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DNA polymerase α and β in the California urchin

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ABSTRACT

DNA polymerase α and β were identified in the urchin, Strongylocentrotus purpuratus. The DNA polymerase β sedimented at 3.4 S, constituted 5% of total DNA polymerase activity, and was resistant to N-ethylmaleimide and high ionic strength. The polymerase α sedimented at 6-8 S, was inhibited by N-ethylmaleimide or 0.1 M $(NH_4)_2SO_4$, and was dependent upon glycerol for preservation of activity. Both the polymerases α and β were nuclear associated in embryos. The DNA polymerase α was markedly heterogeneous on DEAE-Sephadex ion exchange and showed three modal polymerase species. These polymerase α species were indistinguishable by template activity assays but the DNA polymerase associated ribonucleotidyl transferase (Biochemistry 75:3106-3113, 1976) was found predominantly with only one of the DNA polymerase α species.

INTRODUCTION

The sea urchin egg/embryo is a remarkable biological system in regard to DNA synthesis. The egg is essentially inactive, but following fertilization DNA synthesis is activated, resulting in a 1000-fold increase in DNA content per embryo within 24 hours. At the peak of DNA synthesis, the 1 pg genome replicates in no longer than 15 minutes (see 1 and 2 for review of the above). Although de novo synthesis of DNA polymerase could allow for the observed activation of replication, studies with dialyzed homogenates by Fansler and Loeb (3,4) and with DEAE-Sephadex purified preparations by Morris and Rutter (5) demonstrate that the egg contains DNA polymerase, and further, that there is no net increase in assayable DNA polymerase throughout embryogenesis. Inhibition of protein synthesis does not immediately block DNA synthesis (6); and treatment of eggs with ammonical sea water activates extensive chromosome duplication without activating protein synthesis or cytodiaresis (7). Thus, the available evidence suggests that, in addition to the DNA polymerase, the urchin egg is preloaded with all the enzymes required for DNA replication and regulation of replication is likely affected through modulation of the pre-existing egg enzymes. The experimenter may activate DNA

replication at will with chemical or biological means.

In view of this biology of the urchin egg, plus the availability of quantities of the egg, the urchin is attractive as a biochemical system for evaluation of the replicative process in eukaryotes. Multiple DNA polymerases are found in most eukaryotic organisms (8,9) and the presumptive replication enzyme, DNA polymerase α, is frequently quite heterogeneous (10-13). The similarity between the urchin and other eukaryotes is unclear. A single DNA polymerase, apparently polymerase α, has been reported from nuclei of blastula stage embryos (14), while sedimentation and N-ethylmaleimide inhibition revealed a DNA polymerase β activity as well as the α activity (15). Multiple DNA polymerases were observed in Paracentrotus lividus (28) although their correspondence to DNA polymerase α and β was not certain. Thus the similarity between the urchin and other more well studied eukaryotes is unclear. In order to promote the advantages offered by the urchin egg/embryo system to the study of replication, we have characterized both the DNA polymerase β and a heterogeneous DNA polymerase α in the urchin. A preliminary report of this has appeared (16).

MATERIALS AND METHODS

Strongylocentrotus purpuratus were purchased from Pacific Bio-Marine, Venice, California and from R. Bergstrom, Seattle, Washington. Egg collection, embryo development and nuclei isolation were as described previously (17). Nuclear pellets were suspended in 2 volumes 50 mM Tris-HCl pH 7.9, 10% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, 1.5 M KCl, and homogenized 30 sec with the Tekmar homogenizer to shear the DNA prior to chromatography. For preparation of unfractionated extracts, the eggs or embryos were mixed with 1.25 volumes of 0.28 mM EDTA, 140 mM Tris-HCl, pH 7.9, 0.5 mM dithiothreitol, and homogenized for 30 sec at one-half speed with a Tekmar tissue homogenizer, followed by the addition of 1.25 volumes of 1.68 M NaCl in 66% (v/v) glycerol, and 30 sec further homogenization. Debris were removed by centrifugation at 10,000 x g for 10 min.

Sucrose gradients were constructed of 5-20% sucrose in 50 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 0.5 mM dithiothreitol, 25% (v/v) glycerol, and 0.4 M NaCl. Samples (0.2 mL) were layered on 3.9 mL gradients and centrifuged (22 h, 10°C, 60,000 rpm) in an SW60Ti rotor. Gradients were fractionated from the top with an ISCO gradient fractionator into 180 μL fractions.

