Deoxyribonucleic Acid Polymerases of *Euglena gracilis*

PURIFICATION AND PROPERTIES OF TWO DISTINCT DEOXYRIBONUCLEIC ACID POLYMERASES OF HIGH MOLECULAR WEIGHT

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Two DNA polymerases of high molecular weight, pol A (mol.wt. 190000) and pol B (mol.wt. 240000), have been purified 6300-fold and 1600-fold respectively from an extra-mitochondrial supernatant of a bleached strain of *Euglena gracilis*. They have very similar requirements when assayed with an 'activated'-DNA primer-template [the optimum conditions of pH and ionic (K^+ and Mn^{2+}) composition being 7.2, 25 mM and 0.2 mM respectively]. 0.2 mM- Mn^{2+} was about 1.5-2-fold as effective as 2 mM- Mg^{2+} , owing to substrate activation by deoxyribonucleoside 5'-triphosphates in the presence of Mn^{2+} . K_m values for the triphosphates in the absence of activation were about 10^{-6} M with Mn^{2+} and 8×10^{-6} M with Mg^{2+} for both enzymes. They were inhibited to the same extent by *N*-ethylmaleimide, novobiocin and *o*-phenanthroline, but differed in their chromatographic behaviour on DEAE-cellulose and in their electrophoretic mobilities on polyacrylamide gel. No evidence was found for the existence in these cells of a DNA polymerase of low molecular weight, but there were indications that a third enzyme of high molecular weight might exist.

General opinion now favours the existence of two major species of extramitochondrial DNA-dependent DNA polymerase (DNA nucleotidyltransferase, EC 2.7.7.7) in mammalian cells (Craig & Keir, 1974; Loeb, 1974). One of these, the low-molecular-weight enzyme with a sedimentation coefficient of 3.4S, is present within the cell nucleus and also, to a smaller extent, in the cytoplasm, but it shows no clear correlation with DNA synthesis *in vivo* (Craig *et al.*, 1975). The second, heterogeneous, high-molecular-weight (6-8S) species has been shown to consist of a number of distinct entities of different molecular weights that are probably based on a common subunit (Holmes *et al.*, 1974; Yoshida *et al.*, 1974) and is present in greatly increased amounts in cells that are actively engaged in DNA synthesis. For this and other circumstantial reasons (Craig & Keir, 1974), it has been assigned a tentative role in the replication of the nuclear genome of higher eukaryotes. It is, however, generally isolated from the cytoplasmic fraction, but may become associated with the nucleus and released again during the phase of DNA replication. It has been suggested (Lazarus & Kitron, 1973; Hecht & Davidson, 1973) that the 3.4S enzyme is a subunit of the 6-8S species, but this view is not universally favoured (Holmes *et al.*, 1974; Spadari *et al.*, 1974).

Primarily through the use of temperature-sensitive mutants and complementation assay systems *in vitro*,

functions have been assigned to the DNA polymerases of *Escherichia coli* and to a number of other protein factors essential for the replication of prokaryotic DNA (Kornberg, 1974; Tait & Smith, 1974; Tait *et al.*, 1974; Geider & Kornberg, 1974). The lack of comparable mutants of mammalian cells has restricted the development of an understanding of the roles of the eukaryotic DNA polymerases in DNA replication. Such mutants are more readily created and selected in primitive unicellular eukaryotes, and a study of such organisms might prove worth while, but as yet little attention has been focused on their DNA polymerase complement.

One DNA polymerase has been found in extra-mitochondrial extracts of *Tetrahymena pyriformis* (Crerar & Pearlman, 1974) and in *Chlorella pyrenoidosa* (Schönherr & Wanka, 1971), and two high-molecular-weight enzymes with similar properties have been isolated from an extramitochondrial fraction of the yeast *Saccharomyces cerevisiae* (Wintersberger & Wintersberger, 1970; Helfman, 1973). The smut fungus *Ustilago maydis* also appears to contain two DNA polymerases, one of which has been shown to be heat-labile when isolated from a mutant that is temperature-sensitive in DNA replication (Jeggo *et al.*, 1973), thus demonstrating the value of studying such organisms.

In a preliminary communication (McLennan &

Keir, 1973) we reported the separation of four DNA polymerase activities from extracts of the unicellular green flagellate *Euglena gracilis* strain Z. In this and the following paper (McLennan & Keir, 1975b) we describe the purification and properties of two high-molecular-weight DNA polymerases from an extra-mitochondrial fraction of a 'bleached' strain derived from strain Z. These have been designated pol A and pol B in order of their elution from DEAE-cellulose, and correspond respectively to peaks B and C of the previous report (McLennan & Keir, 1973).

In the present paper we demonstrate that the DNA polymerase complement of primitive eukaryotes is significantly different from that of mammalian cells, notably in there being no evidence for an enzyme of the low-molecular-weight 3.4S type.

