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Major DNA Polymerases Common to Different *Xenopus laevis* Cell Types

(oocytes/unfertilized eggs/kidney cells)

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ABSTRACT DNA polymerases from *Xenopus laevis* oocytes, unfertilized eggs, and kidney cells grown in culture have been characterized. The same three major DNA polymerase activities are present in all cell types examined. We attempt to relate the characteristics of amphibian enzymes to those of mammalian polymerases.

Eukaryotic organisms contain multiple DNA polymerases. Although the biochemical properties of the different eukaryotic DNA polymerases have not been definitely established, a considerable amount of information is available about the purification and characterization of the different enzymes.

Three major DNA polymerases have been described in mammalian cells (1–8). McCaffrey *et al.* (1) have suggested a unifying nomenclature: the 6–8S DNA polymerases found mainly in the cytoplasmic fraction of cell extracts is called DNA polymerase "C"; the enzyme sedimenting at 3.3 S, which is generally recovered from the nuclear fraction, is called DNA polymerase "N"; a third enzyme characterized by the ability to synthesize poly(dT) under the direction of poly(A)·oligo(dT) in the presence of Mn^{++} is called DNA polymerase "A."

Xenopus laevis oocytes and eggs represent an interesting system in which to study DNA synthesis. Young oocytes selectively replicate genes that make rRNA (9) whereas older oocytes are unable to carry out any kind of DNA replication (10). Eggs replicate exogenous DNA when it is injected (11), and chromosomal DNA replication occurs at an exceptionally high rate upon fertilization (12).

We are interested in studying the reasons for such variations in the ability of these different cells to synthesize DNA. With this aim, we have looked at the DNA polymerases in *Xenopus laevis* stage 4 and stage 6 oocytes (13), unfertilized eggs, and kidney cells grown in culture; we have also attempted to relate the characteristics of amphibian enzymes to those of mammalian polymerases. Our comparative study indicates that the same pattern of major DNA polymerases is shared by all the cell types examined.

MATERIALS AND METHODS

Materials

Deoxynucleoside-5-triphosphates were obtained from Schwartz Mann, Orangeburg, N.Y. [^{3}H]deoxyguanosine-5-triphosphate (7 Ci/mmol) and [^{3}H]methyl-deoxythymidine-5-triphosphate (15–30 Ci/mmol) were purchased from Amersham. Poly(A)·oligo(dT) and poly(dA)·oligo(dT) were produced by P.L. Biochemicals. Calf thymus DNA (type V, from SIGMA) was "activated" according to Loeb (14).

Cells

Xenopus laevis kidney cells were propagated in Eagle medium with 7% fetal calf serum at 20°.

HeLa cells were grown in suspension in Dulbecco medium with 10% fetal calf serum. Different stage oocytes were collected manually from the ovaries of adult frogs; stage 6 oocytes were defollicolated by collagenase treatment according to Schorderet-Slatkine (15). Germinal vesicles were isolated from stage 6 oocytes with watchmaker forceps; eggs were obtained from adult frogs treated according to Gurdon (16) and dejelled according to Dawid (17).

Isolation of polymerases

All operations were carried out at 0–4°. The purification procedure was the same as that used by McCaffrey *et al.* (1) except when modified as described below.

Oocytes and Eggs. Cells (0.5–1.0 ml) were resuspended in 5 ml of TEMG [Tris·HCl at pH 7.5 50 mM, ethylenediametetraacetate 1 mM, 2-mercaptoethanol 1.4 mM, glycerol 20% (w/v)] with 0.5% Triton X-100 and homogenized. The homogenate was centrifuged at 10,000 × *g* for 10 min (low-speed pellet), the supernatant centrifuged again at 150,000 × *g* for 60 min (high-speed supernatant), and then applied to a phosphocellulose column. In some experiments, the cell homogenate was prepared in TEM buffer (Tris·HCl at pH 7.5 50 mM, ethylenediaminetetraacetate 1 mM, 2-mercaptoethanol 1.4 mM) and the high-speed supernatant brought to pH 5.0 by slow addition of 1 M acetic acid. The suspension was stirred 20 min in the cold and centrifuged at 15,000 × *g* for 15 min. The pellet was carefully resuspended in 5 ml of TEMG buffer, clarified by centrifugation at 15,000 × *g* for 10 min and the supernatant (pH 5.0 fraction) applied to a phosphocellulose column.

