Multiple Deoxyribonucleic Acid Polymerases from Quiescent Wheat Embryos

PURIFICATION AND CHARACTERIZATION OF THREE ENZYMES FROM THE SOLUBLE CYTOPLASM AND ONE FROM PURIFIED MITOCHONDRIA

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(Received 24 November 1978)

Three DNA polymerases (A, B and C) have been purified from the soluble cytoplasm of ungerminated wheat embryos. Mainly on the basis of chromatographic, template-specificity and salt-inhibition evidence, we have characterized the three enzymes. Other physico-chemical and enzymic properties are described. From purified mitochondria we have purified a DNA polymerase that behaves like DNA polymerase B on chromatographic and template-specificity criteria. Only highly purified enzyme B from the soluble cytoplasm showed an exonuclease activity able to degrade 3'- or 5'-labelled polydeoxyribonucleotides, as well as a 'proof-reading' capacity.

Multiple DNA polymerases are found in prokaryotic and eukaryotic organisms. In the case of bacteria, DNA polymerase-I, found in 1956, was the only enzyme having DNA polymerase activity, until the isolation of mutants led to the purification and characterization of two other distinct enzymes, DNA polymerases-II and -III (Kornberg, 1974; de Lucia & Cairns, 1969; Gefter, 1975). In the case of mammalian cells, three DNA polymerases (α , β and γ) have been found (Bollum, 1975; Holmes & Johnston, 1975; Wintersberger, 1977; Weissbach, 1977). DNA polymerase- α is a high-molecular-weight enzyme found mainly in the soluble cytoplasmic fraction. It may have an important role in DNA replication, since it increases dramatically during the S-phase of active DNA synthesis (Chang *et al.*, 1973; Spadari & Weissbach, 1974; Chiu & Baril, 1975). DNA polymerase- β is a low-molecular-weight enzyme having a high affinity for chromatin, very resistant to *N*-ethylmaleimide and whose level is not affected during the cell cycle. DNA polymerase- γ is found in the nucleus and the cytoplasm, and seems to be the only enzyme operating in the mitochondria (Bolden *et al.*, 1977; Bertazzoni *et al.*, 1977; Hubscher *et al.*, 1977; Tarrago-Litvak *et al.*, 1978).

In the case of low eukaryotes, two DNA polymerases have been well characterized in yeast. One of these enzymes has 3'- and 5'-exonuclease activities associated with the polymerizing activity, as with bacterial DNA polymerases (Wintersberger, 1974; Chang, 1977).

Although there are numerous reports on bacterial and animal DNA-dependent DNA polymerases,

only a few articles can be found describing the detailed characterization and/or purification of DNA polymerases from higher plants. DNA polymerase activity has been reported in isolated nuclei of plants (Jerzmanowski *et al.*, 1974; Yamaguchi *et al.*, 1976) and it has been purified and characterized from chromatin (Dunham & Cherry, 1973; Srivastava & Grace, 1974; Stevens & Bryant, 1976; Tymonko & Dunham, 1977) or from the soluble cytoplasm (Stout & Arens, 1970; Mory *et al.*, 1974, 1975; Robinson & Bryant, 1975; Castroviejo *et al.*, 1975; Tarrago-Litvak *et al.*, 1975; Gardner & Kado, 1976; Stevens & Bryant, 1978).

In a previous article (Castroviejo *et al.*, 1975) we reported the purification of two DNA polymerases from the soluble fraction of ungerminated wheat embryos. By changing some chromatographic steps we have been able to show the presence of a third enzyme in this fraction. These three activities have been extensively purified and their properties are described in the present paper. Moreover, we have isolated a DNA polymerase from purified mitochondria. Some properties of this enzyme are also discussed in the present paper, as well as a comparison with the other soluble DNA polymerases. A preliminary account of this work has already been published (Litvak *et al.*, 1978).