Experimental

Materials

ATP, dithiothreitol, 2-mercaptoethanol, novobiocin, salmon testis DNA (type III) and DNAase* I (DN-100) were purchased from the Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K.; pig heart lactate dehydrogenase was from Boehringer Corp., London W5 2TZ, U.K.; CTP, UTP, GTP, dATP, dCTP, dGTP and dTTP were from P-L Biochemicals, Milwaukee, Wis., U.S.A.; calf thymus DNA was from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Acrylamide and *NN'*-methylenebisacrylamide were obtained from the Eastman Kodak Co., Rochester, N.Y., U.S.A., bovine plasma albumin (fraction V) was from the Armour Pharmaceutical Co., Eastbourne, Sussex, U.K., 1-thioglycerol, 2,5-diphenyloxazole, 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and Triton X-100 were from Koch-Light Laboratories, Colnbrook SL3 0BZ, Bucks., U.K., *m*-phenanthroline was from ICN Pharmaceuticals, Cleveland, Ohio, U.S.A., [*Me*-³H]dTTP, [*Me*-³H]dTDP, [⁵-³H]dCTP, [⁵-³H]dCMP and [⁵-³H]UTP were from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K., pancreatic RNAase, Pronase and thymidine were from Calbiochem, Hereford HR4 9BQ, U.K., Whatman DEAE-cellulose (DE52), cellulose phosphate (P11) and GF/C glass-fibre were from Reeve Angel Scientific, London SE1 6BD, U.K., Sepharose 6B was from Pharmacia (G.B.), London W5 5SS, U.K., and cellulose (MN 2200ff) was from Macherey, Nagel and Co., Düren, West Germany. All other chemicals were A.R. grade from BDH Chemicals, Poole, Dorset BH12 4NN, U.K.

* Abbreviations: DNAase, deoxyribonuclease; RNAase, ribonuclease; dNTP, deoxyribonucleoside 5'-triphosphate.

Methods

Preparation of extramitochondrial supernatant from strain SB1/AGM. Cells of the irreversibly streptomycin-bleached strain SB1/AGM derived from *Euglena gracilis* strain Z (McLennan, 1974) were grown in a heterotrophic medium of low pH (Hutner *et al.*, 1966) at 25°C in 2-litre batches and harvested in the exponential phase of growth by centrifugation in the MSE 4L Mistral centrifuge (3100g_{av.} for 10 min at 4°C). All further operations were performed at 4°C. Cells were washed twice in 10mM-Tris-HCl buffer (pH 7.4)–0.25M-sucrose–5mM-2-mercaptoethanol–0.1mM-EDTA (Krawiec & Eisenstadt, 1970), resuspended in 5 vol. of the same buffer adjusted to pH 9.0, and passed through an Aminco French pressure cell at a pressure of 8 MPa (1200 lb·in⁻²). The slurry was shaken gently to disperse aggregates and centrifuged at 1000g_{av.} for 5 min to remove the nuclear and pellicular debris and paramylon granules. The resultant pellet was washed once in 10 vol. of the pH 9.0 buffer and retained; the two supernatants were combined and repeatedly centrifuged at 1000g_{av.} for 5 min each time until no further pellet was produced. The supernatant was then centrifuged twice at 5000g_{av.} for 15 min each time in the 8 × 50 ml fixed-angle rotor of the MSE 18 centrifuge. The crude mitochondrial pellets from each centrifugation were combined and purified further for the isolation of the mitochondrial polymerase, and the supernatant from the final centrifugation was combined with the washed '1000g_{av.}' pellet, the slurry adjusted to pH 7.8 (by the addition of 1M-HCl) and solid KCl added to a final concentration of 1M. The homogenate was then centrifuged in the MSE 65 Superspeed ultracentrifuge (105000g_{av.} for 1 h), the resultant lipid pellicle carefully removed and the supernatant decanted and used for the further purification of pol A and pol B as described in the Results section.

Assay for DNA polymerase. Unless otherwise stated, the standard assay mixture (0.125 ml) contained: 25 μg of 'activated' salmon testis DNA; 0.2mM each of dATP, dGTP and dCTP; 40 μM-[*Me*-³H]dTTP (50 mCi/mmol); 50mM-imidazole-HCl buffer, pH 7.2; 25mM-KCl; 0.2mM-MnCl₂; 10mM-2-mercaptoethanol; 4% (v/v) glycerol; and enzyme protein. 2mM-MgCl₂ replaced 0.2mM-MnCl₂ where stated. After incubation for 1 h at 32°C, the assay mixtures were chilled on ice and 0.125 ml of 10% (w/v) trichloroacetic acid–20mM-Na₄P₂O₇ was added to each. After 20 min, the contents of each assay tube were collected by suction on glass-fibre discs and the tubes were washed out with four 1 ml washes of ice-cold 5% (w/v) trichloroacetic acid–5mM-Na₄P₂O₇. The discs were then washed twice for 10 min each time in the acid-pyrophosphate solution in batches in polypropylene trays, followed by two 5 min washes in ethanol. After drying at 50°C, the precipitated radioactivity was counted in the Inter-

technique SL30 liquid-scintillation spectrometer, with 0.4% (w/v) 2,5-diphenyloxazole and 0.005% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in toluene as scintillator, with a counting efficiency of 41%. A unit of DNA polymerase is that amount which catalyses the incorporation of 1 nmol of [Me - 3H]-dTTP residues into acid-insoluble material under the standard conditions of the assay.

Preparations for column chromatography. DEAE-cellulose (DE 52) was prepared for use according to the manufacturer's instructions (*Whatman Technical Bulletin IE2*), and phosphocellulose was prepared as described by Yoneda & Bollum (1965).

Native DNA-cellulose was prepared by a modification of two methods (Litman, 1968; Alberts & Herrick, 1971). Cellulose powder was washed to remove pyridine (Alberts & Herrick, 1971) and then added slowly with stirring to a solution (3 mg/ml) of native high-molecular-weight calf thymus DNA in 10 mM-Tris-HCl buffer (pH 7.4)-1 mM-EDTA (0.4 g of cellulose/ml of DNA solution). The resultant paste was spread out in a dish and dried at 30°C. Final traces of moisture were removed by grinding the residue to a fine powder and freeze-drying for 5 h. The residue was then suspended in 30 ml of ethanol/g of powder in a shallow beaker and irradiated with stirring 10 cm under a low-pressure mercury u.v. lamp to a total energy dosage of $10^4 J \cdot m^{-2}$. The slurry was filtered and washed with 10 mM-Tris-HCl buffer (pH 7.4)-1 mM-EDTA until the extinction of the filtrate at 260 nm fell to zero. Determination of the DNA released from a sample of the DNA-cellulose by heating at 100°C in buffer for 30 min indicated that approx. 90% of the DNA had become bound to the cellulose.