Gradient sievorptive elution (18) was performed as described previously (5). Columns of DEAE-Sephadex A-25 (Pharmacia) (1.1 x 30 cm), were equili-

brated with 50 mM Tris-HCl, pH 7.9, 25% glycerol, 0.5 mM dithiothreitol (TGD) containing 0.05 M NaCl. A linear gradient of 5.0 mL each of 0.05 M and 0.25 M NaCl in TGD was applied, followed by 0.5-1.0 mL of sample, and then chased with one bed volume of 1.0 M NaCl in TGD. The salt concentration of the elute was determined by conductimetry (Radiometer CDM 2) on 10 µL aliquots of fractions diluted with 1.0 mL distilled water.

DNA polymerase was assayed with activated calf thymus DNA and [^{3}H]-TTP (Amersham/Searle) plus 3 dNTPs as previously described (5) except that the $MgCl_2$ concentration was reduced to 6 mM. DNA was activated with pancreatic DNase (19) and the activation was terminated when the priming activity of the DNA reached a maximum. For synthetic template assays, the DNA was replaced with the indicated template (P-L Laboratories) and the NTP (5) dictated by base complimentarity was used at 0.01 mM and 1400 CPM/pmole; the divalent cation was either 0.6 mM $MnCl_2$ or 6 mM $MgCl_2$. Aliquots of enzyme (20 µL) were assayed at 30°C in a total volume of 50 µL; 40 µL aliquots were spotted onto DE-81 (Whatman) filters and processed as described (5).

RESULTS

Sucrose density gradient centrifugation of crude extracts from urchin eggs (Figure 1a) and blastula (Figure 1b and 1c) resolved two major size classes of DNA dependent DNA polymerase activity. The minor activity peak sedimenting at 3.4 S (fraction 7-8, Figure 1a-c) constituted approximately 5% of the total DNA polymerase activity in all experiments, regardless of embryonic stage; it was identified as DNA polymerase β on the basis of sedimentation coefficient and complete resistance to inhibition by 10 mM N-ethylmaleimide (Figure 1; table 1). The major activity peak (fractions 12-20, Figures 1a-c) sedimented rather heterogeneously in all our observations and was identified as the DNA polymerase α by sedimentation and complete sensitivity to 10 mM N-ethylmaleimide. The DNA polymerase γ was not identified in these extracts from the urchin (table 3 and following paragraphs). Further evidence for dissimilarity of the 3.4 S and 6-8 S DNA polymerase species was shown in the ionic strength preferences and by inhibition with Cu^{+2} and with pyridoxal phosphate (Table 1). The pyridoxal phosphate inhibition was further shown to be completely reversible by the addition of 50 mM ethylenediamine to previously inhibited enzyme; therefore, the reversible formation of an aldimine seems likely to be involved in this inhibition. These phenomena that we have observed with the sea urchin DNA polymerases are quite analogous to other eukaryotic DNA polymerases α and β (8,9,13,15). In agreement with