Materials and Methods

Materials

Two types of wheat seeds were used, the results with both being identical. The variety 'Cesar' was obtained from the Brosse Montceaux Agronomical

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Centre, 77130 Montereau, France and the variety 'Atys G₂' was kindly given by Dr. J. M. Bové from I.N.R.A., 33 Pont de la Maye, France. Commercial wheat-germ was kindly given by Grands Moulins de Bordeaux, 38 Quai Brazza, 33 Bordeaux, France. Labelled products were provided by The Radiochemical Centre, Amersham, Bucks., U.K. Unlabelled nucleoside triphosphates were bought from Sigma (St. Louis, MO, U.S.A.) or Boehringer-Mannheim, France. Poly(dA), poly(dT), poly(dC)-dT₁₂ came from Miles, Lausanne, Switzerland, dG₁₂ was from Pabst Laboratories, Milwaukee, WI, U.S.A., and poly(A), as well as calf thymus DNA, were from Sigma. *Escherichia coli* DNA polymerase-I was kindly given by Dr. G. Brun, Section Biologie, Fondation Curie, Paris, France. DEAE-cellulose (DE-22) and phosphocellulose (P11) were from Whatman (W. and R. Balston), Maidstone, Kent, U.K., and were prepared as described previously (Castroviejo *et al.*, 1975).

Methods

Wheat-embryo isolation. Embryos were separated from the rest of the grain by the mass-isolation technique of Johnston & Stern (1957) and stored at 4°C over anhydrous CaCl₂. They were more than 95% viable, even after several months of storage under the above conditions.

Isolation of mitochondria. The method of Cunningham & Gray (1977) was used. Essentially it consists of recovering the purified mitochondria, after centrifugation, from the interphase between 1.2 and 1.5M-sucrose. In our laboratory the centrifugation step was performed at 90000g in the 6 × 16.5 ml rotor of the MSE ultracentrifuge. The purity of the mitochondrial fraction was monitored by electron microscopy.

Electron microscopy. The mitochondrial pellet was fixed in 2.5% glutaraldehyde in 100mM-potassium phosphate, pH 7.4, for 45 min in the cold, rinsed with buffer and post-fixed in 1% OsO₄ for 60 min at 4°C, dehydrated in ethanol and propylene oxide and embedded in Epon 812 resin. Sections obtained with a Reichert ultramicrotome were stained with uranyl acetate, followed by lead citrate, and observed with a Philips SM 201 electron microscope.

Preparation of activated calf thymus DNA and synthetic double-stranded polynucleotides. Calf thymus DNA was activated by the method of Aposhian & Kornberg (1962). Polynucleotide solutions were 2.0 A₂₆₀ units/ml in 10mM-Tris/HCl, pH 7.5. After mixing the appropriate polynucleotides, the solution was heated at 75°C for 15 min [in the case of poly(C)-dG₁₂ and poly(dC)-dG₁₂ the temperature was 90°C] and left at room temperature (20°C) for 30 min. The template/primer ratio was 5:1, except for poly(dT)·poly(A) (10:1).

DNA polymerase assays. Two types of reaction conditions were used; when activated DNA or double-stranded synthetic polydeoxynucleotides were used, the reaction mixture contained, in a final volume of 0.05 ml: 50mM-Tris/HCl, pH 8.0 [50mM-Mes (4-morpholine-ethanesulphonic acid), pH 7.0, in the case of enzyme B], 10mM-dithiothreitol, 5mM-MgCl₂, 0.1mM each of dATP, dGTP and dCTP, 0.01mM-[³H]TTP (500–1500 c.p.m./pmol), 30 μg of activated DNA/ml (or 0.4 A₂₆₀ unit of the polynucleotide/ml). When manganese was used, the final concentration was 0.5mM.

In the case of enzyme A and when poly(A)-dT₁₂ was used as template in the presence of Mg²⁺, the reaction mixture was identical, except that 0.1 A₂₆₀ unit of poly(A)-dT₁₂/ml was added. When highly purified enzyme was used, 5 μg of bovine serum albumin were added to the incubation mixtures. Incubation at 37°C was carried out for different lengths of time, and the reaction was stopped by the addition of 1 ml of cold 10% (w/v) trichloroacetic acid plus 1% sodium pyrophosphate. The precipitate was filtered through nitrocellulose membranes (Schleicher and Schuell D-3354, Germany), washed with cold 2% trichloroacetic acid, dried and counted for radioactivity in 5 ml of PPO (2,5-diphenyloxazole)/POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]/toluene scintillation mixture.

Sephadex G-150 gel filtration. Gel filtration was carried out at 4°C in a column (1 cm × 17 cm) that had been previously equilibrated in 50mM-Tris/HCl, pH 7.5, with 5mM-2-mercaptoethanol, 0.1mM-EDTA 200mM-KCl and 20% (v/v) glycerol. The column was loaded with 1 ml of sample at a pressure of 20 cm of buffer. For the determination of molecular weight, the column was calibrated with the following standards: *E. coli* DNA polymerase-I (mol.wt. 110000), bovine serum albumin (68000), ovalbumin (45000) and cytochrome c (13000). The void volume (V₀) was determined with Blue Dextran.

