DNA Polymerases from *Chlamydomonas reinhardtii*

PURIFICATION AND PROPERTIES

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(Received 1 September 1977)

Three DNA polymerase activities, A, B and C, were identified in extracts of exponentially growing synchronous cultures of *Chlamydomonas reinhardtii*, and DNA polymerases A and B were characterized in detail. Both enzymes have the same binding affinity for DEAE-cellulose at pH 7.8, but can be distinguished from each other by their behaviour on phosphocellulose and DNA-agarose. 'Activated' calf thymus DNA was used as template, and the pH, K⁺ and bivalent-cation optima were measured. DNA polymerase A sediments at 5.3S in glycerol gradients, with an apparent mol.wt. of 90000–100000. Polymerase B sediments between 8S and 10S in 100mM-KCl, the predominant species having an apparent mol.wt. of 200000. In 200mM-KCl, polymerase B dissociates to a single species, which sediments at 5.8S. A 3S species was found in aged preparations of both enzymes. The activity of polymerase B from cells harvested during nuclear DNA synthesis is twice that found in *Chlamydomonas* at other times during the cell cycle.

Three DNA-dependent DNA polymerases (DNA nucleotidyltransferase, EC 2.7.7.7) have been isolated from *Escherichia coli*, and their precise roles in bacterial DNA replication and repair are becoming apparent (Kornberg, 1974; Gefter, 1975; Schekman *et al.*, 1975; Wickner & Hurwitz, 1976). The measured reaction properties of the purified enzymes, and the comparison of DNA metabolism in wild-type cells and in mutant bacteria lacking individual DNA polymerases has made possible the assignment of separate physiological functions to each enzyme. In addition, most physical and chemical properties of DNA polymerases purified from other bacterial species are remarkably similar to their *E. coli* counterparts (Kornberg & Kornberg, 1974). Similarly, mammalian and other higher eukaryotic species seem to contain a characteristic complement of DNA polymerases (Craig & Keir, 1974; Bollum, 1975; Holmes & Johnston, 1975; Weissbach, 1975; Weissbach *et al.*, 1975). There are three successfully distinguished DNA polymerases apparently concerned with replication and repair of the eukaryotic nuclear genome. DNA polymerases- α have been characterized as enzymes of high molecular weight (greater than 100000) whose activity has been shown to correlate with the cellular DNA replication rate (Chang *et al.*, 1973; Spadari & Weissbach, 1974; Craig *et al.*, 1975). The pre-

dominantly nuclear low-molecular-weight (less than 50000) DNA polymerases- β , however, show no such correlation. The activities of DNA polymerases- γ from HeLa cells have been observed to rise during the time of nuclear DNA synthesis (Spadari & Weissbach, 1974), but this species accounts for only a small part of the total cellular DNA polymerase activity. That little is presently known of the roles of these enzymes *in vivo* is unremarkable since mutant species of higher eukaryotes cannot be made readily available, as in bacterial species.

For this reason, it is profitable to characterize the enzymes of DNA metabolism from unicellular eukaryotes whose short life-cycles can be experimentally controlled. In addition, the production of conditional lethal mutants in DNA synthesis should be possible in these species. As yet, the DNA polymerases have been characterized from few unicellular eukaryotes, including yeast (Wintersberger, 1975), *Euglena gracilis* (McLennan & Keir, 1975a,b), *Tetrahymena pyriformis* (Crerar & Pearlman, 1974) and the smut fungus *Ustilago maydis* (Banks *et al.*, 1976). It is clear, however, that the major DNA polymerase species from these organisms are of high and heterogeneous molecular weight, and some have associated deoxyribonuclease (DNAase) activity. These enzymes therefore do not exactly correspond to the major DNA polymerases of either bacterial or mammalian cells.

We chose to study the DNA polymerase species of the unicellular green alga *Chlamydomonas reinhardtii*,

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an organism that can be grown synchronously in liquid culture. The vegetative and sexual cycles have been well characterized (Sueoka *et al.*, 1967; Johnson & Porter, 1968; Chang, 1976). *Chlamydomonas* has also been used to investigate the nature of cytoplasmic inheritance in eukaryotes (Gillham, 1974; Sager, 1974). In this and the following paper (Ross & Harris, 1978), we describe the purification and properties of the two predominant DNA polymerase activities from *Chlamydomonas* whole-cell extracts, which have been designated DNA polymerases A and B. The preliminary findings of this investigation have been published (Ross & Harris, 1976).

