Bulletin of Aichi Univ. of Education, 59 (Natural Sciences), pp. 43−50, March, 2010

Cytoplasmic Location of DNA Polymerase in Oocytes of the Teleost Fish, *Oryzias latipes*

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ABSTRACT

The intracellular location of DNA polymerase α during oocyte maturation in the teleost fish *Oryzias latipes* was examined. In order to define the optimum assay conditions, DNA polymerase α from the ovaries of the fish was isolated and partially purified by column chromatography before it was characterized. Optimum conditions for the assay of DNA polymerase α activity were Tris-HCl (pH7.8) as a buffer system and 10 − 20 mM Mg^{2+} as a divalent cation when activated DNA was used as a template primer. In fully grown oocytes, almost all of the DNA polymerase α activity was found in enucleated oocytes but not in isolated germinal vesicles. In addition, analysis by isopycnic centrifugation showed that DNA polymerase α was associated with unknown membranes (probably, vesiculated endoplasmic reticulum) appeared in relatively low density fraction, but not with the endoplasmic reticulum in relatively high density fractions obtained from either immature or mature oocytes. These findings suggest that in oocytes of the fish DNA polymerase α exists in the cytoplasm in a different manner from that of the toad (Nagano *et al.,* 1982). No qualitative change in DNA polymerase α activity was observed during the maturation process of the oocyte. However, the level of DNA polymerase α activity in mature oocytes was approximately 2-fold that found in fully grown immature oocytes.

Keywords: teleost fish, DNA polymerase α, oocyte maturation. isopycnic centrifugation

INTRODUCTION

It has so far been reported that almost all DNA polymerase α is located in the germinal vesicles of fully grown oocytes of the toad *Bufo bufo japonicus* (Nagano *et al.,* 1982), the frog *Xenopus laevis* (Fox *et al.,* 1980; Martini *et al.,* 1976; Grippo *et al.,* 1977) and the starfish, *Asterina pectinifera* (Oishi and Shimada, 1983; Haraguchi and Nagano, 1983). Furthermore, the DNA polymerase α in the germinal vesicle is released into the cytoplasm and binds to the endoplasmic reticulum (ER) when the germinal vesicle breaks down (Nagano *et al.,* 1982). This evidence explains the finding that DNA synthesis did not take place in frog oocytes matured after removal of the germinal vesicle (Katagiri and Moriya, 1976). However, we have found that sperm nuclei incorporated into the enucleated oocytes of a teleost fish were able to incorporate [^{3}H]thymidine into their acid-insoluble fraction (unpublished data) in a process that requires DNA polymerase. Therefore, we are interested in determining the location of DNA polymerase α in fish oocytes. It is known that DNA polymerase α in unfertilized eggs of the sea urchin, *Hmicentrotus pulcherimus,* is stored on the rough endoplasmic reticulum (RER) (Shioda *et al.,* 1977, 1980, 1982). We are attempt to confirm whether or not DNA polymerase α is likewise located on the RER of the fish oocyte.

DNA polymerase α of the fish has not been characterized except for that from the spermatocytes of dogfish *Scyliorhinus caniculus* (Phillippe and Chevaillier, 1980). It is necessary to define the optimum conditions for the assay of DNA polymerase α activity to investigate the location of this enzyme in fish oocytes. Thus, the properties of DNA polymerase α and β have been characterized using partially purified DNA polymerase α and β obtained by column chromatography.

In the present study, it was found that DNA polymerase α is located in the cytoplasm but not in the germinal vesicle of fish oocytes and that this enzyme does not bind to the RER with high density in the cytoplasm. We demonstrated that the intracellular location of DNA polymerase α in fish oocytes is different from that of the frog, starfish and sea urchin.

MATERIALS and METHODS

Materials

dATP, dCTP, dGTP and dTTP were purchased from Yamasa, Chiba. [^{3}H] TTP (43Ci/mmol) was obtained from Amersham International Ltd. Synthetic homopolymerases, poly(γA) and oligo(dT)$_{12-18}$ were purchased from PL Biochemicals. Yeast alcohol dehydrogenase, calf thymus DNA and aphidicolin were from Boehringer Manheim, Miles and Wako Pure Industries, Co., Ltd., respectively.