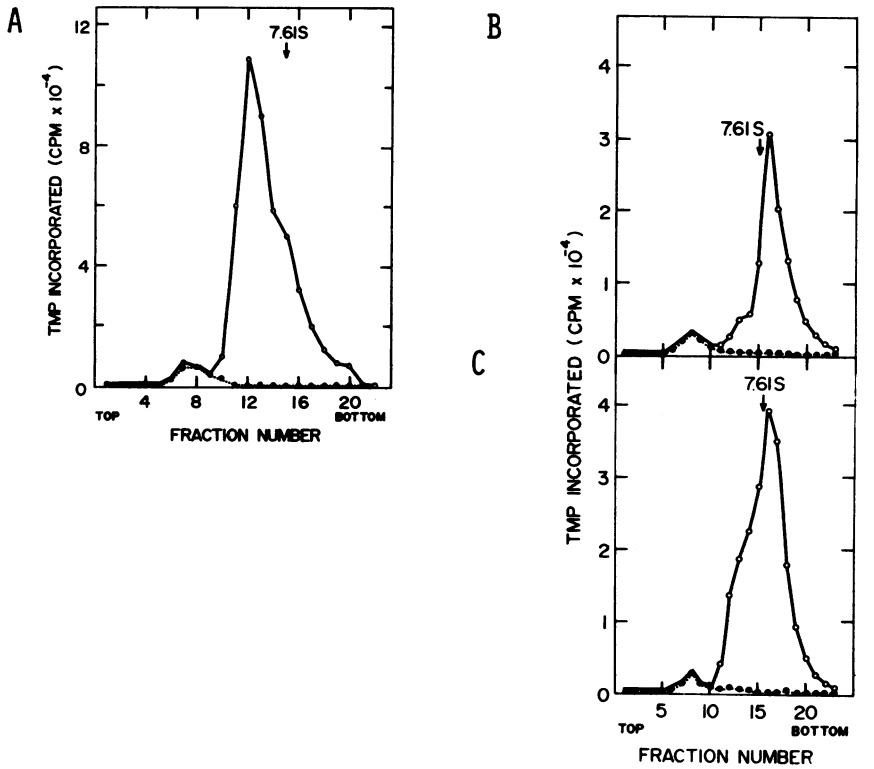

Figure 1: Sucrose density gradient fractionation of crude extracts of S. purpuratus eggs and blastula. Sedimentation was from the left to the right. DNA polymerase was assayed with activated DNA in the absence (open circles, solid line) and presence (close circles, broken line) of 10 mM N-ethylmaleimide. The arrow indicates the internal marker, yeast alcohol dehydrogenase, assayed by the method of Vallee and Hoch (27). Panel A: Unfractionated extract of eggs; Panel B: Unfractionated extract of blastula; Panel C: Nuclear extract of blastulae.

the previous report of Chang (15), we found that DNA polymerase β is a definite, albeit minor, enzyme in the urchin.

During preliminary experiments with the urchin DNA polymerases, we observed a pronounced instability of the DNA polymerase α during centrifugation in sucrose gradients constructed with $(NH_4)_2SO_4$ and containing low glycerol concentration. Results for the recovery of α and β activity from crude egg homogenates are shown in Table 2. The assayable DNA polymerase β was not diminished by alteration of glycerol concentration whereas DNA polymerase α activity was nearly lost by reduction of the glycerol from 21% to 14%. The instability of DNA polymerase α in the absence of glycerol was most noticeable with partially purified samples (e.g., fraction 24-26, Figure 2) containing an

Table 1: Properties of Urchin DNA Polymerase α and β

Parameter[a]	α	β
Sedimentation Coefficient	6-8S	3.4S
Percent inhibition by[b]:		
10 mM N-ethylmaleimide	100%	0%
1 mM Pyridoxal phosphate	98%	0%
0.1 mM Cu^{+2}	89%	0%[c]
Activity ratio in:		
0.2 M NaCl/0.1 M NaCl	0.37	1.0
Poly(dC)/Activated DNA	0.041[d]	<0.0039[e]

[a]Representative data obtained with several DNA polymerase preparations from eggs and embryos.

[b]All enzyme assays used the activated calf thymus DNA directed polymerization [^{3}H]-TTP and 3dNTPs except for the poly(dC) directed polymerization of [^{3}H]-GTP.

[c]As described in reference 24.

[d]4.6 pmoles [^{3}H]-GMP incorporated/20 min assay;
111 pmoles [^{3}H]-TMP incorporated/20 min assay;

[e]<0.1 pmoles [^{3}H]-GMP incorporated/20 min assay;
26 pmoles [^{3}H]-TMP incorporated/20 min assay.

α:β activity ratio of 12:1, after sedimentation in 10% glycerol such samples showed an α:β activity ratio of 1:1. An initial examination of these results suggested conversion of DNA polymerase α to β; however, a balance of applied and recovered activity showed that the DNA polymerase α activity was lost while 95% of the β activity was recovered. There clearly was no evidence for interconversion of the α and β polymerases. The stability of α activity in these sedimentation experiments showed no useful temperature dependency; at temperatures between 2-10°C extensive loss occurred at lower glycerol concentrations. This glycerol dependence was more obvious with $(NH_4)_2SO_4$ but also occurred to a lesser extent with NaCl. Thus we have found the use of 25% glycerol in all buffers to be mandatory.