Experimental

Materials

Wild-type *Chlamydomonas reinhardtii*, mating type + (11/32c) and mating type - (11/32d), were from the Cambridge Culture Collection, Cambridge, U.K. Except for the items mentioned below, all reagents were of AnalaR grade from BDH Chemicals, Poole, Dorset, U.K. Calf thymus DNA (type V), bovine serum albumin, bovine pancreatic deoxyribonuclease I (DN-EP), bovine liver catalase, bovine haemoglobin, Trizma base and Trizma hydrochloride were all from Sigma (London) Chemical Co., Kingston upon Thames KT2 7BH, U.K. dATP, dCTP, dGTP and dTTP were from P-L Biochemicals, Milwaukee, WI, U.S.A. Pig heart lactate dehydrogenase was from the Boehringer Corporation (London), Ealing, London W5 2TZ, U.K. Agarose (for electrophoresis), used in the preparation of DNA-agarose, was supplied by International Enzymes, Windsor, Berks., U.K., and manufactured by Litex, Aarhus, Denmark. DEAE-cellulose (DE-23 and DE-52) and phosphocellulose P11 (Whatman brand) were from Reeve Angel Scientific, London ME14 2L2, U.K., as was the Whatman GF-83 paper from which 2.5 cm glass-fibre discs were cut. Acrylamide, *NN*'-methylenebisacrylamide and Bromophenol Blue were from Eastman (Kodak) Organic Chemicals, Rochester, NY, U.S.A. [*Me*-³H]dTTP (ammonium salt; 30 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Growth and harvesting of cells

Chlamydomonas cells were grown in liquid culture in 0.3 M high-salt medium (Sueoka, 1960). Glass columns holding approx. 2 litres of culture were aerated from below with sterile air/CO₂ (18:1). The surrounding water jacket was maintained at 21°C. Cells were subjected to an alternate cycle of 12 h of light and 12 h of dark, the measured illumination being 3000 lux. Under these conditions, cultures grew exponentially up to a density of just above 5×10^6 cells/ml. Starting material for enzyme preparations was obtained by harvesting cells at densities of 1×10^6 –

2.5×10^6 cells/ml at the required time during the synchronous cell cycle. In each 24 h period, the cells entered S-phase synchronously 15 h from the beginning of the light period (Tait & Harris, 1977). Cells were harvested by centrifugation for 10 min at 2000 *g*_{av.} at 4°C, washed with cold 150 mM-NaCl/100 mM-EDTA, pH 7.0, containing 2 mM-2-mercaptoethanol, and stored at -20°C until required.

Preparation of whole-cell extracts

All buffers used in the preparation of enzymes contained 2 mM-2-mercaptoethanol, 0.1 mM-EDTA and 20% (v/v) glycerol (MEG buffer). All stages of the preparation were carried out at 4°C. Freshly harvested cells were rarely used, since these required longer periods of sonication than previously frozen ones, to produce an acceptable yield of broken cells. Thawed cells were suspended at 2×10^8 cells/ml in an extraction buffer of 20 mM-sodium phosphate, pH 6.5, containing 1% (v/v) Triton X-100. The suspension was decanted into a beaker and sonicated in a NaCl/ice/water bath with a Dawe Soniprobe type 1130A (Dawe Instruments, London W.3, U.K.). The total sonication period was 5 min, the cells being subjected to full power for 30 s at a time. Under these conditions, greater than 95% cell breakage was obtained and this was checked with a microscope. The extract was made 0.6 M with respect to KCl by the addition of solid KCl and stirred for 15 min on ice before centrifugation for 1 h at 38000 *g*_{av.} at 4°C. The pellet, consisting of a white lower layer of starch overlaid with a green layer of cell debris, was washed once with extraction buffer containing 0.6 M-KCl.