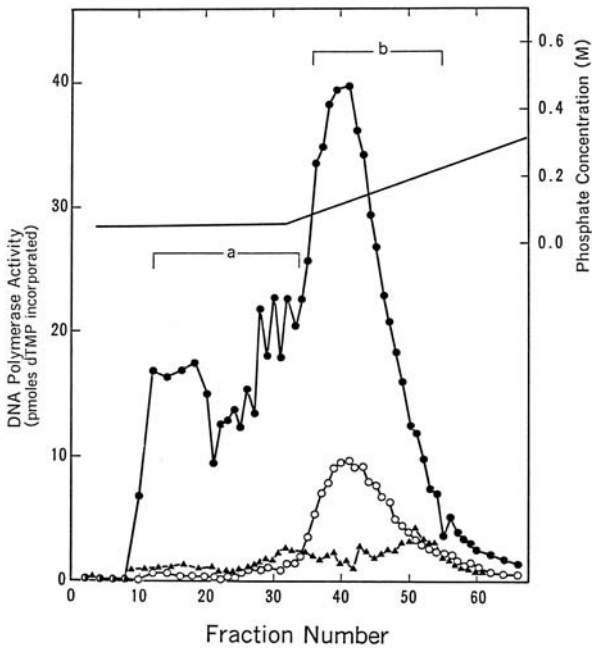

Fig. 1 Elution pattern of DNA polymerase from a DEAE-cellulose column.

Chromatography was performed as described in the Methods. DNA polymerase activity in 5 μl of each fraction was determined with a reaction mixture containing activated DNA as a template-primer in the presence (○) and absence (●) of 20mM MalNEt and a reaction mixture containing poly (γA)-oligo (dT) as a template-primer (△) as described in the Methods. Two types of DNA polymerase activity were observed with respect to the sensitivity to MalNEt when activated DNA was used as a template-primer. The MalNEt-sensitive activity (designated peak a) was eluted at a phosphate concentration of 50mM, and the relatively resistant activity to MalNEt (designated peak b), was eluted at higher phosphate concentrations from 70 to 200 mM. Poly (γA)-oligo (dT)-dependent DNA polymerase activity was very low in both peak a and peak b. Peaks of a and b were isolated and pooled for application to a phosphocellulose column. Concentration of phosphate buffer, solid line.

Preparation of the Fully Grown and the Mature Oocytes

Females of the medaka, *Oryzias latipes,* which spawned every morning (8:00) under a controlled photoperiod of 14 hr light and 10 darkness at a water temperature of 26±0.5℃, were used as source of mature oocytes (metaphase of the second meiosis). Fully grown oocytes (late prophase of the second meiosis) were released into saline from ovaries excised before spawning (21－22 hr) by use of fine forceps under a binocular dissecting microscope. The saline solution consisted of NaCl 650mg, KCl 40mg, $CaCl_2 \cdot 2H_2O$ 15mg and $MgSO_4 \cdot 7H_2O$ 15mg in 100ml of distilled water, adjusted to pH7.3 with N/2 $NaHCO_3$. After they were rinsed in this saline, the collected oocytes were placed in saline containing 25% glycerol, 2mg/ml bovine serum albumin, 1mM 2-mercaptoethanol, 25 mM Tris-HCl (pH8.0) and 10 mM $MgCl_2$ (Fox *et al.,* 1980), and then stored at －80℃.

Enucleation

Preparation of germinal vesicles from the intact and the enucleated fully grown oocytes was performed according to the methods described previously (Nagano *et al.,* 1982)

Sucrose Gradient Centrifugation

Forty fully grown, mature or enucleated oocytes or germinal vesicles were homogenized in 0.5ml of a homogenizing solution containing 25% glycerol, 0.2M potassium phosphate buffer (pH7.4), 1 mM EDTA, 2mM 2-merchaptoethanol, and 0.5M NaCl with a Teflon-glass homogenizer. The homogenate was centrifuged at 10,000x g for 60min and supernatant (0.3 ml) was applied to 4.8ml of a 20－30% sucrose gradient column containing 0.2M potassium phosphate buffer (pH7.4), 2 mM 2-mercaptoethanol and 1mg/ml bovine serum albumin. Centrifugation was carried out for 17 hr at 50,000 rpm using a Beckman preparative ultracentrifuge and an RPS65Ti rotor. Each fraction (10 drops) was collected from the bottom of the tube by puncturing it. Yeast alcohol dehydrogenase (7.5S) was used as the internal marker.