In other experiments, 0.5 ml of cells were homogenized in TEMG buffer without the addition of Triton X-100 and cen-

TABLE 1. *Template specificities of peak I polymerase of stage 6 oocytes*

Template-primer	pmol of dTMP incorporated	
	Mg^{++} 6 mM	Mn^{++} 0.8 mM
Activated DNA	1.2	0.20
poly(dA)·oligo(dT)	1.0	0.25
poly(A)·oligo(dT)	0.5	0.12

Fractions corresponding to peak I of a phosphocellulose column were pooled and dialyzed as described in *Materials and Methods*. The reaction mixture contained in a 0.1 ml volume, Tris·HCl at pH 8.5 50 mM, dithiothreitol 6 mM, KCl 40 mM, [^{3}H]TTP 20 μM (400 cpm/pmol). Template concentration was 50 μg/ml. When activated DNA was the template, the incubation mixture was supplemented with 0.1 mM dATP, dGTP, and dCTP. Either 6 mM $Mg(acetate)_2$ or 0.8 mM $MnSO_4$ was used. Assays were run at 30° for 30 min.

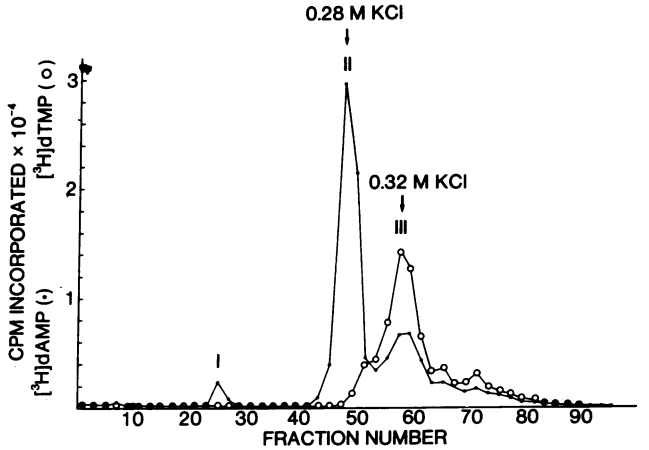

FIG. 1. DNA polymerases from stage 6 oocytes. The high-speed supernatant was applied to a phosphocellulose column. The fractions were assayed with activated DNA (●——●) or with poly(A)·oligo(dT) (○——○) as templates.

trifuged at 10,000 × *g* for 10 min. The supernatant was saved and the low-speed pellet resuspended in 5 ml of TEMG and centrifuged again at 10,000 × *g* for 10 min. The supernatant was combined with the first one and the pellet carefully resuspended in 5 ml of TEMG with 0.5% Triton X-100. The suspension was centrifuged at 10,000 × *g* for 10 min; this last supernatant and the two combined ones prepared without detergent, were centrifuged at 150,000 × *g* for 60 min and then separately applied to two phosphocellulose columns.

Cultured Xenopus laevis Kidney Cells. Cells grown in monolayers were scraped with a rubber policeman, washed three times by centrifugation with Tris·HCl at pH 7.5 20 mM, ethylenediaminetetraacetate 1 mM, NaCl 150 mM). Cell pellet (0.1–0.4 ml) was resuspended in 5 ml of TEMG and sonicated in a Branson sonicator (microtip attachment, setting 4.4 × 10 sec bursts). Triton X-100 (0.5%) was added to the sonicate which was then processed as the homogenate of oocytes and eggs.

HeLa Cells. Cells grown in suspension were collected by centrifugation and washed three times with phosphate buffered saline (NaCl 137 mM, KCl 2.7 mM, $NaHPO_4$ 8.1 mM, KH_2PO_4 1.47 mM). Cell pellet (0.3–0.4 ml) were resuspended in 5 ml of TEM buffer with 0.5% Triton X-100, dounced in a glass homogenizer, and the homogenate processed as for *Xenopus* oocytes.

TABLE 2. *Template specificities of peak II enzyme of stage 6 oocytes and of "N" enzyme of HeLa cells*

	pmol of dTMP incorporated			
	Mg^{++} 6 mM		Mn^{++} 0.8 mM	
Template-primer	peak II	"N"	peak II	"N"
Activated DNA	127	16	31	6
poly(dA)·oligo(dT)	11	4	287	86
poly(rA)·oligo(dT)	0.35	0.57	6	2

The enzymes were assayed as in Table 1. Fractions corresponding to peak II and to "N" polymerases from phosphocellulose columns of oocytes and HeLa cells respectively, were pooled and dialyzed as described in *Materials and Methods*.