Comparison of the DNA polymerase α profiles between egg and blastula (Figure 1) revealed an increase in the sedimentation coefficient for this enzyme from the embryonic cells. The α-activity, either from whole embryos or nuclei (Figure 1b and 1c), sedimented at 7.8 S with a shoulder at 6.3 S; in contrast, the α-activity of the egg sedimented predominantly at 6.3 S with a shoulder at 7.8 S. The trivial possibility that the DNA polymerase of the

Table 2: Dependence on Glycerol for Recovery of DNA Polymerase Activity

Glycerol Concentration	Units of DNA Polymerase/ml of eggs[a]	
	α-Activity	β-Activity
14%	3.25	0.88
17	13.8	0.94
21	17.6	0.88

[a]A unit equals 1 nmole of TMP incorporated/10 min on an activated DNA template. All gradients were centrifuged for 22 hr at 10°C in 5-20% sucrose gradients constructed in 0.1 M $(NH_4)_2SO_4$, 50 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 0.5 mM dithiothreitol, and the indicated glycerol concentration.

embryo was bound to DNA fragments seems unlikely since the polymerase β exhibited no change in sedimentation coefficient. DNA polymerase β bound DNA more tenaciously than polymerase α (20,21) and eluted from DNA-cellulose at higher ionic strength in the present work (data not shown). Further, sedimentation profiles similar to Figure 1b were observed with unfractionated extracts of both 4 cell and gastrula embryos. The nuclear DNA content of the 4 cell embryo is closer to the egg than to the gastrula (1800-2000 cells) and the DNA polymerase activity of the early embryo is still largely cytoplasmic in location (4). For these reasons we consider that fortuitous binding of DNA by the polymerase α cannot account for the observed increase in sedimentation coefficient. The possibility of fortuitous binding of non-DNA entities to the polymerase α was examined by comparing the sedimentation results from whole embryo homogenates of several stages (4-cell, pre-hatching blastula, mesenchyme blastula, and gastrula) and from nuclei of mesenchyme blastula. All these preparations showed the 7.8 S sedimentation profile typical of the embryo. The 4-cell and pre-hatching blastula are similar to the egg in their yolk granule and jelly coat content; the mesenchyme blastula and the gastrula stages are progressively unlike the egg or 4-cell with the loss of the jelly coat and fertilization membrane at hatching and metabolism of the yolk stores. All these embryonic stages have a decreasing cytoplasmic:nuclear volume ratio. Despite the difference in constitution of the samples ranging from the 4-cell homogenate to the gastrula nuclei, the DNA polymerase α was observed to sediment at 7.8 S rather than 6.3 S as in the egg. Because of the diversity of samples contributing to this observation, the fortuitous association of DNA polymerase α with other components does not provide a satisfactory explanation. At the present there is no satisfacotry explanation although we wonder

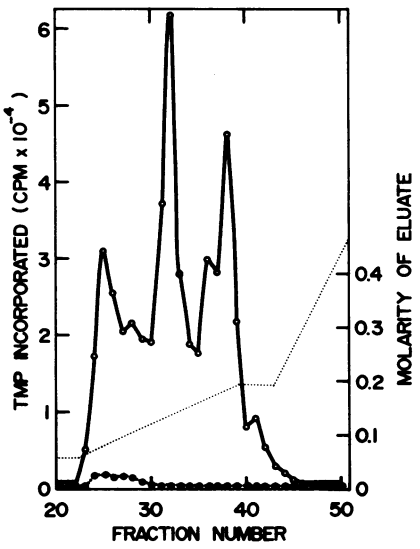

Figure 2: DEAE-Sephadex gradient sievorptive elution profile of unfractionated extract of eggs. DNA polymerase was assayed with activated DNA in the absence (open circles, solid line) or presence (closed circles, broken line) of 10 mM N-ethylmalimide.

whether activation of the egg effects aggregation of several proteins with a replicative function.

Resolution of the DNA polymerases by gradient sievorptive elution on DEAE-Sephadex (Figure 2) showed a complex profile with three major activity peaks; the β-activity was detected as a single minor component by N-ethylmaleimide inhibition. Inspection of the DEAE-Sephadex profiles from several experiments suggested that the DNA polymerase α was quite heterogeneous, perhaps with multiple species giving rise to the modal appearance of three major chromatographic forms. Sequential sedimentation and DEAE-Sephadex chromatography showed that the 8 S component from the egg fractionated into three activity peaks with the major component eluting at 0.14 M NaCl (Figure 3a). The 6 S component of the egg, when subjected to the same protocol, fractionated with the major activity components at 0.07 and 0.19 M NaCl and a minor activity component at 0.14 M (Figure 3b). A similar fractionation of the 6-8 S embryonic DNA polymerase α by sequential sedimentation and DEAE-Sephadex gradient sievorptive elution revealed two major species eluting at 0.07 and 0.19 M NaCl with only a small activity peak apparent at the intermediate ionic strength (Figure 5). Comparison among several egg and embryo preparations led to the conclusion that the 0.14 M NaCl species was enriched in the 8 S fraction from the egg but appeared as a lesser component in embryonic samples where the 0.07 and 0.19 M species sediment around 8 S.

