limited amount of enzyme was recovered without achieving a significant increase in specific activity, all experiments were carried out with the preparations obtained from the first DEAE-Sephadex chromatogram (fraction VI). These final preparations were brought to 10 mM with 2-mercaptoethanol and stored on ice without prior removal of the NaCl. Pols A, B and C were stable for about 4 months, losing only about 20% of activity, whereas pol D lost about 66% of its activity. The protocol for the purification of the DNA polymerases is outlined in Table 1. Each activity was purified at least 1200-fold over that originally present in the crude extract. However, assuming that pols A, B, C and D contribute 34%, 35%, 21% and 10% respectively to the total activity in the original extract from exponentially grown cells, purification factors of 5000-, 8400-, 13450- and 12350-fold respectively are obtained.

The degree of purity of each of the fractions was examined by polyacrylamide-gel electrophoresis. Attempts to analyse the DNA polymerases in native gels have failed, probably because of their extreme lability. SDS/polyacrylamide gels revealed the presence of 3, 7, 3 and 4 distinct bands from pols A, B, C and D respectively (results not shown). This indicates the presence of contaminants accompanying the polymerase activities.

Properties of the DNA polymerase activities

Effect of protein concentration and nuclease treatment. The activities of all four enzymes were linear with respect to protein concentration up to 15 μ g and linear over time up to 2 h when less than 5 μ g of enzyme was used in the assay. Since subsequent incubation of the assay with DNAase I, but not pancreatic RNAase, rendered all of the precipitable radioactive material acid-soluble, the radioactive label was apparently incorporated into DNA. Moreover, heating the product in 0.5 M-HClO₄ for 5 min completely solubilized the product, whereas incubation in 0.3 M-KOH at 37°C for 8 h had little effect.

Metal requirements. All DNA priming activities had an absolute requirement for either Mg²⁺ or Mn²⁺, the preferred bivalent cation being Mg²⁺. In the presence of Mg²⁺, maximum activity for pols A

Table 1. Purification of DNA polymerases from exponential-phase cells of *C. fusiformis*
For details see the text; approx. 55 g (wet wt.) of exponentially growing cells were used as the starting material. Values marked with an asterisk (*) were calculated with respect to units of activity obtained in fraction VI.

Fraction	Total units (nmol/h)	Protein (mg/ml)	Specific activity (units/mg of protein)	Yield (%)
I Crude extract	150	7.5	0.07	100
II 100000g _{av} . supernatant	141	4.0	0.13	95
III Poly(ethylene glycol) 6000/1.5 M-NaCl	204	1.3	0.34	136
IV (NH ₄) ₂ SO ₄ precipitation	225	7.7	0.61	125
V DNA-cellulose	255	0.44	25.30	170
VI DEAE-Sephadex A-50				
Pol A	85	0.060	118	56
Pol B	86	0.065	206	37
Pol C	52	0.045	194	23
Pol D	30	0.035	86	13
VII Rechromatography on DEAE-Sephadex A-50				
Pol A	18	0.039	56	22*
Pol B	22	0.017	150	26*
Pol C	5	0.022	38	10*
Pol D	0	—	—	—

Fig. 4. Effect of Mn²⁺ concentrations on pol A, B, C and D activities
DNA polymerase activities were assayed as described in the text. The assay mixtures contained 0.9 μg of pol A (○), 0.3 μg of pol B (▲), 0.5 μg of pol C (□) and 0.4 μg of pol D (●).

Fig. 5. Effect of K⁺ concentrations on pol A, B, C and D activities
DNA polymerase activities were assayed as described in the text except that each activity was measured in the presence of their optimum pH and Mg²⁺ concentrations. The assay mixtures contained 1.0 μg of pol A (○), 0.4 μg of pol B (▲), 0.5 μg of pol C (□) and 0.6 μg of pol D (●).

and D were identical at 2–3 mM, whereas pols B and C showed maximum activity at 7–8 mM and 5–6 mM respectively. The enzymes displayed unique Mn²⁺ optima. All activities were less than 20% as active in the presence of Mn²⁺ as in that of Mg²⁺. The optimum of pol A was broad, 2–5 mM; that of pol B was 5–6 mM; the requirement of pol C was more stringent at 7 mM; and pol D had high affinity for Mn²⁺ at 0.5 mM (Fig. 4).