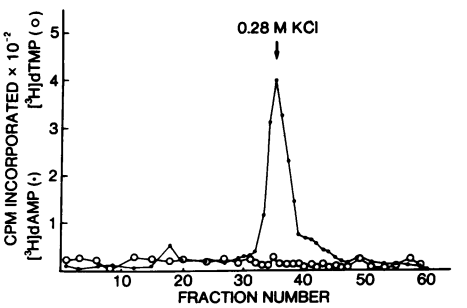

FIG. 2. DNA polymerase from stage 6 oocyte germinal vesicles. A pH 5.0 fraction derived from isolated germinal vesicles was chromatographed on phosphocellulose. The fractions were assayed with activated DNA (●——●) or with poly(A)·oligo(dT) (○——○) as templates.

Phosphocellulose Chromatography. Phosphocellulose (Whatman P 11) was prepared according to Burgess *et al.* (18). From 8 to 10 mg of protein were usually applied to a column (1 cm × 16 cm) equilibrated with TEMG buffer. The column was washed with 10 ml of TEMG buffer containing 1 mg/ml of bovine serum albumin, and eluted with 90 ml of a linear gradient of 0.2–0.8 M KCl in TEMG buffer containing 1 mg/ml of bovine serum albumin. In some experiments, a 0–1.0 M KCl gradient was used. The material was applied and eluted at a flow rate of 12 ml/hr. Fractions (0.6 ml) were collected. KCl concentration in the eluted fractions was measured with a conductimeter.

Assay Conditions for DNA Polymerase Activity. Phosphocellulose fractions were assayed at 37° for 30 min with two different templates, activated DNA and poly(A)·oligo(dT). Both reaction mixtures, in a final volume of 0.1 ml, contained 50 µl of the phosphocellulose fractions, 50 mM Tris·HCl at pH 8.5, 6 mM dithiothreitol. In addition, the activated DNA mixture contained: $Mg(acetate)_2$ 6 mM, dTTP, dGTP, and

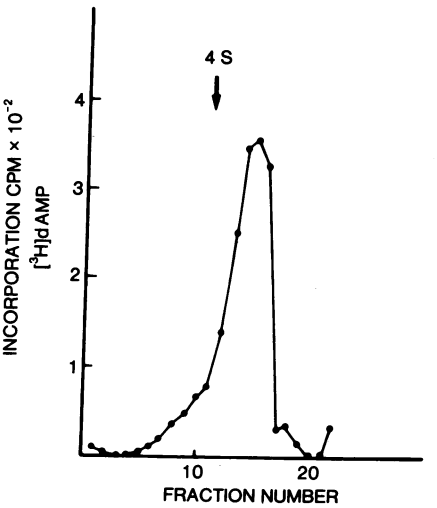

FIG. 3. Sedimentation in glycerol gradient of DNA polymerase from germinal vesicles. A pH 5.0 fraction derived from germinal vesicles was sedimented through a linear 10–30% glycerol gradient containing Tris·HCl at pH 7.5 50 mM, KCl 300 mM, dithiothreitol 10 mM. The centrifugation was in a Spinco rotor SW 50.1 at 43,000 rpm for 16 hr at 4°. [^{32}P]tRNA was used as marker. The fractions of the gradient were assayed with activated DNA as template.

