The Deoxyribonucleic Acid Polymerases from the Diatom *Cylindrotheca fusiformis*

SUBCELLULAR DISTRIBUTION, EXONUCLEASE ACTIVITY AND HETEROGENEITY OF THE ENZYMES

By THOMAS W. OKITA* and BENJAMIN E. VOLCANI†
*Scripps Institution of Oceanography, University of California, San Diego,
La Jolla, CA 92093, U.S.A.*

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Four DNA polymerases from the marine diatom *Cylindrotheca fusiformis*, polymerases A, B, C and D, were further differentiated by their subcellular localization, presence of deoxyribonuclease activity, apparent heterogeneity and molecular weights. Polymerases A, B and D occur in significant amounts in the soluble fraction, suggesting that they were originally localized in the nuclei, whereas polymerase C predominates in the chloroplasts. A mitochondrial DNA polymerase was also isolated and characterized by ion-exchange chromatography. Polymerase D has an associated nuclease activity which prefers denatured DNA and Mg^{2+} , and has a pH optimum higher than that for polymerase activity. Co-elution from a DEAE-Sephadex column and co-sedimentation in glycerol density gradients of deoxyribonuclease and polymerase D activity suggest a molecular association. Polymerases A, B and C are devoid of nuclease activity. Glycerol-gradient-sedimentation analysis showed that all DNA polymerase fractions are heterogeneous at low ionic strengths, with the appearance of a single homogeneous activity at 0.5M-KCl. Estimated molecular weights of 100000, 82000 and 120000 for polymerases A, B and C respectively were obtained from sedimentation analysis and gel filtration. Polymerase D was estimated to have a molecular weight of about 100000 as determined by sedimentation analysis alone.

We have isolated four DNA polymerases (pol A, B, C and D) from the marine photosynthetic diatom *Cylindrotheca fusiformis* and characterized them by a variety of catalytic parameters (Okita & Volcani, 1977). However, the question arose as to whether these activities are distinct entities or are heterogeneous subspecies of the same entity. Aggregation (Bollum, 1975) or proteolysis, as well as possible association with other proteins in the replicative complex (Wickner *et al.*, 1973; Holmes *et al.*, 1976), could be responsible for the observed elution pattern.

The seeming heterogeneity of DNA polymerases has complicated their study in eukaryotic organisms. The high-molecular-weight mammalian enzyme, DNA polymerase- α , has been resolved by column chromatography into two or three species which sediment between 6S and 8S in density gradients (see Craig & Keir, 1974). DNA polymerases from the protists, although differing markedly in properties from their counterparts in higher eukaryotes, also display considerable heterogeneity (McLennan & Keir, 1975a; Banks *et al.*, 1976).

* Present address: Plant Growth Laboratory, University of California, Davis, CA 95616, U.S.A.

† To whom reprint requests should be addressed.

Protist DNA polymerases show an important difference from those of mammalian cells in that they possess an associated nuclease function, i.e. a 3':5'-exonuclease, which is almost ubiquitous in bacterial and bacteriophage-induced DNA polymerases (see Kornberg, 1974). Evidence for a macromolecular association of exonuclease activity has been reported in polymerase B from yeasts (Helfman, 1973) and in the polymerase of the smut fungus *Ustilago maydis* (Banks & Yarranton, 1976). Nuclease activity has also been demonstrated in pol B from *Euglena* (McLennan & Keir, 1975c) and in the single entity in *Tetrahymena pyriformis* (Crerar & Pearlman, 1974). The presence of an associated nuclease activity would further differentiate the various DNA polymerases in *C. fusiformis*.

To resolve the question of multiple activities in the diatom, the DNA polymerases of *C. fusiformis* were analysed by glycerol-gradient sedimentation at different ionic strengths, by gel filtration and by SDS†/polyacrylamide-gel electrophoresis. These activities appear to be distinct entities and not breakdown products of a common oligomer (Holmes *et al.*,

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

1974). Moreover, a DNAase activity is present in pol D, and is apparently associated with polymerase activity, whereas pol C is the predominant activity in chloroplasts.