Glycerol-gradient centrifugation. Sedimentation studies were performed in a 6 × 5 ml rotor of the MSE ultracentrifuge at 400000g for 16 h at 4°C. The glycerol gradient was between 10 and 30% (v/v) and the sample was in a volume of 0.1 ml. Protein standards were those described above for gel filtration. The sedimentation coefficient and molecular weight (assuming that the proteins are of the globular type) were estimated as described by Martin & Ames (1961).

Isoelectric focusing. The isoelectric point (pI) was determined in a 100 ml-capacity LKB 8100 column with 1% Ampholines (pH range 3.5–10) in a 0–60% (v/v) glycerol gradient. The focusing time was at least 72 h at 4°C at 2000 V. The DNA polymerase assay was performed with 10 μl portions of each fraction.

Buffers used in the purification. Buffer TKM contained 50mM-Tris/HCl, pH 7.2, 25mM-KCl, 5mM-

MgCl₂ and 250mM-sucrose. Buffer A contained 50mM-Tris/HCl, pH7.5, 1mM-2-mercaptoethanol, 0.1mM-EDTA and 20% glycerol. Buffer B contained 50mM-Tris/HCl, pH7.9, 1mM-2-mercaptoethanol, 0.1mM-EDTA and 20% glycerol. Buffer C contained 50mM-Tris/HCl, pH7.9, 1mM-EDTA and 20% glycerol.

Protein determination. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Protein determination in the eluate of chromatographic columns was determined by spectrophotometric absorbance at 280nm.

Preparation of double-stranded-DNA-cellulose columns. The column of double-stranded DNA linked to the cellulose matrix was prepared as described by Alberts & Herrick (1971).

Results

Purification of DNA polymerases A, B and C

Crude extract. Wheat-germ (250g) was homogenized in a mortar with 4vol. of buffer TKM. All operations were performed at 4°C. The homogenate was filtered through four layers of gauze and then through one layer of Miracloth. A first centrifugation at 15000g was performed to eliminate nuclei, mitochondria and debris, and the supernatant was centrifuged at 100000g for 2h at 4°C (S₁₀₀).

(NH₄)₂SO₄ fractionation. The S₁₀₀ supernatant was precipitated between 20 and 70%-satd. (NH₄)₂SO₄. The pellet was dissolved in buffer A and dialysed against 50vol. of the same buffer overnight at 4°C. A 3h dialysis was performed against 5vol. of buffer B.

First phosphocellulose column. After absorption in the column (4cm×15cm), the proteins not retained were eluted with buffer B plus 200mM-KCl, and the column washed until the A₂₈₀ was lower than 0.1. Proteins fixed in the phosphocellulose column were eluted with 800mM-KCl in buffer B. All the DNA polymerase activity was pooled, precipitated to 70%-satd. (NH₄)₂SO₄, redissolved in buffer A and dialysed against 100vol. of the same buffer for 12h in the cold. Insoluble material appearing during dialysis was eliminated by centrifugation at 15000g. All the DNA polymerase activity was recovered in the supernatant, whereas almost 30% of the total proteins were eliminated.

DEAE-cellulose. The dialysate from the preceding step was passed through a DEAE-cellulose column (2.5cm×12cm) equilibrated with buffer A. Proteins not retained by the resin were eluted with the same buffer until the A₂₈₀ was lower than 0.1. The retained proteins were eluted with 300mM-KCl. Two peaks of DNA polymerase activity were found. The first was not retained in the DEAE-cellulose column, whereas the second was eluted from the column at 300mM-

KCl. Enzyme C was purified from the fraction not retained, whereas enzymes A and B were purified from the fraction retained on DEAE-cellulose. When DNA polymerase activity not retained on DEAE-cellulose was rechromatographed on DEAE-cellulose, none was retained by the column. Even after six months at -20°C in 50% glycerol none of this fraction was retained on a DEAE-cellulose column.

The DEAE-cellulose-column fractions eluted with 300mM-KCl containing DNA polymerase activity were pooled, diluted with buffer B to a final concentration of 200mM-KCl and applied to a phosphocellulose column.