The response of the DNA polymerases to univalent cations was unique, for all were activated at least 2.5-fold by very high amounts of K⁺. Optimum concentrations for K⁺ were 150 mM, pol A, 90 mM, pol B, 200 mM, pol C, and 110 mM, pol D (Fig. 5).

Silicon may activate the polymerases, thus accounting for the increase of DNA priming activity in crude

extracts obtained during silicon recovery (Sullivan & Volcani, 1973). Hence the effect of silicon (as silicic acid) on the activity of the DNA polymerase was tested. At concentrations of 0.01–5 mM-sodium silicate, all four enzymes showed little response.

pH optima. In the presence of 50 mM-Tris/HCl, the pH optima curves of all activities were similar at 7.9, 7.5, 7.7 and 7.5 for pols A, B, C and D respectively, but less than 20% of the activity was detected at pH 6.0 and 9.5. In the presence of phosphate buffer, the pH optima shifted toward the basic pH, with all four polymerases showing maximum activity at pH 8.0.

Fig. 6. Hofstee plots for pols A (a), B (b), C (c) and D (d)

Assays were conducted for each of the activities at their optimum pH, univalent and bivalent cation concentrations, and at saturating amounts (100 μ g) of activated salmon spermatozoan DNA. Incubations were performed with [3 H]TTP (300 Ci/mol) for 30 min. Incorporation rates were determined as a function of equimolar concentrations of all four deoxyribonucleotide triphosphate species. The assay mixtures contained 1.3 μ g of pol A, 1.0 μ g of pol B, 1.5 μ g of pol C and 1.5 μ g of pol D.

Effects of thiols and thiol-blocking agents. The reducing agents, 2-mercaptoethanol and dithiothreitol, at concentrations above 0.1 mM, stimulated all DNA polymerase activities by 3–6-fold. The role of free thiol groups in catalysis is further demonstrated by the effect of thiol-blocking agents, *N*-ethylmaleimide and *p*-chloromercuribenzoate. All four enzymes showed about 80% inhibition of activity at an *N*-ethylmaleimide concentration of 1 mM; an increase to 5 mM had no further effect. On the other hand, total inhibition occurred in the presence of 0.1 mM-*p*-chloromercuribenzoate.

Effect of other inhibitors. All four activities were inhibited by the chelating agent, *o*-phenanthroline with 50% and 95% inhibition for all activities at 0.25 mM and 1 mM respectively. This suggests a role for Zn^{2+} as a component of the enzyme necessary for catalysis (Slater *et al.*, 1971). All of the diatom's DNA polymerases were inactivated by novobiocin (95% inactivation at 3 mM), which has been used to differentiate DNA polymerase- α and - β from rat brain (Sung, 1974). The enzymes were also tested for their sensitivity toward ethidium bromide and acriflavin, dyes used in plasmid elimination (Kornberg, 1974) and mitochondrial mutagenesis (Goldring *et al.*, 1970). For all polymerases, concentrations of 5–10 μ M of either drug inhibited activity by about 50%.

V_{max} and K_m values of DNA polymerases. There were marked differences in the affinities of the four enzymes for the deoxyribonucleoside triphosphates

in the presence of Mg^{2+} . Kinetic plots revealed K_m values for pols A, B, C and D of 1.9 μ M, 6.4 μ M, 15.8 μ M and 6.5 μ M respectively with V_{max} values of 174, 426, 400 and 177 nmol/h per mg of protein (Fig. 6). No apparent substrate activation of the DNA polymerases, as reported in *Euglena gracilis* (McLennan & Keir, 1975a), was detected.

All reactions depended on the presence of DNA. Pols A, B, C and D (all at 0.1 unit of activity) were saturated with activated salmon spermatozoan DNA at 5 μ g, 8 μ g, 15 μ g and 6 μ g respectively; none of the activities were inhibited by DNA in amounts as high as 200 μ g/assay. Inhibition did occur, however, when equal parts of native and activated DNA (10 μ g of each) were used; incorporation of dTMP was decreased by 70–80% for all activities.

The maximum incorporation rate of [3 H]dTTP was obtained only in the presence of all four nucleotides (Table 2). In the absence of one or two nucleotides, the responses of the polymerases differed. Pol A showed the greatest variation in the absence of one nucleotide, retaining about 37% of its original activity, whereas for the other enzymes, rates were only 17–27% of the original value. Significant incorporation of dTMP occurred for pol B in the presence of dCTP or dATP.