Assay for DNA polymerase activity

The assay measured the incorporation of mononucleotidyl residues from [*Me*-³H]dTTP into an acid-insoluble product. The template consisted of calf thymus DNA activated by a modification of the method of Loeb (1969).

DNA polymerase A system 1. This contained the following components in a total volume of 0.125 ml: 18 µg of 'activated' DNA, 50 µM each of dATP, dCTP, dGTP, 50 µM-[*Me*-³H]dTTP (50 µCi/µmol), 50 mM-Tris/HCl, pH 8.5, 50 mM-KCl, 2 mM-2-mercaptoethanol, 4% (v/v) glycerol, 2 mM-MgCl₂ and enzyme protein. DNA polymerase B system 1 was identical except that the Tris/HCl buffer was at pH 7.5 and the KCl concentration was raised to 150 mM.

The assay mixtures were incubated for 1 h at 32°C, then chilled on ice, made 1.5 mg/ml with respect to acetylated bovine serum albumin and 0.2 ml of 10% (w/v) trichloroacetic acid/20 mM-tetrasodium pyrophosphate was added to each tube. After at least 20 min at 4°C, the contents of each assay were collected under suction on Whatman GF/C filter discs. The assay tubes were rinsed with 2 × 1 ml portions of 5% (w/v) trichloroacetic acid/20 mM-

tetrasodium pyrophosphate, followed by 5 ml of 0.5M-HCl and ethanol. Discs were then dried and counted for radioactivity in 2 ml of non-aqueous scintillation fluid [0.5% (w/v) 2,5-diphenyloxazole and 0.03% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in toluene] in a Packard Tri-Carb model 3385 liquid-scintillation spectrometer.

Assay system 2. This was used to monitor column-chromatographic fractions, and contained, in an assay volume of 0.125 ml: 18 μ g of 'activated' calf thymus DNA, 50 mM-Tris/HCl, pH 8.5, 100 mM-KCl, 2 mM-2-mercaptoethanol, 2 mM-MgCl₂, 4% (v/v) glycerol and enzyme protein.

Column chromatography

Ion-exchange chromatography. DEAE-cellulose DE-23 and DE-52 were prepared as directed in the Whatman Information Leaflet IL2. Phosphocellulose (P11) was precycled by immersion in 0.1M-HCl for 5 min and washing under suction with water until the eluate reached pH 4.0, followed by washing with 0.1M-NaOH/1 mM-EDTA for a maximum of 5 min and washing again with water until neutrality.

Affinity chromatography. Single-stranded-DNA-agarose columns were prepared by the method described by Schaller *et al.* (1972). The final DNA concentration in the gel was 2.5 mg/ml. Although no covalent linkage existed between the DNA and the agarose, the complex was stable and little DNA was released on being stored at 4°C in 10 mM-Tris/HCl, pH 7.8, containing 1 M-NaCl.

Polyacrylamide-gel electrophoresis and recovery of DNA polymerase from gels

The method was based on that of McLennan & Keir (1975a). The 4% and 7% separating gels were prepared essentially as described by Davis (1964), with the addition of 100 μ g of 'activated' DNA/ml to each gel. The gels were electrophoresed at a constant current of 2 mA/gel for 1 h at pH 8.9 to remove persulphate ions. After polymerization of stacking gels, the tubes were cooled to 4°C. Subsequently, the gels were removed and sliced lengthwise, one half being stained for protein, the other half being used for assay of DNA polymerase. After several washes in 50 mM-Tris / HCl (pH 8.5) / 2 mM-2-mercaptoethanol, the half gels were placed in a DNA-free DNA polymerase assay system 2, containing [³H]dTTP at 5 μ Ci/ml and 30 Ci/mmol. Incubation at 32°C was carried out for 1 h, and the gels were washed overnight with several 1-litre changes of 5% (w/v) trichloroacetic acid/20 mM-tetrasodium pyrophosphate. The gels were sliced (1.5 mm width), covered with 0.5 ml of Soluene-100, incubated at 50°C for 3 h, and 5 ml of scintillation fluid [toluene/Triton X-100 (2:1) containing 4 g of 2,5-diphenyloxazole and 0.05 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/

litre] was added. Samples were analysed after 24 h at 4°C in the dark.