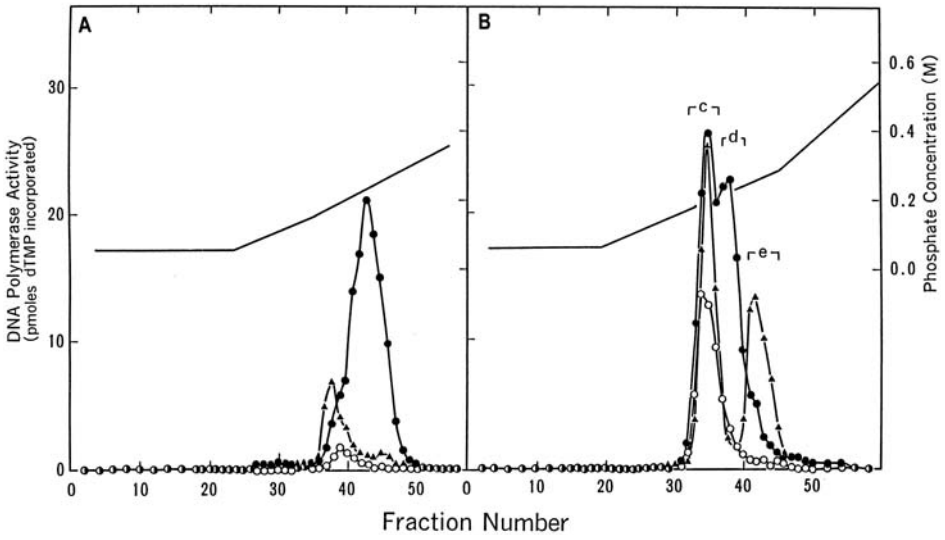

Fig. 2 Elution patterns of DNA polymerase from a phosphocellulose column for peak a (A) and peak b (B) fractions shown in Fig. 1.

Chromatography was performed as described in the Methods. DNA polymerase activity was assayed using the reaction mixture containing activated DNA as a template-primer in the presence (○) and absence (●) of 20mM MalNEt and the reaction mixture containing poly(γA)-oligo(dT) as a template-primer (▲), as described in the Methods. As shown in B, the MalNEt-resistant and poly(γA)-oligo(dT)-dependent activity designated peak c was eluted at phosphate concentrations from 160 to 190mM. The MalNEt-sensitive activity designated peak d was eluted from 200 to 500mM, and poly(γA)-oligo(dT)-dependent activity designated peak e was eluted from 240 to 270mM. The elution pattern of DNA polymerase was essentially the same in A as in B. The MalNEt-sensitive activity shown in A (fraction numbers 45 and 46) and peaks c, d and e were isolated and pooled. Concentration of phosphate buffer, solid line.

Isopycnic Sucrose Gradient Centrifugation

Forty fully grown, mature or enucleated oocytes were homogenized in 0.3ml of a solution containing 0.25M sucrose, 4 mM EDTA or 10mM $MgCl_2$ and 5mM 2-mercaptoethanol. After incubation for 1hr at 4℃, a 0.3ml-aliquot was applied to a column containing 4.9ml of a 10－70% linear sucrose gradient with 1mM Tris-HCl (pH8.0), 10mM $MgCl_2$, 5mM 2-mercaptoethnol, as described previously. Centrifugation was carried out for 5hr at 55,000rpm using a Hitachi RPS65T rotor. After fractionation, the free EDTA of each fraction was adjusted to 2mM.

Assay of DNA Polymerase Activity

Total activity of DNA polymerase α and β was assayed using a reaction mixuture (50μl) containing 50mM Tris-HCl (pH 8.0), 10mM $MgCl_2$, 40mM NaCl, 5 μg activated DNA, 20μg bovine serum albumin, 50μM each of dATP, dGTP and dCTP, 1 μCi of [^{3}H] dTTP and 10μM dTTP. After incubation at 37℃ for 60min, the acid insoluble radioactivity was determined, as described previously (Haraguchi and Nagano, 1983). For determination of DNA polymerase β activity, a reaction mixture containing either 2 mM N-ethylmaleimide (MalNEt) or 10μg/ml aphidicolin was used.

Poly(γA)-oligo(dT)-dependent DNA polymerase activity was assayed using a reaction mixture (50μl) containing 50mM tris-HCl (pH7.5), 2 mM $MgCl_2$, 20μg bovine serum albumin, 10μM dTTP. One μCi of [^{3}H] dTTP and 4 μg poly(γA)-ologo(dT) (base ratio; A/T＝4) as a template-primer.

