CHARACTERIZATION OF THE TWO MAJOR DNA POLYMERASE ACTIVITIES IN OOCYTES AND EGGS OF *XENOPUS LAEVIS*

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1. Introduction

We have previously shown [1,2] that in the full-grown oocyte of *Xenopus laevis* only a major DNA-polymerase activity can be detected by electrofocusing columns. On the other hand, in the ovulated egg a second major form of DNA polymerase activity appears which accounts at least in part for the several fold increase of the overall enzyme activity during oocyte maturation. It was suggested that the new activity arises during maturation either as a result of the synthesis of a new enzymatic protein or of the activation of a pre-existing, inactive enzyme. However, the possibility of an artifact had to be taken into consideration. In fact, the second DNA polymerase activity could derive from the interaction of a fraction of the oocyte enzyme with nucleic acids and/or nucleo-proteins, synthesized during maturation, thus showing an altered isoelectric point.

The partial purification and characterization of the two major DNA polymerase activities (two additional smaller ones having been identified in the course of this work) described in the present paper, lends support to the view that the DNA polymerase that appears in the egg during maturation is in fact a different enzyme from the one present in the oocyte of *Xenopus laevis.*

2. Materials and methods

Synthetic polymers polyd(A-T), polydT.polyrA, polydC.polyrG,polydG.polydC were purchased from Miles Laboratories and polyrAd $(pT)_9$ from P.L. Biochemicals Inc. Salmon sperm DNA was obtained from Calbio-chem. Unlabelled T7 DNA was prepared according to Richardson [3]. Unlabelled deoxynucleoside-5′-triphosphates and [^{3}H] dCTP, [^{3}H] dATP, [^{3}H] dTTP were purchased from Schwarz Mann; Pancreatic DNase and Exonuclease III were obtained from Worthington and Boeringer, Mannheim, respectively.

2.1. *Assay of DNA polymerase*

The standard reaction mixture contained in a total volume of 0.30 ml: 50 mM potassium phosphate buffer (pH 7.4), 10 mM $MgCl_2$, 3.3 mM 2-mercaptoethanol, 80 μg of 'gapped' (see below) salmon sperm DNA, 0.33 mM of each dCTP, dGTP, dATP, dTTP (one of which was tritium labelled with a specific activity 2×10^4 cpm/nmol) and 0.1 to 1 unit of enzyme. After incubation at 37°C for 20 min, the reaction was stopped by the addition of 1 ml of cold 0.1 M sodium pyrophosphate and 2 ml of cold 10% trichloroacetic acid. The acid-insoluble material was collected by filtration on a Whatman GF/C glass filter and then washed 5 times with 3 ml of cold 1% trichloroacetic acid. The glass filter was dried and the radioactivity measured in a liquid scintillation counter. One unit of enzyme activity is defined as the amount catalyzing the incorporation of 1 nmol of [^{3}H] dTTP into an acid-insoluble product in 20 min.

2.2. *Preparation of heat denatured DNA*

Heat denatured DNA was obtained by heating a solution of native salmon sperm DNA (2 mg per ml in 20 mM Tris–HCl pH 7.6) at 100°C for 5 min and cooling immediately at 0°C.

2.3. *Preparation of 'nicked' DNA*

'Nicked' salmon sperm DNA was prepared by digestion of native salmon sperm DNA (2 mg per ml) with

pancreatic DNase I (0.011 units per ml) in the presence of 0.5 mg per ml of bovine serum albumin, 5 mM $MgCl_2$ and 50 mM Tris–HCl buffer (pH 7.6), at 37°C for 15 min. The reaction was stopped by addition of EDTA (final concentration 5 mM) and heating at 65°C for 10 min.

2.4. *Preparation of 'gapped' DNA*

'Gapped' salmon sperm DNA was prepared by digestion of the 'nicked' salmon sperm DNA (1 mg per ml) with Exonuclease III (2.4 units per ml) in the presence of 20 mM 2-mercaptoethanol at 37°C for 60 min. The reaction was stopped by addition of EDTA (final concentration 5 mM) and heating at 65°C for 15 min. The extent of hydrolysis was calculated by measuring the optical densities at 260 nm made acid soluble.

2.5. *Preparation of oocytes and dejellified unfertilized eggs*

Oocytes at stage 6 of oogenesis were isolated as previously described [1]. Ovulation in *Xenopus* females was induced as described by Gurdon [4] and the jelly, which surrounds the ovulated eggs, was removed as described by Dawid [5].

