# PARTICULATE DNA POLYMERASE FROM THE CYTOPLASM OF XENOPUS LAEVIS OOCYTES

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SUMMARY: A DNA polymerase has been partially purified and characterized from Xenopus laevis stage 6 oocytes. The enzyme is present only in the cytoplasm and has been shown to be able to copy Poly(A).oligo(dT), to be sensitive to N-ethylmaleimide, and to sediment faster than 4 S in high salt glycerol gradient. The enzyme can be extracted from particulate material which has a density in sucrose gradient ranging from 1.200 to 1.225 g/cc. This particulate material is identified by its ability to use Poly(A).oligo(dT) as template in an exogenous DNA polymerase reaction and by its endogenous DNA synthesizing capacity.

Three major DNA polymerases have been described in eucaryotic cells, $\alpha$ $\beta$ and $\gamma$. Polymerases $\alpha$ and $\beta$ have been shown to be nuclear enzymes (1). Polymerase $\gamma$ has been found in all cell types examined and has been reported to be present both in the nucleus and the cytoplasm of HeLa cells (2).

Tatò et al. (3) have observed that, in Xenopus laevis oocytes, the use of detergent was necessary to release in the high speed supernatant a DNA polymerase capable of reading Poly(A).oligo(dT). Our report presents data on partial purification and characterization of a particulate DNA polymerase from Xenopus laevis oocytes which also can use Poly(A).oligo(dT) as template. As a possible approach to understanding the function that the enzyme performs in the cell we have examined its cellular localization. Certain characteristics of the particulate material from which the enzyme can be solubilized have also been studied.

MATERIALS AND METHODS: Materials. Deoxynucleotide-5-triphosphates were obtained from Schwartz Mann, Orangeburg, N.Y. [$^3$H] methyl-deoxythymidine-5-triphosphate (40-50 Ci/mmol) was purchased from Amersham. Poly(A).oligo(dT) and Poly(dA).oligo(dT) were from P.L. Biochemicals. Calf thymus DNA (Type V, from Sigma) was "activated" according to Loeb (4).

Cells. Ovaries were collected from adult frogs and stage 6 oocytes were defolliculated by collagenase treatment according to Schorderet-Slatkine (5) and separated from oocytes at different stages of maturation with tea strainers of different

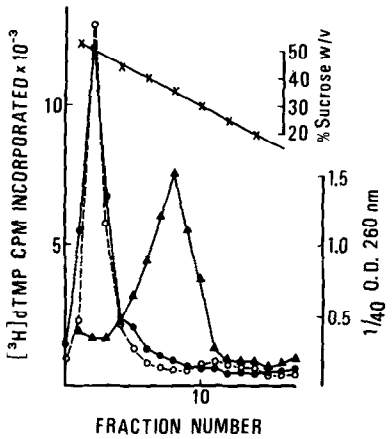


Fig. 1 - Isopycnic centrifugation of the "particle". The fractions of the gradients were assayed for endogenous DNA synthesis (o——o). Each assay, in a final volume of 50 µl, contained 25 µl of the fraction. Tris-HCl pH 8.5 50 mM, KCl 50 mM, MgAc 10 mM, dithiothreitol 5 mM, dATP, dCTP, dGTP 10 µM, [$^3$H] TTP 1 µM (5 x $10^3$ cpm/mol). The fractions of the gradient were assayed in an exogenous DNA polymerase assay using Poly(A).oligo(dT) as template (.——.). Each assay in a final volume of 50 µl, contained 25 µl of the fraction Tris-HCl pH 8.5 50 mM, MgAc 10 mM, dithiothreitol 5 mM, [$^3$H]TTP 1 µM (5 x $10^4$ cpm/pmol), Poly(A).oligo(dT) 10 µg/ml. The optical density of the fractions was also determined (▲——▲).

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sizes, washed with Barth's solution (6) and stored at -20°C in B buffer (sucrose 0.25 M, Tris-HCl pH 7.5 mM, ethylenediaminetetraacetate 1 mM, dithiothreitol 5 mM). Nuclei and cytoplasms were separated with watchmaker forceps under a dissecting microscope and stored frozen in B buffer.