Rechromatography by DEAE-Sephadex of the 0.07 and 0.19 M species from the egg showed that each eluted at the same ionic strength as before (Figure 4a

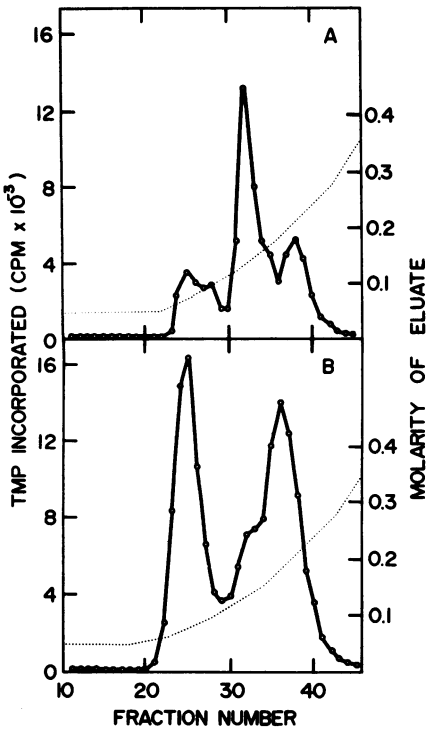

Figure 3: DEAE-Sephadex gradient sievorptive profiles of the 6 S and 8 S DNA polymerase components of eggs. Fractions from 6 identical sucrose density gradients, corresponding to either the 6 S (fraction 11, Figure 1a) or the 8 S (fraction 16, Figure 1a) were pooled and 0.5 mL samples chromatographed as described. In both cases the recovery of enzyme activity ≈120%. Panel A: 8 S component. Panel B: 6 S component. Significant cross-contamination of the 6 S and 8 S seems unlikely since widely separated fractions were chosen for these experiments. Enzyme instability during re-sedimentation prevented a direct assessment of cross-contamination.

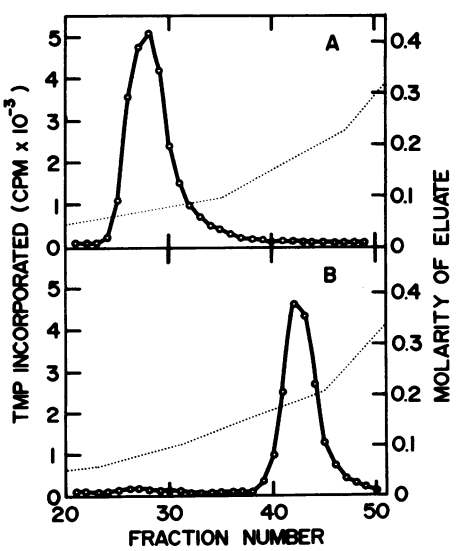

Figure 4: DEAE-Sephadex recromatography of peak fractions of the 6 S DNA polymerase component from eggs. Fractions were pooled and rechromatographed with conditions identical to the first DEAE-Sephadex chromatography. Panel A: Fractions 23-26 from Figure 3b. Panel B: Fractions 36-38 from Figure 3b.

and b); likewise, these two species from the embryo (Figure 5) eluted at the same ionic strength when rechromatographed (data not shown). In all cases the 0.07 and the 0.19 M NaCl species rechromatographed unchanged regardless of developmental stage or initial sedimentation coefficient. In contrast, the 0.14 M species, whether from the egg (e.g., fractions 31-33, Figure 3a) or the embryo (e.g., fractions 35-37, Figure 5), was not stable to rechromatography. The 0.14 M, 8 S species from the egg consistently eluted at 0.07 M with ≃60% activity recovery. This recovery of activity plus the original low cross-contamination by the 0.07 M species (Figure 3a) suggests that the 0.14 M form may be converted to the low salt form by rechromatography. The low activity present in the 0.14 M species of both embryo and 6 S egg fractions precluded rechromatography of these without excessive loss. Multi-step purifications resulted in the loss of the 0.14 M species, either by apparent conversion to the 0.07 M species or by frank activity loss (see Figure 1 of the following manuscript). It appears likely that this DNA polymerase α may be derived from the 0.07 M species while both the 0.07 and the 0.19 M species appear to be stable DNA polymerases α of unknown structural relatedness. Unlike other workers (22), we have not found conditions that yield a single DNA polymerase α species other than by extensive activity loss.