2.6. *Preparation of extracts from oocytes and eggs of Xenopus*

Dejellified eggs or oocytes were ground in a Dounce

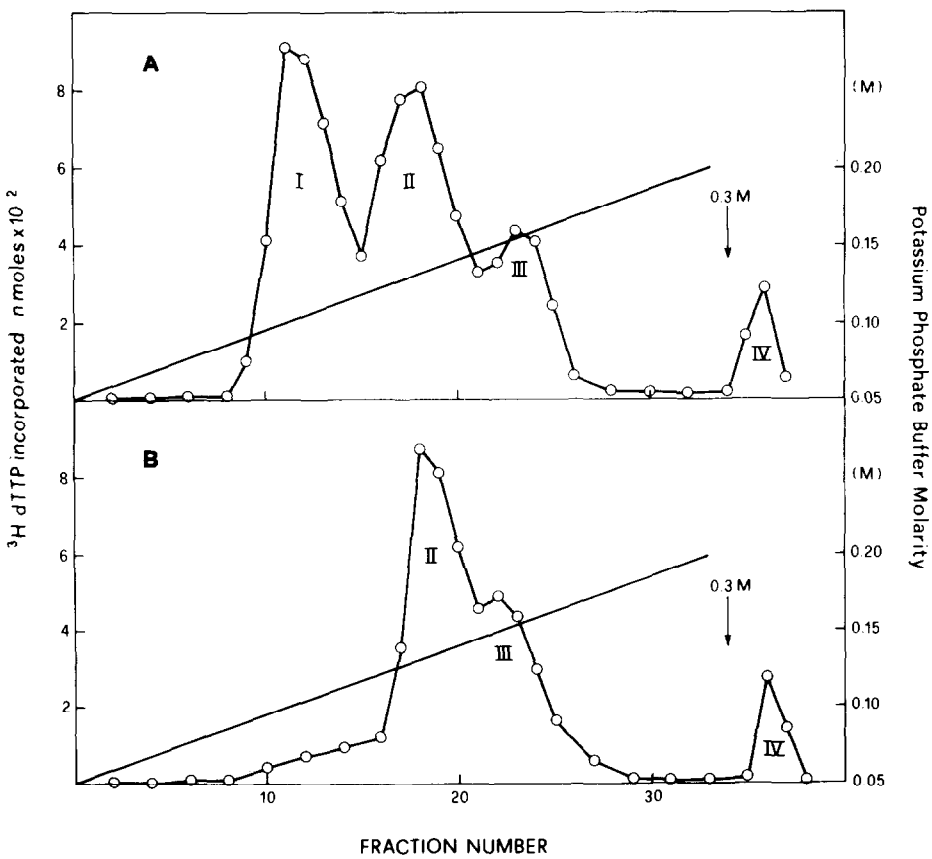

Fig. 1. Chromatography on DEAE-cellulose column of egg extract (A) and oocyte extract (B). (A) 3 ml of egg extract (90 units) were applied to a DEAE-cellulose column (1.8 cm^2 X 9 cm) and DNA polymerase activities were eluted with a linear gradient (100 ml) of potassium phosphate (pH 7.4) in buffer B.2.9 ml fractions were collected and 25 μl samples were assayed in a standard reaction mixture for 20 min. (B) 2 ml of oocyte extract (35 units) were applied to a DEAE-cellulose column eluted and assayed as above.

