SEPARATION AND PARTIAL CHARACTERIZATION OF DNA POLYMERASES IN SEA URCHIN PARACENTROTUS LIVIDUS EGGS.

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SUMMARY

Paracentrotus lividus eggs contain three separable DNA polymerases (deoxynucleoside triphosphate: DNA deoxynucleotidyltransferase, EC 2.7.7.7.). The two main peaks of activity, designated form I and form II, differ in the following features: 1) form I is able to use poly(dA) as primer-template more efficiently than form II; 2) the initial rate of incorporation of dTTP or dCTP in the absence of other deoxynucleosidetriphosphates (dNTPs) is higher with form I than with form II when the template is DNA or poly(dA,dT); 3) form II is preferentially inhibited by KCl; 4) the two forms show a different optimal Mn^{2+} concentration for their maximal activity.

DNA polymerase is present in the unfertilized sea urchin egg and its overall activity does not change until the pluteus stage (1,2). On the other hand when the embryos are dissociated into individual cells, and are then cultured under conditions which prevent their reaggregation, the DNA polymerase activity undergoes a striking increase (2). In the unfertilized eggs the enzyme has been found to be located in the cytoplasm and, as development proceeds, it moves to the nucleus and associates with chromatin (1,3). The presence of multiple forms of DNA polymerase in eukaryotic tissues has been reported by several authors. Most of these studies were carried out on mammalian cells in which cytoplasmic, mitochondrial, and nuclear DNA polymerase has been described (4-15).

Little information is available about DNA polymerase in sea urchin embryos. The purification of an enzyme from embryo nuclei has been reported (17), and the existence of multiple forms of DNA polymerase has been inferred by other authors (18). We have found that in extract of unfertilized eggs of Paracentrotus lividus three forms of DNA polymerase are separated by DEAE-cellulose chromatography. The two major peaks of activity have been characterized on the basis of their template specificity and cation requirements. This study represents a necessary prerequisite to an investigation now being carried out on the changes the various forms of this enzyme undergo in the course of development.

MATERIALS AND METHODS

Unlabeled deoxyribonucleoside triphosphates were obtained from Sigma; [^{3}H]dTTP (15-17 Ci/mmole) and [^{3}H]dCTP (25 Ci/mmole) from New England Nuclear; pancreatic DNase I (RNase free) from Worthington Biochemical Corp. or from Boeringer; DEAE-cellulose (DE-52) from Whatman; synthetic polynucleotides were

from Miles Laboratories or P.L. Biochemicals Inc. DNA was prepared from sea urchin sperm by the method of Marmur (19). All other chemicals were reagent grade. Sea urchin were obtained from the Zoological Station of Naples.

Sea urchin eggs and sperm were collected as previously described (20). Activated DNA was prepared using pancreatic DNase I (21). DNA was denatured by heating at 100°C for 10 min followed by rapid cooling in an ice-water bath. Protein concentration was determined by the procedure of Lowry et al. (23).

Preparation of extracts for column chromatography were carried out at 0-4°C. Sea urchin eggs were packed by low speed centrifugation, washed twice with filtered sea water and twice with 0.63M NaCl. They were then homogenized in buffer