Experimental

Buffers

Buffer I, 20mM-Hepes/NaOH (pH 7.6)/0.4M-sucrose/5mM- Na_2EDTA /1mM-dithiothreitol; buffer II, 20mM-Hepes/NaOH (pH 7.6)/0.4M-sucrose/0.01% bovine serum albumin/50mM- NaCl /1mM-dithiothreitol; buffer III, 20mM-Hepes/NaOH (pH 7.6)/0.6M-sucrose/1mM-dithiothreitol; buffer IV, 20mM-Tris/HCl (pH 7.6)/1mM-dithiothreitol; buffer V, 20mM-Tris/HCl (pH 7.8)/1mM- Na_2EDTA /1mM-2-mercaptoethanol/50mM- NaCl .

Materials

Diatom DNA polymerase activities, pols A, B, C and D were obtained from fraction VI of the purification protocol (Okita & Volcani, 1977), and were used throughout this study. Materials were obtained as follows: Sephadex G-200 and Dextran Blue 2000 were purchased from Pharmacia, Piscataway, NY, U.S.A.; rabbit muscle lactate dehydrogenase, ovalbumin, and horse heart cytochrome *c* were from Sigma Chemical Co., St. Louis, MO, U.S.A.; bovine haemoglobin was obtained from Miles Laboratories, Kankakee, IL, U.S.A.; bovine serum was from Pentex, Kankakee, IL, U.S.A.; myoglobin was obtained from Miles Seravac (Pty.) Ltd., Maidenhead, Berks., U.K.; Aquasol was from New England Nuclear, Boston, MA, U.S.A.; *Escherichia coli* [^3H]DNA was generously given by Dr. D. W. Smith, Dept. of Biology, University of California, San Diego, CA, U.S.A. For all other chemicals used in this study see Okita & Volcani (1977).

Methods

Culture conditions. Axenic cultures of *C. fusiformis* were maintained and cultured as described previously (Okita & Volcani, 1977). Exponentially grown cells were collected with a Sharples centrifuge (Sharples Corp., Philadelphia, PA, U.S.A.) at 5°C.

Organelle separation. About 75g (wet wt.) of cells was washed in buffer I, resuspended in 225ml of buffer II, and disrupted in an ice-cold pressure cell (Yeda Research and Development Co Ltd., Rehovot, Israel) at 32.1 MPa (4500 lb/in²). Fractions enriched with chloroplasts and mitochondria were obtained by differential centrifugation as described by Mehard *et al.* (1974) modified as follows: cell walls and chloroplasts were sedimented together at 2500g_{av.} for 10min. The organelle fractions were washed once with buffer III.

To further purify the chloroplasts, they were

resuspended in buffer IV containing 1.3M-sucrose, layered over 35ml of 1.6M-sucrose in a 50ml centrifuge tube, and centrifuged at 30000g_{av.} for 30min at 4°C. The material at the interface and the top layer were collected and diluted with buffer IV to a final sucrose concentration of 0.8M, after which the chloroplasts were sedimented at 20000g_{av.} for 10min. Electron micrographs showed that no mitochondria or other organelles were present but that almost 90% of the chloroplasts suffered disruption of the outer membranes (M. Borowitzka & T. W. Okita, unpublished work).

Mitochondria were purified by using a linear sucrose gradient (Paul & Volcani, 1975). The lower half of the gradient was collected and the mitochondria were sedimented at 60000g_{av.} for 60min after dilution with buffer IV.

Soluble and microsomal fractions were obtained by centrifuging the postmitochondrial supernatant fluid at 100000g_{av.} for 1.5h.

Preparation of polymerases from organelles. Enzyme activity was extracted from the different organelles by resuspending the organelles in buffer IV at 1mg of protein/ml, and disruption by sonic oscillation (Branson Sonic Power Co., Danbury, CO, U.S.A.) at 4°C by using 2 × 5s bursts at a setting of 5. Solid NaCl was then added to 1.5M and the polymerases were extracted by slowly stirring at 4°C for 60min. The microsomal fraction was extracted without sonication. Particulate material from all organelle fractions was then removed by centrifugation at 100000g_{av.} for 2h. Since the crude preparations of DNA polymerase could not be examined by direct application on DEAE-Sephadex because of interference from nucleic acids and lipid, the activities from each of the organelle fractions were partially purified as described previously (Okita & Volcani, 1977).

