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Participation of Deoxyribonucleic Acid Polymerase α in Amplification of Ribosomal Deoxyribonucleic Acid in *Xenopus laevis*

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Aphidicolin, a known inhibitor of eucaryotic deoxyribonucleic acid (DNA) polymerase α, efficiently inhibited amplification of ribosomal DNA during oogenesis in *Xenopus laevis*. DNA polymerase α, but not DNA polymerase γ, as isolated from ovaries, was sensitive to aphidicolin. DNA polymerase β was not detectable in *Xenopus* ovary extracts. Therefore, DNA polymerase α plays a major role in ribosomal ribonucleic acid gene amplification.

During oogenesis in many different species, a large number of ribosomes are accumulated. These are needed for the extensive protein synthesis in the rapidly proliferating cells of the embryo. In some organisms, this process is facilitated by selective amplification of the genes that code for ribosomal ribonucleic acid (RNA) (9), thus providing additional templates for the massive ribosomal RNA synthesis which occurs during the development of the oocyte.

In the South African clawed frog, *Xenopus laevis*, diploid somatic cells contain some 900 copies of the ribosomal RNA gene (2, 5) clustered together in the chromosomal nucleolus organizer regions (22). This repetitive deoxyribonucleic acid (DNA) contains, per repeat unit, the sequences for one copy each of 28S, 18S, and 5.8S ribosomal RNA and transcribed and nontranscribed spacers (2, 5, 7, 17, 26). During the early meiotic prophase in the oocyte (1, 6), the ribosomal RNA genes are amplified more than 1,000-fold and stored extrachromosomally in the nucleoplasm, forming hundreds of nucleoli (for review see reference 9).

The mechanism of ribosomal DNA amplification in *Xenopus* proceeds by the selective replication of one of the repeat units of the ribosomal RNA genes, which exist in *Xenopus* as a multigene family whose members differ in the length of the nontranscribed DNA region (30, 31). During amplification, a circularized copy of the ribosomal DNA is multiplied by a rolling-circle mechanism (13, 23, 31), producing multiple identical copies, tandemly arranged in circular and linear molecules (31). To date, it is not known which of the DNA polymerases is involved in ribosomal DNA amplification. We, therefore, investigated the role of DNA polymerase α in this process by using aphidicolin as a specific α polymerase inhibitor (14). From our data, we conclude that DNA polymerase α plays a crucial role in ribosomal RNA gene amplification in *X. laevis*.

MATERIALS AND METHODS

X. laevis tadpoles (stage 63) and adult females were obtained from Nasco, Ft. Atkinson, Wis.; Amphibian Culture Medium was from GIBCO; [*methyl*-^{3}H]thymidine and [5-^{3}H]deoxycytidine were purchased from New England Nuclear Corp.; [*methyl*-^{3}H]thymidine 5'-triphosphate ([*methyl*-^{3}H]TTP) was from Amersham; deoxyribonucleotides and salmon sperm DNA type III were from Sigma; oligodeoxythymidylic acid [$(dT)_{12-18}$] was from Schwarz/Mann; 2',3'-dideoxy-TTP was obtained from P-L Biochemicals; polyadenylic acid $(rA)_n$ and bovine serum albumin were from Miles; ribonuclease A was ordered from Worthington, proteinase K was from Merck; endonucleases *Eco*RI and *Hin*dIII and λ virus DNA were from Bethesda Research Laboratories. Agarose was obtained from Seakem; CsCl was from E. Merck, and ethidium bromide was from Calbiochem. Diethylaminoethyl (DEAE)-cellulose (DE-52) and phosphocellulose (P11) were obtained from Whatman, England. Aphidicolin was a gift from B. Hesp (Imperial Chemical Industries, England), and DNA from *Rhodopseudomonas sphaeroides* was kindly supplied by K. Gibson, Roche Institute of Molecular Biology.