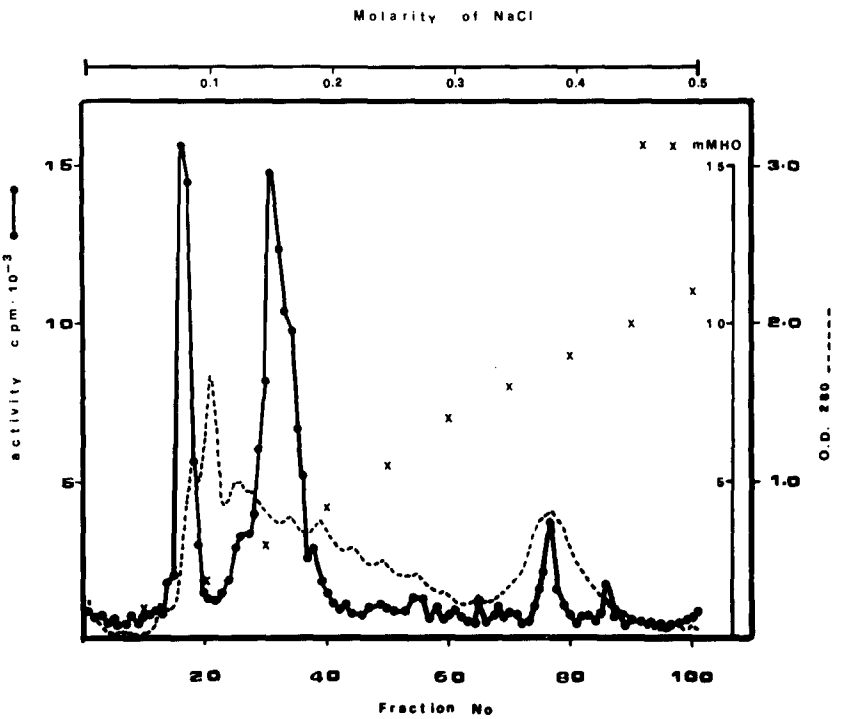

Fig. 1 - DEAE-cellulose chromatography of DNA polymerase from unfertilized eggs of Paracentrotus lividus.
10 ml of the extract containing 180 mg of protein were absorbed on a 2.5 x 25 cm DE-52 column previously equilibrated with buffer B. After washing in buffer until absorbance reached the base line, a 400 ml gradient from 0 to 0.5 M NaCl in buffer B was applied. 50 µl of each fraction were assayed with a reaction mixture containing in 250 µl: 64 mM phosphate buffer pH 7.4, 12 mM $MgCl_2$, 4 mM β-mercaptoethanol, 0.2 mM dNTPs (specific activity of [^{3}H]dCTP 180,000 cpm/nmole), 100 µg of activated DNA from sea urchin sperm. After 20 min at 37°C the reaction was stopped by the addition of 0.5 ml of 0.5 M NaCl and 2 ml of 1 M TCA containing 0.1 M $Na_4P_2O_7$. The precipitate was collected on a fiber-glass filter (GF/A Whatman) and washed with 30 ml of 0.1 M TCA containing 0.01 M $Na_4P_2O_7$, and with 10 ml of ethanol-ether (3:1 v/v). The filters were then dried and counted in toluene omnifluor scintillation fluid. The recovery from the column was higher than 90% of the initial activity.

A (20mM Tris-HCl pH 8.5, 25% glycerol, 1mM EDTA, 1mM 2-mercaptoethanol and 0.3M NaCl). The homogenate was intermittently sonicated with a Branson sonicator, model J-17 A, for 1 min and centrifuged at 27,000xg for 30 min. The supernatant was centrifuged again at 100,000xg for 1hr and dialyzed overnight against 40 volumes of buffer B (20mM Tris-HCl pH 8.5, 25% glycerol, 1mM EDTA, 1mM 2-mercaptoethanol). The dialyzed supernatant was diluted with buffer B to a protein concentration of 10 mg/ml, and streptomycin sulfate was added to a final concentration of 0.5 mg/ml. After equilibration for 20 min the precipitate was removed by centrifugation at 27,000xg for 30 min. The supernatant was fractionated by ammonium sulfate precipitation; the precipitate between 35-75% saturation was dissolved in buffer B and dialyzed against a large volume of the same solution. The extract was further fractionated by DEAE-cellulose chromatography.

RESULTS AND DISCUSSION

Three peaks of activity were separated by DEAE-cellulose chromatography

TABLE I

Template and substrate requirements of DNA polymerase form I and form II.

Primer-Template	Substrate	pmoles incorporated in 20 min. Form I	Form II
None	[^{3}H]dTTP + 3 other dNTPs	7	7
"Activated" DNA	[^{3}H]dTTP + 3 other dNTPs	46	37
	[^{3}H]dTTP + dATP	48	30
	[^{3}H]dTTP	40	15
	[^{3}H]dCTP + 3 other dNTPs	48	30
	[^{3}H]dCTP	37	14
Native DNA	[^{3}H]dTTP + 3 other dNTPs	13	17
Denatured DNA	[^{3}H]dTTP + 3 other dNTPs	19	18
poly(A)oligo(dT)$_{10}$	[^{3}H]dTTP + 3 other dNTPs	18	17
	[^{3}H]dTTP	11	10
	[^{3}H]dCTP	7	6
poly(dA)	[^{3}H]dTTP	40	16
poly(dA,dT)	[^{3}H]dTTP + dATP	136	120
	[^{3}H]dTTP	114	48
	[^{3}H]dCTP	8	7