Assay for deoxyribonuclease. The conversion of [^3H]DNA into acid-soluble material was measured: the reactions were incubated at 32°C in a final volume of 0.125ml and contained the following: 55mM-Tris/HCl; 1mM-2-mercaptoethanol; 100mM-KCl; 10% (v/v) glycerol; 3nmol of [^3H]DNA (18400c.p.m.); bivalent cation; protein. The pH and concentration of the bivalent cations are given in the Results section. After incubation for 60min, 100μg each of bovine serum albumin and heat-denatured DNA were added to each assay and the reaction was terminated by the addition of 0.5ml of 10% (w/v) trichloroacetic acid and 20mM-sodium pyrophosphate. Reaction mixtures were kept on ice for 15min and then centrifuged at 5000g_{av.} for 10min. Supernatant fluid (0.5ml) was added to 10ml of Aquasol and counted for radioactivity in a Beckman LS-250 spectrometer.

Assay for DNA polymerase. DNA polymerase activity was measured with activated DNA as described previously (Okita & Volcani, 1977), except that each of the activities was at its optimum pH

and ion concentration. In addition, for pol D, 2 mM-spermine was added to the assay mixture. One unit of DNA polymerase activity is defined as the amount of enzyme that catalyses the incorporation of 1 nmol of dTMP into acid-insoluble material in 1 h.

Glycerol-density-gradient centrifugation. Linear 10–30% (w/v) glycerol gradients were prepared in 50 mM-Tris/HCl, pH 7.8, and 5 mM-2-mercaptoethanol containing 0.05, 0.15, or 0.5 M-KCl in a 5 ml cellulose nitrate tube. The gradients were equilibrated overnight at 5°C and then layered with 0.15 ml of enzyme. Samples were prepared for sedimentation analysis by dialysis against the same buffer as that used in the glycerol density gradients. Lactate dehydrogenase (15 units/gradient) was the routine molecular-weight standard used (Martin & Ames, 1961), and was added to the samples before dialysis. The gradients were centrifuged in a Beckman SW 50L rotor at $130000g_{av}$ for 17–19 h and the patterns determined by upward displacement using 50% (w/v) glycerol in an ISCO (Lincoln, NE, U.S.A.) gradient fractionator. About 33 five-drop fractions were collected and assayed for polymerase activity by using [Me^3H]dTTP at 19 Ci/mmol (0.25 μ Ci/assay).

Gel-filtration chromatography. Sephadex G-200 was washed and swollen according to the manufacturer's recommendations. Protein samples were concentrated 4-fold by reverse dialysis against solid sucrose and dialysed against 50 mM-Tris/HCl (pH 7.8)/5 mM-2-mercaptoethanol/10% (w/v) glycerol/0.5 M-KCl for at least 3 h. The samples (0.5 ml) were then layered on to a calibrated column (1 cm $\times$ 56 cm) of Sephadex G-200 which had been equilibrated with the same buffer used in dialysis. Fractions (0.5 ml) were collected at a flow rate of 5 ml/h and assayed for polymerase activity. The column was calibrated for the determination of molecular weights by using Dextran Blue 2000 (void volume) and the following protein standards: lactate dehydrogenase [rabbit muscle, mol.wt. 142000 (Worthington Enzyme Manual, Worthington Biochemical Corp., Freehold, NJ, U.S.A.)]; haemoglobin [bovine, mol.wt. 64500 (Braunitzer *et al.*, 1964)]; ovalbumin [hen, mol.wt. 45000 (Cunningham *et al.*, 1963)]; and cytochrome *c* [horse heart, mol.wt. 12400 (Margoliash, 1962)].

Lactate dehydrogenase was assayed by the method of Kornberg (1955). Dextran Blue and ovalbumin were spectrophotometrically monitored during column elution at 280 nm, and a haemoglobin and cytochrome *c* were measured at 412 nm.