Labeling of ovarian DNA. Ovaries at the amplification stage were obtained from 3- to 5-week-old *X. laevis* froglets maintained at 19 to 20°C. Three to six ovary halves were incubated at 24°C in 5% CO_2 for 6 h in 1 ml containing 0.8 ml of Amphibian Culture Medium (supplemented, per ml, with 50 U of penicillin and 50 μg of streptomycin) and 100 μCi of [^{3}H]thymidine (77 Ci/mmol) plus 100 μCi of [^{3}H]deoxycytidine (26.8 Ci/mmol) in 200 μl of water (23). For restriction enzyme analysis of ribosomal DNA, ovarian DNA was labeled with 800 μCi of [^{3}H]thymidine (77 Ci/mmol) per ml. Aphidicolin dissolved in dimethyl sulfoxide (DMSO) (1 mg/ml) was added 0.5 to 1 h before onset of the pulse. Controls received the same amount of DMSO (final concentration, 0.5%, vol/vol).

Isolation and characterization of ribosomal DNA. After incubation, the radioactive media were removed, and the ovaries were washed two times with 5 ml of ice-cold phosphate-buffered saline and digested overnight in 2 ml of 0.5% sodium dodecyl sulfate–10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.4)–10 mM ethylenediaminetetraacetate (EDTA) with 100 μg of proteinase K per ml. A 5-μg sample of *R. sphaeroides* DNA (density, 1.730 g/ml; 27) was added to serve as a density marker for subsequent CsCl buoyant density gradient analysis. The viscous solution was then extracted with an equal volume of phenol-chloroform (1:1) saturated with 10 mM Tris-hydrochloride (pH 7.9)–1 mM EDTA. After extraction, the phenol phase was reextracted with 0.5 ml of 10 mM Tris-hydrochloride (pH 7.4)–10 mM EDTA. The combined aqueous phases were adjusted to 0.3 M sodium acetate (pH 4.8), and DNA was precipitated with 3 volumes of ethanol at −70°C for 1 to 3 h.

The ovarian DNA used for restriction enzyme analysis (see below) was washed with 70% ethanol in 10 mM Tris-hydrochloride (pH 7.4)–10 mM EDTA and incubated in 10 mM Tris-hydrochloride (pH 7.5)–10 mM NaCl with 100 μg of ribonuclease A per ml at 37°C for 1 h. The DNA was subsequently ethanol precipitated, digested with proteinase K, extracted with phenol-chloroform, and again precipitated with ethanol as described above.

For separation of ribosomal from chromosomal DNA, DNA pellets were dissolved in 1 ml of 0.1× SSC (SSC = 0.15 M NaCl–0.015 M sodium citrate, pH 7.0), mixed with 4 ml of 1.9 g of CsCl per ml in 0.1× SSC (resulting final density, 1.70 g/ml), and centrifuged at 40,000 rpm in a VTi65 Spinco rotor for 18 h at 20°C. Fractions were collected from the top of the gradient, and their optical density at 254 nm was determined. Portions of alternate fractions were spotted onto GF/C glass fiber filters (Whatman), and trichloroacetic acid-insoluble radioactivity was determined in a liquid scintillation counter. For further purification of ribosomal DNA, fractions were combined as indicated, the CsCl concentration was adjusted to ~1.5 M by addition of 0.1× SSC, and the DNA was precipitated with 2 volumes of ethanol. Subsequent CsCl density gradient centrifugation and measurement of radioactivity were performed as described above.

For restriction endonuclease analysis of ribosomal DNA, the fractions containing the lower DNA band were combined, and the CsCl density was adjusted to 1.70 g/ml. Density gradient centrifugation was performed as described before. The gradient was fractionated, and samples were used for determination of trichloroacetic acid-insoluble radioactivity. The fractions containing ribosomal DNA were pooled, and after dilution of the CsCl solution, the DNA was precipitated with ethanol. The DNA was washed with 70% ethanol, dissolved in 200 μl of 100 mM Tris-hydrochloride (pH 7.2)–5 mM $MgCl_2$–2 mM 2-mercaptoethanol—60 mM NaCl, and digested with 20 U of *Eco*RI at 37°C. After 30 min the same amount of endonuclease was added and incubated for another 30 min.