Isolation and Partial Purification of DNA Polymerase α and β

All column chromatography procedures were performed at 4℃. The ovaries (1.1g) were homogenized by several strokes with a Teflon-glass homogenizer in 5 volumes of a buffer solution containing 0.2M potassium phosphate (pH7.4), 0.5M NaCl, 2 mM EDTA, 10mM 2-mercaptoethanol and 0.5mM phenylmethane sulfonyl fluoride. The homogenate was subjected to centrifugation at 10,000x g for 30min. The supernatant was dialyzed against a solution A (20% glycerol, 2 mM EDTA and 10mM 2-mercaptoethanol) containing 50mM potassium phosphate buffer (pH7.4), overnight, and then applied to a column (1.4x 5 cm) of DEAE-cellulose equilibrated previously with the same buffer. Elution was carried out with 40ml of a 50－400 mM phosphate linear gradient followed by 15ml of solution A containing 0.6M potassium phosphate buffer (pH7.4). Fractions of 1 ml were collected. Two fractions of DNA polymerase activity were obtained: one was sensitive to MalNEt and the other was relatively resistant to MalNEt. Each fraction was extensively dialyzed against the solution A containing 50mM po-

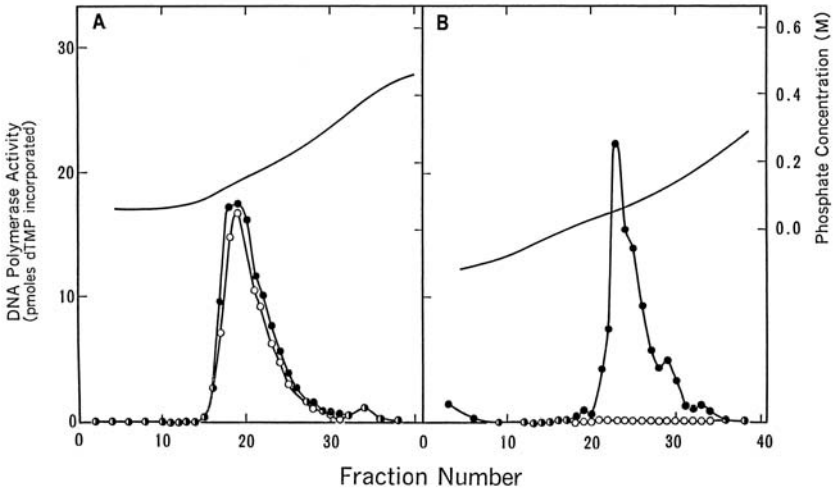

Fig. 3 Elution pattern of DNA polymerases from a hydroxyapatite column for the peak c fraction of Fig. 2B (A) and from a second phosphocellulose column for the peak d fraction of Fig. 2B (B).

Chromatography was performed as described in the Methods. DNA polymerase activity was assayed using a reaction mixture containing activated DNA as a template-primer in the presence (○) and absence (●) of 10μM dCTP, which was used in stead of 20μM dCTP. As shown in A, the aphidicolin-resistant activity was eluted at phosphate concentrations from 120 to 170mM. The aphidicolin-sensitive activity was eluted at phosphate concentrations from 230 to 250mM in B. The aphidicolin-resistant activity shown in A (fraction numbers18−21) and the MalNEt-sensitive activity shown in B (fraction numbers 23−25) was isolated and pooled. Concentration of phosphate buffer, solid line.

tassium phosphate buffer (pH7.4) before it was applied to a column (1.6x5.3) of phosphocellulose equilibrated with the same buffer. Linear gradient elution was performed under the same conditions, as described above. Using relatively MalNEt-resistant fractions from the DEAE-cellulose column chromatography, three peaks of DNA polymerase activity were revealed. The first enzyme fractions were applied to a hydroxyapatite column (0.5x5.5cm) and eluted under the same conditions as mentioned above except that 12ml of a 50−300mM potassium phosphate linear gradient followed by 4 ml of solution A containing 0.6M potassium phosphate buffer (pH7.4) were used. The second fraction, which was MalNEt-sensitive, was applied to a second phosphocellulose column (0.5x5.5cm) and eluted under the same conditions as described above except that 14ml of a 100−300mM potassium phosphate linear gradient followed by 4 ml of solution A containing 0.6M potassium phosphate buffer (pH7.4) were used. The resulting enzyme prepatations were stored at 4℃ and used for characterization studies. The resulting enzyme preparation was inadequate for characterization because it had a low level of the activity.