Polyacrylamide-gel electrophoresis. The 7% separating gels were prepared as described by Davis (1964), but containing 100 μ g of 'activated' DNA/ml, and were subjected to electrophoresis at 2 mA/gel for 1 h in separating-gel buffer, pH 8.9 (1:8 dilution of stock solution A), to remove persulphate ions. Stacking gels were then polymerized on top of the separating gels (Davis, 1964), and the gels were cooled to 4°C. Samples were applied in duplicate and run at pH 8.3 and 4°C at a current of 1 mA/gel. After being run, the gels were measured and one of each pair was stained with Bromophenol Blue and scanned at 590 nm with the Unicam SP.1809 gel scanner. The duplicate gels were incubated for 1 h at 4°C in two 50 vol. changes of 50 mM-imidazole-HCl buffer, pH 7.2, then covered with a DNA-free DNA polymerase assay mixture containing [Me - 3H]dTTP at 4 μ Ci/ml and 24 Ci/mmol. In calculating the composition of the assay mixture, the interstitial volume of the gel was taken to be 75%. After incubation for 1 h at 32°C the gels were washed free of excess of assay mixture with 5% (w/v) trichloroacetic acid, and then washed overnight with two 2-litre changes of

5% (w/v) trichloroacetic acid-20 mM- $Na_4P_2O_7$ -1 mM-thymidine per gel. The gels were then frozen, measured and cut into 1 mm slices with a Joyce-Loebl gel slicer. The slices were placed in scintillation vials, covered with 30% (v/v) H_2O_2 and incubated at 50°C until they had dissolved.

The solubilized slices were counted for radioactivity in 10 ml of toluene-Triton scintillator [the standard toluene-based scintillation fluid containing 67% (v/v) of Triton X-100].

Other methods. Protein concentrations were determined by evaporating samples to dryness *in vacuo*, dissolving in 0.5 M-NaOH and measuring as described by Miller (1959). DNA was determined by the method of Burton (1956), and 'activated' DNA was prepared essentially as described by Loeb (1969), the period of incubation with DNAase I being increased to 2 h to achieve maximum template activity. Lactate dehydrogenase activity was assayed by using the Boehringer Biochemica Test Combination lactate dehydrogenase assay kit (Boehringer Corp.).

Results

Purification of pol A and pol B

All operations were performed at 4°C, and after dialysis all fractions were clarified by centrifugation at 38000 g_{av} for 30 min in the 8 $\times$ 50 ml or 16 $\times$ 15 ml fixed-angle rotor in the MSE 18 centrifuge. Buffer A was 10 mM-Tris-HCl buffer (pH 7.8)-20% (v/v) glycerol-5 mM-2-mercaptoethanol.

Phosphocellulose. A sample of extramitochondrial supernatant was prepared from the homogenate (fraction I) derived from the disruption of 1.2×10^{10} exponentially growing cells in the French pressure cell, and was dialysed overnight against two 100 vol. changes of 0.1 M-potassium phosphate buffer (pH 7.5)-20% (v/v) glycerol-5 mM-2-mercaptoethanol to yield fraction II. This was applied to a column (30 cm $\times$ 2.1 cm) of phosphocellulose that had previously been equilibrated in the same buffer, and the column was washed at a flow rate of 60 ml/h with buffer until the extinction of the eluate at 280 nm reached zero. It was found that 98% of the DNA polymerase activity (fraction III) was eluted with a step of 0.4 M-potassium phosphate buffer (pH 7.5)-20% (v/v) glycerol-5 mM-2-mercaptoethanol.

Sephacrose 6B. Fraction III was dialysed for 3 h against 300 vol. of 20 mM-potassium phosphate buffer (pH 7.5)-20% (v/v) glycerol-5 mM-2-mercaptoethanol, and the protein was precipitated by the addition, with stirring over a 20 min period, of solid $(NH_4)_2SO_4$ to give 70% saturation. If the dialysis step was omitted, potassium phosphate was also precipitated from solution. After stirring for 30 min the suspension was centrifuged at 38000 g_{av} for 30 min in the 8 $\times$ 50 ml fixed-angle rotor of the MSE 18 centrifuge and the resultant pellet was dissolved in 3 ml of buffer

A made 0.5M with respect to KCl and 10% (w/v) with respect to sucrose. This was applied to a column (1.2cm×86cm) of Sepharose 6B that had previously been equilibrated in buffer A made 0.5M with respect to KCl and chromatographed at a flow rate of 10ml/h, and 2.1 ml fractions were collected. The activity was eluted as a broad heterogeneous peak after the void volume, and the active fractions were pooled and dialysed overnight against two 100vol. changes of buffer A containing EDTA at 1mM to yield fraction IV.

