STUDIES ON A DNA POLYMERASE γ-LIKE ENZYME FROM WHEAT EMBRYOS

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1. Introduction

Multiple DNA polymerases have been found in eukaryotic cells differing in properties and intracellular location.

In the case of vertebrates at least four different DNA polymerases are found [1]. Besides the mitochondrial enzyme three other cellular DNA polymerases, named with Greek letters, are well studied. DNA polymerase α found in the cytoplasm and nuclei is the most abundant, (80–90% of the total enzymatic activity of the cell). It has a mol. wt between 110–220 000 and is sensitive to sulfhydryl reagents. DNA polymerase β is a low mol. wt protein (about 45 000) confined to the nuclear fraction. It is insensitive to SH-reagents and recognizes activated DNA and at a lesser extent poly rA-dT_{12}. DNA polymerase γ is the latest found in animal cells. It was first described in HeLa cells [2] and recently found in other organisms [3–7]. Its main property is to recognize poly rA-dT_{12} at a much higher rate than a deoxyribonucleic acid template like poly dA-dT_{12}. Its mol. wt in the case of HeLa is 110 000 and requires sulfhydryl groups for maximal activity.

We have purified two distinct DNA polymerases from the cytoplasmic fraction of ungerminated wheat embryos called A and B according to the order of elution from a phosphocellulose column [8]. The main difference between both enzymes is that enzyme A is able to recognize poly rA-dT_{12} better than activated DNA while the B enzyme uses only activated DNA and polydeoxyribonucleic acid templates. Other differences concern a more basic optimal pH for enzyme A and the sensitivity of enzyme A to high ionic strength and sulfhydryl reagents, while enzyme B is not affected.

In this article we want to describe the purification and some further properties of DNA polymerase A. Several templates have been tested and the K_M for TTP with poly rA-dT_{12} and activated DNA as templates were determined. Moreover the protection of the enzyme from thermal denaturation by synthetic polydeoxynucleotides is described.

2. Materials and methods

2.1. *Materials*

Commercial wheat germ was a gift of Les Grands Moulins de Bordeaux. Wheat seeds were a kind gift of Dr J. M. Bové.

[^{3}H]TTP, [^{3}H]GTP, [^{3}H]CTP, [^{3}H]dATP were obtained at The Radiochemical Center, Amersham. [^{3}H]UTP was from the Commissariat à l'Energie Atomique (Saclay).

Unlabelled nucleoside triphosphates came from Boehringer, Manheim GmbH. Single stranded polynucleotides were from Miles, Pabst Laboratories and Biopolymers.

2.2. *Methods*

2.2.1. Buffers

(a) Buffer A: Tris–HCl 50 mM pH 7.5, β-mercaptoethanol 1 mM, EDTA 0.1 mM and glycerol 20%.
(b) Buffer B: Tris–HCl 50 mM pH 7.9, β-mercaptoethanol 1 mM, EDTA 0.1 mM, glycerol 20%.

2.2.2. Enzyme purification

A detailed account of the purification procedure will be described elsewhere [8]. All steps were performed at 4°C. Wheat germs or wheat embryos prepared according to Johnston and Stern [9] were

homogenized with 4 vol of buffer A in a mortar and filtered through cheesecloth and Miracloth. The filtrate was centrifuged at 10 000 *g* for 10 min and the supernatant centrifuged at 105 000 *g* for 1h. The supernatant was precipitated between 20 and 70% ammonium sulphate and the pellet dialyzed against buffer A. A DEAE-cellulose chromatography was carried out with a KCl gradient between 0–0.4 M KCl in buffer A. The tubes containing the activity were pooled and precipitated at 70% with ammonium sulphate. After dissolving the precipitate in buffer A plus 100 mM KCl, a Sephadex G-150 chromatography was performed. The tubes with DNA polymerase activity were pooled and concentrated with ammonium sulphate, dissolved and dialyzed against buffer B plus 200 mM KCl. A phosphocellulose chromatography between 0.2 and 0.8 M KCl in buffer B was performed, and the two peaks containing DNA polymerase activity were concentrated separately with ammonium sulphate.