Preparation of the "low speed pellet". 15-20 ml of packed oocytes were thawed and homogenized in 5 volumes of B buffer. All subsequent operations were performed at 0-4°C. The homogenate was centrifuged at 300 x g for 10 min and the supernatant centrifuged at 12, 000 x g for 20 min; the pellet was resuspended in B buffer and centrifuged again at 12, 000 x g for 20 min. This pellet is the starting material for the enzyme preparation from the "low speed pellet".

Preparation of the "particle". 15-20 ml of packed oocytes were thawed and homogenized in B buffer in the presence of 0.5% of NP40. The homogenate was centrifuged at 8, 000 x g for 20 min and the supernatant centrifuged at 38, 000 rpm in a 50.1 Spinco rotor for 75 min. The high speed pellet, washed and resuspended in B buffer, was layered on 15-60% (w/v) linear sucrose gradients containing Tris-HCl pH 7.5 10 mM, ethylenediaminetetraacetate 1 mM, dithiothreitol 5 mM, and centrifuged at 37, 000 rpm in a SW 41 Spinco rotor for 16 hours. The gradients

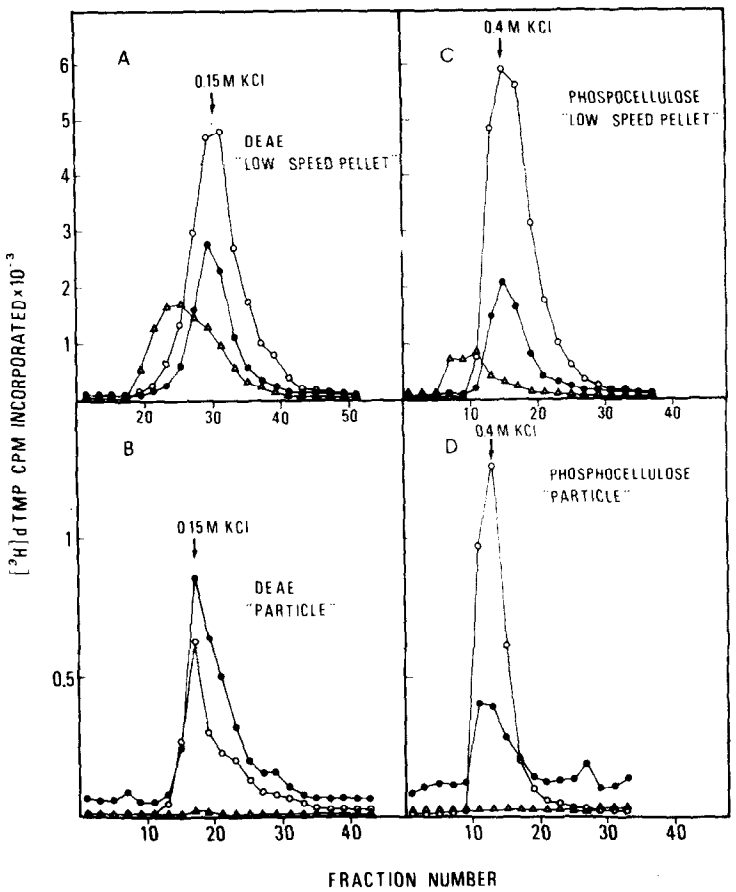


Fig. 2-A and B: DEAE-cellulose chromatograms
C and D: Phosphocellulose chromatograms
5 µl of the fraction(1.0 ml) were assayed as in Materials and Methods. Poly(A).oligo(dT)(.—.), Poly(dA).oligo(dT)(o—o), activated DNA(Δ—Δ) were used as templates. [$^3$H]TTP was used as label with a specific activity of 4500 cpm/pmol in the assay with Poly(A).oligo(dT) and 450 cpm/pmol in the other assays.

were fractionated and fractions assayed for exogenous and endogenous DNA polymerase activities. The active fractions were pooled, diluted to a sucrose concentration of 10% and pelleted at 38,000 rpm in a 50.1 Spinco rotor for 75 min. The resulting pellet is the starting material for the enzyme preparation from the "particle".