The standard assay of fig.1 was used with the following modifications: specific activity of the labeled dNTP was 90,000-100,000 cpm/nmole, the amount of the synthetic polynucleotides was 50 µg per assay, 50 µg of protein from peak I or II were used in each assay. The assays carried out in triplicate showed an error contained within 5%.

from extracts of unfertilized eggs (fig.1). The first peak (form I) was eluted at 0.075 M NaCl, and the second peak (form II) at 0.15:0.20 M NaCl. The third peak, eluted at 0.40 M NaCl, was very unstable and therefore no further attempt was made to characterize it.

The activity of DNA polymerase form I and form II with different templates and substrates are summarized in Table I. With "activated" DNA, both enzymes displayed comparable levels of activity which were higher than those observed with denatured and native DNA.

Poly(dA,dT) proved to be an even better template for both forms I and II. The two polymerases showed the same ability to use this copolymer as template when both dATP and dTTP were provided as substrates. Both forms I and II showed the same limited ability to use poly(A)oligo(dT) as template. With poly(dA), however, the activity of form I was more than twice that of form II. In the presence of DNA-template and only one dNTP, the level of activity of form I was similar to that observed in the presence of all the four dNTPs. On the contrary, the activity of form II was reduced to about 1/2 when three dNTPs were omitted. The same results were obtained when the template used was poly(dA.dT).

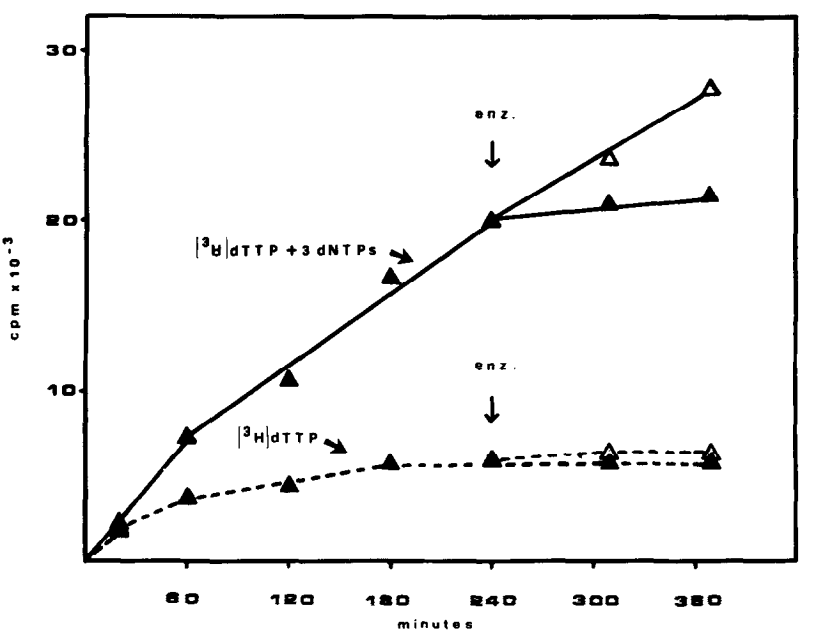

Fig. 2 - Kinetics of incorporation of 4 dNTPs (▲——▲)versus 1 dNTP (▲- -▲) by DNA polymerase form I from sea urchin eggs.
The reactions were as those of Table I with 100 µg of activated DNA as template. At 240 min, 50 µg of new enzyme were added to each reaction mixture.

In this case, with form II the incorporation was about 3-fold greater when both dATP and dTTP were present than when dTTP alone was present. With form I the extent of incorporation was similar irrespective of whether dATP and dTTP were provided as substrates or whether dTTP alone was present.

The high level of incorporation of one dNTP, observed especially with form I, was not due to the presence of a terminal nucleotidyl transferase. In fact neither of the two enzymes were able to incorporate dCTP to a significant level in the presence of poly(dA,dT), thus showing that incorporation was template-directed. The extent of the reaction with a single dNTP was dependent on the duration of incubation (fig.2). The rate of incorporation of [^{3}H]dTTP alone by polymerase form I was comparable to that observed in the presence of all four dNTPs during the first 20 min of incubation. Thereafter the rate of incorporation began to decline and reached a plateau at 180 min. The reaction could not be resumed by addition of new enzyme. In contrast, when all four dNTPs were present the rate of incorporation was almost linear for 240 min, and then sharply declined; however, the initial rate was resumed following addition of new enzyme. At 370 min the extent of incorporation with [^{3}H]dTTP was only 20% of that with all four dNTPs. This substantial incorporation in the presence of only a single dNTP has been reported for other eukaryotic DNA polymerases (7).