Glycerol-density-gradient-sedimentation analysis

Glycerol gradients (10–30%; v/v) were prepared by layering successive 0.9 ml samples of 30, 25, 20, 15 and 10% glycerol in 10 mM-Tris/HCl (pH 7.8) / 2 mM-2-mercaptoethanol/0.1 mM-EDTA, containing 100, 200 or 500 mM-KCl as required. After at least 2 h, 250 μ l samples, containing lactate dehydrogenase as molecular-weight marker, were layered on the top of gradients, and centrifugation was performed for 17 h at 130 000 *g*_{av.} at 4°C in the Beckman Spinco L2 ultracentrifuge with the SW50L rotor. Fractions (0.15 ml) were collected by upward displacement of the gradient with 70% (w/v) sucrose containing Trypan Blue dye. Gradients prepared in this manner were linear with respect to glycerol concentration as determined by measurement of refractive index of alternate fractions. Fractions were assayed for DNA polymerase activity in assay system 2, and for lactate dehydrogenase by the Boehringer Test Combination lactate dehydrogenase assay kit.

Protein concentration was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Purification of DNA polymerases A and B

All stages of purification were carried out at 4°C as far as possible, and all buffers contained 2 mM-2-mercaptoethanol/0.1 mM-EDTA/20% glycerol (MEG buffer). The purification scheme is summarized in Table 1. Chromatographic fractions were collected as a routine in tubes containing acetylated bovine serum albumin. Cells (wet wt. 12 g) were harvested at mid-exponential phase of culture growth, after 3 h in the light of the synchronous cell cycle for the preparation of DNA polymerase A, and after 3 h in the dark for the preparation of DNA polymerase B. The latter is the time of nuclear DNA synthesis (Tait & Harris, 1977). Whole-cell extracts (fraction I) were prepared as described in the Experimental section, and the supernatants made 0.3 M with respect to KCl (fraction II). These were passed through a column (3.2 cm $\times$ 10.4 cm) of DEAE-cellulose DE-23 equilibrated in MEG/20 mM-potassium phosphate buffer, pH 6.5 (fraction III).

Fraction (III) was loaded on to a phosphocellulose column (3.2 cm $\times$ 19.5 cm for DNA polymerase A; 3.2 cm $\times$ 28 cm for DNA polymerase B) equilibrated in MEG/0.15 M-potassium phosphate buffer, pH 6.5. The flow rate was 20 ml/h and 7.5 ml fractions were collected. Fractions containing unbound material were pooled, dialysed overnight against 2 $\times$ 2 litres of MEG/10 mM-Tris/HCl buffer, pH 7.8, and served as the starting material for the preparation of DNA

Table 1. *Purification of DNA polymerases A and B from C. reinhardtii*

A unit of enzyme is the amount that converts 1 nmol of dTMP residues into acid-insoluble product under the conditions of assay system 1 in 1 h.

Fraction	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)
DNA polymerase A				
(I) Crude homogenate	35	674	5.65	—
(II) Supernatant	102	597	10.0	—
(III) DEAE-cellulose	100	593	38.9	—
(IV) Phosphocellulose	80	315	68	0.22
(V) DNA-agarose	42	0.63	144	228
(VI) DEAE-cellulose	16	0.41	112	272
DNA polymerase B				
(I) Crude homogenate	50	880	—	—
(II) Supernatant	150	782	7.1	—
(III) DEAE-cellulose	125	632	7.6	—
(IV) Phosphocellulose	85	130	475	3.6
(V) DNA-agarose	60	27	97	3.6
(VI) DEAE-cellulose	15	0.55	77	140

Fig. 1. *Column chromatography of DNA polymerases on DNA-agarose*

Fractions (IVa) from DNA polymerase A (a) and DNA polymerase B (b) preparations were eluted with a gradient of 0.1–1.0M-NaCl. The total yield of DNA polymerase activity from (a) and (b) were 228% and 68% respectively.