RESULTS

Isolation and Characterization of DNA Polymerase α and β

DNA polymerases were isolated by column chromatography, as described in the Methods and the legends of Figs. 1, 2 and 3, and then characterized. The fractions of the two peaks of the enzyme eluted by first phosphocellulose column (Fig. 2B) and by the second phosphocellulose column (Fig. 3B) were classified as eukaryotic DNA polymerase α, judging from the preference for activated DNA as a template-primer, the sensitivity to MalNEt, the sensitivity to aphidicolin and the relatively large molecular size of 6.5S (data not shown). The fractions of the enzyme eluted by a hydroxyapatite column were classified as DNA polymerase β judging from the reference to activated DNA and synthetic homopolymer, poly(γA)-oligo(dT), as a template-primer, the resistance to MalNEt and the resistance to aphidicolin. As shown in Figs. 4 and 5, DNA polymerase α and β were further investigated as to the optimum conditions for the assay when activated DNA was used as a template-primer. Both fractions of DNA polymerase α activity had essentially the same optimum conditions for the activity (data not shown). The optimum conditions for DNA polymerase α were Tris-HCl (pH8.0) as a buffer system and 10−20mM $MgCl_2$ as a divalent cation (Figs. 4A and 5). On the other hand, the highest activity of DNA polymerase β was obtained at 50mM Tris-HCl (pH8.5) as a buffer system over the tested range from pH6.5 to pH8.5 (Fig. 4B). The higher activity of both DNA polymerase α and β was observed in the assay mixture containing Tris-HCl buffer Tris-Maleate buffer. The optimum concentration

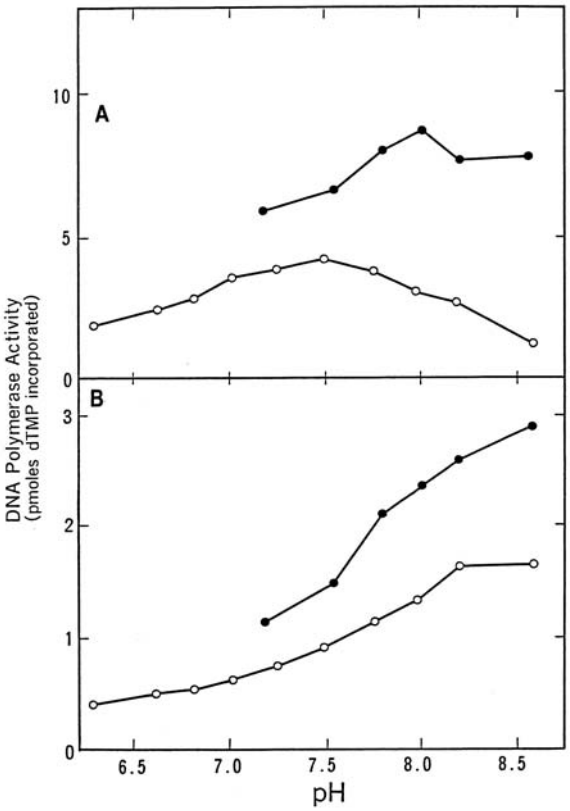

Fig. 4　Dependence of DNA polymerase activity on pH.

A: DNA polymerase activity was assayed in 5 μl of the MalNEt-sensitive fractions eluted by the first phosphocellulose column (Fig. 2A) using the reaction mixture containing 7 mM $MgCl_2$, 20μM each of ATP, dGTP and dCTP, 40mM NaCl, 0.1μg/ml activated DNA, 0.4mg/ml bovine serum albumin, 5 μM dTTP, 0.5μCi of [^{3}H] dTTP and 50mM Tris-HCl (●) or Tris-Maleate (○) buffer at different pH, as described in the Methods. B: DNA polymerase activity was assayed in 5 μl of the aphidicolin-resistant fractions eluted by a hydroxyapatite column (Fig. 3A) using the reaction mixture as described in A except for in the presence of 50μg/ml aphidicolin.

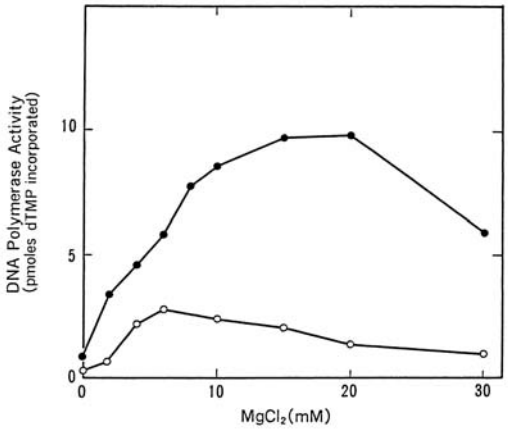

Fig. 5　Divalent cation requirements.

The same fraction of the enzyme as used in Fig. 4 was used in these experiments. DNA polymerase activity was assayed in 5 μl of the MalNEt-sensitive fractions (●) and the aphidicolin-resistant fraction (○) according to the methods that were described in the legend of Fig. 4 in the presence of indicated concentrations of $MgCl_2$.

of $MgCl_2$ for the enzyme activity was 6 mM (Fig. 5). Due to these results, a reaction mixture containing 50mM Tris-HCl (pH8.0), 10mM $MgCl_2$ and the other components as described in the Methods was used for the assay of DNA polymerase α activity.