DNA polymerase forms I and II were differently affected by KCl (fig.3). Form I was little affected by 200mM KCl and not affected by lower concentration.

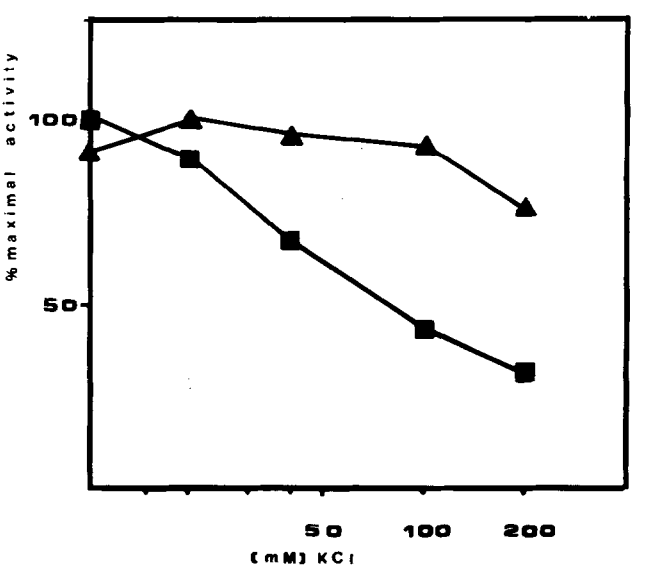

Fig. 3 - Inhibitory effect of KCl on DNA polymerase form I (▲—▲) and form II (■—■). The reactions were carried out as described in the legend of Table I in the presence of the indicated concentrations of KCl.

On the other hand, form II showed a 40% inhibition at 40mM KCl and 70% at 200mM KCl. An even more striking difference between the two forms was observed when the reaction was carried out in the presence of Mn^{2+} ions and poly(dA,dT) (fig.4). At low concentration of Mn^{2+} (0.4-0.8 mM), form II displayed no activity; the enzyme gradually became activated by rising the concentration from 1.0 to 2.0 mM. Form II was fully active in the whole range of Mn^{2+} concentrations used in the assay. The optimal Mg^{2+} concentration was between 6 and 12mM for both forms I and II.

These results suggest that the two peaks of activity present in the extracts of unfertilized sea urchin eggs are in fact distinct enzymes. The presence of multiple forms of DNA polymerases in sea urchin eggs raises, once again, the question of their biological role in the processes of DNA replication. It is possible that, as in other eukaryotic systems, the three forms found in sea urchin eggs have different intracellular localization. DNA polymerases from eukaryotes have been recently classified with the Greek letters α, β and γ (23). The α enzyme corresponds to the high molecular weight DNA polymerase, while the β enzyme is the low molecular weight DNA polymerase found exclusively in the nuclei. Due to the lack of information on the molecular weights of forms I and II, we have preferred, for the moment, not to follow this classification. How-