The reaction mixture was extracted with phenol-chloroform, and DNA was precipitated with ethanol. The pellet was dissolved in electrophoresis buffer (40 mM Tris-hydrochloride–5 mM sodium acetate–1 mM EDTA, pH 7.8) containing 25% glycerol and 0.01% bromphenol blue, and the DNA fragments were separated by electrophoresis in a 1% horizontal agarose gel for 15 h at 50 V (25 mA). *Hin*dIII-cut λ DNA was used as marker and run in an adjacent lane.

Marker DNAs were visualized by soaking the gel in 1 μg of ethidium bromide per ml of water for 30 min and viewing under ultraviolet light. For determination of radioactivity, the slab gel was cut into 1-mm slices. The slices were dissolved in 75 μl of 5 M sodium perchlorate at 60°C for 30 min. After addition of 100 μl of water and 3.5 ml of ACS (Amersham), the radioactivity in each slice was counted in a liquid scintillation spectrometer.

Preparation of DNA polymerases α and γ from *X. laevis* ovaries. DNA polymerases α and γ were isolated from ovaries according to Pedrali Noy and Weissbach (21) and Grippo et al. (10). The ovary from one female adult frog was removed, minced with scissors, and homogenized in 4 volumes of homogenization buffer (0.4 M potassium phosphate, pH 7.4–0.5 mM EDTA–1 mM 2-mercaptoethanol–25% glycerol). After centrifugation at 27,000 × *g* for 20 min at 4°C, the pellet was suspended in 2 volumes of homogenization buffer, sonicated 4 times for 15 s at setting 3-low (Branson Sonifier) at 0°C, and centrifuged as described above. More than 95% of the DNA polymerase γ activity, as determined in the DNA polymerase γ assay (see below), was found in the pellet of the first centrifugation, whereas most of the DNA polymerase α activity (more than 95%) was found in the first supernatant. Both supernatants were combined, and the potassium phosphate concentration was adjusted to 0.36 M by addition of homogenization buffer.

For removal of nucleic acids, the extract (62 ml) containing 1,562 mg of protein was placed on a 100-ml DEAE-cellulose column, previously equilibrated with 0.35 M potassium phosphate (pH 7.4)–0.5 mM EDTA–5 mM 2-mercaptoethanol–25% glycerol, and eluted with 1 column volume of the same buffer. The effluent was dialyzed overnight against 0.02 M potassium phosphate (pH 7.4)–5 mM 2-mercaptoethanol–25% glycerol (buffer A). The precipitate that formed, which contained less than 10% of total DNA polymerase activity, was removed by centrifugation at 15,000 × *g* for 20 min at 4°C and discarded.

The phosphate concentration of the supernatant was adjusted to 0.04 M potassium phosphate, and the solution was absorbed to a 62-ml DEAE-cellulose column which had been equilibrated previously with buffer A. After a wash with 2 column volumes of buffer A, a linear gradient of 7.5 column volumes between 0.02 and 0.2 M potassium phosphate (pH 7.4) containing 5 mM 2-mercaptoethanol–25% glycerol was applied. This was followed by a 0.3 M potassium phosphate elution step. The activities eluting from DEAE-cellulose at 0.08, 0.12, and 0.16 M phosphate were pooled separately and dialyzed overnight against buffer A. After addition of bovine serum albumin to 1.3 mg/ml, each of the pooled peaks was applied separately to a 5-ml phosphocellulose column previously equilibrated with buffer A. After a wash with 3 column volumes of the same buffer, DNA polymerase activities were eluted with a linear gradient of 12 column volumes between 0.02 and 0.5 M potassium

phosphate (pH 7.4), containing 5 mM 2-mercaptoethanol, 25% glycerol, and 0.25 mg of bovine serum albumin per ml. The phosphate concentrations of the fractions were calculated from their conductivity. The fractions containing the highest DNA polymerase activities were combined and stored in liquid nitrogen.

DNA polymerase assays. DNA polymerases were assayed by a modification of those procedures described by Pedrali Noy and Weissbach (21). For these determinations, 5-μl aliquots of column fractions were added to 45 μl of reaction mixture and incubated for 30 min at 37°C. During this time period the reaction kinetics were linear. Forty-microliter portions of the reaction mixtures were spotted onto GF/C glass fiber filters and washed with trichloroacetic acid as described by Bollum (3).