DNA-cellulose. A 10ml (packed wet volume) portion of cellulose that had been equilibrated in buffer A containing EDTA at 1mM was added to fraction IV and the slurry stirred for 1h. The suspension was then centrifuged (1000*g*_{av.} for 5min; MSE Mistral 4L), the supernatant decanted and the pellet washed by centrifugation with two 10ml portions of buffer A containing 1mM-EDTA. The supernatants were combined (fraction V) and added to 22ml (packed wet volume) of native calf thymus DNA-cellulose. The slurry was gently stirred for 2h, then poured into a column (16cm×1.5cm) that was fitted with an extension and that had a 10ml bed of DNA-cellulose at the base. After the suspension had settled, the unbound protein (which contained only 3% of the applied activity) was pumped through at a flow rate of 35ml/h, the extension was removed and the column was washed with buffer A containing EDTA at 1mM until the extinction of the eluate at 280nm reached zero. A 150ml linear gradient of 0–0.5M-KCl in buffer A containing EDTA at 1mM was then applied at a flow rate of 20ml/h, and 2.1 ml fractions were collected (Fig. 1).

DEAE-cellulose. The active fractions were pooled and dialysed overnight against 100vol. of buffer A containing KCl at 50mM (fraction VI), then applied at a flow rate of 25ml/h to a column (5cm×1cm) of DEAE-cellulose that had been equilibrated in buffer A containing KCl at 50mM. After the unbound protein had been washed through, a 60ml linear gradient

of 0.05–0.4M-KCl in buffer A was applied at a flow rate of 25ml/h, and 1ml fractions were collected (Fig. 2a). The separation of pol A and pol B can clearly be seen. The peak fractions of each activity were pooled separately and dialysed against 100vol. of

Fig. 2. Column chromatography of (a) fraction VI, (b) fraction VIIA and (c) fraction VIIB on DEAE-cellulose

Gradient elution chromatography was performed as described in the text. (a) The contents of tubes 18–21 were pooled and dialysed to give fraction VIIA, and the contents of tubes 26–36 were pooled and dialysed to give fraction VIIB; (b) the contents of tubes 19–27 were pooled to give fraction VIIA; (c) the contents of tubes 21–30 were pooled to give fraction VIIB. ●, DNA polymerase activity; —, KCl gradient.

Fig. 1. Column chromatography of fraction V on DNA-cellulose

Gradient elution chromatography was performed as described in the text. The contents of tubes 14–33 were pooled and dialysed to give fraction VI. ●, DNA polymerase activity; ---, E_{280} ; —, KCl gradient.

Table 1. Purification of pol A and pol B from exponential-phase *Euglena gracilis* strain SB1/AGM

For details see the text; 1.2×10^{10} exponentially growing cells were used. The values marked with an asterisk (*) were calculated by assuming pol B/pol A activity ratio of 9:1 in exponentially growing cells (A. G. McLennan & H. M. Keir, unpublished work) and no preferential loss of either enzyme during purification.

Fraction	Step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)	Purification
I	Initial homogenate	110	3750	865	0.23	100	1
II	Extramitochondrial supernatant	125	920	830	0.90	96	3.9
III	Phosphocellulose	34.6	54	1306	24.2	151	105
IV	Sephacrose 6B	35.5	28	1055	37.7	122	163
V	Cellulose	58	22	813	37.0	94	160
VI	DNA-cellulose	42	2.4	597	249	69	1078
VIIA	DEAE-cellulose	20.7	0.84	233	277	27	1202
VIIIB	DEAE-cellulose						
VIIIA	Rechromatography on DEAE-cellulose	5.8	0.16	26	162.5	27*	6340*
VIIIB	Rechromatography on DEAE-cellulose	6.4	0.35	117	334	15*	1630*

Fig. 3. Polyacrylamide-gel electrophoresis of pol A and pol B

Samples (0.1 ml) of (a) pol A and (b) pol B were submitted to electrophoresis in duplicate as described under 'Methods' in the Experimental section. One gel of each pair was stained and scanned at 590 nm and the other was assayed for DNA polymerase activity. After compensation for the different changes in gel length due to the staining and assaying procedures, the protein and activity profiles were superimposed. ●, DNA polymerase activity; —, E_{590} .

buffer A containing KCl at 50 mM to give fractions VIIA and VIIIB respectively.

Rechromatography on DEAE-cellulose. In order to separate further the two enzymes, they were individually chromatographed on separate columns (4 cm $\times$ 0.8 cm) of DEAE-cellulose at a flow rate of 15 ml/h. Pol A was eluted with 25 ml of a linear gradient of 0.05–0.20 M-KCl in buffer A (Fig. 2b) and pol B with 25 ml of a linear gradient of 0.05–0.30 M-KCl in buffer A (Fig. 2c). Separation from the remaining amounts of the other polymerase was achieved in each case. The gradient fractions (0.65 ml) were collected directly into small portions of solutions of ovalbumin or bovine serum albumin such that the final albumin concentration was about 0.25 mg/ml. If they were not collected in this way, they lost up to 75% of their activity before they could be assayed and the fractions of highest specific activity pooled.

The final purified preparations (fractions VIIIA and VIIIB) were divided into suitable small volumes and stored at -70°C without prior removal of KCl, because the latter procedure resulted in almost total loss of activities. Notwithstanding, pol A was still very unstable and sometimes lost up to 70% of its activity on freezing and thawing once. Pol B retained full activity for more than 3 months at -70°C . All experiments were performed with these preparations.

Purity of fractions VIIIA and VIIIB

The purification scheme is outlined in Table 1. The degree of purity of these fractions was studied by polyacrylamide-gel electrophoresis. Gels were scanned for protein bands, and DNA-containing gels were assayed *in situ* for DNA polymerase activity (Fig. 3). Apart from the main peak corresponding to

the bovine serum albumin, pol A produced one major band and one minor band close to the origin, but attempts to associate DNA polymerase activity with either of these bands were unsuccessful (Fig. 3a). Pol B exhibited three protein bands (apart from the bovine serum albumin), the major one coinciding with the polymerase activity (Fig. 3b). If the major band in Fig. 3(a) is indeed pol A, its mobility is what one would expect from a consideration of the behaviour of the two enzymes during chromatography on DEAE-cellulose. At pH values of 7.8 and 8.3 pol B appeared to be more negatively charged than pol A. Attempts to elute active enzymes from the gels failed. The enzyme protein concentration in fractions VIIIA and VIIIB used to calculate the final purifications in Table 1 were estimated from the gel scans by comparison with standards of known protein concentration. It is possible that further enzyme protein bands migrated to the same position as the bovine serum albumin, but this was not taken into account in the calculations.