We wondered whether these three modalities of DNA polymerase α might have enzymatic differences indicative of differing catalytic functions. Partial results from template preferences experiments are summarized in Table 3; in general, there were few significant differences among the three α-activities regardless of source. For reason of lack of difference, the majority of the data is not presented (i.e., for reactions on poly(dC)·oligo(dG), poly d-(C-G), poly d(A-T), poly(dA)·oligo(dT), and poly(dT)·oligo(dA)). Evidence for a DNA polymerase γ in these α polymerases was not obtained; Table 3 shows that all three species were less active on poly(A)·oligo$(dT)_{15}$ than on poly-(dA)·oligo$(dT)_{15}$. Similar results were found with extracts fractionated only by sedimentation. Thus the DNA polymerase γ, if similar to the mammalian enzyme in preference for poly(A)·oligo(dT) template (8,9), is not readily observed in these urchin extracts after fractionation by sedimentation alone or in combination with chromatography.

The DNA polymerase α was found to be strongly dependent upon DNase I digestion of the calf thymus DNA for maximal activity (Table 3), but all three of the DNA polymerase α species do not show equally low activities with the native calf thymus DNA. Whether this activity difference has any meaning to replicative function is unknown; one recognizes, however, that a nuclease

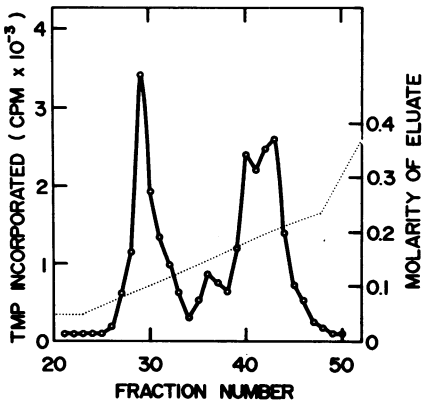

Figure 5: DEAE-Sephadex gradient sievorptive profile of the 6-8 S DNA polymerase from blastulae. Fractions from 3 identical sucrose density gradients (fractions 13-18 Figure 1b) are pooled and a 0.5 ml sample is chromatographed as described. Recovery of enzyme activity is 76%.

Table 3: Template Preferences of DNA Polymerase α

Template	Relative Activity of DNA Polymerase α Species[a]		
	0.07M	0.14M	0.19M
Activated Calf Thymus DNA	1.00 (22)[b]	1.00 (17)[b]	1.00 (19)[b]
Non-Activated Calf Thymus DNA	0.02	0.06	0.08
Poly(dC), dGTP + Mg^{+2}	1.10	1.03	0.96
Poly(dC), GTP + Mn^{+2}	0.12[c] (0.17-0.078)	0.03[c] (0.07-0.00)	0.048[c] (0.12-0.00)
Poly(A)·oligo(dT), TTP + Mn^{+2}	0.38	0.35	0.30
Poly(A)·oligo(dT), TTP + Mg^{+2}	0.14	0.13	0.11
Poly(dA)·oligo(dT), TTP + Mn^{2}	1.76	1.81	1.71

[a]The modal species of DNA polymerase α are identified by the NaCl concentration at which they elute from DEAE-Sephadex. Egg extracts were fractionated by density gradient centrifugation followed by DEAE-Sephadex chromatography of the 6-8 S pool. These data were obtained from pooled peak fractions of each DNA polymerase α from a single experiment with the exception stated in footnote c.

[b]The value in parenthesis is the pmoles of [^{3}H]-TMP incorporated/20 min assay; the limit of detection is 0.1 pmole/assay for all the data in this table. The DNA directed assays were as described in MATERIALS AND METHODS; the synthetic template directed assays used the stated NTP and divalent metal ion.

[c]The average values from five separate enzyme preparations with the range of values in parenthesis.

mixed with the DNA polymerase could yield template activation simultaneously with the polymerization reaction.

