Partial purification and characterization of two cytoplasmic DNA polymerases from ungerminated wheat

M. Castroviejo, L. Tarragó-Litvak and S. Litvak

Laboratoire de Biochimie, Université de Bordeaux II, 351 Cours de la Libération, 33405 Talence, France

Received 5 September 1975

ABSTRACT. Two DNA polymerases have been purified from the 105,000 x g supernatant of ungerminated wheat. The purification stages included: high speed centrifugation, salt fractionation, DEAE-cellulose chromatography, Sephadex G-150 filtration and phosphocellulose chromatography.

Several properties of the two enzymes (called A and B according to the order of elution from the phosphocellulose column) have been studied. Enzyme A has a sedimentation coefficient of about 7 S, utilizes activated DNA and synthetic polydeoxynucleotides as well as poly rA-dT, while B has a sedimentation coefficient of about 6.2 and uses only activated DNA and synthetic polydeoxynucleotides as templates.

Other parameters like KCl effect, MnCl₂ effect, optimum pH, etc. allow us to distinguish clearly between both DNA polymerases.

INTRODUCTION. The existence of multiple forms of DNA polymerases in eukaryotic tissues is well established. (For two recent reviews see references 1 and 2).

In the case of mammalian DNA polymerases, when aqueous extraction methods are used, more than 50% of the DNA polymerase activity is found in the cytoplasmic fraction associated with the mitochondria, microsomes and soluble fraction. Similar results have been described in chicken embryos (3) and sea urchin embryos (4).

Although a great deal of information exists in animal systems concerning the multiple DNA polymerases, very little is known on the DNA synthesizing enzymes from plant organisms.

DNA synthesis in maize seeds has been described (5). The purification of an enzyme from wheat embryos has been achieved (6) and a high molecular weight enzyme has been found in tobacco cell cytoplasm (7).

The wheat embryo system is a beautiful example of a quiescent organism, that under determined conditions (water imbibition) starts a cascade reaction for the biosynthesis of RNA, proteins and DNA that leads to a
highly proliferative system. It seemed to us that the study of DNA synthesis in this system could answer two types of questions. First, the problem concerning the relationship between cellular distribution and physiological activities of the multiple DNA polymerases, and second, the question of the biosynthesis and level of the multiple DNA polymerase that could give some clues on the enzyme(s) involved in the replication of DNA and in its repair.

In this paper, we want to describe the study of the cellular distribution of DNA polymerases in ungerminated wheat embryos and the purification and properties of the two most conspicuous enzymes found in the soluble fraction of this organism.

**MATERIALS.**

Commercial wheat germ was kindly supplied by Les Grands Moulin de Bordeaux and wheat seeds were a kind gift of Dr. J. Bové (INRA, 33 Pont de la Maye).

Ion exchange resins (DEAE-cellulose DE-22 and phosphocellulose P-11) were from Whatman. They were prepared following the technique described by Burgess.

Labelled products were provided by The Radiochemical Center Amersham. Unlabelled nucleoside triphosphates were bought from Sigma and Boehringer.


Poly A, poly C and poly U were from Biopolymers.

Deoxyribonuclease I and ribonuclease I were products of Sigma.

E. coli DNA polymerase was provided by Boehringer Mannheim GmbH.

**METHODS.**

1. Protein determination. Protein in the eluates of chromatographic columns was determined at 280 nm or by the Lowry method.

2. Buffer A: Tris-HCl 50 mM pH 7.5, β-mercaptoethanol 1 mM, EDTA 0.1 mM and glycerol 20%. Buffer B: Tris-HCl 50 mM pH 7.9, β-mercaptoethanol 1 mM, EDTA 0.1 mM, glycerol 20%. Buffer TKM: Tris-HCl 50 mM pH 7.2, KCl 25 mM, MgCl2 5 mM, sucrose 250 mM.

3. Preparation of wheat embryos. The method of Johnson and Stern was used.
4.- Cellular Fractionation. The method used was essentially the same as that described by Baril et al.  