2.2.3. Enzyme assay

In a final vol of 0.05 ml the assay mixture contained: 50 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 100 mM DTT, 0.05 mM [^{3}H]TTP (50 to 150 counts × min^{-1} × $pmol^{-1}$), 0.05 A_{260} unit of poly rA-dT_{12}. When activated DNA [10] was used as template (1.5 μg), 0.1 mM each of dATP, dCTP and dGTP were also added. When the phosphocellulose enzyme was used 5 μg of bovine serum albumin were included. Incubation at 37°C was carried out for different lengths of time. The reaction was stopped by addition of 0.1 ml of ice cold 20% TCA plus 1% sodium pyrophosphate. The precipitate was filtered through nitrocellulose filters (Schleicher and Schüell), washed with cold 5% TCA, dried and counted in 5 ml PPO–POPOP–toluene scintillation mixture.

2.2.4. Preparation of synthetic double-stranded polynucleotides

Polynucleotide solutions were made 2.0 A_{260} unit/ml in 10 mM Tris pH 7.5, 5 mM $MgCl_2$. 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 min. The ratio of template to initiator was 5:1.

2.2.5. Protein determination

The method of Lowry et al. [10] was used.

3. Results

3.1. *Purification and purity of the enzyme*

As seen in Table 1 the activity of DNA polymerase A using poly rA-dT_{12} is extremely low in the first

Table 1
Purification of DNA polymerase A

PROCEDURE	Vol. (ml)	Units / ml	Total Units	Protein mg/ml	Units / mg prot.	Purification
Crude Extract	200	0.30	60.0	90	0.0033	1.0
Ammonium Sulphate	60	0.27	16.2	59	0.0457	13.8
DEAE-cellulose	160	0.32	51.2	4	0.0800	24.2
Sephadex G-150	25	5.04	126.0	18	0.2800	84.8
Phosphocellulose	4	13.74	55.0	1.6	8.5900	2603.0

A unit of enzyme is defined as the amount of DNA polymerase able to polymerize 1 nmole of [^{3}H]TMP per minute at 37°C.

three steps of purification. This is due to a very active ribonuclease that hydrolyses poly rA as tested with [^{3}H]poly rA. This RNase is completely eliminated in the phosphocellulose column, since it is not retained by the resin, while enzyme A is eluted at about 0.45 M KCl.

No contaminations with RNase, RNase H, DNase [12], or RNA polymerase [13] was observed after phosphocellulose chromatography.

Under non-denaturing conditions the purified enzyme does not enter to a 4% acrylamide gel, however, using a 10% SDS gel according to the method of Weber and Osborn [14], a main band corresponding to about 70% of the total protein is obtained. This band has a mol. wt of approx. 85 000.

3.2. *Sedimentation coefficient*

By sucrose gradient centrifugation it has been possible to determine the sedimentation coefficient and an approximative molecular weight for the enzyme, using the method of Martin and Ames [15]. As seen in fig.1 the enzyme has a sedimentation coefficient of about 7.2, corresponding to a mol. wt of 150 000, assuming that the protein has a globular conformation. The sedimentation profile was symmetrical in fresh preparations but with some aged enzymes a second peak sedimenting at 4.5 S was observed. No difference was observed when 0.5 M KCl was included in the sucrose gradient.