The most obvious activity difference among the three DNA polymerase α

species was the ribonucleotide polymerization in response to poly(dC) (Table 3). The poly(dC) directed GTP polymerization was consistently observed in DNA polymerase α preparations; however, this activity, relative to activated DNA, was observed to vary among preparations, particularly in the 0.14 M and 0.19 M NaCl forms of DNA polymerase from egg or embryo. In two preparations (egg) these latter forms lacked any poly(dC) directed GTP polymerization while the 0.07 M NaCl form of egg and embryo never failed to show such activity. The dGTP polymerization was nearly equal among the DNA polymerase α species and did not exhibit the variation observed with GTP regardless of whether Mg^{+2} (Table 3) or Mn^{+2}(not shown) was used as the divalent cation. This was the single catalytic activity that showed substantial differences among the DNA polymerase α species and is the sole piece of evidence that argues for enzymatic function as opposed to fortuitous aggregation as the basis for the heterogeneity of DNA polymerase α.

DISCUSSION

The results presented here show that the high and low S coefficient DNA polymerases from the sea urchin have enzymatic properties similar to the DNA polymerases α and β of higher eukaryotes (8,9) and to eukaryotes in general (15). These distinctive properties include N-ethylmaleimide resistance and a 3.5 S coefficient for the polymerase β and N-ethylmaleimide inhibition and a heterogeneous 6-8 S coefficient for the polymerase α. The lesser extent of activity on poly(A)·oligo(dT) than on poly(dA)·oligo(dT) leads to the presumption that the 6-8 S polymerase population is largely DNA polymerase α rather than γ. Sedimentation in high ionic strength did not reveal a 4 S, N-ethylmaleimide sensitive polymerase such as reported with the mitochondrial or γ polymerases of HeLa cells (23). The present activities were also seen in nuclei purified via sedimentation thru 1.9 M sucrose. For these reasons we consider that the N-ethylmaleimide sensitive DNA polymerases are the polymerase α and not a mitochondrial polymerase. However, all of the present chromatographic forms of urchin polymerase α exhibit appreciable activity on poly(A)·oligo(dT) in either Mn^{+2} or Mg^{+2} and therefore, are different from the polymerase α of mammals but are similar to the 7.3 S Drosophila DNA polymerase that was approximately one-fifth as active on poly(rA)·oligo(dT) as on poly(dA)·oligo(dT) (29). In this latter case the same enzyme was indicated as catalyzing the polymerization from both templates.

Further evidence for the dissimilarity of the DNA polymerases α and β was provided by metal ion and pyridoxal phosphate inhibition. The polymerase

β was completely insensitive to either Cu^{+2} or Fe^{+2} in several liganded forms (present work and 24) while the α was quite sensitive to inhibition by these metal ions. Catalytically essential lysine residues in DNA polymerase α was indicated by the reversible inhibition with pyridoxal phosphate; the polymerase β was resistant to this inhibitor also. Pyridoxal phosphate is known to react with essential lysine residues in several RNA polymerases including the E. coli, yeast polymerase I, and the rat liver polymerases I and II (25,26); also, all three classes of the sea urchin RNA polymerases are inhibited by pyridoxal phosphate (P.W. Morris, unpublished). It is not unexpected that lysine would form part of the active site in enzymes which bind a polyanionic nucleic acid. However, the finding that polymerase β is insensitive to pyridoxal phosphate suggests that lysine may not occur in the active site of this enzyme and emphasizes the fundamental difference between the α and β classes of DNA polymerases.

Inasmuch as eukaryotic DNA polymerases are well described from numerous other sources, it is scarcely heuristic to describe yet another DNA polymerase source unless it offers some unique property. The general uniqueness of egg/embryo systems such as the urchin is that the haploid cell contains sufficient replicative enzymes for many cycles of DNA replication (3-7). The specific uniqueness in this instance is the DNA polymerase α associated ribonucleotidyl transferase activity that is observed with poly(dC) or with DNA (5, present work and following manuscript). This particular ribonucleotidyl transferase was found with DNA polymerase α but not β after sedimentation resolution and further was most reliably associated with the low salt form of DNA polymerase α. As argued from inhibitor data (5) and purification behavior (following manuscript) this ribonucleotidyl transferase has no apparent relation to the DNA dependent RNA polymerases. The pertinent question then is whether the DNA polymerase α associated ribonucleotidyl transferase is indicative of in vivo function and, if so, what function. As developed in the following manuscript, this ribonucleotidyl transferase has some enzymatic properties reminiscent of the E. coli primase, an enzymatic function which is certainly contained but which is not described in eukaryotes.

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