Purification of the enzyme. The enzyme was solubilized from either one of the two sources by resuspending the pellet in Tris-HCl pH 7.5 10 mM, ethylenediaminetetraacetate 1 mM, dithiothreitol 5 mM, KCl 1M, NP40 2%. The extract after standing at 4°C for 30 min was dialyzed against TEMG buffer.° The enzyme was purified from the dialyzed extract through a DEAE-cellulose

° Abbreviation: TEMG buffer: Tris-HCl pH 7.5 50 mM, ethylenediaminetetraacetate 1 mM, 2-mercaptoethanol 1.4 mM, glycerol 20% w/v.

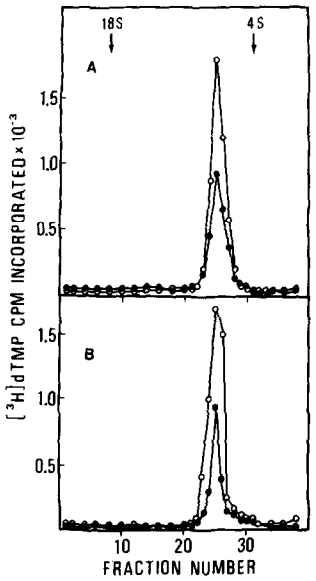


Fig. 3 - Sedimentation in glycerol gradient of DNA polymerase from "low speed pellet"(A), and from the "particle"(B). A linear 10-30% glycerol gradient containing Tris-HCl pH 7.5 50 mM, KCl 300 mM, dithiothreitol 10 mM was centrifuged in a Spinco rotor SW 41 at 39,000 rpm for 18 hours at 4°C. 4S RNA and 18S RNA were used as markers in a separate gradient. 10 µl of each fraction were assayed as in Materials and Methods with Poly(A).oligo(dT)(.____.) and Poly(dA).oligo(dT)(o____o) as templates, using [$^3$H]TTP as label with a specific activity of 4500 cpm/pmol for Poly(A).oligo(dT) and 140 cpm/pmol for Poly(dA).oligo(dT).

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chromatography followed by a phosphocellulose chromatography. The columns were equilibrated in TEMG buffer and the activity eluted with KCl gradients in TEMG buffer. The most active fractions from the phosphocellulose columns were dialyzed against TEMG buffer and used for enzyme characterizations and size determination in glycerol gradients.

DNA Polymerase assay. DNA polymerase assays, unless otherwise stated, were run for 30 min at 37°C. Each assay, in a final volume of 50 µl, contained Tris-HCl pH 8.5 50 mM, KCl 50 mM, MgAc 10 mM, dithiothreitol 2.5 mM, dATP, dCTP, dGTP 0.1 mM, [$^3$H]TTP 5 µM with a specific activity as indicated under the figures. Synthetic templates were used at 10 µg/ml, activated DNA at 60 µg/ml. Bovine serum albumin was present at 500 µg/ml.

RESULTS: Fig. 1 shows a sucrose gradient analysis of the "particle" prepared from stage 6 oocytes (see Materials and Methods). It should be noted that (a) after 7 hours of centrifugation the "particle" has already reached the equilibrium position; (b) the density of the "particle" has varied in different