Phosphocellulose chromatography of fractions A+B. The phosphocellulose column (2.5cm×4cm) was washed with buffer B plus 200mM-KCl until A₂₈₀ was less than 0.1. A gradient of 200–450mM-KCl was established (40ml total volume). As soon as DNA polymerase activity was detected, the gradient flow was stopped and the column washed with 35ml of 300mM-KCl in buffer B. When the eluting activity had clearly decreased, a second gradient was established between 300 and 700mM-KCl in buffer B. The elution profile of such column is shown in Fig. 1. Enzyme A eluted at about 300mM-KCl, whereas enzyme B was eluted at about 450mM-KCl.

Phosphocellulose chromatography of fraction C. The phosphocellulose column (2.5cm×4cm) was equilibrated with buffer B plus 200mM-KCl. Once the sample was adsorbed, the column was washed with the same buffer until the A₂₈₀ was less than 0.1. A continuous gradient of KCl between 200 and

Fig. 1. Separation of DNA polymerases A and B by phosphocellulose chromatography

The DNA polymerase peak obtained from DEAE-cellulose (see Results section, under 'DEAE-cellulose') was diluted to 200mM-KCl and applied to a phosphocellulose column. Elution was performed as described in the Results section under 'Phosphocellulose chromatography of fractions A+B', and portions of the fractions were incubated 30min in the presence of activated DNA and Mg²⁺ as described under 'Methods'.

700mm in buffer B was established. The enzyme was eluted between 250 and 300mm-KCl.

Double-stranded-DNA-cellulose. In some instances DNA polymerase C was submitted to a further purification step by affinity chromatography on a double-stranded-DNA-cellulose column. The column (1 cm × 5 cm) was equilibrated with buffer C. About 10mg of purified enzyme protein was dialysed against buffer C and adsorbed on the column. After the column had been washed extensively with the same buffer, DNA polymerase activity was eluted with a gradient between 200 and 500mm-KCl. A unique peak of activity between 250 and 300mm-KCl was obtained.

In general, fractions containing enzymic activity were pooled and concentrated by dilution and passage through a small column (0.5 cm × 2 cm) of phosphocellulose. Elution was performed with 500mm-KCl. The concentrated fraction having DNA polymerase activity was dialysed against buffer B plus 50% glycerol. Enzymes were kept at -20°C in 50% glycerol for several months without apparent loss of activity.

In Table 1 a summary of the purification of enzymes A, B and C is shown. As in the case of the purification of many other nucleic acid-polymerizing enzymes, an increase in the total activity of wheat DNA polymerase is observed in the first steps of purification. This may be due to the loss of an inhibitor, probably a nuclease. Although we have not studied the nature of this inhibitor, it is noteworthy that the 'wash-out' of the first phosphocellulose column contains a very powerful inhibitor of DNA polymerase activity as tested with enzymes A, B and C *in vitro* (not shown).

Purification of mitochondrial DNA polymerase

Electron micrographs of purified mitochondria (Cunningham & Gray, 1977) from quiescent wheat

embryos showed numerous intact as well as some ruptured organelles. Little non-mitochondrial material was observed. The mitochondrial pellet was suspended in buffer A plus 200mm-KCl and 0.15% (v/v) Triton X-100 and sonicated in an Annemasse disintegrator at 4°C, three times for 10s each time, at maximal intensity, with intervals of 50s. The homogenate was centrifuged at 18000g for 10min and the resultant supernatant was used for purification. This supernatant was submitted to DEAE-cellulose chromatography (see Fig. 2). Fig. 2(a) shows the DEAE-cellulose chromatographic profile of a cytoplasmic extract as described under 'DEAE-cellulose' above. Enzyme C is not retained, whereas enzymes A+B are eluted with 300mm-KCl. In Fig. 2(b) the same type of chromatography is shown for the mitochondrial extract. No activity was found in the wash-out of the column, whereas a peak of DNA polymerase activity eluted with the 300mm-KCl step. In Fig. 2(c) a phosphocellulose column of the fractions A+B from the soluble cytoplasm is shown; conditions are those described under 'Phosphocellulose chromatography of fractions A+B' above, except that a continuous gradient of KCl between 200 and 800 mm was used. In the case of the cytoplasmic extract, two overlapping peaks (DNA polymerases A and B) were obtained, whereas in the case of the mitochondrial enzyme (Fig. 2d) only one peak coinciding with cytoplasmic enzyme B was obtained.