DNA polymerase α assay. The DNA polymerase α assay was carried out in a solution containing: 50 mM Tris-hydrochloride (pH 7.9); 7.5 mM $MgCl_2$; 0.5 mM dithioerythritol, 140 μg of bovine serum albumin per ml; 250 μg of activated salmon sperm DNA (21) per ml; and deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate, each at 10 μM (2,500 cpm/pmol) or 100 μM (250 cpm/pmol) [^{3}H]TTP. When DNA polymerase γ activity was assayed on activated DNA as a template, 0.25 M KCl was added to suppress any DNA polymerase α activity, which, under these conditions, is less than 2% of the activity measured in a standard DNA polymerase α assay.

DNA polymerase β assay. The assay was performed as previously described (21).

DNA polymerase γ assay. The reaction was carried out in 50 mM Tris-hydrochloride (pH 8.5), 50 mM potassium phosphate (pH 8.5), 0.13 M KCl, 0.5 mM $MnCl_2$, 2.5 mM dithioerythritol, 140 μg of bovine serum albumin per ml, 50 μg of $(rA)_n \cdot (dT)_{12-18}$ (5:1, wt/wt) per ml, and 100 μM [^{3}H]TTP (250 cpm/pmol).

When the effect of 2′,3′-dideoxy-TTP was measured, the TTP concentration was 10 μM and the 2′,3′-dideoxy-TTP was added with an equimolar amount of $MgCl_2$ to maintain a constant free Mg^{2+} concentration.

RESULTS

Influence of aphidicolin on ribosomal DNA amplification in *X. laevis* ovaries. To measure ribosomal DNA synthesis, ovary halves from young frogs were incubated for 6 h in the presence of [^{3}H]thymidine and [^{3}H]cytidine. DNA was isolated, and the high guanine-cytosine-containing ribosomal DNA (density, 1.729 g/cm^3) was separated from labeled chromosomal DNA (density, 1.700 g/cm^3) by equilibrium density centrifugation in a CsCl gradient (4). Most of the labeled DNA banded at a density characteristic for *Xenopus* chromosomal DNA (Fig. 1A), with the ribosomal DNA appearing as a minor component (Fig. 1A). To enhance the purity of the ribosomal DNA, fractions of the CsCl gradient were combined as indicated (Fig.