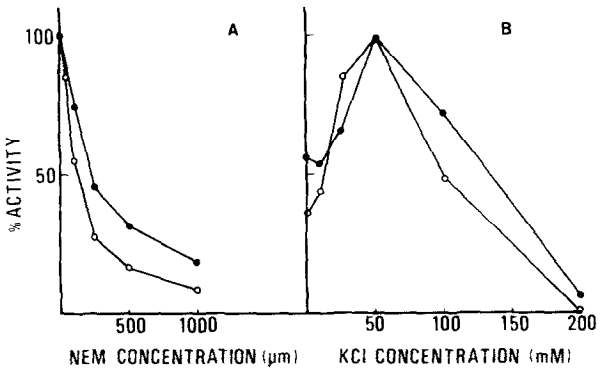


Fig. 4 - Effect of N-ethylmaleimide (A) and of KCl concentration (B) on "low speed pellet" enzyme using Poly(A).oligo(dT) (.——.) and Poly(dA).oligo(dT) (o——o) as templates. Reactions were run as described in Materials and Methods. Dithiothreitol was omitted in the N-ethylmaleimide experiment. [$^3$H] TTP was used as label with a specific activity of 4500 cpm/pmol.

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experiments from 1.200 to 1.225 g/cc, probably because of different degrees of homogenization; (c) the endogenous reaction is completely dependent upon the presence of all four deoxytriphosphates. The chromatographic behavior on DEAE-cellulose column, on phosphocellulose column, and the sedimentation in high salt glycerol gradients of the enzyme prepared from the "low speed pellet" are compared to those of the enzyme prepared from the "particle" in Figures 2 and 3. It can be observed that the main DNA polymerase activity which is solubilized from the "particle" is not distinguishable from the activity in the "low speed pellet" which is able to use Poly(A).oligo(dT) as template.

Up to 50% of the activity present in the "low speed pellet" can be recovered from the "particle" when Poly(A).oligo(dT) is used as template. The variability and low recovery are probably due to the difficulty of homogenizing the oocytes in a complete and reproducible manner. We have observed that at any given detergent concentration, part of the enzyme is solubilized, part is found in the "low speed pellet", and part is found in the high speed pellet from which the "particle" is prepared (data not shown). In general the use of 0.5% NP40 gave the best reproducibility and "particle" recovery. Fig. 4 shows N-ethylmaleimide sensitivity and salt dependence of the enzyme derived from the "low speed pellet"; table 1 shows template specificities of the same enzyme. The same results have been obtained with the enzyme extracted from the

Table 1 - Utilization of different templates by the enzyme from "low speed pellet"

| Template-primer | $Mg^{++}$ 10 mM | $Mn^{++}$ 0.8 mM |
|---|---|---|
| Activated DNA | 38 | 23 |
| Poly(dA).oligo(dT) | 570 | 94 |
| Poly(A).oligo(dT) | 100 | 100 |

The reaction mixture contained Tris-HCl pH 8.5 50 mM, KCl 50 mM, dithiothreitol 5 mM, dATP, dCTP, dGTP 0.1 mM, [$^3$H]TTP 5 $\mu$M(4500 cpm/pmol) templates at 200 $\mu$g/ml and 10 $\mu$l of dialysed phosphocellulose fraction. Reactions were run for 10 min at 37°C. Values are expressed as % of the activity with Poly(A).oligo(dT). Assays were proportional to enzyme amount.

"particle". There are apparent discrepancies between template preferences shown in Table 1 and those shown by the other experiments. This is due to the fact that to characterize the enzyme 200 $\mu$g/ml of template were used and the reactions run for 10 min, while in all other experiments 20 times less template was used and the reactions were run for 30 min.

Martini et al. (7) have shown that in Xenopus laevis oocytes it is possible to avoid nuclear leakage by manually separating nuclei from cytoplasms. Using the same procedure by which they have found $\alpha$-polymerase only in the nucleus, we have been able to show that the enzyme we have characterized is present only in the cytoplasms. The insert in fig. 5 shows DEAE-cellulose chromatograms of extracts derived from the "low speed pellet" prepared either from nuclei or from cytoplasms. An enzyme able to use Poly(A).oligo(dT) as template can be solubilized from the "low speed pellet" of cytoplasms but not from that of nuclei. The fractions eluting from DEAE-cellulose columns with 0.4 M KCl have been further analysed in two 10-30% glycerol gradients. Fig. 5 shows again that the activity using Poly(rA).oligo(dT) as template is present only in the 0.4 M KCl eluate of the column loaded with the extract derived from cytoplasms and that this activity sediments at the same rate as the enzyme derived from the whole cell (see fig. 3).