Vertical-slab SDS/polyacrylamide-gel electrophoresis. DNA polymerases and protein standards were prepared for electrophoresis by adding 5% (v/v) 2-mercaptoethanol/0.5% SDS and immersing the mixture in boiling water for 2 min. For gel electrophoresis, 0.8 mm-thick vertical-slab gels were used at an acrylamide concentration of 10% (w/v) (Laemmli, 1970; Ames, 1974). Protein samples (5 μ g) were

applied to each sample well; electrophoresis was initially carried out at 7.5 mA until the Bromophenol Blue entered the separating gel and then the current was increased to 20 mA. The slab was fixed and stained with Coomassie Brilliant Blue as described (Fairbanks *et al.*, 1971). The following standard proteins were also electrophoresed for the determination of molecular weights (Weber & Osborn, 1969): phosphorylase *b* (94000), bovine serum albumin (68000), ovalbumin (48000), lactate dehydrogenase (35000), myoglobin (17200) and cytochrome *c* (11700).

Results

Subcellular localization of the diatom's DNA polymerases

Chloroplast polymerases. Polymerase activity from a chloroplast fraction was resolved into one predominant peak of material with two minor ones (Fig. 1*a*). Their chromatographic behaviour corresponded to pol C for the major entity and pols A and B for the minor species as previously described (Okita & Volcani, 1977). Since pols A and B have an affinity for membranous structures (see Fig. 1*d*), fortuitous binding of pols A and B could have occurred during the isolation of the chloroplasts. About 13% of the total activity of the pol C present in exponential cells is recovered in the chloroplast fraction, but less than 1% of pol A or pol B is present. Alternatively, the two minor peaks may be subspecies of pol C, shown to be extremely heterogeneous on glycerol gradients (see under 'Molecular weights of the DNA polymerases').

Mitochondrial polymerase. Chromatography of the polymerase activity for purified mitochondria showed a single symmetrical peak of material eluted between pol A and pol B (Fig. 1*b*). If it is assumed that only 10% of the mitochondria can be recovered from the diatom by the methods described (Paul *et al.*, 1975) with 100% extraction and recovery of the polymerase thereafter, then the mitochondrial polymerase contributes less than 3% of the total DNA-priming activity found in exponential-phase cells. This value agrees with that obtained for the mitochondrial polymerase in *Euglena* (McLennan & Keir, 1975*d*).

Proteins in the soluble protein fraction. We have been unable to isolate intact nuclei from this diatom, hence an unequivocal assignment of the nuclear polymerases is not possible. An alternative approach is to examine the polymerase activities that are present in the soluble protein fraction, e.g., the high-speed supernatant fluids. In mammalian cells the bulk of the polymerase is not chromatin bound and is present in the soluble fraction when aqueous buffers are used (see Craig & Keir, 1974). We therefore examined the polymerases from a soluble fraction,

Fig. 1. DEAE-Sephadex separation of DNA polymerases from (a) chloroplasts, (b) mitochondria, (c) soluble protein fraction and (d) microsomal fraction

Subcellular fractions were isolated and the polymerases isolated as described in the text. A column (1 cm $\times$ 7 cm) was used for all samples and eluted with a 100 ml 0.10–0.35M-NaCl gradient; 1 ml fractions were collected. ●, DNA polymerase activity; —, NaCl gradient.

isolated at low ionic strength, on the assumption that the predominant entities are of nuclear origin.

DEAE-Sephadex chromatography of the polymer-

ases from the soluble fraction showed four distinct activities corresponding to pols A, B, C and D (Fig. 1c). Only about 4% of the total pol C present in exponential-phase cells is found in this fraction, suggesting that it is merely a contaminant. Pols A and B are recovered at 24–28% of the total activity of each enzyme present in exponentially grown cells, but only 14% of pol D is found.