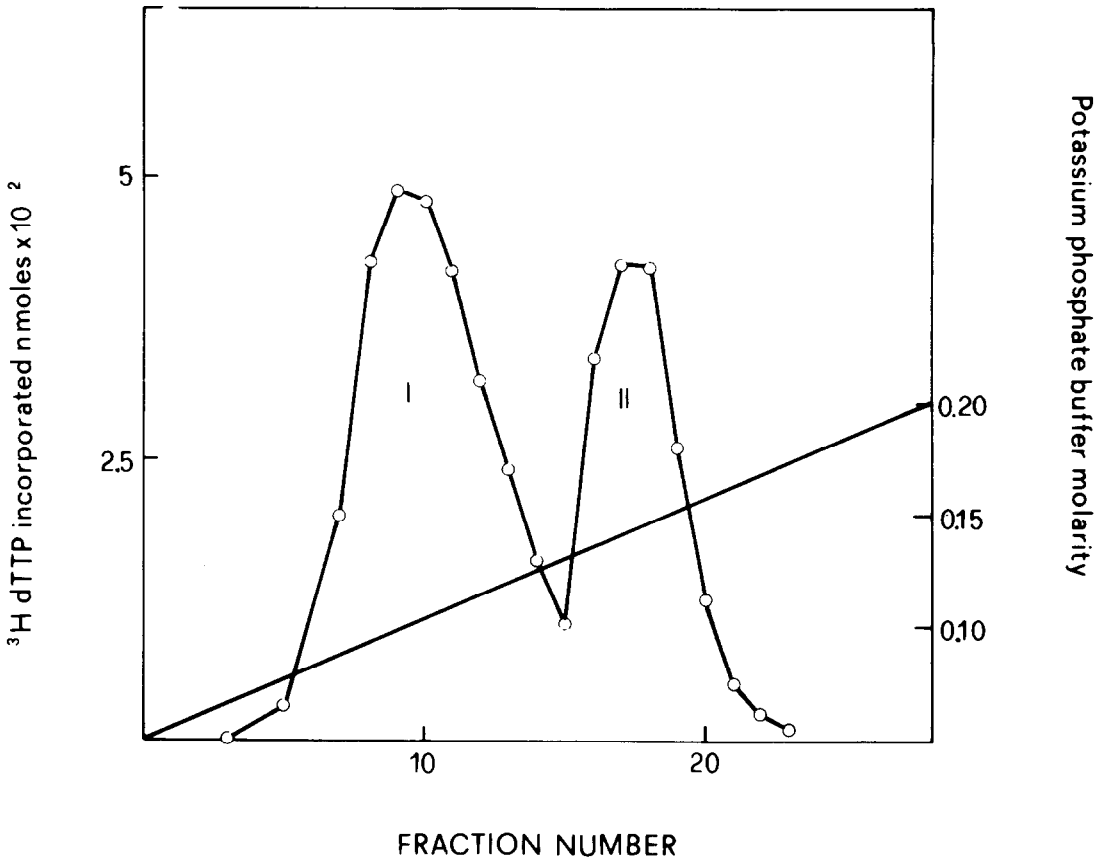

Fig. 2. Rechromatography on DEAE-cellulose column of peak I from DEAE cellulose column (fig. 1A) and Peak II (fig. 1B). Active fractions of the peak I (fig. 1A) and peak II (fig. 1B) were pooled and dialyzed against buffer B. 14 units of peak I and 10 units of peak II were mixed and loaded onto a DEAE-cellulose column (1.8 cm^2 × 10 cm). Elution of DNA polymerases were performed as in fig. 1. 50 μl samples were assayed in the standard reaction mixture.

homogenizer in two volumes of 0.2 M potassium phosphate buffer (pH 7.4) containing 25% sucrose, 10^{-3} M EDTA. The homogenate was centrifuged at 4000 *g* for 5 min. The floating fatty layer removed with a spatula, the supernatant was collected and centrifuged again for 20 min at 27 000 *g*. The pellet was discarded and the supernatant centrifuged for 1 hr at 105 000 *g*. The supernatant was collected and dialyzed overnight against 0.5 M potassium phosphate buffer (pH 7.4) containing 25% glycerol, 10^{-3} M 2-mercaptoethanol and 10^{-3} M EDTA (buffer A). After dialysis the enzyme was mixed with DEAE-cellulose previously equilibrated with 0.05 M potassium phosphate buffer (pH 7.4) containing 25% glycerol, 5×10^{-3} M 2-mercaptoethanol (buffer B). The suspension was stirred for 20 min, centrifuged for 20 min at 27 000 *g* and the supernatant fluid discarded. DEAE-cellulose was resuspended in one vol of buffer B in a glass column and washed with buffer B until the optical density at 280 nm was less than 0.05. DNA polymerase activity was eluted from the column with buffer B made 0.3 M in potassium phosphate (pH 7.4). The active fractions were pooled and dialyzed overnight against buffer B.

3. Results

3.1. *Separation of multiple DNA polymerase activities by DEAE-cellulose columns*

Figs. 1A and 1B show the fractionation, on DEAE-

cellulose column, of egg extract (see Methods) and of oocyte extract respectively. In the oocyte extract (fig. 1B) three peaks of DNA polymerase activity can be separated: a major peak (peak II) is eluted at a potassium phosphate concentration corresponding to about 0.13 M (pH 7.4), while two minor peaks peak III and IV, are eluted at 0.15 M and 0.3 M potassium phosphate respectively. In the case of egg extract (fig. 1A), a new major peak (peak I) is eluted at about 0.1 M potassium phosphate while the two minor peaks (IIIand IV) are still eluted at 0.15 M and 0.3 M. When the two major peaks (peak I (14 units) from column 1A and peak II (10 units) from column 1B) were mixed and rechromatographed under the same conditions (fig. 2), they were eluted at the same ionic strengths as in the first DEAE-column. Peak I (fig. 1A) and peak II (fig. 1B) were analyzed by electrofocusing column in order to compare the two major DNA polymerase activities separated by DEAE-cellulose column with the two DNA polymerase activities previously fractionated by electrofocusing column [1]. The isoelectric points measured, pH 6.95 and pH 6.30, (data not shown) are very close to the values previously reported (pH 7 and 6.3 respectively).