Molecular weights

Samples of freshly purified pol A and pol B were subjected to sedimentation analysis on glycerol density gradients in 50mM-KCl as previously described (McLennan & Keir, 1975a). Sedimentation coefficients of 8.7S (pol A) and 10.3S (pol B) were estimated by comparison with a lactate dehydrogenase standard (7.2 S), and molecular weights of 190000 and 240000 respectively were calculated by the method of Martin & Ames (1961). These values were confirmed by using glutamate dehydrogenase as the reference. As the preparations aged, species of lower molecular weight appeared (McLennan & Keir, 1975a).

Properties of the reaction with 'activated' DNA as primer template

Effects of protein concentration, time of incubation and nuclease digestion. The activities of both enzymes were linear with respect to protein concentration up to the maximum amounts ever used in an assay (1.4 μ g of pol A protein and 2.7 μ g of pol B protein), and with respect to incubation period at 32°C up to 60 min. The label was all incorporated into DNA, since subsequent incubation of an assay with pancreatic DNAase (but not with pancreatic RNAase or Pronase) rendered all the precipitable radioactive material acid-soluble.

pH optima. Imidazole-HCl buffer (50mM) was used over the pH range 6.4–7.4, Tris-HCl buffer (50mM) over the range 7.6–8.6 and glycine-NaOH buffer (50mM) over the range 8.8–9.8. The optimal pH for the reaction was shown to be 7.2 for both purified enzymes (Fig. 4), this requirement being seemingly more stringent for pol A. Pol A also exhibited a slight activation at alkaline pH. Both enzymes retained less

Fig. 4. pH optima of pol A and pol B

The assay mixtures contained 0.28 μ g and 0.55 μ g of pol A (●) and pol B (○) protein respectively, and 100% activity was 171 and 325 nmol of dTMP residues incorporated/h per mg of protein for pol A and pol B respectively.

than 1% of their activity when assayed over the pH range 9.2–9.8.

Effects of univalent and bivalent cations. Pol A and pol B displayed an identical response to univalent cations. They were both activated 4-fold by 25mM- Na^+ , K^+ or NH_4^+ and retained 50 and 20% activity at concentrations of 50mM and 100mM respectively.

Both enzymes exhibited an absolute requirement for a bivalent cation. The optimal Mg^{2+} concentration was 2mM in each case, pol A retaining 55, 40 and 5% of its activity and pol B 70, 42 and 19% of its activity at Mg^{2+} concentrations of 0.5, 10 and 20mM respectively. Mn^{2+} , however, at a concentration of 0.2mM promoted a 1.5–2-fold higher activity with both enzymes than that sustained by 2mM- Mg^{2+} . At Mn^{2+} concentrations above 2mM, both enzymes showed less than 1% of their maximum activity. Ca^{2+} and Zn^{2+} were unable to support the incorporation of dTMP residues over the same concentration ranges. A study of less highly purified enzyme fractions has provided evidence that may indicate the presence of a third high-molecular-weight DNA polymerase. After a whole-cell extract of strain Z was chromatographed directly on DEAE-cellulose (McLennan & Keir, 1973) and the resultant pol A fraction examined, it was found to have a less stringent requirement for Mg^{2+} than has purified pol A (80% activity retained with 10mM- Mg^{2+}) and to exhibit a secondary activation response by K^+ at a concentration of 150mM (100, 48 and 73% activity at 25mM-, 50mM- and 150mM-KCl respectively; McLennan, 1974). These results could not be due to the chloroplast or mitochondrial DNA polymerases that have now been shown to correspond to peaks A and D respectively obtained in previous work

Fig. 5. Effect of *N*-ethylmaleimide on the activity of pol A

Pol A (0.35 μ g of protein) was assayed without 2-mercaptoethanol in the presence of either 50mM-imidazole-HCl (●) or 50mM-Tris-HCl (○) buffer and various amounts of *N*-ethylmaleimide. The 100% activity was 64 nmol of dTMP residues incorporated/h per mg of protein.

(McLennan & Keir, 1973; A. G. McLennan & H. M. Keir, unpublished work).

Effects of thiols and *N*-ethylmaleimide. 2-Mercaptoethanol, 1-thioglycerol and dithiothreitol at concentrations above 0.5mM stimulated the activities of pol A and pol B by 5–8-fold, suggesting that free thiol groups are present in the active configurations of the enzymes. This being the case, the thiol-binding reagent *N*-ethylmaleimide might be expected to inhibit the enzymes, but, when it was added to the standard assay without 2-mercaptoethanol, a 3-fold stimulation of the activities of pol A and pol B was found at a concentration of *N*-ethylmaleimide of about 0.5mM. However, when the imidazole-HCl buffer in the assay was replaced by Tris-HCl buffer (50mM), the expected inhibition was observed in each case. Fig. 5 depicts the effect of *N*-ethylmaleimide on pol A. Pol B behaved identically (McLennan, 1974). A possible explanation for the lack of inhibition by *N*-ethylmaleimide in the presence of imidazole-HCl buffer arises from the fact that imidazole can specifically catalyse the rapid polymerization of *N*-ethylmaleimide to *N*-ethyl-(*N*-ethylmaleamyl)_n-maleamic acid, which is then precipitated from solution (Smyth *et al.*, 1960), but the reason for the observed activation is unknown.