Polymerases from the protists are unusual, since they have a tendency to associate with membranous material. A majority of the nuclear polymerases from yeasts (Wintersberger, 1974) and *Euglena* (McLennan & Keir, 1975d) are found in the microsomal fraction when isolated at low ionic strength. Analysis of the 100000g_{av} pellet from *C. fusiformis*, isolated at low ionic strength, reveals that about 45% of the total polymerase activity in exponential-phase cells is found in this fraction (Fig. 1d). Pols A and B are present in about the same amounts in the membrane fraction as in the soluble fraction, whereas the concentration of pol D is 3-fold higher in the membranes. Each of these three enzymes is recovered at a yield of 50–60% in the soluble and microsomal fractions; the remaining activities are presumably lost during the isolation of organelles. Conversely, 86% of the total amount of pol C is present in these two fractions.

Deoxyribonuclease activity

Analysis of the four polymerase fractions for exonuclease activity showed that it is associated only with pol D (Fig. 2) and is coincident with DNA polymerase activity; the ratios of the two activities were fairly constant across the peak. Further analysis was conducted on glycerol gradients in the presence of 0.5M-KCl; DNAase and pol D activity again coincided (Fig. 3).

Fig. 2. Activities of DNA polymerase and DNAase during DEAE-Sephadex chromatography

Sample preparation and conditions for chromatography were described previously (Okita & Volcani, 1977). Fractions (2 ml) were collected; 0.02 ml of each was assayed for DNA polymerase activity for 30 min and 0.05 ml samples were assayed for nuclease activity for 60 min. ●, DNA polymerase; Δ, DNAase.

Fig. 3. Co-sedimentation of DNAase and DNA polymerase activities on glycerol gradient
Pol D (10µg), fraction VI (Okita & Volcani, 1977), was layered on a 10–30% glycerol gradient containing 0.5M-KCl; 0.15ml fractions were collected. DNA polymerase was assayed by incubating 0.05ml from each fraction for 60min as described (Okita & Volcani, 1977). A sample (0.1 ml) from each fraction was assayed for nuclease activity for 90min in a total volume of 0.15ml. Arrow indicates sedimentation position of lactate dehydrogenase. ●, DNA polymerase; △, DNAase.

Table 1. Exodeoxyribonuclease activity of pol D
Each assay mixture, as described in the Materials and Methods section, contained about 3nmol of [³H]-DNA and 1.25µg of pol D, and incubated for 60min at 32°C. A 100% activity is 64nmol/h per mg of protein.

pH	DNA substrate	Bivalent cation (mM)	Exo-DNAase activity (% of maximum)
7.5	Denatured	Mg ²⁺ (0.5)	21
		Mg ²⁺ (5.0)	54
		Ca ²⁺ (5.0)	14
8.2	Denatured	Mg ²⁺ (0.5)	35
		Mg ²⁺ (5.0)	100
		Ca ²⁺ (5.0)	22
9.0	Denatured	Mg ²⁺ (0.5)	26
		Mg ²⁺ (5.0)	61
		Ca ²⁺ (5.0)	16
8.2	Denatured	Mg ²⁺ (5.0)	12
8.2	Native	–2-Mercaptoethanol + N-ethylmaleimide (5.0)	
		Mg ²⁺ (5.0)	21

Conditions for the optimum hydrolysis of deoxyribonucleotides were then studied. Assays performed at pH 7.5, 8.2 and 9.0, using native or heat-denatured DNA in the presence of Mg²⁺ or Ca²⁺, showed no detectable hydrolysis by pols A, B and C under any of these conditions. Pol D, with a preference for heat-denatured DNA as a substrate, showed optimum hydrolysis at pH 8.2 in the presence of 5mM-Mg²⁺

Fig. 4. Glycerol-gradient centrifugation of pol A (a), pol B (b), pol C (c) and pol D (d) in 0.05M-KCl
The conditions for sample preparation, centrifugation and DNA polymerase assay are described in the text. Pol A (12µg), pols B and C (6µg of each) and pol D (5µg) were layered on each gradient; 15ml fractions were collected. ●, DNA polymerase activity; ○, lactate dehydrogenase marker.

(Table 1). DNAase activity is dependent on a free thiol group, since N-ethylmaleimide inhibits the net hydrolysis.