Location of DNA Polymerase α in the Cytoplasm But Not in the Germinal Vesicle

Location of DNA polymerase activity was determined with whole fully grown oocytes, isolated germinal vesicles and enucleated oocytes (Fig. 6). The DNA polymerase activity was mainly due to DNA polymerase α judging from the large molecular size of 6.5S and the sensitivity to aphidicolin (data not shown). No appreciable amount of DNA polym-

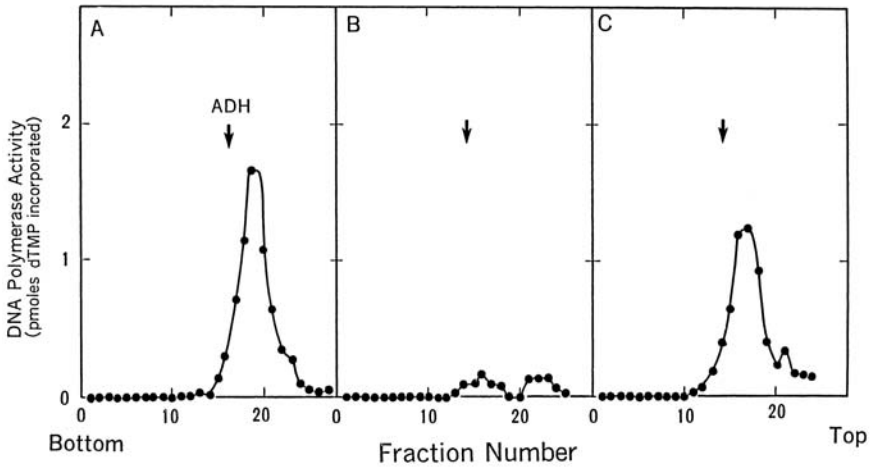

Fig. 6　Isokinetic linear sucrose gradient centrifugation.

Supernatants equivalent to 17 fully grown oocytes (A), 17 isolated germinal vesicles from fully grown oocytes (B) and 17 enucleated full grown oocytes (C) were subjected to sucrose gradient centrifugation, as described in the Methods. An arrow indicates the position of yeast alcohol dehydrogenase in each panel (7.5S).

erase β activity could be detected under our experimental conditions. A high level of DNA polymerase α activity was observed in enucleated oocytes (Fig. 6C). The level of DNA polymerase α activity was, however, very low in isolated germinal vesicles (Fig. 6B).

Approximately ninety percent of the DNA polymerase α activity found in whole fully grown oocytes was retained in the enucleated oocytes. Recovery of DNA polymerase α activity by enucleation was approximately one hundred percents.

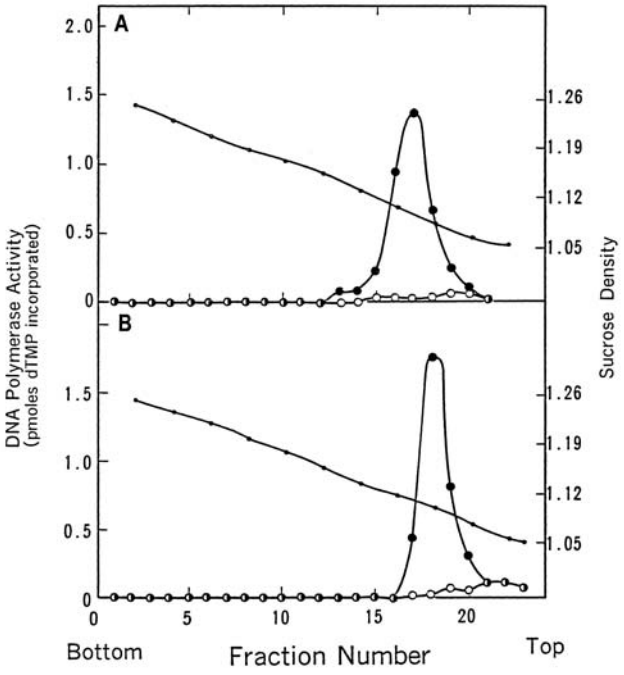

Fig. 7 Isopycnic centrifugation using a linear sucrose gradient containing 2 mM EDTA.

Homogenates equivalent to 7 intact (A) and 7 enucleated fully grown oocytes (B) were subjected to 10–70% linear sucrose gradient centrifugation, as described in the Methods. DNA polymerase activity in the absence (●) and presence (○) of 20mM MalNEt was determined in the presence of $MgCl_2$ at a final concentration of 10mM. Sucrose concentration, nodulated line.