●, DNA polymerase activity; ----, A_{280} ; —, NaCl gradient.

polymerase A (fraction IVa). The majority of the DNA polymerase activity was subsequently eluted from the phosphocellulose with MEG/0.5M-phosphate buffer, pH 6.5. Fractions (7.5 ml) were pooled and dialysed against 100mM-Tris/HCl(pH 7.8)/100mM-NaCl/MEG buffer. This resulted in a doubling of enzyme activity due to removal of inhibitory phosphate ions. This material served as the source of DNA polymerase B (fraction IVb).

Fractions (IVa) and (IVb) were separately applied to DNA-agarose columns (1.5cm×25cm; bed vol.

25ml) previously equilibrated in MEG/10mM-Tris/HCl, pH 7.8, containing 100mM-NaCl. The columns were washed with 200ml of equilibration buffer (flow rate 25 ml/h) and 7.5 ml fractions collected. A gradient (300 ml) of 100mM–1.0M-NaCl in 10 mM-Tris/HCl, pH 7.8, was then applied and 5 ml fractions were collected. Figs. 1(a) and 1(b) describe the respective elution profiles of DNA polymerases A and B. The unbound activity in the DNA polymerase A preparation (Fig. 1a) was not further investigated. Fractions eluted between 0.3 and 0.4M-NaCl were dialysed

extensively against MEG/50mM-Tris/HCl buffer, pH 7.8 (fraction Va), or dialysed against this buffer containing 50% glycerol and stored at -20°C . Two peaks of activity are revealed from the DNA polymerase B preparation (Fig. 1b). The unbound activity served as the source of DNA polymerase B, and was dialysed against MEG/50mM-Tris/HCl, pH 7.8 (fraction Vb), and that eluted by the NaCl gradient was designated DNA polymerase C.

Fractions (Va) and (Vb) were separately applied to DEAE-cellulose and Fig. 2 shows that a single peak of DNA polymerase activity was recovered for both enzyme preparations. The KCl concentrations at which polymerases A and B were eluted were only marginally different, reflecting the initial difficulties in resolving polymerase activities from cell extracts on this exchanger. Enzyme activity after DEAE-cellulose separation proved somewhat unstable and assay times were decreased to 15 min. Active fractions were stored at -50°C (fractions VIa and VIb).

Stability of purified enzymes

DNA polymerases A and B, stored in 20% (v/v) glycerol at -50°C retained full activity for more than 9 months. The enzymes were free from proteolysis during that time as judged by glycerol-gradient centrifugation of aged preparations. At 0°C , the activity of DNA polymerase A could be stabilized by increasing the glycerol concentration to 50%.

Under these conditions, however, the activity of DNA polymerase B was decreased by more than 50% overnight.

Purity of fractions (VIa) and (VIb)

The purification scheme is outlined in Table 1. On 4% polyacrylamide gels, DNA polymerase A was recovered as a single peak of material that had migrated at a rate of 40% that of acetylated bovine serum albumin. No band other than bovine serum albumin was detected on half-gels stained for protein. Analysis of DNA polymerase B preparations on 7% polyacrylamide gel gave rise to only one protein-staining band in addition to acetylated bovine serum albumin and this coincided with the recovered DNA polymerase activity. It is possible that further protein migrated to the same position as the acetylated bovine serum albumin.

General properties and optimum assay conditions

Reaction rate was linear with respect to protein concentration up to $1.0\mu\text{g}$ of DNA polymerase A and $1.2\mu\text{g}$ of DNA polymerase B under standard assay conditions, although the amount of enzyme protein present in assays was usually considerably less than this. The optimum temperature for both enzymes was 32°C . DNA polymerase A was more sensitive to higher temperatures. At 39°C for 1 h, DNA polymerase A had only 5% of optimum activity compared with 70% for DNA polymerase B. At

Fig. 2. DEAE-cellulose chromatography of DNA polymerases A and B

Fractions (V) of the DNA polymerase A and B preparations were separately applied to a column ($0.9\text{cm} \times 14.5\text{cm}$) of DEAE-cellulose (DE-52) (bed vol. 6 ml) equilibrated with MEG/50mM-Tris/HCl buffer, pH 7.8, and washed overnight with the same buffer. A gradient (0–400 mM) of KCl was applied, the flow rate was 20 ml/h and 2 ml fractions were collected. ●, Polymerase A; ○, polymerase B.