Ten grams of embryos were homogenized in an ice cold mortar with 5 volumes of TKM buffer. The homogenate was filtered through four layers of cheesecloth and the filtrate was passed through one layer of Miracloth. The filtrate was centrifuged at 600 x g for 10 minutes. The supernatant (S₁) was put aside for further fractionation (see below). The pellet (P₁) was resuspended in buffer TKM, layered over one volume of buffer TKM in sucrose 2.3 M and centrifuged at 90,000 x g for one hour. The pellet was resuspended in TKM, and Triton X-100 was added to a final concentration of 1%. After stirring for 15 minutes at 4°C, the nuclear fraction was centrifuged at 600 x g for 15 minutes and the pellet washed twice in buffer TKM. The final pellet (Nuclei) was resuspended in buffer TKM.

The first supernatant (S₁) was centrifuged at 10,000 x g for 15 minutes and the supernatant (S₂) put aside (see below). The pellet washed with TKM and centrifuged at the same speed three times. The final pellet (Mitochondrial fraction) was resuspended in buffer TKM.

The first mitochondrial supernatant (S₂) was centrifuged at 105,000 x g for 1 hour and the pellet (Microsomes) were suspended in buffer TKM. The microsomal supernatant (S₃) was centrifuged at 80,000 x g for 15 hours and the pellet (Ribosomes) resuspended in buffer TKM. The ribosomal supernatant corresponds to the soluble cytoplasmic fraction.

5.- DNA polymerase assay. In a final volume of 0.05 ml the assay mixture contained: 2.5 umoles Tris pH 8.0 (2.5 umoles MES pH 7.0 in the case of B), 0.25 umoles MgCl₂, 5 umoles DTT, 5 nmoles of dATP, dCTP, dGTP each, 2.5 nmoles [³H]-TTP (0.15 C/mmol), 1.5 ug activated DNA. After phosphocellulose chromatography, 5 ug of bovine serum albumin were added to each enzymatic assay. Incubation was carried out at 37°C for different lengths of time. The reaction was stopped by addition of 0.1 ml of ice cold 20% TCA with 1% pyrophosphate carrier. The mixtures were filtered over nitrocellulose filters (Schleicher and Schuell), washed with 5% cold TCA, dried and counted in a scintillation mixture containing 4 g PPO, 0.2 g POPOP in 1 l toluene.

6.- Sucrose gradient centrifugation. The sedimentation coefficient was determined as described by Martin and Ames. Sucrose gradient centrifugation was run in the MSE ultracentrifuge (rotor: 6 x 5 ml) at 400,000 x g for 6 hours at 4°C. (5 to 20% sucrose).

7.- Preparation of synthetic double stranded polynucleoti-
des. Polynucleotide solutions were made 0.2 O.D. 260/ml in 10 mM Tris pH 7.5, 5 mM MgCl₂, and 100 mM NaCl. After mixing the appropriate polynucleotides, the solution was heated at 75°C for 10 minutes (for poly rC-poly dG the temperature was 90°C) and left at room temperature for 30 minutes. The template-initiator ratio was 5:1.

8.- DNA activation. The method of Aposhian and Kornberg was used with pancreatic DNase I.

9.- Nuclease activity assay. The assay mixture contained in a final volume of 0.1 ml: 50 mM Tris pH 7.5, 10 mM MgCl₂, 20-30 μg of protein and the following amounts of nucleic acid: 5-10 nmoles of [3H]-poly rA or [3H]-poly rA-poly dT and 2-3 μg of [3H]labelled DNA. After 30 minutes at 37°C the reaction was stopped and the remaining radioactivity counted as described for the polymerase assay.

10.- Polyacrylamide gel electrophoresis. Electrophoresis under non denaturating conditions was performed as described by Davis. SDS gel electrophoresis was done using the method of Weber and Osborn.

RESULTS.