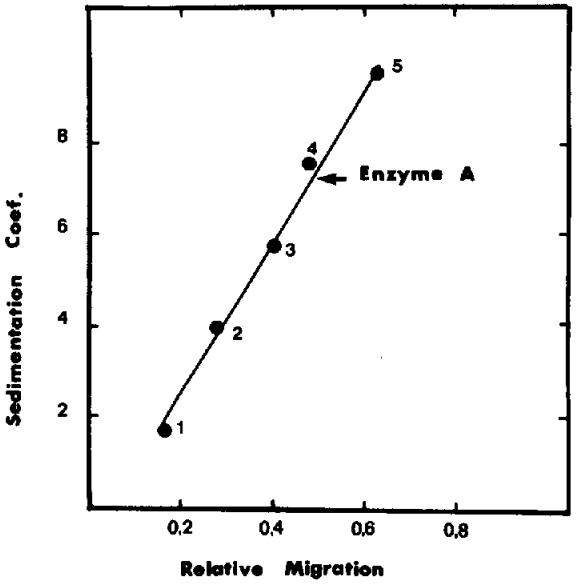

Fig.1. Sucrose gradient centrifugation of DNA polymerase A. Enzyme protein (100 μg) was centrifuged on a 10–30% sucrose gradient in 50 mM Tris pH 7.5 for 15 h at 250 000 g at 4°C in the MSE ultracentrifuge (rotor 6 × 5 ml). The standards used were: (1) cytochrome, (2) bovine serum albumin monomer, (3) *E. coli* DNA polymerase I, (4) yeast alcohol dehydrogenase, (5) pyruvate kinase.

3.3. *Template specificity*

As seen in Table 2 the enzyme prefers the RNA–DNA hybrid poly rA-dT_{12} to all the other synthetic template-primers used. The synthetic hybrid poly rC-poly dG is also used, but to a much lower extent than poly rA-dT_{12} or poly rA-poly dT, poly rC-dG_{12} is not recognized by enzyme A in our assay conditions. The enzyme is also able to use the poly rA strand of poly rA-poly dT as an initiator for the polymerization of dAMP, although at a lesser rate than poly rA-dT_{12}.

Preliminary experiments indicate that when globin messenger RNA was used as template and oligo dT_{12} as primer [20], no incorporation of deoxynucleotides triphosphates was observed (not shown).

Table 2
Template specificity of DNA polymerase A

TEMPLATE SPECIFICITY		
Template	^{3}H Substrate	pmoles
Poly rAdT 12	TTP	18.2
Poly dA-dT12	TTP	9.8
Poly rA-rU 8	TTP	<1.0
Poly rA	TTP	1.8
Poly dG-Poly rC	dGTP	2.5
Poly rC	dGTP	<1.0
Poly rI-Poly rC	dGTP	<1.0
Poly dG-Poly rC	dCTP	1.2
Poly rI-Poly rC	dCTP	<1.0
Poly rA-Poly dT	dATP	4.3
Activated DNA	TTP	8.7
Poly rC-dG 12	dGTP	<1.0

Reaction mixture contained 5 μg of enzyme protein and 0.05 A_{260} unit of template. Incubation was carried out for 30 min at 37°C as described in Methods.

3.4. *Kinetic parameters*

The K_M for TTP using the method of Lineweaver and Burk has been determined for the enzyme A using poly rA-dT_{12} and activated DNA as templates. In the first case a value of about 1.7 μM was observed, while in the case of activated DNA a value approximately of 12 μM was determined, as seen in fig.2.

3.5. *Thermal denaturation studies*

As shown in fig.3 the A enzyme activity is diminished to less than 10% of the control, when preincubated at 50°C for 15 min in the absence of a polynucleotide. In the presence of poly rA or poly rA-dT_{12} a strong protection against thermal inactivation is observed, while in the case of poly dA or poly dA-dT_{12} the protection is less important than in the former case.