32°C, DNA polymerase B activity was linear with time for 60 min and DNA polymerase A linear for 2 h. The substrate was completely incorporated into DNA since subsequent incubation of assays with pancreatic deoxyribonuclease (but not ribonuclease or Pronase) rendered all the acid-precipitable radio-activity acid-soluble (results not presented).

pH optima

Potassium phosphate buffer (50 mM) was used over the pH range 5.8–7.2, Tris/HCl buffer (50 mM) over the range 7.0–9.0, and glycine/NaOH (50 mM) over

the range 8.2–9.2. Both enzymes had much greater activity when assayed in Tris/HCl compared with other buffers of the same ionic strength. DNA polymerase A had an alkaline pH optimum of 8.5, whereas DNA polymerase B was most active at pH 7.5. In neither case, however, did the change of pH produce any drastic alteration of activity over the ranges measured.

Effects of bivalent and univalent cations

Neither enzyme was active in the absence of bivalent metal cations, but the response of DNA

Fig. 3. Effect of bivalent cations on DNA polymerases A and B

The effect of Mg²⁺ and Mn²⁺ on DNA polymerase A (a and b) and DNA polymerase B (c and d) is expressed as a percentage of total activity under standard conditions with 2 mM-Mg²⁺. A 100% activity is 240 and 330 nmol of dTMP residues incorporated/mg of protein for DNA polymerases A and B respectively.

polymerases A and B to these cofactors was different (Fig. 3). Both enzymes had maximum activity with Mg^{2+} ions. DNA polymerase B had little activity with Mn^{2+} , and in some cases no activity was detected. The reason for this is not known. Ca^{2+} and Zn^{2+} at 2mM were unable to support the incorporation of dTMP residues, but both enzymes were active to some extent in 2mM- Fe^{2+} ions. DNA polymerase A retained 95% of activity in Fe^{2+} whereas DNA polymerase B was only 20% as active in Fe^{2+} as in Mg^{2+} .

Fig. 4 shows that the activity of DNA polymerase A was relatively unaffected by K^+ ions until the concentration exceeded 100mM, whereas DNA polymerase B was strongly activated by K^+ ions, showing maximum activity at 100–200mM- K^+ .

Molecular weights

In 10–30% (v/v) glycerol gradients in 100mM-KCl, DNA polymerase A sedimented as one symmetrical peak (Fig. 5a) of sedimentation coefficient 5.3S, which corresponds to an apparent mol.wt. of 90000, calculated by the method of Martin & Ames (1961). This species was stable on storage at $-50^{\circ}C$ for up to 6 months, but older preparations showed the presence of a DNA polymerase activity that sedimented at 3S (Fig. 5b). Increasing the KCl concentration within the glycerol gradients to 500mM had no effect on the sedimentation profile (Fig. 5c). Freshly prepared DNA polymerase B on the other hand produced a less uniform profile with peaks of activity sedimenting at 7.9S, 8.9S and 10S (Fig. 6a), corresponding to apparent mol.wts. of 160000, 210000 and 245000 respectively. On storage, the peak of activity sedimenting at 8.9S became predominant, with a minor peak at 3S (Fig. 6b). Increasing the KCl concentration within gradients to 200mM caused the 8.9S activity to sediment at 5.8S (Fig. 6c),

Fig. 4. Effect of KCl on DNA polymerases A and B. Assay conditions for the two enzymes were as described in the Experimental section, with 2mM- Mg^{2+} in addition to various KCl concentration. ●, DNA polymerase A; ○, DNA polymerase B.

Fig. 5. Glycerol-gradient sedimentation of DNA polymerase A.