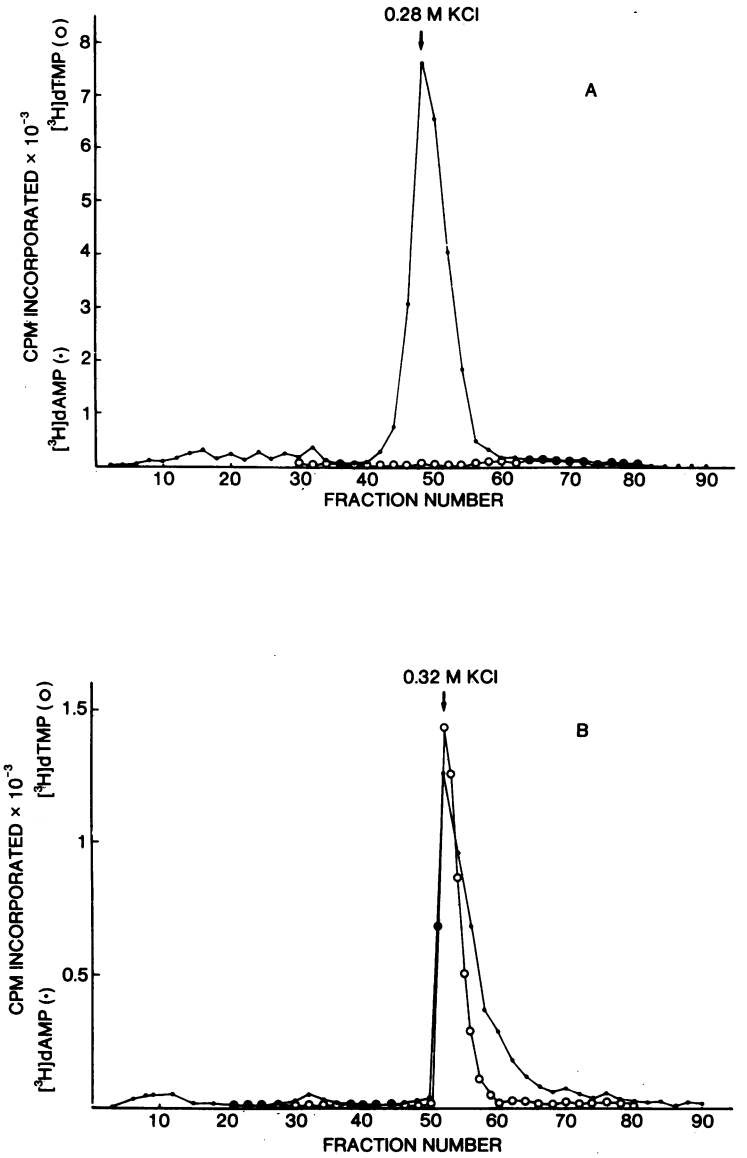

FIG. 4. Effect of Triton X-100 on the extraction of DNA polymerases from stage 6 oocytes. (A) The high-speed supernatant obtained using an extraction buffer deprived of Triton X-100 (see *Materials and Methods*), was applied to a phosphocellulose column. (B) The high-speed supernatant derived from a low-speed pellet extracted with Triton X-100 was applied to a phosphocellulose column. Activated DNA (●——●) and poly(A)·oligo(dT) (○——○) were used as templates.

dCTP 20 μM, [³H]dATP 0.75 μM (3000 cpm/pmol), activated DNA 6.25 μg/(0.1 ml assay). The poly(A)·oligo(dT) assay contained $MnSO_4$ 0.8 mM, [³H]dTTP 0.33 μM (6500 cpm/pmol) and 1 μg of template-primer/0.1 ml of assay. To compare template specificities, fractions of the phosphocellulose column corresponding to the three peaks, were dialyzed against modified TEMG buffer (pH 8.5), containing 0.1 mM ethylenediaminetetraacetate instead of 1 mM, and assayed as indicated in the legends of the tables.

RESULTS

DNA polymerases in stage 6 oocytes

The chromatographic pattern of stage 6 oocytes shows three major DNA polymerase activities when the column is assayed with activated DNA as template (Fig. 1). The first peak (I) elutes as soon as the 0.2–0.8 M KCl gradient is applied. When a 0–1.0 M KCl gradient was used, the first peak eluted at 0.15 M KCl. The subcellular localization of peak I enzyme appears to be mainly cytoplasmic since it was found in eluates from a phosphocellulose column of oocytes from which the germinal vesicles had been manually removed (data not shown). Peak I prefers activated DNA as template (see Table 1) and this characteristic allows us to relate this enzyme to polymerase I described by Smith and Gallo in human lymphocytes (6), polymerase "C" of McCaffrey *et al.* (1), maxipolymerase of Yoneda and Bollum (2). In "L" cells in culture, it has been shown that the amount of "C" enzyme varies according to the rate of cell growth and DNA synthesis (19). Peak I showed variable, small amounts of activity and in some preparations was almost undetectable. We observed this variability both in oocytes and eggs in which DNA replication is absent, and also in kidney cells growing in culture. The low yield of peak I did not allow a better characterization nor the determination of its sedimentation coefficient.