Utilization of natural template primers. All of the diatom's polymerases had an absolute requirement for a DNA template primer in the reaction (Table 3). Activated salmon spermatozoan DNA produced

Table 2. *Effect of deoxyribonucleoside triphosphate composition of the reaction mixture on DNA polymerase activities*

All deoxyribonucleotides were present at 0.1 mM each. The 100% activities were those obtained in the presence of all four deoxyribonucleotide triphosphate species, and were 186, 450, 393 and 156 nmol of labelled nucleotide incorporated/h per mg of protein for pol A, B, C and D respectively. Each polymerase was present at about 1 µg/assay.

Nucleotides	DNA polymerase activities (%)			
	A	B	C	D
Complete mixture	100	100	100	100
-dCTP	20	15.5	23	20
-dGTP	37	17	20	27
-dATP	15.5	16	12.5	15
-dATP and dGTP	11.5	24	10.5	13
-dATP and dCTP	9.5	4	4.5	5
-dGTP and dCTP	3.5	14	4	5
-dATP, dGTP and dCTP	1	1	1	1

Table 3. *Activities of the DNA polymerases on various DNA template primers*

All assays were performed at optimum pH and univalent cation concentrations under standard conditions; 100% activities are given by 109, 210, 195 and 78 nmol/h per mg of protein for pol A, B, C and D respectively.

DNA template	DNA polymerase activities (%)			
	A	B	C	D
Activated salmon spermatozoan	100	100	100	100
Native calf thymus	1	3	2	1
Denatured calf thymus	54	66	54	57
Native salmon spermatozoan	15	13	16	14
Denatured salmon spermatozoan	55	77	87	71

maximum activity for all four enzymes. Native salmon spermatozoan DNA was a more effective substrate than was native calf thymus DNA, probably because of the presence of more 'nicks', although both supported the polymerization reaction rather poorly. Heat-denatured DNA was also an effective template primer for all activities, supporting the incorporation of deoxyribonucleoside triphosphates at 55–87% of the rate of activated DNA. The relatively high activity with heat-denatured DNA suggests that all four polymerase activities can efficiently utilize primers that are adjacent to long single-stranded template regions.

Utilization of synthetic template primers. The ability of the diatom's polymerases to utilize the synthetic template primers was examined at a single concentration under standard conditions with the addition of 0.5 mM-Mn²⁺ or -Mg²⁺ at their optimum concentrations. Mg²⁺ was the preferred bivalent cation in the polymerization reaction except when the polyribonucleotide template, poly(A)·oligo[d(T)₁₀], was used (Table 4). When Mg²⁺ was used, all of the polymerases were able to incorporate [*Me*-³H]TMP on to poly[d(A-T)] at about the same rate as that in the presence of activated DNA, although it was still not as effective as activated DNA if one considers the total deoxyribonucleotide residues incorporated. All the polymerases used poly[d(A)]·oligo[d(T)₁₀] poorly; however, pols B, C and D were 3–7-fold more efficient than pol A with this template primer. No incorporation of deoxyribonucleotides on poly(A)·oligo[d(T)₁₀] was detected for pols A, B and C, whereas limited incorporation was observed for pol D in the presence of Mn²⁺.

Other studies. In the early stages of the purification of the DNA polymerases, 2 mM-ATP was required for maximum incorporation of [*Me*-³H]TMP. However, after the DNA-cellulose step, ATP did not enhance incorporation. Spermine (0.1–5 mM) was also tested for possible stimulatory effects. Pol D

Table 4. *Activities of DNA polymerases on various synthetic template primers*

All assays contained 5 µg of template primer in a total assay volume of 75 µl and were performed at 25°C. The conditions of the assay were: pol A, 2.5 mM-Mg²⁺ and 0.5 or 3.5 mM-Mn²⁺; pol B, 7.5 mM-Mg²⁺, 0.5 and 5.5 mM-Mn²⁺; pol C, 55 mM-Mg²⁺, 0.5 and 6 mM-Mn²⁺; pol D, 2.5 mM-Mg²⁺ and 0.5 mM-Mn²⁺. Since negligible activity was evident with the artificial template primers at the higher Mn²⁺ concentration, only the values obtained at 0.5 mM-Mn²⁺ are shown. Activities of 100% were obtained with activated DNA and Mg²⁺; they were 103, 168, 110 and 65 nmol/h per mg of protein for pol A, B, C and D respectively.