1.- Intracellular distribution of DNA polymerases. As seen in Table I, when ungerminated wheat embryos are fractionated as described in Methods, 99% of the activity is found in the cytoplasm. Most of this activity does not sediment after 15 hours at 80,000 x g. Nuclei prepared by this method although inactive in DNA synthesis were active in RNA synthesis (unpublished results). Only a very small contamination with mitochondria was observed in the microsomal and ribosomal fraction when examined by electron microscopy.

2.- Purification procedure. All manipulations were performed at 0-4°C. Preparation of the crude homogenate. Ungerminated wheat embryos or commercial wheat germ were homogenized in a mortar with 4 volumes of TKM buffer and then filtered through 4 layers of cheesecloth. The filtrate was centrifuged at 15,000 x g for 10 minutes. The supernatant was centrifuged in the MSE ultracentrifuge (rotor: 8 x 50 ml) at 105,000 x g for 60 minutes. The proteins precipitating between 20 and 70% ammonium sulphate were collected. They were dissolved in 5 volumes of buffer A and dialyzed against 500 volumes of this buffer overnight. The solution was adsorbed onto a DEAE-cellulose column equilibrated with buffer A. The co-
Table I.
Distribution of DNA polymerase activity in the subcellular fraction prepared from ungerminated wheat.

<table>
<thead>
<tr>
<th>Site</th>
<th>% Total Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>0.4</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1.9</td>
</tr>
<tr>
<td>Microsomes</td>
<td>10.5</td>
</tr>
<tr>
<td>Ribosomes</td>
<td>7.4</td>
</tr>
<tr>
<td>Soluble</td>
<td>79.8</td>
</tr>
</tbody>
</table>

Fractions were prepared as described in Methods. Reactions were performed with 10-20 μg of enzyme protein as described for the assay system.

Lumn was washed with the same buffer until the absorbance at 280 nm was less than 0.2. A KC1 gradient from 0 to 0.4 M in buffer A was applied. As seen in Fig. 1A the main peak eluates at about 0.15 M KC1. Another peak is obtained at lower concentrations of KC1; this peak corresponds to enzyme(s) attached to ribosomes that have not sedimented in 1 hour at 105,000 x g. (See discussion for more details on this enzyme). The peak eluting at about 0.15 M KC1 was precipitated with 70% ammonium sulphate, dissolved in buffer B containing 100 mM KC1 and filtered through a Sephadex G-150 column equilibrated with the same solution. The tubes containing the activity were pooled and precipitated with 70% ammonium sulphate. The pellet was dissolved in buffer B containing 200 mM KC1 and dialyzed extensively against this buffer.

The "Sephadex-Enzyme" was layered over a phosphocellulose column equilibrated with buffer B plus 200 mM KC1. Most of the protein eluted in the first tubes as shown in Fig. 1B. No polymerase activity was found associated with the bulk of the protein. A gradient of KC1 between 0.2-0.8 M in buffer B was established and two peaks of DNA polymerase activity were obtained, one eluting at about 0.48 M KC1 and the other at 0.56 M KC1. Both peaks were pooled and concentrated separately with ammonium sul-
Figure 1. Purification of DNA polymerase by column chromatography on DEAE-cellulose (1A) and phosphocellulose (1B).

1A. The volume of the column was 520 ml and each fraction had a volume of approximately 20 ml. Protein concentration was determined at 280 nm and polymerase activity was determined with 10 μl of each fraction as described in the text.

1B. The volume of the column was 215 ml. Protein concentration and enzyme activity was determined as in 1A, but 5 μg of bovine serum albumin was added for DNA polymerase activity.
phate and dialyzed against buffer B plus 200 mM KCl. The enzymes were kept at -20°C in 50% glycerol without loss of activity for a period of 6 months.

Table II shows the purification scheme of DNA polymerases A and B. This Table and Fig. 1A and 1B correspond to a purification from 400 g of wheat germ.

Table II.
Purification of DNA polymerases A and B.