Molecular weights of the DNA polymerases

Analysis by glycerol-density-gradient sedimentation. Since freeze-thawing destroys about 50% of the DNA polymerase activity (Okita & Volcani, 1977), we examined the enzymes on glycerol gradients using buffer with KCl at different ionic strengths. At low ionic strength (0.05M-KCl) subspecies of all four activities were present (Fig. 4). Pol A sedimented as two subspecies (Fig. 4a) at 3.4S and 5.7S corresponding to mol.wts. of 46000 and 99000 respectively,

when compared with lactate dehydrogenase. Pol B sedimented with a main component at 5.0S (82000 mol.wt.) and two minor peaks at 7.3S (144000 mol.wt.) and 8.7S (187000 mol.wt.) (Fig. 4b). Pol C displayed the greatest heterogeneity of all, yielding peaks at 3.5S (48000 mol.wt.), 5.4S (92000 mol.wt.) and 6.5S (121000 mol.wt.), as well as a shoulder at 7.1S (138000 mol.wt.) (Fig. 4c). Pol D sedimented as two entities at 7.3S (144000 mol.wt.) and 8.7S (187000 mol.wt.) (Fig. 4d). Recovery for enzyme activity was 21%, 54%, 30% and 31% for pols A, B, C and D respectively. Heterogeneity of subspecies of each polymerase fraction was not due to aging of the preparations (3 weeks), since fresh preparations yielded identical results.

Intermediate salt concentration (0.15M) produced new activity profiles (Fig. 5). Pol A retained the main peak at 5.7S but the smaller satellite peak sedimented

at 4.0S (58000 mol.wt.) instead of 3.4S (Fig. 5a). Pol B no longer exhibited the 8.7S peak (Fig. 5b), suggesting an aggregation of subunits of low ionic strength which is disrupted at the higher salt concentration. This appears to be true for pols C and D also: pol C contained only two peaks at 5.3S and 6.3S (Fig. 5c), but pol D sedimented as a single entity at 7.3S (Fig. 5d). Recovery of enzyme activity was 76%, 71%, 92% and 37% respectively for pols A, B, C and D.

In the presence of high-ionic-strength buffer (0.5M) each of the polymerases sedimented as a single entity, showing sedimentation values of 5.6S (97000 mol.wt.), 5.1S (84000 mol.wt.), 6.3S (115000 mol.wt.) and 5.6S (97000 mol.wt.) for pols A, B, C and D respectively (Fig. 6). The recovery for pols A, B and C was above 93%, but for pol D it was only 55%. Identical results were obtained when the polymerases were

Fig. 5. Glycerol-density-gradient-sedimentation analysis of pol A (a), pol B (b), pol C (c) and pol D (d) in 0.15M-KCl. For details see legend to Fig. 4. ●, DNA polymerase; ○, lactate dehydrogenase marker.

Fig. 6. Glycerol-density-gradient-sedimentation analysis of pol A (a), pol B (b), pol C (c) and pol D (d) in 0.5M-KCl. For details see legend to Fig. 4. ●, DNA polymerase; ○, lactate dehydrogenase marker.

Fig. 7. Gel filtration of pol A (a), pol B (b) and pol C (c) on Sephadex G-200 in 0.5M-KCl

DNA polymerases from fraction VI (Okita & Volcani, 1977) were analysed. Pol A (120 μ g) and pols B and C (60 μ g of each) were used. About 0.5 ml fractions were collected and analysed for DNA polymerase activity. Pol D was completely labile on this column. The arrows indicate the void volume (V_0) and the elution volume of the protein standards: LDH, lactate dehydrogenase; Hb, haemoglobin; Ov, ovalbumin; Cyt c, cytochrome c.

sedimented in 1M-KCl. Unlike those from *Euglena* (McLennan & Keir, 1975a), the diatom polymerases are stable at high ionic strength; this may reflect their marine environment.