Template primer	Polymerase activities (% of activated DNA)							
	Pol A		Pol B		Pol C		Pol D	
	Mg ²⁺	Mn ²⁺	Mg ²⁺	Mn ²⁺	Mg ²⁺	Mn ²⁺	Mg ²⁺	Mn ²⁺
Activated salmon spermatozoan DNA	100	6	100	8	100	9	100	17
Poly[d(A-T)]	123	27	77	22	116	28	85	8
Poly[d(A)]·oligo[d(T) ₁₀]	5	3	16	10	36	23	15	5
Poly(A)·oligo[d(T) ₁₀]	0	0	0	0	0	0	2	13

Fig 7. DEAE-Sephadex separation of DNA polymerases from cell extracts at different growth stages

Polymerase activities were obtained from 4×10^{10} cells and resolved on a column (1 cm $\times$ 7 cm) of DEAE-Sephadex which was eluted with an 80 ml 0.10–0.35 M-NaCl gradient; 1 ml fractions were collected. (a) Exponential phase; (b) late-exponential phase; (c) late-stationary phase. ●, DNA polymerase activity; — NaCl gradient.

increased its incorporation rate by about 43 % in the presence of 2 mM-spermine; the other polymerases were not affected.

DNA polymerases in exponential-, late-exponential- and stationary-phase cells. In an attempt to elucidate the function of the DNA polymerases, their relative activities during three growth states were assayed, by using the stationary culture as the basis of comparison. The total polymerase activity per cell of the extramitochondrial supernatants increased 2.4-fold in exponentially growing cells and 1.7-fold in late-exponential-phase cells over that observed in stationary-phase cells.

The elution profiles from the DEAE-Sephadex column show that changes in pols A and D were far greater during exponential growth than during the late-exponential phase (Fig. 7): the increases in pol A were 2.3- and 1.6-fold in the exponential and late-exponential phases respectively, whereas increases in pol D were 2.0 and 1.2. Pol B and the chloroplast enzyme, pol C, were less affected by the growth state, rising only 1.2–1.4-fold.

Cells from the late stationary phase exhibited at least one unresolved activity which was eluted between

pol A and B (Fig. 7c). This peak may be due to an increase in the synthesis of the mitochondrial polymerase (Okita & Volcani, 1977). The number of mitochondria rises during silicon starvation of *C. fusiformis* (Lewin *et al.*, 1966), which would produce a corresponding rise in the amount of polymerase activity. On the other hand, this activity may be a product of proteinase and/or nuclease activity which might be induced as the culture ages (McLennan & Keir, 1975d).

Discussion

An extramitochondrial supernatant was used as the starting material to minimize the possible interference of the mitochondrial DNA polymerase. Since at least 90 % of the chloroplasts are damaged by pressure-cell disruption of the cells, it is likely that one of the DNA polymerases resolved by DEAE-Sephadex was originally located in the chloroplast. An important aspect of this purification protocol was the use of poly(ethylene glycol) 6000 extraction and nuclease treatment, which not only removes some of the nucleic acid but also precipitates almost 99 % of the lipid from the cell extract. Lipid appears to interfere with the binding of the enzymes to the DNA-cellulose column. Equally important was the use of pancreatic DNAase and RNAase to remove any possible nucleic acid, which interferes with the resolution of the enzymes on DNA-cellulose and on DEAE-Sephadex. Loading the cell extract on to the ion-exchange column before nuclease treatment caused the enzymes to smear when eluted from the column. The high yield of activity in fractions III and V (Table 1) is probably in part due to the removal of nucleic acid and lipid, and in part to the removal of phosphatases, which also produces interference as shown by the lack of ATP stimulation during the later stages of the purification protocol (Keller *et al.*, 1973). The specific activities of 140, 246, 228 and 86 units/mg of protein for pols A, B, C and D respectively are at least an order of magnitude lower than those of comparable enzymes from yeast (Wintersberger, E., 1974), reflecting the fact that only about 10 % of the protein from each fraction is comprised of DNA polymerase activity. This is also reflected in our slab polyacrylamide-gel electrophoretic studies.