The second peak (II) elutes at 0.28 M KCl and represents the major activity. This enzyme is associated with the germi-

TABLE 3. *Template specificities of peak III polymerase of stage 6 oocytes*

Template-primer	pmol of dTMP incorporated	
	Mg^{++} 6 mM	Mn^{++} 0.8 mM
Activated DNA	10	2.5
poly(dA)·oligo(dT)	36	9
poly(rA)·oligo(dT)	2	11

Fractions from peak III of a phosphocellulose column were pooled and dialyzed as described in *Materials and Methods*. Enzyme was assayed as in Table 1 except that the KCl concentration was 150 mM when Mn^{++} was present.

nal vesicles. Fig. 2 shows the phosphocellulose pattern of a germinal vesicles-extract in which only peak II is detected in appreciable amount. Peak II activity is undetectable in column eluates derived from cytoplasms which have been deprived of nuclei (data not shown). In a glycerol gradient (Fig. 3), this enzyme sediments slower than the 4S marker. The sedimentation properties and the subcellular localization of the enzyme correspond to those of the "N" enzyme (1). Table 2 shows the template specificities of peak II and those of the "N" enzyme isolated from HeLa cells under the same conditions. Both enzymes markedly prefer poly(dA)·oligo (dT) in presence of Mn^{++} as does the DNA polymerase II described by Smith and Gallo in human lymphocytes (6) [minipolymerase of Chang and Bollum (4)].

The third peak (III) elutes at 0.32 M KCl with a broad tail; we occasionally observed preparations that yielded double peaks. Fig. 1 shows that when the column is assayed with poly(A)·oligo(dT) as template in presence of Mn^{++}, the only poly(dT) synthesizing activity observed, coincided with peak III. The template specificities of peak III (Table 3) indicate that in the presence of Mn^{++} the best template is poly-(A)·oligo(dT). This property and the fact that peak III was detected in cytoplasmic extracts of anucleated oocytes (data not shown) allows us to suggest that the poly(dT) synthesizing activity is the amphibian equivalent of the R-DNA polymerase (20,21) [polymerase "A" of McCaffrey *et al.* (1)].

When we fractionated a HeLa cell extract, which was prepared following the same procedure, the "A" enzyme and the "C" polymerase chromatographed close together as has been previously observed (1). Other fractionation methods yielded the separation of the two enzymes (22). The fact that peak III activity is observed when both activated DNA and poly(A)·oligo(dT) are used as templates, could indicate that different activities overlap in our chromatogram and we are investigating this possibility. We did not detect along the chromatogram any terminal deoxynucleotidyl-transferase activity (26).

The elution profile of DNA polymerases described above is characteristic of extracts derived from stage 6 oocytes either with or without removing follicle cells with collagenase. The same elution profile results if a pH 5.0 fraction is chromatographed.

When Triton X-100 is not added to the extraction buffer (see *Materials and Methods*), the phosphocellulose chromatogram of the high-speed supernatant shows only peak II; activity requiring poly(A)·oligo(dT) as template is not detectable (Fig. 4A). Peak III activity can be recovered from