Properties of cytoplasmic DNA polymerases A, B and C

Some properties of DNA polymerases A, B and C are summarized in Table 2. The three enzymes are found mainly in the soluble cytoplasmic fraction. The size of enzymes B and C are very similar (110000), whereas enzyme A has a mol.wt. of 140000. The

Table 1. *Summary of wheat DNA polymerase purification*

A unit of enzyme is the amount able to catalyse 1 nmol of dGMP [with poly(dC)-dG₁₂ as template] in 1 h at 37°C. The specific activity of [³H]dGTP was 500 c.p.m./pmol.

	Volume (ml)	Protein (mg)	Activity		Purification
			Total units	Units/mg	
Crude extract	380	22800	3192	0.14	1.0
S ₁₀₀ supernatant	315	15120	5745	0.38	2.7
Ammonium sulphate ppt.	140	8400	5208	0.62	4.4
Phosphocellulose	60	288	14688	51.00	364.0
Dialysis	60	210	17640	84.00	600.0
DEAE-cellulose (fraction not retained)	63	79	16906	214	1528
DEAE-cellulose (fraction retained)	64	109	11772	108	771
Phosphocellulose (A)	5	4	1800	450	3214
Phosphocellulose (B)	13	8	2080	260	1857
Phosphocellulose (C)	19	3.4	4620	1360	9714

Fig. 2. Chromatographic study of mitochondrial and postmitochondrial wheat DNA polymerases

(a) Chromatography of soluble cytoplasmic DNA polymerases on a column of DEAE-cellulose as described in the Results section under 'DEAE-cellulose.' (b) Chromatography of mitochondrial DNA polymerase on a column of DEAE-cellulose. Conditions of the chromatography were the same as above. The mitochondrial extract was prepared as described in the Results section under 'Purification of mitochondrial DNA polymerase'. (c) Phosphocellulose chromatography of DNA polymerase A and B. Conditions were the same as in Fig. 1, except that a continuous gradient (----) was used, as shown in the Figure. (d) Phosphocellulose chromatography of mitochondrial DNA polymerase. Conditions for the phosphocellulose chromatography were the same as given above. The enzymic activity from the different fractions was measured in the presence of activated DNA and Mg^{2+} ; the incubation time was 30 min.

Table 2. Properties of wheat DNA polymerases A, B and C

The assays were done with activated DNA as template as described under 'Methods'. The incubation time was 30 min. The conditions for the determination of the isoelectric point, the sedimentation coefficient and the molecular weight are given under 'Methods'. The sensitivity to *N*-ethylmaleimide was determined by preincubating the enzymic fraction with 5 or 10 mM-*N*-ethylmaleimide at 0°C for 30 min, followed by a 30 min incubation at 37°C, in the presence of activated DNA as described under 'Methods'.

Property	Polymerase ... A	B	C
pI	6.8	5.2	7
$s_{20,w}(S)$	7.5	6.3	5.9
Molecular weight	140000	110000	110000 (50000)
Optimum pH	8	7	8
Inhibition by <i>N</i> -ethylmaleimide	Yes	Yes	Yes
Optimum $[Mg^{2+}]$	5 mM	5 mM	5 mM
Optimum $[Mn^{2+}]$	Inhibition	0.1 mM	Inhibition
Inhibition by KCl	No (up to 200 mM)	Yes	Yes

isoelectric points of enzymes A and C are very similar (about 7), whereas the pI of enzyme B is 5.2. The optimum pH for the three enzymes is about 7–8, and all are greatly inhibited by 2 mM-*N*-ethylmaleimide.

The optimum $[Mg^{2+}]$ for the three enzymes is 5 mM. Only enzyme B exhibits an optimal $[Mn^{2+}]$, at 0.1 mM; the two other enzymes are inhibited at all concentrations of the metal. In the presence of synthetic double-stranded polydeoxynucleotides an optimal $[Mn^{2+}]$ of about 0.5 mM was obtained for the three enzymes. KCl inhibits enzymes B and C, whereas no inhibition up to 200 mM was observed with enzyme A.

Table 3 shows the template specificity of wheat DNA polymerases. Enzymes A, B and C are able to recognize activated DNA, poly[d(AT)], poly(dA)-dT₁₂, poly(dC)-dG₁₂, although with different efficiencies. The template poly(A)-dT₁₂ is recognized by enzymes A and B in the presence of Mn^{2+} , whereas only DNA polymerase A is active with this template in the presence of Mg^{2+} . The template poly(C)-dG₁₂ is not recognized by any of the three enzymes; only enzyme A is able to use to a significant extent the template poly(dT)·poly(A) (10:1), where poly(dT) is the template and poly(A) the primer.