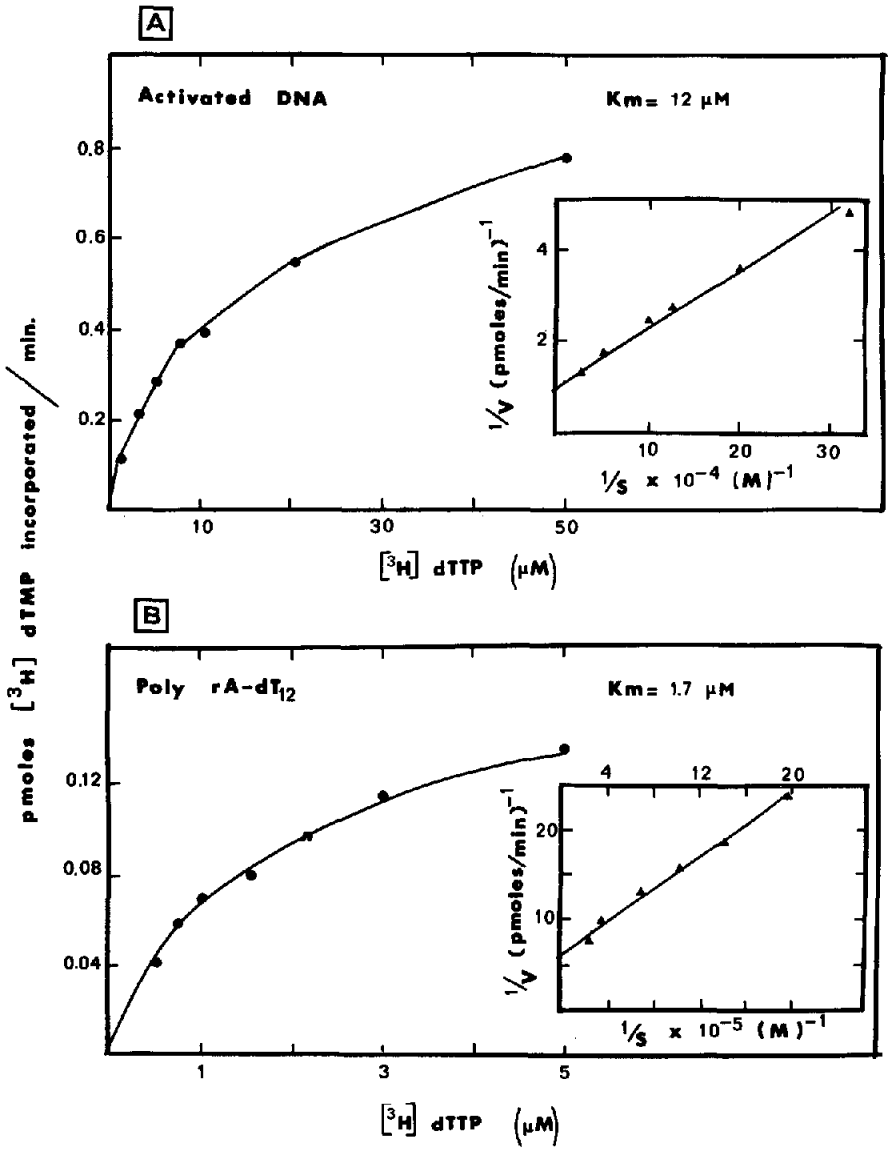

Fig.2. Determination of the K_M for TTP of DNA polymerase A in the presence of activated DNA (A) and poly rA-dT_{12} (B). Concentrations of the templates are given under enzyme assay.