Fraction (VI) of DNA polymerase A preparation was analysed in the presence of lactate dehydrogenase as marker. (a) Freshly prepared enzyme; (b) enzyme after storage at $-50^{\circ}C$ for 7 months; (c) freshly prepared enzyme sedimented in the presence of 500mM-KCl. Sedimentation was from left to right. ----, Lactate dehydrogenase activity; ●, DNA polymerase activity.

the major sedimenting species thus changing from 210000 to 107000. No further dissociation of the enzyme occurred in higher ionic strengths (Fig. 6d). It should be noted that the 'dissociated' form of DNA polymerase B (5.8S) does not have the same sedimentation coefficient as DNA polymerase A (5.3S).

DNA polymerase C

The DNA polymerase activity recovered by elution with NaCl from the DNA-agarose column of DNA polymerase B preparations (Fig. 1b) was subsequently also purified on DEAE-cellulose. This third enzyme, DNA polymerase C, was very similar to DNA polymerase B, having the same molecular-weight distribution in glycerol gradients, similar responses to KCl and similar inhibition characteristics. DNA polymerases B and C differ, however, in their heat-denaturation characteristics and may show slight variation in utilization of synthetic primer-template homopolymers (Ross & Harris, 1978).

Occurrence of enzymes throughout the growth cycle

Table 2 summarizes the activities of fraction (V) of each preparation at different phases in the vegetative life cycle. For wild-type cells, DNA poly-

merases B and C always accounted for the greater part of the total activity. The total DNA polymerase activity/ 10^{10} cells at the period of nuclear DNA synthesis (15 h from beginning of light period) was almost twice that of cells harvested earlier in the cell cycle (compare line 1 with line 2 in Table 2). At this time, the specific activity of DNA polymerase B was doubled. Of several u.v.-sensitive mutants examined in this laboratory, the strain *uvs-4* had been observed to be deficient in dark repair DNA synthesis (J. M. Cameron, unpublished work). The relative activities of the three DNA polymerases in these u.v.-sensitive cells showed no difference from wild-type.

Discussion

Three DNA polymerases, A, B and C, have been isolated from whole-cell extracts of the green alga *Chlamydomonas reinhardtii*. Sonication was chosen as the method of cell breakage since this consistently produced a high yield of broken cells. Under these conditions, however, few intracellular organelles remained intact. Attempts to obtain organelles from extracts produced by gentler methods of cell breakage were unsuccessful. Low values for total DNA polymerase activity in crude extracts of *Chlamydomonas* were measured as a result of the high deoxyribonuclease activity in this species (Schonherr & Keir, 1972). This prevented any realistic measurement of the yield of DNA polymerase species. The specific activities of the most purified fractions of DNA polymerases A and B were 272 and 140 units/mg of protein respectively. These values were lower than those generally reported for higher eukaryotic enzymes, but comparable with those quoted for higher-molecular-weight DNA polymerases isolated from lower eukaryotes *Euglena gracilis* (McLennan & Keir, 1975a), *Paramecium macronuclei* (Tait & Cummings, 1975) and *Ustilago maydis* (Banks *et al.*, 1976).

The behaviour of the DNA polymerases A and B on DEAE-cellulose showed them to be very alike in overall charge, but they behaved differently from each other on the matrices of phosphocellulose and

Fig. 6. Glycerol-gradient sedimentation of DNA polymerase B

Fraction (VI) of DNA polymerase B preparation was analysed in the presence of lactate dehydrogenase as marker. (a) Freshly prepared enzyme in 100 mM-KCl; (b) enzyme after storage at -50°C for 10 weeks in 100 mM-KCl; (c) freshly prepared enzyme sedimented in the presence of 200 mM-KCl; (d) freshly prepared enzyme sedimented in the presence of 500 mM-KCl. ----, Lactate dehydrogenase activity; ●, DNA polymerase activity.

Table 2. Concentrations of DNA polymerases A, B and C during the vegetative growth cycle

Fraction (V) preparations were prepared at various times during the vegetative cell cycle. A unit of enzyme activity is the amount of enzyme that catalyses the incorporation of 1 nmol of dTMP residues into acid-insoluble product under the standard conditions of assay system 1 in 1 h.