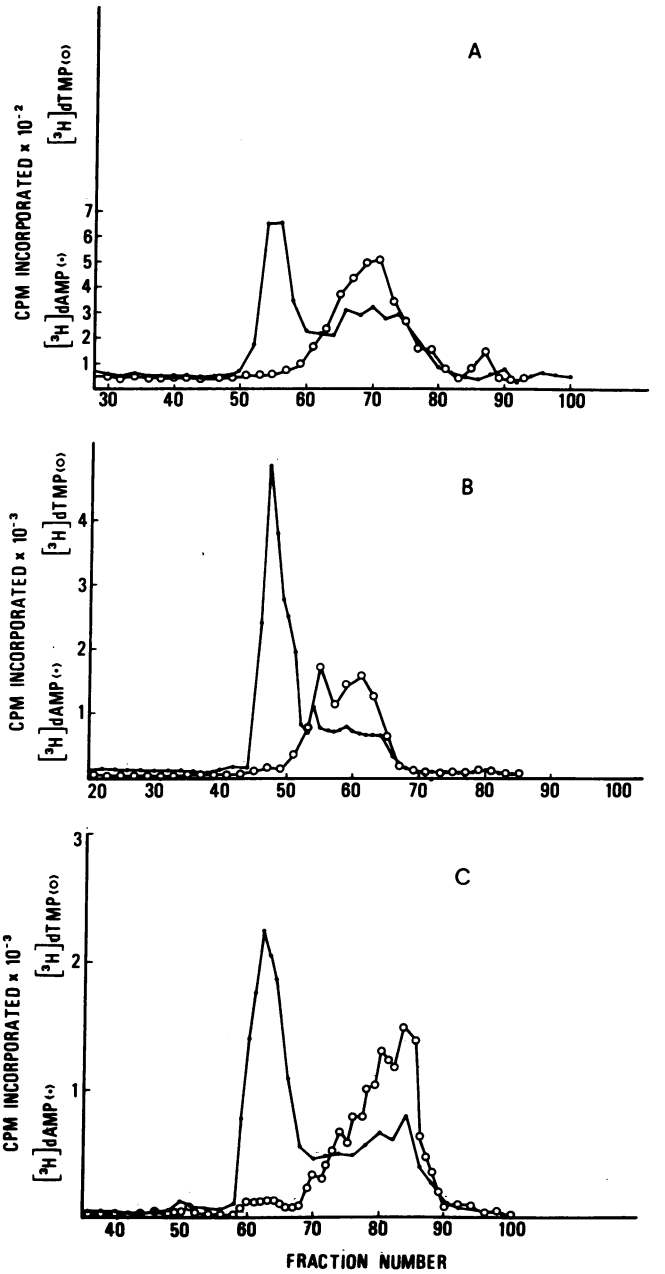

FIG. 5. DNA polymerases from stage 4 oocytes (A), eggs (B), and kidney cells (C). The high-speed supernatants, prepared from the various cell types, were applied to three phosphocellulose columns. Activated DNA (●——●) and poly(A)·oligo(dT) (○——○) were used as templates.

the low-speed pellet by reextraction with Triton X-100 containing buffer (Fig. 4B). In this experiment, peak I activity was too low to ascertain whether the detergent is necessary to solubilize the enzyme.

DNA polymerases in other cell types

In order to compare the DNA polymerases from stage 4 oocytes, eggs, and kidney cells, with those from stage 6 oocytes, we used the same procedure in treating the various cell types. Fig. 5A, B, and C shows that the elution profile of DNA polymerase activities from these other cell types was very similar to the profile obtained from stage 6 oocytes. Peak I, which was detectable in other experiments, was only minimally active

in these preparations; peak II and III were instead very evident.

The template specificities of the enzyme preparations from the various cells types were also studied and found identical to those from stage 6 oocytes.

Stage 6 oocytes and eggs were found to contain comparable amounts of overall DNA polymerase activity per cell.

The fact that the same enzymes are observed in all of these cell types (kidney cells, oocytes, and eggs) which contain very different amount of mitochondria, indicates that we are not detecting the mitochondrial DNA polymerase (23). This is probably due either to the inefficient solubilization obtained using our procedure, or to improper assay conditions.

DISCUSSION

These data demonstrate that the same elution profile of DNA polymerases was obtained from all *Xenopus laevis* cells examined. The cell types we have examined are oocytes stage 4 and 6, eggs, and kidney cells grown in culture.

We have identified three activities: peak I which is present in variable amounts and in some preparations is undetectable; peak II which is associated with germinal vesicles in oocytes and, therefore, corresponds to the mammalian "N" enzyme; peak III which is the only poly(dT) synthesizing activity we have detected. We suggest that this last activity corresponds to the "A" enzyme in mammals. The amphibian "A" enzyme elutes at higher ionic strength than the "N" enzyme; the order of elution of the two enzymes in mammals is reversed.

In our attempt to relate *Xenopus laevis* enzymes to mammalian polymerases, we were unable to determine the amphibian equivalent of the "C" enzyme. Peak I is a possible candidate since its template specificity corresponds to that of the "C" enzyme, but the low activity which we detected, did not allow us to determine the sedimentation coefficient. Since peak III uses both poly(A)·oligo(dT) and activated DNA as templates, it is possible that another activity cochromatographs with the "A" enzyme. This activity may correspond to the mammalian "C" enzyme.

The detergent Triton X-100 is necessary to release the oocyte "A" activity which appears to be associated with a particulate fraction; consequently, the omission of the detergent allows the selective extraction of the "N" enzyme.

Gurdon *et al.* (24) have reported that injected DNA is replicated in eggs, but not in stage 6 oocytes. The presence, in stage 6 oocytes and in eggs, of the same major DNA polymerase activities suggests that a difference, alone, in the major polymerases here described, does not explain their findings. The two cell types could differ in minor DNA polymerase activities, since the presence of minor enzymes have been reported (25), or in other factors required for DNA replication.

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