<table>
<thead>
<tr>
<th>PROCEDURE</th>
<th>VOL (ml)</th>
<th>Units ml</th>
<th>Total Units</th>
<th>Protein mg/ml</th>
<th>Units mg Prot</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>200</td>
<td>1.80</td>
<td>360.00</td>
<td>90</td>
<td>0.020</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Ammonium Sulphate</td>
<td>60</td>
<td>3.24</td>
<td>200.40</td>
<td>59</td>
<td>0.057</td>
<td>56</td>
<td>2.8</td>
</tr>
<tr>
<td>DEAE - cellulose</td>
<td>100</td>
<td>2.94</td>
<td>470.40</td>
<td>4</td>
<td>0.735</td>
<td>130</td>
<td>3.58</td>
</tr>
<tr>
<td>Sephadex G-150</td>
<td>25</td>
<td>19.10</td>
<td>492.50</td>
<td>18</td>
<td>1.094</td>
<td>137</td>
<td>5.47</td>
</tr>
<tr>
<td>Phosphocellulose A</td>
<td>4</td>
<td>10.16</td>
<td>40.94</td>
<td>16</td>
<td>6.350</td>
<td>113</td>
<td>317.0</td>
</tr>
<tr>
<td>Phosphocellulose B</td>
<td>8</td>
<td>11.32</td>
<td>90.56</td>
<td>0.7</td>
<td>16.171</td>
<td>25.1</td>
<td>808.5</td>
</tr>
</tbody>
</table>

A unit of enzyme is defined as the amount of DNA polymerase able to polymerize 1 nmole of [3H]-TMP per minute at 37°C. Activated DNA was used in the reaction mixture.

3.- Purity of enzymes A and B. The DNA polymerases A and B obtained as described above are not homogeneous. By polyacrylamide gel electrophoresis under non denaturing conditions, both enzymes entered poorly into a 4% acrylamide gel. After 5 hours at 4 mA per tubular gel, only one band at about 2 mm from the origin was observed. In SDS gels (10% acrylamide), A gave a main band, corresponding to 70-80% of the total stained proteins. This band of 85,000 daltons, coincided with the peak of DNA polymerase activity obtained in a 5-20% sucrose gradient centrifugation (unpublished results).

Enzyme B gave 3 bands of equal importance in SDS gels.

We were not able to detect the following activities in the A and B preparations: RNase, RNase H, DNase I and RNA polymerase II (21).

Table III shows some of the properties of the system. Both enzymes are completely inactive in the absence of DNA or in the presence of pancreatic DNase, while the addition of pancreatic RNase does not
Table III.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>11.2</td>
<td>13.0</td>
</tr>
<tr>
<td>Minus DNA</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Minus Protein</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Plus DNase I</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Plus RNase I</td>
<td>10.7</td>
<td>12.8</td>
</tr>
<tr>
<td>Minus DTT</td>
<td>2.9</td>
<td>11.0</td>
</tr>
<tr>
<td>Minus unlabelled dATP</td>
<td>2.4</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Some properties of DNA polymerases A and B.

When indicated reaction mixture contained 1 ug of RNase I and DNase I.

affect the incorporation of TMP into DNA.

The omission of DTT markedly affects A while B activity is less inhibited. Both enzymatic activities are drastically affected by the omission of unlabelled dATP, dCTP and dGTP.

The Michaelis constant for TTP has been determined by the double reciprocal method of Lineweaver and Burk (17): in both cases they were similar and in the range of 1.2-2.5 x 10^{-5} M.

4.- Sedimentation behaviour. As seen in Fig. 2, enzyme A has a sedimentation coefficient of about 7 and B gave an S value of approximately 6.2. Assuming a globular conformation and using yeast ADH and bovine serum albumin as standards, we obtained a molecular weight of 150,000 for A and 110,000 for B. The formula of Martin and Ames:

\[
\frac{S_1}{S_2} = \left(\frac{MW_1}{MW_2}\right)^{2/3}
\]

was used for this purpose (13).