Effects of *o*-phenanthroline and novobiocin. Both pol A and pol B were inhibited by the chelating agent *o*-phenanthroline (15, 80 and 98% at concentrations of 0.1, 0.5 and 1.0mM respectively), but not by its non-chelating analogue *m*-phenanthroline. Such an inhibition suggests a role for a heavy-metal ion in the

catalytic reaction, and it has been shown that zinc is present at the catalytic centre of a number of DNA polymerases (Slater *et al.*, 1971). Mg^{2+} at a concentration of 5mM was used in the assay to counteract excessive chelation of the bivalent cation, and the concentration of 'activated' DNA was decreased 10-fold, since it competes with the *o*-phenanthroline for the zinc-containing site (Slater *et al.*, 1971).

Sung (1974) has reported that the 6–8S DNA polymerase from rat brain is inhibited by novobiocin in the presence of Mg^{2+} ions whereas the 3.4S enzyme is not. Both pol A and pol B were inhibited by the drug when assayed with 2mM- Mg^{2+} (50, 75 and 95% at concentrations of 0.5, 1.0 and 2.0mM respectively).

Activities of pol A and pol B as a function of the concentration of deoxyribonucleoside 5'-triphosphates in the presence of Mn^{2+}

Since the metal ion complexes of the dNTP species are the probable substrates for the polymerase reaction rather than the free triphosphates (Mildvan, 1970), a difference in dependence on dNTP concentration might be envisaged in the presence of Mn^{2+} and Mg^{2+} owing to their different affinities for nucleotides. It is apparent from Fig. 6 that two kinetically different reactions occur with both enzymes at different dNTP concentrations in the presence of Mn^{2+} . At concentrations up to 10 μ M, classical 'Michaelis-Menten' kinetics are obeyed (Fig. 6a). Suitable dNTP concentrations were chosen to construct double-reciprocal plots by the procedure of Lineweaver & Burk (1934), the assays performed and the results plotted. The K_m values calculated from these plots for the dNTP- Mn^{2+} complex were 1.1×10^{-6} M and 1.2×10^{-6} M for pol A and pol B respectively and the maximal velocities (V_{max}) attainable assuming 'Michaelis-Menten' kinetics to hold throughout were 16 and 33 nmol of dTMP residues incorporated/30min per mg of protein respectively.

As the concentration of each dNTP was raised between 10 and 100 μ M, a dramatic rise in the reaction rate of both polymerases occurred (Fig. 6b), presumably owing to some form of substrate activation. The decrease in enzyme activity observed at dNTP concentrations above 100 μ M is probably due to competition for the triphosphate-binding site by uncomplexed dNTP, since Mn^{2+} was added to a concentration of only 0.2mM.

Activities of pol A and pol B as a function of the concentration of deoxyribonucleoside 5'-triphosphates in the presence of Mg^{2+}

Initial examination of the results of a similar experiment performed with 2mM- Mg^{2+} suggested that 'Michaelis-Menten' kinetics were followed up to dNTP concentrations of 0.5mM (Fig. 7); however, a double-reciprocal plot (for pol B) revealed that, above 20–30 μ M, slight substrate activation also occurred,

Fig. 6. Activities of pol A and pol B in the presence of Mn^{2+} as a function of the concentration of deoxyribonucleoside 5'-triphosphates

Pol A (●) (0.70 μg of protein) and pol B (○) (1.34 μg of protein) were assayed for 30 min with 0.2 mM- Mn^{2+} and 50 mCi/mmol of [3H]dTP. (a) $0 \leq [dNTP] \leq 10 \mu M$; (b) $5 \leq [dNTP] \leq 500 \mu M$. The total dNTP concentrations are 4 times the values quoted on the abscissa.

since at low values of $1/[S]$ the kinetics became of a higher order (Fig. 8). Pol A behaved similarly. The inhibition at high dNTP concentrations was not observed, since there was sufficient Mg^{2+} to complex all the dNTP species (Fig. 7b). From the lower values of dNTP concentration, K_m values of $8.0 \times 10^{-6} M$ and $8.6 \times 10^{-6} M$ for the dNTP- Mg^{2+} complex were calculated for pol A and pol B respectively, the corresponding values for V_{max} in the absence of activation being 25 and 100 nmol of dTMP residues incorporated/30 min per mg of protein respectively. Thus, in the absence of substrate activation, for pol B V_{max} was 3-fold higher in the presence of Mg^{2+} than Mn^{2+} and for pol A it was 1.5-fold higher. However, at a dNTP concentration of 200 μM the velocities of the pol A-catalysed reactions were 100 and 40 nmol of dTMP residues incorporated/30 min per mg of protein with Mn^{2+} and Mg^{2+} respectively, whereas those for

pol B were 225 and 160 nmol of dTMP residues incorporated/30 min per mg of protein with Mn^{2+} and Mg^{2+} respectively. Thus the preference for Mn^{2+} in the standard assay is a result of the relative magnitudes of substrate activation produced by the dNTP- Mg^{2+} and dNTP- Mn^{2+} complexes. The apparent values of V_{max} for both the pol A- and pol B-catalysed reactions are increased about 7-fold by activation due to the dNTP- Mn^{2+} complex but only by about 1.7-fold by dNTP- Mg^{2+} . ATP was unable to simulate the activation effect.