Pols A, B and D are found in high-speed supernatant fluids, which suggests that these are the enzymes responsible for nuclear DNA replication or repair. Pol C is the predominant enzymic activity residing in the chloroplasts (Okita & Volcani, 1977). The finding of three DNA polymerases of the diatom agrees with the number of activities found during balanced growth of *Paramecium* (Tait & Cummings, 1975); on the other hand, only two activities occur in *Euglena gracilis* (McLennan & Keir, 1975a) and in

yeasts (Wintersberger & Wintersberger, 1970; Helfman, 1973), and only one activity has been reported in the smut fungus *Ustilago maydis* (Banks *et al.*, 1976) and in *Tetrahymena* (Crerar & Pearlman, 1974). However, the presence of a third DNA activity in *Euglena* has been suggested (McLennan & Keir, 1975a). The differences in the number of DNA polymerases in these eukaryotic micro-organisms might be due to the fact that the enzymes were separated on DEAE-cellulose which resolves proteins rather poorly as compared with DEAE-Sephadex. For example, Schutz & Hall (1976) showed that in yeast cells only two RNA polymerase activities are resolved by DEAE-cellulose, whereas the activities are resolved into three well-separated peaks by DEAE-Sephadex.

The presence of four DNA polymerase activities that can be separated by ion-exchange chromatography does not necessarily imply that these proteins are distinct macromolecules, but may reflect the presence of unknown factors which alter both their properties and their chromatographic behaviour. Such a situation has been described for the single DNA polymerase in *Ustilago maydis* which, in the presence of a DNA-binding protein, increases its maximum velocity and affinity for trinucleotides (Yarranton *et al.*, 1976). On the other hand, the multiplicity of enzyme activities may be due to multiples of a single subunit (Holmes *et al.*, 1974; McLennan & Keir, 1975c).

The diatom's pols A, B and D are similar to the two nuclear DNA polymerases from yeasts (Wintersberger & Wintersberger, 1970; Helfman, 1973; Wintersberger, U., 1974), in *Euglena* (McLennan & Keir, 1975a,d) and *Paramecium* (Tait & Cummings, 1975) with regard to pH, template-primer utilization, susceptibility toward thiol-blocking agents, and affinity toward deoxyribonucleotides. The two polymerases from each of these organisms display almost identical enzymic properties, but in *Paramecium* a third polymerase activity has a less stringent Mg^{2+} requirement and higher K^+ optima. The two activities in *Chlamydomonas* are unusual in that they are widely disparate in their physical and catalytic properties (Ross & Harris, 1976). This similarity in catalytic properties (except for the *Chlamydomonas* polymerases) suggests again that pols A, B and D are of nuclear origin; pol C which resides in the chloroplasts differs markedly from the rest of the activities with respect to ion requirements, K_m and template-primer utilization.

A unique property displayed by all of the diatom's polymerases is their requirement for high amounts (≥ 100 mM) of univalent cations for maximum activity. Normally the polymerases from other protists require about 25–50 mM- K^+ , although the polymerase C from *Paramecium* and polymerase B from *Chlamydomonas* require optimum concentrations

of 100 mM and 150 mM respectively. These comparably high concentrations would not be surprising for the marine diatom, since it is normally bathed in about 0.5 M-salt and might have the same intracellular concentration as shown for the diatom *Coscinodiscus walsbyi* (Gutknecht & Dainty, 1968).

The diatom pol D shares some unusual properties in common with the pol B activity of *Euglena* (McLennan & Keir, 1975b) and yeast (Wintersberger, E., 1974). These enzymes have some activity on the synthetic template primers, poly[d(A)]-oligo[d(T)₁₀] and poly(A)-oligo[d(T)₁₀]. In addition, the *Euglena* and yeast pol B exhibits an exonuclease activity. This associated enzyme function has also been found with pol D from the diatom (Okita & Volcani, 1977).

Although silicate had no apparent effect on the diatom polymerases, it may still act as an effector of enzymic activity. The diatom is known to contain pools of silicon (Si) (Azam *et al.*, 1974) which may be released during cell disruption, thus allowing an interaction with the polymerase. The binding sites of the polymerase may be saturated with Si, making it difficult to detect an effect on polymerase with exogenous Si. Also no precautions were taken to avoid contamination of the polymerases by Si during the purification. The trace amount of Si that is leached from glassware may be sufficient to maintain enzyme activity. The interaction of Si with proteins *in vitro*, mainly by hydrogen-bonding or ionic interactions (Holt & Bowcott, 1954; Holt & Went, 1959) suggests such a possibility. Nor can the possibility that Si may be integrated with the polymerase during translation be ruled out; evidence that Si is covalently bonded to biological macromolecules has been reported (Schwarz, 1973).