Properties of mitochondrial DNA polymerase

As Table 3 shows, the mitochondrial enzyme is active with activated DNA, poly[d(AT)], poly(dA)-dT₁₂ and poly(dC)-dG₁₂. It is active with poly(A)-dT₁₂ only in the presence of Mn^{2+} , and it is not able to recognize poly(C)-dG₁₂ or poly(dT)·poly(A) (10:1). The effect of salt is shown in Fig. 3. When activated DNA is used as template, an important stimulation with a peak at 150 mM-KCl was observed. No such effect was observed when poly(dA)-dT₁₂ was used as template.

Exonuclease and 'proof-reading' activities of wheat DNA polymerases

As Table 4 shows, only enzyme B gives a positive answer when the exonuclease activities at the 3'- and

Fig. 3. Effect of KCl on wheat embryo mitochondrial DNA polymerase

Incubations were for 30 min as described under 'Methods', with various KCl concentrations. The assays were carried out in the presence of activated DNA and Mg^{2+} (○) or poly(dA)-dT₁₂ and Mg^{2+} (●). 100% activity with poly(dA)-dT₁₂ = 120 pmol of TMP incorporated/30 min; 100% activity with activated DNA = 68 pmol of TMP incorporated/30 min.

Table 3. Template specificity of DNA polymerases A, B, C and mitochondrial polymerase

Results are expressed in pmol incorporated/30 min. About 1 μ g of enzyme protein was used in the case of DNA polymerases A, B and C; 10 μ g of mitochondrial enzyme protein was used.

Template	Primer	Polymerase	Metal ion Precursor	Incorporation (pmol/30 min)							
				A		B		C		Mitochondrial	
				Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
Activated DNA			TTP	1.23	0.87	1.05	0.52	12.65	5.52	0.05	0.09
Poly[d(AT)]			TTP	1.77	1.55	1.95	1.05	17.76	9.22	0.04	0.05
Poly(A)	dT ₁₂		TTP	8.83	12.94	<0.01	5.85	<0.01	<0.01	0.03	<0.01
Poly(dA)	dT ₁₂		TTP	4.03	18.20	1.90	6.86	6.46	7.51	0.10	0.35
Poly(dC)	dG ₁₂		dGTP	8.36	18.14	4.49	4.77	12.12	14.31	0.15	0.25
Poly(C)	dG ₁₂		dGTP	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.025	<0.01
Poly(dT)	Poly(A)		dATP	3.70	—	0.80	—	<0.01	—	<0.01	<0.01

Table 4. *Exonuclease (a) and 'proof-reading' activities (b) on wheat DNA polymerases*

For the exonuclease assay the incubation mixture contained, in a final volume of 0.1 ml: 50 mM Tris/HCl, pH 7.9, 2 mM-MgCl₂, 2 mM-2-mercaptoethanol, 10 µg of bovine serum albumin, 0.5 mM-[³H] (dpA)₄-d(pA)₃₀₀ (3'-labelled substrate) or 0.3 mM-(dpA)₄₅₀·[¹⁴C] (dpa)₁₉₀ (5'-labelled substrate); when poly(dT) was present, the final concentration was one-fifth that of poly(dA). One unit of DNA polymerase was used. Incubation was carried out for 30 min at 30°C. Residual radioactivity was determined as for the DNA polymerase assay. For the 'proof-reading' experiments the incubation mixture contained in a final volume of 50 µl the following components: 50 mM-Tris/HCl, pH 7.9, 5 mM-MgCl₂, 5 mM-DTT, 5 µg of bovine serum albumin, 4 µM-[¹⁴C]TTP (110 c.p.m./pmol) and 1 unit of DNA polymerase. Incubation was carried out at 37°C for 30 min. Radioactivity was determined in an Intertechnique SL 4000 liquid-scintillation counter.