Activities of pol A and pol B as a function of the concentration of 'activated'-DNA primer-template

Both reactions were completely dependent on the presence of DNA but could not be saturated with 'activated' DNA up to 85 μg/assay mixture (=680 μg/ml) (Fig. 9). For pol B three reproducibly distinct phases of rate of increase of reaction rate with DNA concentration were observed from 0–1.5 μg, 1.5–30 μg and 30–70 μg of DNA/assay. Only the second and

Fig. 7. Activities of pol A and pol B in the presence of Mg^{2+} as a function of deoxyribonucleoside 5'-triphosphate concentration

Details are as given in the legend to Fig. 6 except that 2 mM- Mg^{2+} replaced the Mn^{2+} .

third phases were apparent for pol A. These results were obtained in the presence of Mn^{2+} , but similar results have been found with Mg^{2+} . Without knowledge of the exact structure of the DNA it is difficult to interpret the observed kinetics of the two reactions.

Precursor utilization by pol B

The nature of the radioactively labelled dNTP was unimportant, dTTP and dCTP (also dATP) serving equally well as precursors (Table 2). A high degree of incorporation of label in the absence of a full complement of dNTP was observed, indicating that the

Fig. 8. Double-reciprocal plot of pol B activity as a function of the concentration of deoxyribonucleoside 5'-triphosphates

Pol B (1.34 μg of protein) was assayed for 30min with 50mCi/mmol of [3H]dTTP, 2mM- Mg^{2+} and various equimolar amounts of all four dNTP species.

Fig. 9. Dependence of reaction rate on the concentration of 'activated'-DNA primer-template

Pol A (●) (0.28 μg of protein) and pol B (○) (0.55 μg of protein) were assayed for 30min with various amounts of 'activated' DNA. Where the amount of DNA was less than 25 μg/assay mixture it was increased to this amount after incubation and before precipitation by the addition of a suitable amount of native DNA solution.

Table 2. Utilization of precursors by pol B

The 100% activity was that obtained in the presence of all four dNTP species; 100% (Mn^{2+}) = 518 nmol of labelled nucleotide incorporated/h per mg of protein; 100% (Mg^{2+}) = 330 nmol of labelled nucleotide incorporated/h per mg of protein. Each assay mixture contained 0.55 μg of pol B protein. N.T., Not tested.

Precursors (0.2mM each, 0.25 μCi of 3H /assay mixture)	Activity of pol B (% of control with each bivalent cation)	
	0.2mM- Mn^{2+}	2mM- Mg^{2+}
[3H]dTTP, dATP, dGTP, dCTP	100	100
[3H]dTTP, dATP, dGTP	26	47
[3H]dTTP, dATP	6	20
[3H]dTTP	4	9
No triphosphates	0	0
[3H]UTP	0	0
[3H]UTP, ATP, GTP, CTP	0	0
[3H]UTP, dATP, dGTP, dCTP	0	0
[3H]dCTP, dATP, dGTP, dTTP	112	103
[3H]dCMP, dATP, dGTP, dTTP	0	0
[3H]dTDP, dATP, dGTP, dCTP	N.T.	13
[3H]dTDP, dATP, dGTP, dCTP+0.75mM-ATP	N.T.	12

template contained short tracts deficient in one or more nucleotides that could direct complementary synthesis (Craig & Keir, 1975). The apparently more stringent requirement for all four dNTP species with Mn^{2+} may indicate that the substrate activation produced by the dNTP- Mn^{2+} complex was dependent on all four dNTP species. The preparation contained no DNA-dependent RNA polymerase activity, since ribonucleotides were not incorporated, and the enzyme did not incorporate [3H]UTP even in the presence of the other three dNTP species. [3H]dTDP (but not [3H]dCMP) did, however, appear to serve as a substrate for DNA synthesis, at least with Mg^{2+} , being 12–13% as efficient as dTTP, suggesting that pol B may have an associated nucleoside diphosphokinase activity (McLennan & Keir, 1975b). The extent of incorporation of label from [3H]dTDP was not increased by the inclusion of 0.75 mM-ATP in the assay mixture. Pol A was not tested for precursor utilization.

Discussion

An extramitochondrial supernatant of a chloroplast-free derivative of strain Z was used as the starting material for the purification of pol A and pol B to remove possible interference from the chloroplast- and mitochondria-related polymerases. The high yield of activity in fraction III (Table 1) may have been due to the removal of an inhibitor present in fraction II, probably endogenous DNA. Single-stranded DNA is a potent inhibitor of both enzymes (McLennan & Keir, 1975b), and fraction II contained 6 mg of DNA. Fraction III had only 120 μ g of DNA, and this was completely removed by gel filtration through Sepharose 6B in 0.5 M-KCl. The usual methods of nucleic acid removal (phase separation, differential precipitation and enzymic digestion) were found to decrease drastically the yields of polymerase activity and were regarded as unnecessary in conjunction with the phosphocellulose and Sepharose steps. About 25% of the protein of fraction IV could be removed by treatment with cellulose before DNA-cellulose chromatography, the yield of activity usually being higher than that shown in Table 1. The specific activities of 163 and 334 units/mg of protein were lower than those generally reported for eukaryotic DNA polymerases but could be increased 3–4-fold by raising the concentration of 'activated' DNA in the assay mixture (Fig. 9).