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References

- Alberts, B. & Herrick, G. (1971) *Methods Enzymol.* **21**, 198–217
- Ames, G. F.-L. (1974) *J. Biol. Chem.* **249**, 634–644
- Azam, F., Hemmingsen, B. B. & Volcani, B. E. (1974) *Arch. Microbiol.* **97**, 103–114
- Banks, G. R., Holloman, W. K., Kairis, M. V., Spanos, A. & Yarranton, G. T. (1976) *Eur. J. Biochem.* **62**, 131–142
- Chang, L. M. S. & Bollum, F. J. (1972) *J. Biol. Chem.* **247**, 7948–7955
- Chang, L. M. S., Brown, McK. & Bollum, F. J. (1973) *J. Mol. Biol.* **74**, 1–8
- Crerar, M. & Pearlman, R. E. (1974) *J. Biol. Chem.* **249**, 3999–4005
- Darley, M. & Volcani, B. E. (1969) *Exp. Cell Res.* **58**, 334–342
- Golding, E. S., Grossman, L.-J., Kupnick, D., Cryer, D. R. & Marmur, J. (1970) *J. Mol. Biol.* **52**, 323–335

- Gutknocht, J. & Dainty, J. (1968) *Oceanogr. Mar. Biol. Annu. Rev.* **6**, 163-200
- Helfman, W. B. (1973) *Eur. J. Biochem.* **32**, 42-50
- Holmes, A. M., Hesslewood, I. P. & Johnston, I. R. (1974) *Eur. J. Biochem.* **43**, 487-499
- Holt, P. F. & Bowcott, J. E. L. (1954) *Biochem. J.* **57**, 471-475
- Holt, P. F. & Went, C. W. (1959) *J. Chem. Soc., Faraday Trans. Soc.* **5**, 1435-1440
- Keller, S. J., Beidenbach, S. A. & Meyer, R. R. (1973) *Biochem. Biophys. Res. Commun.* **50**, 620-628
- Kornberg, A. (1974) *DNA Synthesis*, W. H. Freeman, San Francisco
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685
- Lehman, I. R. & Uyemura, D. G. (1976) *Science* **193**, 963-969
- Lewin, J. C., Reimann, B. E., Busby, W. F. & Volcani, B. E. (1966) in *Cell Synchrony Studies in Biosynthetic Regulation* (Cameron, I. L. & Padilla, G. M., eds.), pp. 169-188, Academic Press, New York
- Loeb, L. A. (1969) *J. Biol. Chem.* **244**, 1672-1681
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- McLennan, A. G. & Keir, H. M. (1975a) *Biochem. J.* **151**, 227-238
- McLennan, A. G. & Keir, H. M. (1975b) *Biochem. J.* **151**, 239-247
- McLennan, A. G. & Keir, H. M. (1975c) *Nucleic Acid Res.* **2**, 223-237
- McLennan, A. G. & Keir, H. M. (1975d) *Biochim. Biophys. Acta* **407**, 253-262
- Okita, T. W. & Volcani, B. E. (1977) *Biochem. J.* **167**, 611-619
- Ross, C. A. & Harris, W. J. (1976) *Biochem. Soc. Trans.* **4**, 806-807
- Schutz, L. D. & Hall, B. D. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1029-1033
- Schwarz, K. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1608-1612
- Slater, J. P., Mildvan, A. S. & Loeb, L. A. (1971) *Biochem. Biophys. Res. Commun.* **44**, 37-43
- Sullivan, C. W. & Volcani, B. E. (1973) *Biochim. Biophys. Acta* **308**, 212-229
- Sullivan, C. W. & Volcani, B. E. (1976) *Exp. Cell Res.* **98**, 23-30
- Sung, S. C. (1974) *Biochim. Biophys. Acta* **361**, 115-117
- Tait, A. & Cummings, D. J. (1975) *Biochim. Biophys. Acta* **378**, 282-295
- Tait, R. C. & Smith, D. W. (1974) *Nature (London)* **249**, 116-119
- Tait, R. C., Harris, A. L. & Smith, D. W. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 675-679
- Warburg, O. & Christian, W. (1941) *Biochem. Z.* **310**, 384-421
- Weissbach, A. (1975) *Cell* **5**, 101-108
- Wintersberger, E. (1974) *Eur. J. Biochem.* **50**, 41-47
- Wintersberger, U. (1974) *Eur. J. Biochem.* **50**, 197-202
- Wintersberger, U. & Wintersberger, E. (1970) *Eur. J. Biochem.* **13**, 11-19
- Yarranton, G. T., Moore, P. D. & Spanos, A. (1976) *Mol. Gen. Genet.* **145**, 215-218