Gel filtration on Sephadex G-200. The size and shape of the DNA polymerases were analysed by gel filtration (Andrews, 1969). At lower ionic strengths (0.05–0.15M-KCl) the polymerase activities were labile. In the presence of 0.5M-KCl only about 16–32% of the activity was recovered for pols A, B and C, but no activity was observed for pol D. Filtration of pols A, B and C yielded a single homogeneous peak of activity between lactate dehydrogenase and haemoglobin (Fig. 7). The gel-filtration results corroborate those obtained from sedimentation analysis in that they provide no evidence for the presence of more than a single component in each of the DNA polymerase samples.

Estimation of the molecular weights of pols A, B and C by standard plot yields values of 100000,

80000 and 122000 respectively, which agree with those obtained from sedimentation analysis.

SDS / polyacrylamide - slab - gel electrophoresis. Although the DNA polymerases from the diatom have not been purified to homogeneity, some information may be gained on their macromolecular structure by analysis with SDS/polyacrylamide-gel electrophoresis. We feel justified in assuming that the polymerase is a major constituent of each fraction, since the comparable enzymes from *Euglena* (McLennan & Keir, 1975b), which are in about the same state of purity, show few proteins on disc polyacrylamide gels. In each of the diatom polymerase samples, protein staining with Coomassie Brilliant Blue showed at least one major polypeptide (results not shown). Pol A had two bands at 98000 and 80000 mol.wt.; pol B contained seven bands between 120000 and 70000 mol.wt., with one broad faint band at 37000 mol.wt.; pol C had a doublet at 65000 and 68000 mol.wt., and pol D had bands at 87000, 79000 and 74000 mol.wt. No distinct bands could be found at 35000–50000 mol.wt. except in pol B, but a broad faint band at approx. 24000 mol.wt. was present in all fractions.

Discussion

Pol C is the predominant enzyme in purified chloroplasts; this suggests that it is the principal enzyme involved in DNA replication/repair in chloroplasts, and agrees with our earlier suggestion (Okita & Volcani, 1977) that pol C is of chloroplast origin. Pol C differs from the other polymerases in extramitochondrial supernatants in its higher ion requirements, its affinity for deoxyribonucleotides, and its artificial-template-primer utilization.

The presence of significant amounts of pols A, B and D in the soluble protein fraction points to a role in nuclear DNA replication/repair. Ion-exchange chromatography of polymerase activities from the nuclei and soluble fraction of yeast (Wintersberger, U., 1974) and *Euglena* (McLennan & Keir, 1975d) has shown that their elution profiles are similar and suggests that the two enzyme preparations are identical. Further analysis of these enzymes may indicate which of the activities are responsible for silicon-dependent DNA replication in the diatom.

A DNAase activity is co-eluted with pol D on DEAE-Sephadex and co-sediments on glycerol gradients, which suggests that the two enzyme activities are associated. However, since the pol D preparation has not been purified to homogeneity, it may be that the two activities can be separated by other methods.

Both the presence of a DNAase associated with the diatom pol D, and its properties, agree with characterizations reported for other protists: presumably a 3':5'-exonuclease, the DNAase in yeasts

(Helfman, 1973), *Euglena* (McLennan & Keir, 1975c), *Tetrahymena* (Crerar & Pearlman, 1974), *U. maydis* (Banks & Yarranton, 1976), as well as in the diatom, has a requirement for Mg^{2+} , a pH optimum higher than that for polymerase activity, and a preference for single-stranded DNA (except in *U. maydis* where its preference is for native DNA).

An exonuclease activity is commonly associated with the above properties in polymerase preparations from prokaryotic organisms. This 3':5'-exonuclease may function as a 'proof reader' during DNA replication by removing mismatched deoxyribonucleotides from the 3'-end of the growing chain and thereby minimizing the possibility of mutations during DNA replication (Brutlag & Kornberg, 1972). The presence of a similar activity in the diatom pol D suggests that this enzyme is actively involved in DNA replication and is not just a repair enzyme. A preliminary report indicates that polymerase b in *Chlamydomonas reinhardtii*, which has an associated nuclease activity with the above-mentioned properties, is the predominant DNA-priming activity during nuclear DNA synthesis (Ross & Harris, 1976). Evidence for a similar role for the diatom pol D has been presented (Okita & Volcani, 1977).