(a) Exonuclease activity

Substrate ...	Activity (pmol of residual label)			
	3'-Labelled poly(dA)		5'-Labelled poly(dA)	
	Minus poly(dT)	Plus poly(dT)	Minus poly(dT)	Plus poly(dT)
None	3.29	3.21	587	604
A	2.95	2.78	549	548
B	2.78	2.10	383	606
C	3.30	3.23	564	588
<i>E. coli</i> I	2.62	1.02	33	485

(b) 'Proof-reading' activity

Enzyme	Activity	
	(pmol of residual ³ H radioactivity)	(pmol of [¹⁴ C]TMP incorporated)
None	19.7	0
A	16.1	1.78
B	5.8	10.6
C	17.1	0.4
<i>E. coli</i> I	2.0	13.75

5'-end of labelled polynucleotides were tested. For determining the 'proof-reading' capacity of the poly(dA)-poly(dT) enzymes, template having a [³H] guanosine at the 3'-end of the primer poly(dT) was used. The 'proof-reading' capacity was measured by the ability to excise the unmatched base and the capacity of using the free 3'-end of the poly(dT) primer for [¹⁴C]TMP polymerization. As Table 4 shows, enzyme B is again the only one able to use such a template. *E. coli* DNA polymerase-I was used as control.

Discussion

Properties of cytoplasmic DNA polymerases A, B and C

We have reported previously the purification and characterization of two DNA polymerases, A and B, from the soluble fraction of ungerminated wheat embryos (Castroviejo *et al.*, 1975; Tarrago-Litvak *et al.*, 1975). The purification consisted of the following steps: salt fractionation, DEAE-cellulose chromatography, Sephadex G-150 gel filtration and a phosphocellulose step. By changing some of the purification steps we have been able to find a third

DNA polymerase that we have called C. The main improvement is a first phosphocellulose column after the (NH₄)₂SO₄ step. Most of the protein is not retained, whereas all the DNA polymerase activity is retained and can be eluted with high salt. A dramatic purification of about 100-fold is accomplished in this step. When the DEAE-cellulose step is done, most of the activity is not retained in the column (Fig. 2a). By using our previously published method (Castroviejo *et al.*, 1975), we had found only a small amount of the DNA polymerase activity that was not retained in the DEAE-cellulose column. These results can be tentatively explained by the presence of an inhibitor not retained in DEAE-cellulose and phosphocellulose columns. In our current purification procedure the inhibitor would thus be eliminated in the first phosphocellulose column, and enzyme C is then found in the DEAE-cellulose 'wash-out'. This would imply that enzyme C, in our previous method, was present in the DEAE-cellulose step, but was strongly inhibited. We have found that the 'wash-out' of the first phosphocellulose column contains a strong inhibitory activity against DNA polymerase activity. Whether this inhibitor is a nuclease, proteinase or a more specific factor remains to be established.

Mory *et al.* (1975) have described a high-molecular-weight DNA polymerase from wheat-germ. After only one chromatographic step they obtained a homogeneous enzyme. We have not been able to obtain similar results, and in our hands at least four chromatographic steps are necessary before a highly purified enzyme is yielded. The DNA polymerase of Mory *et al.* (1975) resembles DNA polymerases A, B and C purified in our laboratory in its high molecular weight and high activity in quiescent embryos. Many of the properties of DNA polymerase C are similar to those of the enzyme purified from the periwinkle *Vinca rosea* (Gardner & Kado, 1976).

The specific activities of DNA polymerases A, B and C shown in Table 1 are, to our knowledge, the highest described for DNA polymerases from higher plants. Only the purified enzyme from *Vinca rosea* has a comparable specific activity.

The molecular weights of the three enzymes are higher than 100000, as judged by sucrose-gradient centrifugation and Sephadex gel filtration, both in the presence of high-ionic-strength buffers. However, after several months at -20°C in 50% glycerol, DNA polymerase C, although still fully active, had mol. wts. between 40000 and 60000. Proteolytic cleavage, already described in animal cells (Hesslewood *et al.*, 1978), could explain this finding.

Besides the distinct chromatographic behaviour, enzymes A, B and C can be distinguished by their template specificities. As Table 3 shows, only enzyme A is able to recognize a template poly(A)-dT₁₂ in the presence of Mg²⁺ or Mn²⁺, whereas enzyme B will use it only in the presence of Mn²⁺ and enzyme C will not recognize it at all. Moreover, only enzyme A will use a synthetic RNA primer [poly(dT)·poly(A) (10:1)] to a significant extent, and it is the only enzyme resistant to 200 mM-KCl.