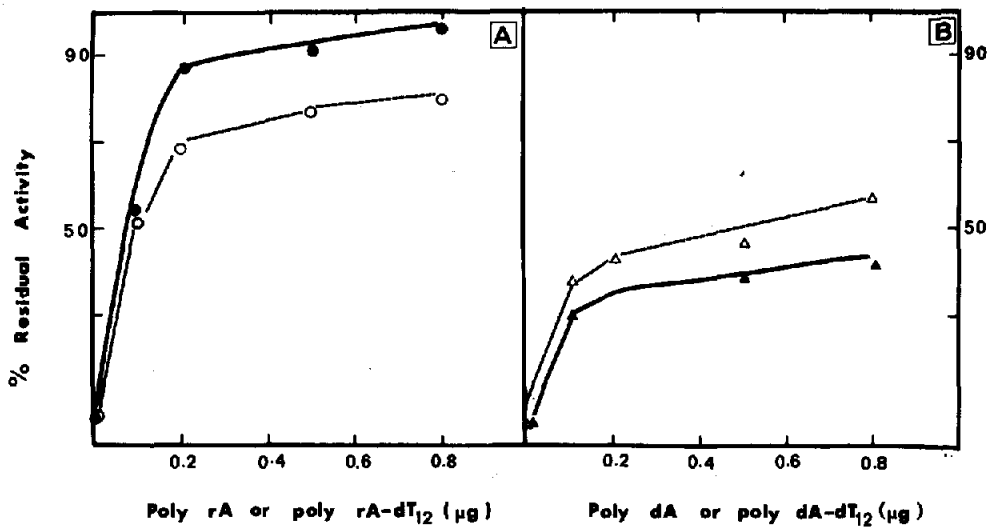

Fig.3. Study of the effect of different polynucleotides on the thermal inactivation of DNA polymerase A. A preincubation of 15 min at 50°C was performed in the presence of 5 μg of enzyme protein and different amounts of template. The incubation mixture was then completed by adding the components of the enzymatic assay mixture described under Methods. In fig.3A, poly rA-dT_{12} or poly rA were added to a final amount of 0.8 μg. In (B) poly dA-dT_{12} or poly dA were added to a final amount of 0.8 μg. The incubation was carried out for 30 min at 37°C as described for the enzyme assay under Methods. The control (100%) corresponds to the incorporation at 37°C for 30 min. (A). (●——●) Poly rA. (○– – –○) Poly rA-dT_{12}. (B) (▲——▲) Poly dA. (△– – –△) Poly dA-dT_{12}.

4. Discussion

Although considerable information exists on animal DNA polymerases very little is known concerning the plant enzymes.

A cytoplasmic enzyme having a sedimentation coefficient of 7.0 S has been described in tobacco cell cultures [17] as well as a DNA polymerase from germinated wheat embryos having a mol. wt of 230 000 [18]. None of these enzymes is able to recognize poly rA-dT_{12} as a template.

It is difficult and premature to insert DNA polymerase A purified from wheat embryos in the general scheme of the animal DNA polymerases (see the Introduction for a description of these enzymes).

DNA polymerase A is capable of recognizing poly rA-dT_{12} as in the case of DNA polymerases β and γ, but it differs markedly from polymerase β since the wheat enzyme has a mol. wt of approx. 150 000 and requires sulfhydryl groups for maximal activity. Enzyme A uses also poly rC-poly dG but at a lesser rate than poly rA-dT_{12}. As seen in table 2 polymerase A does not recognize poly rC-dG_{12} nor a natural messenger RNA plus oligo dT_{12} (not shown), indicating that most probably it does not behave as a reverse transcriptase-type of enzyme.

As in the case of HeLa cells DNA polymerase γ the wheat DNA polymerase A localisation is mainly cytoplasmic although in the case of HeLa some nuclear activity is also found [1]. Moreover the K_M for TTP using poly rA-dT_{12} as template is about 10 times lower than the K_M for the same substrate in the presence of activated DNA; this is also the case in HeLa cells.

It seemed interesting to us to make a comparative study of enzyme A using ribo- and deoxynucleotide templates, although it is not yet clear whether both enzymatic activities belong to the same protein. It has been shown for reverse transcriptase from oncogenic virus that double stranded hybrids DNA–RNA highly protect the enzyme against thermal denaturation, while the effect of natural or synthetic single stranded RNA is less important [19]. We have not found such differences as seen in fig.3; moreover poly rA seems to be a better protecting agent than poly rA-dT_{12}. However an interesting observation is that

at the same concentration poly rA-dT_{12} protects better the enzyme against thermal denaturation than poly dA-dT_{12} reflecting thereby the specificity of the enzyme for both templates.

It is evident from these results that DNA polymerase A purified from the cytoplasmic fraction of wheat embryos ressembles more to DNA polymerase γ than to the other animal DNA polymerases described up to now. However it must be pointed out that DNA polymerase γ corresponds to 1–2% of the total activity of the cell while DNA polymerase A activity level is much higher (about 30–40%, not shown).

The question concerning the physiological role of DNA polymerase γ is still unanswered. No experiments ruling out the possibility that this enzyme is an artifact arising during the purification procedure or a viral vestige have been described. On the other hand the natural messenger RNA's used in most cases for testing reverse transcriptase activity are 'foreign' macromolecules like AMV–RNA or globin messenger RNA. It would be interesting to test whether the plant messenger RNAs having a poly A sequence at their 3′ end are able to be used by the enzyme in the presence of oligo dT.

In the case of wheat embryo germination several authors have shown that very shortly after water imbibition, a heterodisperse nuclear RNA is synthesized followed by protein biosynthesis and followed only several hours later by the onstart of DNA replication [20,21]. Work is in progress in our laboratory to show whether this early RNA is related to DNA replication and whether the DNA polymerase A described in this article is related to the cytoplasmic DNA synthesis reported in the literature [22].

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