Strain	Cell concentration at harvest (no./ml)	Time after beginning of light period (h)	Total DNA polymerase activity (units/ 10^{10} cells)	Percentage of total activity		
				A	B	C
WT	2×10^6	15	425	10	72	18
WT	5×10^6	3	232	25	62	13
<i>uvs-4</i>	5×10^6	3	193	21	59	20

DNA-agarose, where separation is based on the architecture of the catalytically functional parts of the molecule. They differed markedly in their response to univalent cations (Fig. 4), though both were remarkable in that they retained 50% of maximum activity in the presence of 300mM-KCl. DNA polymerases generally are not noted for their tolerance to salt in enzyme assays. In particular, *E. coli* DNA polymerase III and mammalian DNA polymerases- α are both markedly inhibited at univalent concentrations greater than 50mM. The yeast mitochondrial DNA polymerase behaves similarly to DNA polymerase A, neither enzyme being much affected by 100mM univalent cation. At high salt concentrations, however, the yeast enzymes are substantially inhibited (Wintersberger & Wintersberger, 1970). Some DNA polymerases have an absolute requirement for Mn^{2+} ions (Stavrianopoulos *et al.*, 1972; Chandra *et al.*, 1975) or have similar activity with activated DNA in the presence of either Mg^{2+} or Mn^{2+} (Byrnes *et al.*, 1973; McLennan & Keir, 1975b). However, both DNA polymerases A and B were more active with Mg^{2+} ions than with Mn^{2+} when the template was activated DNA, though DNA polymerase A seemed able to utilize a wider range of bivalent metal ions, the activity being unaffected by the replacement of 2mM- Mg^{2+} by 2mM- Fe^{2+} .

All three DNA polymerases were of high molecular weight. No species comparable in size with the mammalian DNA polymerase- α was found. The absence of low-molecular-weight species from lower eukaryotic species has been noted before (Wintersberger, 1974; McLennan & Keir, 1975c), and a phylogenetic survey of the occurrence of DNA polymerase- β -type enzymes in eukaryotic systems suggests that DNA polymerase- β is present only in multicellular organisms (Chang, 1976). Aged preparations of both DNA polymerases A and B revealed low amounts of DNA-polymerizing activity that had a sedimentation coefficient of about 3.0S (Figs. 5b and 6b). An enzyme species at 3.0S has also been found in preparations of the two similar high-molecular-weight enzymes from *Euglena gracilis* (McLennan & Keir, 1975c). The relationship between these dissociation products and the 5S DNA polymerase A or 9S DNA polymerase B is not known, but it is possible that both high-molecular-weight enzymes may comprise of a number of 3S subunits.

KCl concentrations of 200mM and above appear to decrease the apparent molecular weight of DNA polymerase B from 200000 to 100000. In addition, the three peaks of polymerase B activity appearing in glycerol gradients (Fig. 6a) have sedimentation coefficients that show an apparent molecular weight difference of 50000. This apparent difference, however, in Fig. 6(a) may be an artifact due to the equilibrium between differently aggregated forms of the enzyme resulting in an overestimation of the

actual number of sedimenting species. But a structure based on a 50000-mol.wt. subunit has been suggested for calf thymus DNA polymerase- α (Holmes *et al.*, 1975) and for the *Ustilago maydis* DNA polymerase (Banks *et al.*, 1976). The properties of DNA polymerase C were very similar to those of DNA polymerase B, the only consistent difference being the binding to DNA-agarose. Rechromatography of DNA polymerases B and C on DNA-agarose suggested that the two forms were to some extent interconvertible at least in the direction B to C. Microheterogeneity in the population of mammalian DNA polymerase has been well documented (Holmes & Johnston, 1975; Matsukage *et al.*, 1976), and the three DNA polymerases from *Paramecium* macronuclei could be distinguished only by the time of their appearance in the cell cycle (Tait & Cummings, 1975). Also, the DNA polymerases A and B of *Luglena* differ only in their behaviour on DEAE-cellulose and relative abundance in resting and exponential-phase cells. Table 2 shows that the DNA polymerase C proportion of the total DNA polymerase activity is always less than DNA polymerase B, the ratio of these two species ranging from 1:4 to 1:5, which may reflect an equilibrium between the two forms.

C. A. R. was supported by a Medical Research Council Studentship, and the research was supported in part by a grant from the Science Research Council.

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