3.2. *Requirements for DNA synthesis and template specificity*

The requirements for DNA synthesis and the template specificity of peak I and peak II (after re-chromatography on DEAE-cellulose were further investigated. As shown in table 1, maximal activity for both enzymes depends on the presence of all four deoxyribonucleoside-5′-triphosphates, DNA and Mg^{2+}. Sulphydryl-group-blocking agents such as *N*-ethylmaleimide and *p*-chloromercuribenzoate inhibit both enzymes although to a different extent. Mn^{2+} can substitute for Mg^{2+}. The optimal concentration for the two ions is reported in fig. 3. Table 2 shows that native salmon sperm DNA and native T7 DNA are not used as templates, thus suggesting that the two enzymes have an absolute requirement for a primer and that both enzymes lack 3′-5′ exonucleolytic activities. Indeed, salmon sperm DNA treated with pancreatic DNase ('nicked' DNA) is a template, while subsequent hydrolysis with Exonuclease III of the 'nicked' DNA ('gapped' DNA) enhances the template properties of DNA polymerase I only. Heat denatured salmon sperm DNA is not used as template by the purified polymerases whereas less purified preparations can; this

Table 1
Requirements for DNA synthesis

Reaction mixture	Relative activity	
	peak I	peak II
	%	%
Complete	100	100
-dTTP	20	25
-dTTP, dGTP, dCTP	7	10
-DNA	0.5	0.5
-Mg^{2+}	0.5	0.5
-2-Mercaptoethanol	95	95
-Mg^{2+} + Mn^{2+} (1 mM)	60	80
-2-Mercaptoethanol + NEM (0.1 mM)	65	15
-2-Mercaptoethanol + PCMB (0.015 mM)	30	5

DNA polymerases (peak I and II of fig.2) were incubated for 20 min at 37°C in the standard reaction mixture containing [^{3}H]dTTP as labelled nucleotide (see Methods), or in reaction mixtures from which the indicated components had been omitted or added. In the experiments with NEM and PCMB the enzymes were dialyzed against buffer A in which 2-mercaptoethanol was omitted. [^{3}H]dATP was used as labelled nucleotide in the reaction mixtures from which dTTP was omitted.

Table 2
Template specificity

Template	peak I		peak II	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
	pmol*		pmol*	
Native Salmon sperm DNA	16	–	8	–
Native T_7 DNA	10	–	–	–
'Nicked' Salmon sperm DNA	190	130	255	200
'Gapped' Salmon sperm DNA	400	240	260	210
Denatured Salmon sperm DNA	20	–	12	–
polyd(A-T)	104	70	18	25
polydT.polyrA	2	2	< 0.1	< 0.1
PolyrA.d(pT)$_9$	4	3	< 0.1	< 0.1
polydC.polyrG	3	2	< 0.1	< 0.1
PolydG.polydC	6	5	8	7

* Total pmoles of nucleotide incorporated per 20'.
Assay conditions were the same as described in Methods except that different templates (80 μg) were substituted for 'gapped' salmon sperm DNA. 1 mM $MnCl_2$ was present instead of $MgCl_2$ as indicated. The enzymes used were peak I and II of the second DEAE column (fig.2).