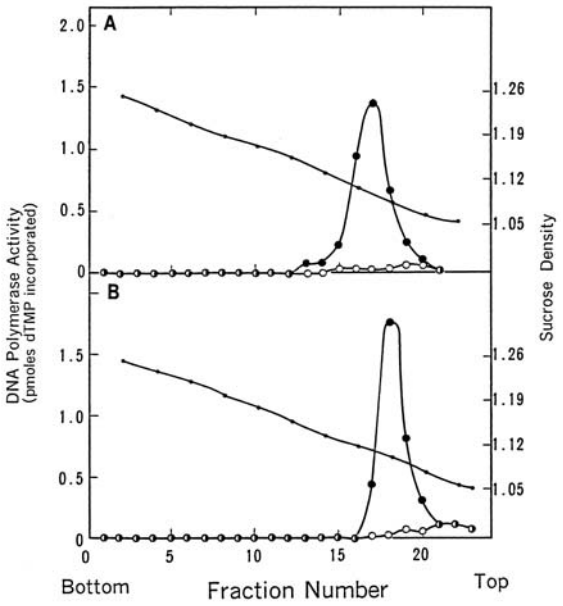

Fig. 8 Isopycnic centrifugation using a linear sucrose gradient containing 10mM $MgCl_2$.

The collected oocytes as described in the Methods were rinsed with 0.25M sucrose, and the solution was removed. Homogenates equivalent to 7 fully grown oocytes (A) and 7 mature oocytes (B) were subjected to 10–70% linear sucrose gradient centrifugation, as described in the Methods. DNA polymerase activity in the absence (●) and presence (○) of 20mM MalNEt was determined in the presence of EDTA at a final concentration of 2 mM. Sucrose concentration, nodulated line.

No Association of DNA Polymerase α with the Rough Endoplasmic Reticulum (RER)

DNA polymerase α is associated with the RER in sea urchin eggs (Katagiri and Moriya, 1976; Shioda *et al.,* 1977, 1980) and in toad eggs (Nagano *et al.,* 1982). The location of DNA polymerase α on microsomes was determined with the intact, enucleated fully grown and the mature oocytes of the teleost fish by isopycnic centrifugation. As shown in Fig. 7, the DNA polymerase α activity (6.5S and MalNEt-sensitive) of intact fully grown, enucleated fully grown and mature oocytes was observed in the fractions at a buoyant density of 1.110g/cm^3 (25% sucrose) in the presence of 10mM $MgCl_2$, but not in those of 1.229g/cm^3 (62% sucrose) corresponding to the position of RER. It has, so far, been reported that in the medaka oocyte, the vesiculation of the lamella structure (ER) occurs when the germinal vesicle breaks down (Iwamatsu *et al.,* 1976). Judging from its density, the fractions containing DNA polymerase activity were composed of the membranous fragments with the buoyant density, probably vesiculized ER. At a lower $MgCl_2$ concentration, the same results were obtained as in the case of 10 mM $MgCl_2$ (Fig. 8). From these results, it is concluded that DNA polymerase α in oocytes of the teleost fish is not associated with the RER with the high density. DNA polymerase β activity (MalNEt resistant) could not be found in this experiment.

Change of DNA Polymerase α Activity during Maturation Process

The qualitative change in DNA polymerase α activity during oocyte maturation was investigated using sucrose gradient centrifugation as shown in Fig. 9. The homogenate was inadequate to determine the level of DNA polymerase α activity because the MalNEt-sensitive activity is sometimes very low even in a crude extract. The level of DNA polymerase α activity is expressed as a relative rate from the peak area of DNA polymerase activity. The level of DNA polymerase α activity in mature oocytes was approximately 2-fold higher than that of fully grown immature oocytes.

DISCUSSION

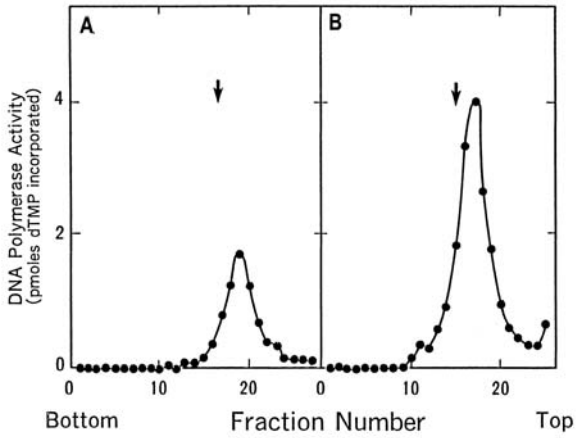

Fig. 9 Isokinetic linear sucrose gradient centrifugation.
Supernatants equivalent to 17 fully grown oocytes (A) and 17 mature oocytes (B) were subjected to a sucrose gradient centrifugation as described in the Methods. An arrow in each panel indicates the position of yeast alcohol dehydrogenase (7.5S).