Properties of mitochondrial DNA polymerase from ungerminated wheat embryos

Mitochondria obtained by the method mentioned above (under 'Purification of mitochondrial DNA polymerase') gave no indication of contamination with plastids when monitored by electron microscopy. The DNA polymerase activity was only released after sonication and treatment of the organelles with detergent. By the criteria of chromatography and template-specificity, the mitochondrial enzyme resembles DNA polymerase B from the soluble cytoplasm. The mitochondrial activity is fully retained in a DEAE-cellulose column and elutes from a phosphocellulose column at the same ionic strength as does enzyme B (Fig. 2). The mitochondrial enzyme is active with poly(A)-dT₁₂ only in the presence of Mn²⁺, and it is not able to use a synthetic RNA primer, as Table 3 shows. Enzyme B and the mitochondrial DNA polymerase are thus quite similar.

Whether soluble DNA polymerase B is a release product of broken mitochondria or it is an enzyme present in the nuclear and extramitochondrial cytoplasmic fractions (as for DNA polymerase- γ from animal cells) remains to be established. As mentioned in the introduction, animal DNA polymerase- γ can be found in the nuclei as well as in the mitochondria. Although both enzymes are similar in many respects, they differ in salt-stimulation (Bolden *et al.*, 1977; L. Tarrago-Litvak, unpublished work). The same phenomenon is observed in wheat, since enzyme B is inhibited by KCl and the mitochondrial enzyme is strongly stimulated when activated DNA is used as template. Although there is no explanation for these results, we can speculate by assuming that part or all the mitochondrial DNA polymerase is coded in the nuclear genome, and that the passage of the enzyme into the mitochondria may involve some structural changes (loss or gain of subunits) affecting the enzyme activity.

It is noteworthy that although the wheat enzyme most similar to DNA polymerase- γ from animal cells is enzyme A (Tarrago-Litvak *et al.* 1975), we have not been able to detect this enzyme in wheat mitochondria.

Exonuclease and 'proof-reading' activities of wheat DNA polymerases

All our attempts to find a low-molecular-weight DNA polymerase similar to the β -enzyme in freshly purified preparations of quiescent embryos have failed (Castroviejo *et al.*, 1975). A phylogenetic study performed by Chang (1976) showed that an enzyme of the β -type appeared only in multicellular animals (sponges and higher organisms). No β -enzyme was found in bacteria, low eukaryotes and higher plants (wheat and *Vinca rosea*). A low-molecular-weight DNA polymerase has been described in tobacco, (Srivastava & Grace, 1974), beet (Tymonko & Dunham, 1977) and pea (Stevens & Bryant, 1976). Besides its size, the enzyme from beet has a high resistance to *N*-ethylmaleimide and an important affinity for chromatin, properties that are typical of DNA polymerase- β from animal cells. In our case we have not found a low-molecular-weight DNA polymerase in quiescent embryos, and, moreover, all the wheat polymerases are inhibited by *N*-ethylmaleimide. No DNA polymerase activity is found in purified nuclei from ungerminated embryos (results not shown). A detailed search for a low-molecular-weight DNA polymerase in germinated wheat has not been carried out.

Results obtained with yeast and other low eukaryotes showed that one of the two high-molecular-weight DNA polymerases has a 3'- and 5'-exonucleolytic activity associated with the poly-

merizing capacity (Chang, 1977). It has been speculated that from the point of view of evolution, DNA polymerase- β appears when the exonuclease activity associated with the DNA polymerase is lost (Chang, 1976). This idea is supported by the results shown in Table 4. Wheat enzyme B has a 3'- and 5'-exonuclease activity, as well as a 'proof-reading' capacity. This kind of activity is not shown by enzymes A and C. The latter two enzymes seem to be antigenically related, as is shown in the following paper (Castroviejo *et al.*, 1979). Another interesting similarity with the yeast enzyme is that the enzyme having exonuclease activity in yeast is not able to use synthetic RNA primers for DNA synthesis. As seen in Table 3, enzyme A is the only one able to use the primer RNA and does not possess the exonuclease activity. Although it is tempting to compare plant DNA polymerases with animal or bacterial enzymes, we think it is too early to draw any conclusion at this stage of our work. In the case of quiescent wheat embryos the situation seems closer to that of lower eukaryotes, since we have not detected low-molecular-weight DNA polymerases, we have not found a chromatin-associated activity, and one of the high-molecular-weight DNA polymerases from wheat has an exonuclease-associated activity.

We are deeply grateful to Dr. L. M. Chang for a gift of exonuclease and 'proof-reading' substrates and to Dr. M. Duvert for electron microscopy. This work was supported by C.N.R.S. and the University of Bordeaux II.

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