Estimates of the molecular weight of diatom polymerases by both sedimentation analysis and gel filtration showed that at high ionic strength, correspondence between the two techniques is good. This indicates that the polymerases are not very asymmetrical and hence suggests that they are globular. Similar results have been obtained in *Euglena* (McLennan & Keir, 1975d) and *U. maydis* (Banks *et al.*, 1976), which, in this respect, differ drastically from the highly asymmetric complements in mammalian tissues (Holmes *et al.*, 1974). If the diatom polymerases are globular, sedimentation analysis should give good estimates of molecular weights of the heterogeneous subspecies present at low ionic strength.

Each of the DNA polymerase fractions yielded a single species at high ionic strength with molecular weights between 80000 and 120000. Because none of the fractions showed a polypeptide between 38000 and 50000 mol.wt. on SDS/polyacrylamide gels, it is unlikely that pols A, B and D occur as oligomers of identical subunits under these conditions (McLennan & Keir, 1975a). However, the presence of polypeptides with molecular weights of 80000–92000 on SDS/polyacrylamide gels suggest that they exist as monomers at high ionic strength. The doublet yielded by pol C on SDS/polyacrylamide gels suggests a mixed subunit composition or a dimer.

If pols B and D are present as monomers at high ionic strength, the existence of the 7.3S–7.6S (144000–153000 mol.wt.) and 8.7S (187000 mol.wt.) entities found at low ionic strength is difficult to explain simply by aggregation. Dimerization of pols B and D

would give globular structures with molecular weights of 160000–168000 and 194000 respectively, but this could account for only one of the entities observed on glycerol gradients. However, the anomaly could be explained by the existence of a second protein associated with these polymerases at low ionic strength but subsequently lost at high ionic strengths by disaggregation. In fact, the association with other proteins is an essential feature of polymerases involved in the elongation step of DNA synthesis, as evidence by the pol III holoenzyme of *Escherichia coli* (Wickner *et al.*, 1973; Wickner & Kornberg, 1973). In eukaryotic systems, evidence for a protein factor has been reported for pol B of *Euglena* to explain a similar situation (McLennan & Keir, 1975a). Holmes *et al.* (1976) have shown the oligomer structure of DNA polymerase- α to be composed of a polypeptide of 150000 mol.wt. possessing polymerase activity and a smaller subunit of 50000–70000 mol.wt. devoid of activity. In addition, structural studies of the DNA polymerase from yeast (Wintersberger, U., 1974) and *U. maydis* (Banks *et al.*, 1976) suggest a composition with dissimilar subunits. Thus pols B and D may aggregate with another protein(s) in globular structures or the values may be anomalous owing to asymmetry formed during aggregation.

The presence of subspecies that sediment around 3.5S (48000 mol.wt.) in pols A and C at low ionic strength, suggests that the main component of each polymerase is comprised of subunits (McLennan & Keir, 1975a). However, the absence of a polypeptide of this molecular weight on SDS/polyacrylamide gels, and the low recovery of activity at low ionic strengths, indicates that these subspecies are probably produced by an unfolding of the tertiary conformation which decreases the sedimentation rate in glycerol gradients. The shift in the apparent sedimentation rate of these subspecies in pol A from 3.4S to 4.0S on increase of ionic strength suggests a conformation change, a condition that has been described for the pol A12 mutant of *E. coli* (Uyemura & Lehman, 1976). Although the molecular weight and charge density of pol A12 is identical with that of polymerase I, its migration in native polyacrylamide gels and sedimentation on sucrose velocity gradients is slower than that of polymerase I.

McLennan & Keir (1975c) have suggested that the protist polymerases may be more closely related to their counterparts in bacteria than to those in higher eukaryotes. This is supported by the diatom polymerases, i.e., the presence of a DNAase activity apparently associated with pol D, molecular weights of about 100000, and lack of asymmetry, all properties common to most prokaryotic polymerases. It is further supported by the absence from the diatom of DNA polymerase- β , the low-molecular-weight enzyme, which is characteristic of mammalian cells (Bollum, 1975).

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