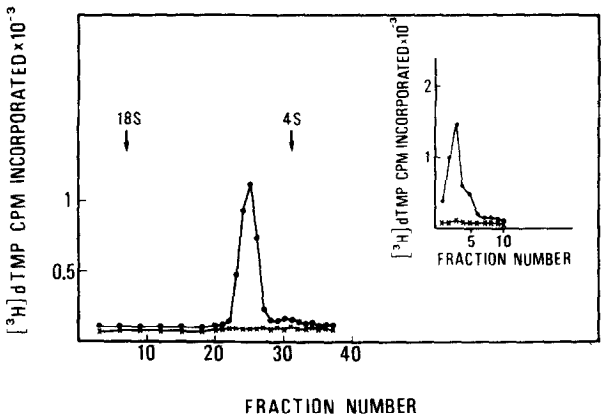


Fig. 5 - DEAE-cellulose chromatograms (insert) and sedimentation in glycerol gradients of extracts from nuclei (x——x) and from cytoplasms(.——.). From 600 nuclei and 600 cytoplasms "low speed pellet" extracts were prepared as described in Materials and Methods. Each extract was adsorbed on a separate DEAE-cellulose column and eluted with 0.4 M KCl step. The 0.4 M KCl eluates were assayed and layered on two separate 10-30% glycerol gradients and centrifuged as in fig. 3. The fractions of the DEAE-cellulose columns and of the gradients were assayed, as described in Materials and Methods, with Poly(A).oligo(dT) as template and using [$^3$H]TTP as label with a specific activity of 4500 cpm/pmol.

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DISCUSSION: In Xenopus laevis oocytes as in other systems polymerase $\alpha$ has been shown to be present in the nucleus (7) and to prefer activated DNA as template (4). The enzyme we have purified is not polymerase $\alpha$ because it is cytoplasmic and uses Poly(A).oligo(dT) better than it uses activated DNA. Two major DNA polymerases that are able to use Poly(A).oligo(dT) as template have been described in eucaryotic cells, namely the $\beta$ and $\gamma$ polymerases (1). Because of its size and N-ethylmaleimide sensitivity our enzyme is clearly not a $\beta$ -polymerase. It could be the equivalent in Xenopus laevis oocytes of $\gamma$-polymerase of other systems, although in other systems it has been shown that $\gamma$ -polymerase has a higher activity with Poly(A).oligo(dT) than with Poly(dA).oligo(dT) (8, 9). Another possibility is that the enzyme is mito - chondrial, because it is cytoplasmic and large amounts of mitochondria are present in Xenopus laevis stage 6 oocytes. Since mitochondrial DNA polymerase has not been purified from Xenopus laevis, our enzyme can be only compared with Mt-DNA polymerase of other systems. The behavior on DEAE-cellulose and the template specificity of our enzyme are different from those

found for Mt-DNA polymerase in HeLa cells (10, 11). However from our and other available data on Xenopus laevis oocytes it is impossible to state conclusively whether or not the enzyme is a mitochondrial enzyme.

The enzyme is clearly present only in the cytoplasm. This fact tends to suggest that the enzyme is not involved in DNA replication. It should be kept in mind, however, that Xenopus laevis stage 6 oocytes do not synthesize DNA and that before DNA replication starts, germinal vescicles are broken and components of nucleus and cytoplasm become mixed.

Our work also shows that the enzyme is associated with particulate material that can be banded to equilibrium in sucrose gradient. We have shown that the same fractions from the sucrose gradient that are able to use Poly(A).oligo(dT) in an exogenous polymerase reaction are also able to perform endogenous DNA synthesis. This finding is interesting because it suggests that the enzyme could be associated with a template. Though there is no direct evidence that this enzyme is responsible for the endogenous reaction, the enzyme which is able to use Poly(A).oligo(dT) is clearly the main DNA polymerase extracted from the "particle". The nature of the template present in the "particle" is under investigation.

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