Both enzymes were of high molecular weight, and in their responses towards pH, univalent and bivalent cations, *N*-ethylmaleimide and novobiocin in the presence of 'activated' DNA behaved in an almost identical manner typical of the eukaryotic 6–8S species (Craig & Keir, 1974; Loeb, 1974). The anomalous activation of both enzymes by *N*-ethylmaleimide in the presence of imidazole demonstrates the

importance of control experiments in such studies. This may be a phenomenon independent of the proposed enzyme thiol groups. Differences between the enzymes were in their molecular weights, their overall response to pH and their behaviour on DNA-cellulose and DEAE-cellulose chromatography and polyacrylamide-gel electrophoresis. Pol A and pol B from yeast both have a pH optimum of 7.5, and yeast pol A, which is eluted first from DEAE-cellulose (Wintersberger & Wintersberger, 1970), also exhibits the alkaline pH response shown by pol A of *Euglena* but to a much greater extent. The response of the yeast enzymes to univalent cations is also similar, though the optimum concentration is 50 mM rather than 25 mM.

Pol A and pol B are unusual in exhibiting a preference for Mn^{2+} over Mg^{2+} in the 'activated'-DNA-dependent reaction. Rabbit bone-marrow cytoplasmic DNA polymerase (Byrnes *et al.*, 1973) and the nuclear calf thymus enzyme isolated by Bekkering-Kuylaars & Campagnari (1972) are the only other eukaryotic polymerases reported to prefer Mn^{2+} when assayed with a natural DNA primer-template. Pol I of *Esch. coli* has also been reported to be more active in the presence of 0.07 mM- Mn^{2+} and 'activated' DNA than when assayed at its Mg^{2+} optimum concentration of 8 mM (Slater *et al.*, 1972). This preference was evident only at higher concentrations of dNTP species and appeared to be a consequence of substrate activation in the presence of Mn^{2+} . By using values of [dNTP] below those that induced activation, K_m values of about 10^{-6} M and 8×10^{-6} M were calculated for the 'unmodified' enzymes (both identical) in the presence of Mn^{2+} and Mg^{2+} respectively. K_m values for the triphosphates in the presence of Mg^{2+} of between 0.8×10^{-6} and 160×10^{-6} M have been found for different DNA polymerases (Sedwick *et al.*, 1972; Momparler *et al.*, 1973; Yoshida *et al.*, 1974; Crerar & Pearlman, 1974), whereas Byrnes *et al.* (1974) report the K_m for triphosphates in the presence of Mn^{2+} to be 0.5×10^{-6} M for the rabbit bone-marrow 6–8S enzyme. This low value agrees with the lower K_m found for pol A and pol B with Mn^{2+} and is the probable reason for the Mn^{2+} optimum concentration being one-tenth of that for Mg^{2+} . The degree of activation may depend on the presence of all four dNTP species, since the extent of nucleotide incorporation catalysed by pol B in the absence of one or more of the dNTP species was significantly and reproducibly lower in the presence of Mn^{2+} than with Mg^{2+} . The exact mechanism of this process will not become clear without further investigation, but, owing to the widespread occurrence of enzymes under allosteric control, such a possibility is not unreasonable in this case. Since both enzymes have a subunit structure (McLennan & Keir, 1975a), a model involving an oligomer-protomer relationship may be applicable (Whitehead, 1970). Since a higher V_{max} is attain-

able in the absence of activation with Mg^{2+} , each bivalent cation may have a separate role in substrate binding and activation. Four Mn^{2+} -binding sites apart from the substrate site where dNTP molecules can bind have been demonstrated on the molecule of pol I of *Esch. coli* (Slater *et al.*, 1972). Similar sites on the molecules of pol A and pol B could account for the activation phenomenon.

In conclusion, we have isolated two high-molecular-weight DNA polymerases from *Euglena gracilis* that are so like each other with respect to the DNA-repair reactions that they catalyse that one must infer a close structural relationship between them. Similarities were noted between the *Euglena* polymerases and the corresponding enzymes from yeast. Another important similarity between these systems is the complete lack of evidence for an enzyme of the 3.4S type. Despite repeated attempts with a wide variety of extraction methods we were unable to demonstrate the existence of such a species. Until the relationship, if any, between the mammalian 6-8S and 3.4S enzymes is firmly established, any correspondence between the low-molecular-weight class of polymerase and the 3.0S entity produced by a time-dependent breakdown of pol B at low temperatures (McLennan & Keir, 1975a) must remain in doubt. A 3.4S polymerase is also lacking in *Tetrahymena* (Crerar & Pearlman, 1974), in *Chlorella* (Schönherr & Wanka, 1971), in *Physarum polycephalum* (Schiebel & Bamberg, 1973) and in prokaryotes (Kornberg, 1974), so its function may be outside the fundamental process of DNA replication. It could be of interest to see at which point in the evolutionary scale the 3.4S polymerase manifests itself and perhaps to correlate its presence with the appearance of a new nuclear function.

Tait & Cummings (1975) have isolated three high-molecular-weight DNA polymerases from *Paramecium* macronuclei; the polymerases A and B that they describe may correspond to pol A and pol B of *Euglena*, but polymerase C has a broader Mg^{2+} concentration optimum and is maximally active in 100mM-KCl. The presence of an enzyme of this type chromatographing on DEAE-cellulose with pol A, as does polymerase C (and also polymerase B) of *Paramecium*, might explain the difference in properties between crude and purified pol A. Such an enzyme must presumably be lost at an earlier stage in the purification scheme outlined in the present paper and must also be of high molecular weight, since whole-cell extracts chromatographed on Sepharose 6B yield only high-molecular-weight polymerase activity (McLennan & Keir, 1975a). Further examination of this possibility will be necessary.

A study of their synthetic primer-template utilization and associated enzymic activities should yield further information on the functions of pol A and pol B and their relationships with other DNA poly-

merases, and, in these respects, important differences have been found between the two enzymes (McLennan & Keir, 1975b).

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