5.- Template specificity. Table IV shows the behaviour of enzymes A and B with different templates. Both enzymes are very active with DNase I-activated calf thymus DNA, while native DNA (calf thymus or wheat germ) is a very poor template for both polymerases.

Although both enzymes recognize the synthetic templates poly dA-dT and poly (dAT)-poly d(AT), the ratio of the relative activities, activated DNA: poly dAT, poly dAT-poly d(AT) is 1: 1.16: 0.78 for A and 1: 1.67: 1 for B.

While poly dA-rU can serve as template for both enzymes with similar efficiency, a synthetic double stranded RNA, poly rA-rU is completely inactive as template. A very interesting property of enzyme A
Figure 2. Glycerol gradient centrifugation of DNA polymerases A and B;

Conditions are described in the text. Approximately 100 μg of enzyme protein.

Figure 3. The effects of reaction mixture pH upon the activity of DNA polymerases A and B;

Reactions contained 5 μg of enzyme protein. Other components of the incubation mixture are described in the text.

A: △ 50 mM Tris-HCl.
B: ■ 50 mM MES.
△ 50 mM Tris-HCl.

Figure 4. The effect of KCl on the activity of DNA polymerases A and B.

Reactions containing 5 μg of enzyme protein were incubated for 30 minutes as described in the text.

A: △ 0.1 to 0.2 M KCl
B: ○ 0.1 to 0.2 M KCl
Template specificity of DNA polymerases A and B.

Reaction mixtures contained 0.01 OD\textsubscript{590} of the synthetic template. Other components were as described in Methods.

Table IV.

<table>
<thead>
<tr>
<th>Template</th>
<th>Polymerase A</th>
<th>Polymerase B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly A-dT\textsubscript{12}</td>
<td>10.1</td>
<td>10.1</td>
</tr>
<tr>
<td>Poly d(A-T)</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Poly dA-poly dC</td>
<td>11.2</td>
<td>12.3</td>
</tr>
<tr>
<td>Activated DNA</td>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td>Poly dA-dT\textsubscript{12}</td>
<td>6.4</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Poly dA</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Poly dA-rU</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Poly dA-rU</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Activated DNA</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

Table V.

Study of the effect of some inhibitors on the activity of DNA polymerases A and B.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>% Residual activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethidium bromide 4 mM</td>
<td>70</td>
</tr>
<tr>
<td>Orthophenanthroline 1.2 mM</td>
<td>45</td>
</tr>
<tr>
<td>Ethanol 50%</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>NEM 3 mM</td>
<td>37</td>
</tr>
</tbody>
</table>

is its capacity to recognize poly rA-dT\textsubscript{12}, while B does not use this template. A more detailed study on this property will be published elsewhere (L. Tarrago-Litvak, M. Castroviejo and S. Litvak, in preparation).

6. Comparative studies between A and B.

a) Optimum pH. Enzyme A has an optimum pH between 8-8.5, while enzyme B has a more acidic optimum pH around 7 (Fig. 3).

b) Effect of monovalent cations. As seen in Fig. 4, polymerase A is insensitive to KCl up to a concentration of 0.2 M, while enzyme B is inhibited to 20% of the control activity in the presence of a concentration of 0.1 M KCl.

c) Effect of divalent cations. As shown in Fig. 5A both enzymes have a maximal activity between 2 and 6 mM Mg\textsuperscript{++}. In the case of Mn\textsuperscript{++} (Fig. 5B) polymerase A is inhibited even at very low concentrations, while enzyme B has an optimum at 0.1 mM Mn\textsuperscript{++} and is inhibited at concentrations higher than 0.3 mM.

d) Effect of inhibitors. Table V shows the effect of some inhibitors on the activity of both DNA polymerases. Ethidium bromide and orthophenanthroline have a stronger effect on enzyme B; ethanol and NEM (N-ethyl maleimide) drastically inhibit enzyme A, while B is affected to a lesser extent.
Figure 5. The effect of divalent cations on the activity of DNA polymerases A and B.