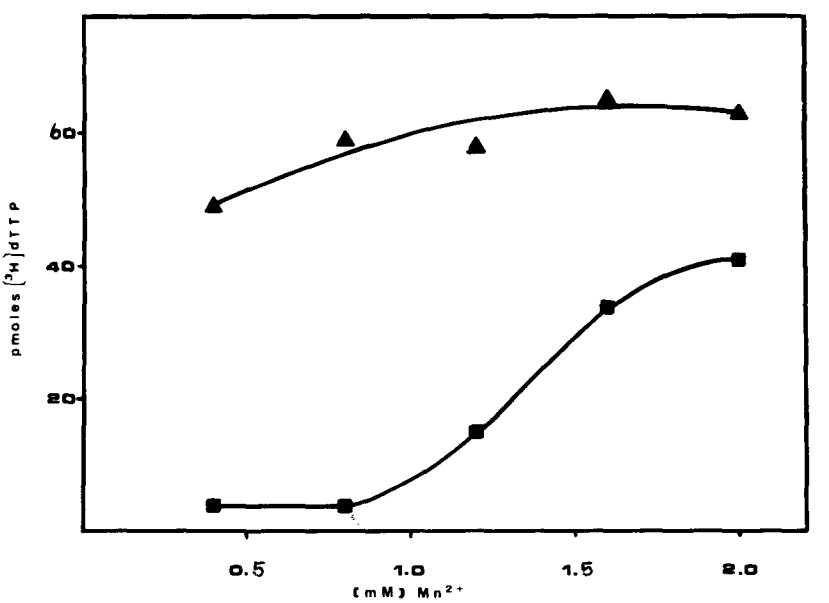

Fig. 4 - Effect of Mn^{2+} ions on DNA polymerases form I (▲—▲) and form II (■—■). The conditions of the reaction were those described in Table I, with 50 µg of poly(dA,dT) as template and the indicated concentrations of $MnCl_2$ instead of $MgCl_2$.

ever, on the basis of their behaviour on DEAE-cellulose, we might tentatively identify the more basic form I as a β-like DNA polymerase and the more acidic form II as a DNA polymerase α. Preliminary results, obtained in our laboratory, indicate that nuclei from embryos contain a single DNA polymerase activity that on DEAE-cellulose behaves as form I from eggs. Studies are in progress to further characterize the DNA polymerases found in sea urchin eggs and to establish their intracellular localization.

REFERENCES

1. Fansler,B. and Loeb,L.A., (1969) Exptl.Cell Res., 57, 305-310.
2. De Petrocellis,B. and Vittorelli,M.L., (1975) Exptl.Cell Res., 94, 392-400.
3. Fansler,B. and Loeb,L.A., (1972) Exptl.Cell Res., 75, 433-441.
4. Wallace,P.G., Hewish,D.R., Venning,M.M. and Burgoyne,L.A. (1971) Biochem.J., 125, 47-54.
5. Weissbach,A., Schlabach,A., Fridlender,B. and Bolden,A., (1971) Nature New Biol., 231, 167-170.
6. Haines,M.E., Holmes,A.M. and Johnston,I.R., (1971) FEBS Letters, 17,63-67.
7. Sedwick,W.D., Shu-Fong Wang,T. and Korn, D., (1972) J.Biol.Chem., 247, 5026-5033.
8. Holmes,A.M., Hesslewood,I.P. and Johnston,I.R., (1974) Eur.J.Biochem., 43, 487-499.
9. Grippo,P. and Lo Scavo,A., (1972) Biochem.Biophys.Res.Comm., 48, 280-285.
10. Spadari,S. and Weissbach,A., (1974) J.Biol.Chem., 249, 5809-5815.
11. Brun,G., Rougeon,F., Lauber,M. and Chapeville,F., (1974) Eur.J.Biochem., 41, 241-251.
12. Benbow,R.M., Pestell,R.G.W. and Ford,C.C., (1975) Dev.Biol., 43, 159-174.
13. Claycomb,W.C., (1975) J.Biol.Chem., 250, 3229-3235.
14. Lazarus,L.H. and Kitron,N., (1975) Biochim.Biophys.Acta, 402, 309-322.
15. Soriano,L., (1972) FEBS Letters, 22, 310-314.
16. Probst,G.S. and Meyer,R.R., (1973) Biochem.Biophys.Res.Comm., 5o, 111-117.
17. Loeb,L.A., (1969) J.Biol.Chem., 244, 1672-1681.
18. Slater,I., and Slater,D.W., (1972) Nature New Biol., 237, 81-85.
19. Marmur,J., (1961) J.Mol.Biol., 3, 208-218.
20. Grippo,P., Iaccarino,M., Parisi,E. and Scarano,E., (1968) J.Mol.Biol., 36, 195-208.
21. Aposhian,H.V. and Kornberg,A., (1962) J.Biol.Chem., 237, 519-525.
22. Lowry,P.H., Rosebrough,N.J., Farr,A.L. and Randall,R.J., (1951) J.Biol.Chem. 193, 265-275.
23. Weissbach,A., Baltimore,D., Bollum,F., Gallo,R. and Korn,D., (1975) Eur.J. Biochem., 59, 1-2.