Starfish oocytes contain four distinctive DNA polymerases, i.e. two types of DNA polymerase α, DNA polymerase β and DNA polymerase γ (Oishi and Shimada, 1983). In the present study, two species of DNA polymerase were isolated from the ovaries of a teleost fish. One is classified as eukaryotic DNA polymerase α and another as eukaryotic DNA polymerase β. Other types of DNA polymerase α and γ were not isolated in these experiments. The peak of the enzyme eluted by the first phosphocellulose column (Fig. 2) might be classified as DNA polymerase γ from the reference to poly(γA)-oligo(dT) as a template-primer and sensitivity to MalNEt. Generally, approximately 5 −10mM of $MgCl_2$ has been used for the assay of DNA polymerase α activity in eukaryotic cells (Matsukage, 1976). The concentration of $MgCl_2$ required for the assay of DNA polymerase α activity in the fish was higher than that for the other species. It has been reported that the optimum pH for eukaryotic DNA polymerase α activity is approximately 7.2 (Matsukage, 1976). Compared with this, a higher pH was required for the assay of the activity of DNA polymerase α from the fish oocyte.

It has previously been reported that almost all of the DNA polymerase α activity in fully grown oocytes of the toad (Nagano *et al.,* 1982; Fox *et al.,* 1980; Martini *et al.,* 1976) and the starfish (Oishi and Shimada, 1983) is contained in the germinal vesicles. In contrast,in the present study it was found that ninety percents of the DNA polymerase α activity is located in the cytoplasm of fully grown oocytes but not in the germinal vesicle. The reason why the location of DNA polymerase α in oocytes of the teleost fish is different from that of the toad and the starfish was not investigated in the present study. It is assumed that in *Oryzias latipes,* the components required for DNA synthesis are dispersed into the cytoplasm when the germinal vesicle breaks down. If the assumption is correct, the difference might be explained by consideration that the machinery of DNA synthesis or assembly of the enzymes related to DNA synthesis is formed in the cytoplasm but not in the germinal vesicle in the case of the fish. Further experimental investigations are necessary to find the explanation. The significance of the location of DNA polymerase α in the cytoplasm in fully grown oocytes of the fish remains unknown.

In other animals, it has been reported that DNA polymerase α dispersed into the cytoplasm during germinal vesicle breakdown is stored on the RER (Shioda *et al.,* 1980). In the present experiments, it is apparent that the membranous components corresponding to the RER migrated to the position of 62% sucrose in the presence of 10mM $MgCl_2$ during isopycnic centrifugation. This is the same position as that reported previously (Nagano *et al.,* 1982; Fox *et al.,* 1980; Martini *et al.,* 1976; Grippo *et al.,* 1977). However, DNA polymerase α activity was not detected at the position of the RER. These findings suggest that DNA polymerase α is not or is only loosely associated with the RER in fish oocytes. It is possible that DNA polymerase α in the fish oocyte is associated with any other intracellular components in the cytoplasm. On the other hand, it is assumed that DNA polymerase α stored on the ER is transported to the nucleus when the nuclear membrane is formed from the ER. In this respect, it is possible that DNA polymerase α in the fish oocyte is associated with the membranous components of which the nucleus is composed.

In the case of oocyte maturation, it has been reported that DNA polymerase α activity increases and a new species of the enzyme appears during oocyte maturation. On the other hand, we have previously reported that quantitative and qualitative changes in DNA polymerase α are not observed during oocyte maturation in the toad (Nagano *et al.,* 1982). From the results shown in Fig. 9, we infer that DNA polymerase α activity increases 2-fold during maturation in fish oocytes. Isopycnic centrifugation is not adequate to determine the level of the activity because DNA polymerase α is labile and easily denatured under low ionic conditions. DNA polymerase α activity from enucleated fully grown oocytes processed by isopycnic centrifugation was always low in comparison with that recovered by isokinetic centrifugation (data not shown). Therefore, we determined the level of DNA polymerase α activity based on the results of isokinetic centrifugation.

In the present study, we have demonstrated that DNA polymerase α in oocytes of the teleost fish, *Oryzias latipes,* exists in the cytoplasm in an unique manner, different from that of the toad, starfish or sea urchin. Our findings are important for further

investigations to clarify the mechanism of translocation of DNA polymerase α to the nucleus and DNA synthesis during early development.

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（Received August 18, 2009）