Reactions were performed as described in Methods except for the indicated concentrations of Magnesium chloride (5A) and Manganese chloride (5B). In each experiment were added 5μg of enzyme protein (20 min. incubation).

Enzyme A: Δ-----Δ
Enzyme B: O---O
DISCUSSION.

Two forms of DNA polymerases, in addition to the mitochondrial enzyme, have been found in the cytoplasmic fraction of ungerminated wheat embryos. A very low activity was recovered in the nuclear fraction; this low level did not allow us to characterize the polymerase(s) associated with the nuclei. Preliminary results obtained in our group indicate that after 36 hours of germination, nuclear activity is increased. This situation is similar to the one described for lymphocytes stimulated with phytohemagglutinin. Interestingly, this latter effector has been widely used in animal cells, while its role in plant systems is as yet not clear. In the case of wheat germ agglutinin, this protein is synthesized at the beginning of germination and it would be tempting to speculate that it may have something to do with the transport of polymerase(s) from the cytoplasm to the nucleus.

Enzyme activity associated with ribosomes and microsomes is released with 0.2 M KCl. The activity eluting at low salt concentration from the DEAE-cellulose column (Fig. 1A) corresponds to polymerase associated with ribosomes that did not sediment in 1 hour at 105,000 x g. This enzyme activity when released from ribosomes with KCl behaves like enzyme A as judged by phosphocellulose chromatography, effect of KCl and the use of poly rA-dT as template.

We have not been able to find a low molecular weight DNA polymerase (3-4 S) in fresh preparations, although in some aged preparations of enzyme A a peak sedimenting at 4.5 to 5.0 S has been obtained. It is interesting to point out that both in yeast and slime mold no low molecular weight DNA polymerase has been found.

The enzymes we have purified from ungerminated wheat seem to be different from the one purified by Mory et al. These authors reported the isolation of an enzyme of 230,000 daltons as determined by sucrose gradient centrifugation and 17,000 daltons in SDS gels; as they used germinated wheat embryos the difference may be ascribed to the physiological stage of their material.

It is interesting to point out that a cytoplasmic DNA polymerase of sedimentation coefficient 7 S, has been described in tobacco cell culture; as this enzyme is not able to use poly rA-dT as template, it may be compared with our polymerase B.

By several criteria we can affirm that polymerase A and polymerase B are not able to use poly dA-dU as template, indicating that an RNA primer might be involved; this finding may be important if the role of RNA as initiator as
observed in prokaryotes, is extended to eukaryotes (19).

The pH optimum, KCl effect, manganese effect, etc. can clearly distinguish between both enzymes. Orthophenanthroline, a specific chelating agent for Zn, can inhibit both polymerases as described before for other DNA polymerases (1), but the inhibitory effect is more important with enzyme B. DTT is practically not needed for optimal activity by enzyme B. This confirms the result with NEM which does not affect B, while decreasing markedly the activity of A.

This is to our knowledge the first report on two different cytoplasmic DNA polymerases in plant systems. In animal cells multiple soluble polymerases have been described in several cases (1, 2).

Our aim is to study the behaviour of these multiple DNA polymerases during the germination of wheat embryos. It is too early to say whether these DNA polymerases play a role in DNA synthesis in the cytoplasm itself and the evidence presented in that sense requires confirmation (20).

We think that one of the early events in germination is the passage to the nucleus of DNA polymerases previously stocked in the cytoplasm. The distribution, purification and characterization of DNA polymerases after different times of germination is currently under study in our laboratory.

ABBREVIATIONS.


ACKNOWLEDGMENTS.

The authors are grateful to Dr. G. Brun for many fruitful discussions and to Dr. A.L. Haenni for revising the manuscript.

This work was supported by grants from the C.N.R.S. (A.I. 996083 and A.T.P. 1897). The initial part of this work was supported by grant 73-7-1881 from the D.G.R.S.T. to B.Labouesse.

REFERENCES.

Nucleic Acids Research