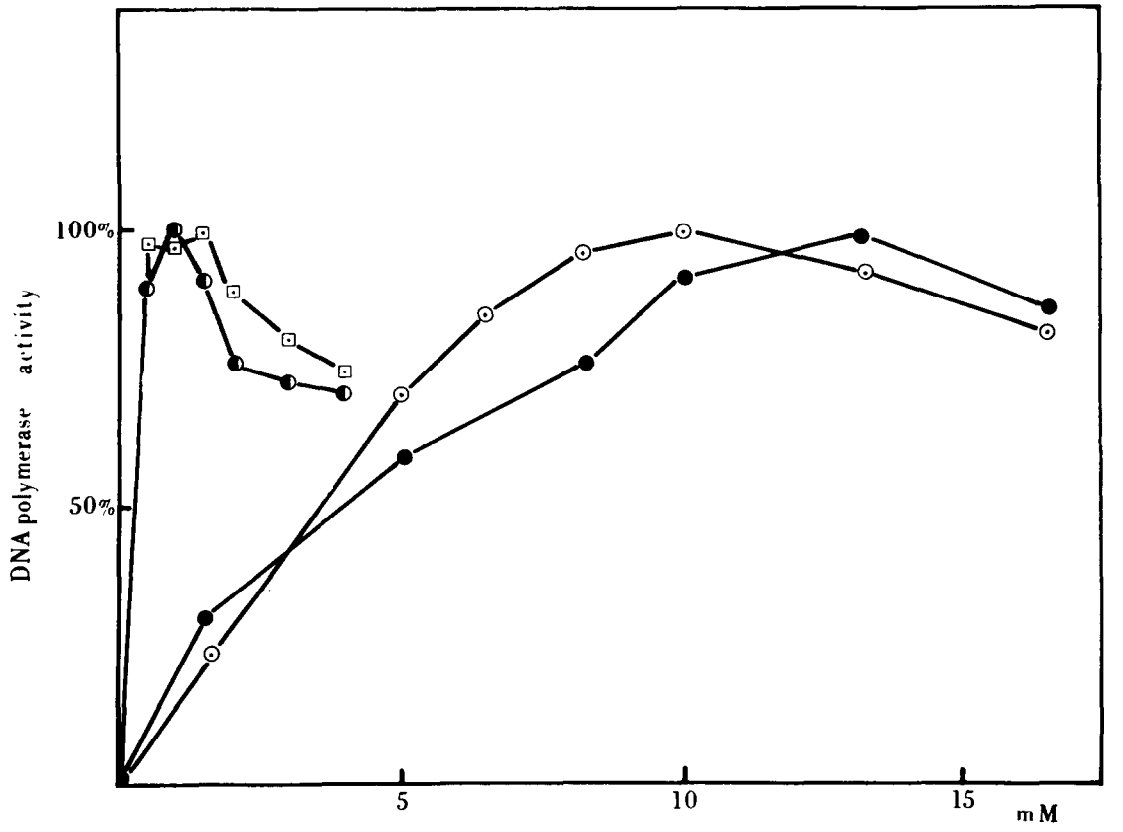

Fig. 3. Dependence of the DNA polymerase activities on the concentration of $MnCl_2$ and $MgCl_2$. Assay conditions were as described in the Methods except for $MgCl_2$ which was present or substituted with $MnCl_2$ at the indicated concentrations. (o–o–o) peak I (fig. 1A) assayed in the presence of $MgCl_2$ or in the presence of $MnCl_2$ (□–□–□). (•–•–•) peak II (fig. 1B) assayed in the presence of $MgCl_2$ or in the presence of $MnCl_2$ (o–o–o).

suggests that nucleolytic activities that somehow activate DNA are present in the latter as contaminants. A synthetic alternating copolymer such as polyd(A-T) serves as template for both enzymes. Synthetic hybrids polydeoxy-polyribonucleotides such as polydT. PolyrA, polyrA.d$(pT)_9$, polydC.polyrG, are not templates. PolydC. polydG is a very poor template for both enzymes. The activity with polyd(A-T) as template is 26% for peak I and 7% for peak II (taking the activity with 'gapped' salmon sperm DNA as 100%).

4. Discussion

The experiments reported in this paper confirm our previous findings that two major DNA polymerases are present in *Xenopus* egg while only one is detectable in the immature oocyte. The fractionation procedures used in this work have also revealed the presence of two minor DNA polymerase activities both in the oocyte and in the egg; their characterization is currently in progress. Furthermore, the demonstration (i) that the two major forms of DNA polymerase activity (peak I and II) can be separated by DEAE cellulose columns; (ii) that even after two chromatographic runs on DEAE cellulose their isoelectric points are not changed; (iii) that though sharing a number of properties, the two polymerases can be distinguished from one another on the basis of some of their properties; supports the view that they are in fact two different enzymes. Hence our data represent the first evidence that, concurrently with the completion

of meiosis, a new type of DNA polymerase appears in the egg of *Xenopus laevis*. Results to be described in a forthcoming paper further indicate that the new enzyme activity is present also in the nuclei of the embryo and remains virtually unchanged throughout development to the neurula stage (Rether and Grippo, in preparation).

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