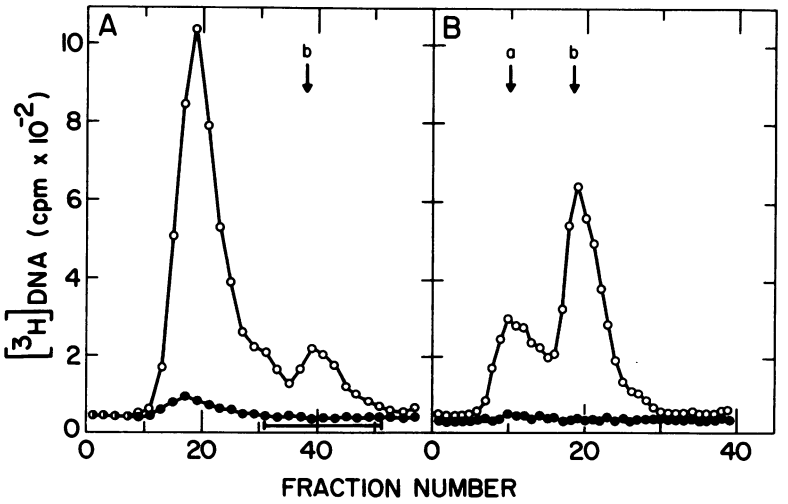

FIG. 1. *Separation of ribosomal DNA and nuclear DNA from ovaries incubated in the absence or presence of aphidicolin. (A) Three ovary halves were incubated for 6.5 h at 24°C with 5 μg of aphidicolin per ml in DMSO (●) or with an equivalent volume (final concentration, 0.5%, vol/vol) of DMSO alone (○). During the last 6 h of incubation, ovaries were pulse-labeled with [^{3}H]deoxycytidine and [^{3}H]thymidine. Total DNA was isolated as described in the text, and ribosomal DNA was separated from nuclear DNA by equilibrium centrifugation in a VTi65 Spinco rotor at 40,000 rpm for 20 h at 20°C in CsCl. Twenty-microliter volumes of alternate fractions were assayed for radioactivity. (B) The ribosomal DNA-containing fractions (fractions 31 to 50) were combined as indicated. DNA was precipitated by ethanol and recentrifuged in CsCl at 50,000 rpm for 18 h at 10°C. Total fractions were used for determination of radioactivity. The density of the CsCl gradients increases from left to right. The positions of marker DNAs are marked by arrows. a, Calf thymus DNA (density, 1.700 g/cm^3); b, DNA from R. sphaeroides (density, 1.730 g/cm^3).*

1A) and rerun on a CsCl density gradient. Some 75% of the DNA rebanded at the same position in the gradient relative to the marker DNAs, whereas the remainder of the DNA had the same density as *X. laevis* chromosomal DNA (4), which is identical in base composition to calf thymus DNA (Fig. 1B; 25).

In a separate experiment, the opposite halves of the ovaries which had been used for the control experiment were exposed to 5 μg of aphidicolin per ml 30 min before incubation with [^{3}H]thymidine and [^{3}H]cytidine. The [^{3}H]DNA was isolated and analyzed identically to the control samples. In the presence of aphidicolin, both nuclear DNA replication in the follicle cells and ribosomal DNA synthesis in the oocytes are inhibited more than 95% (Fig. 1). Half-maximal inhibition of ribosomal DNA synthesis was observed at an aphidicolin concentration between 0.2 and 1 μg/ml (data not shown).

To further prove the identity of ribosomal DNA, a parallel experiment was carried out in which ovaries were labeled with [^{3}H]thymidine and their DNAs were separated on a CsCl gradient. The ribosomal DNA of the denser lower band was recentrifuged to equilibrium in CsCl and finally treated with restriction endonuclease

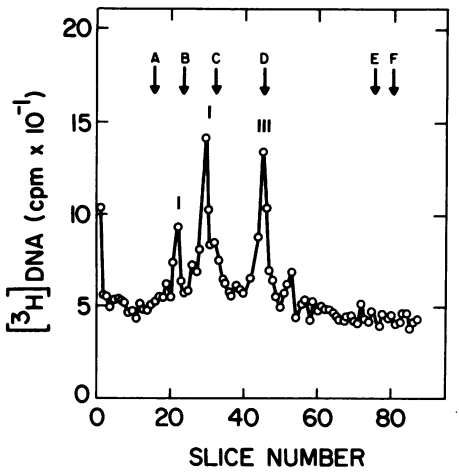

FIG. 2. *EcoRI restriction enzyme pattern of [^{3}H]-thymidine-labeled amplified ribosomal DNA. Three ovaries were pulse-labeled for 6 h with [^{3}H]thymidine. Ribosomal DNA was isolated by two successive CsCl density gradient centrifugations as described in the legend to Fig. 1. Purified ribosomal DNA was digested with the restriction enzyme EcoRI, and the DNA fragments were separated on a 1% horizontal agarose gel. The gel was sliced and analyzed for radioactivity. Migration was from left to right, and the positions of HindIII restriction fragments of λ DNA are indicated by arrows. The sizes in megadaltons are 16.6 (A), 6.62 (B), 4.67 (C), 2.98 (D), 1.58 (E), and 1.37 (F).*

*Eco*RI. Figure 2 shows the pattern of restriction enzyme fragments obtained by agarose gel electrophoresis. In addition to a characteristic DNA fragment with a molecular weight of 3×10^6 (peak III), which is derived from the constant region of the repeating unit of the ribosomal DNA (31), two major bands could be observed having molecular weights of 5.4×10^6 (peak II) and 8.1×10^6 (peak I) (Fig. 2). These DNA fragments contain the heterogeneous nontranscribed spacers of the ribosomal RNA gene (31).

Inhibition of DNA polymerase α from *X. laevis* ovaries by aphidicolin. To demonstrate that *Xenopus* DNA polymerase α is inhibited by aphidicolin as shown in a number of higher animal species (16, 18–20), DNA polymerases α and γ were isolated from ovaries of adult frogs. The ovarian extract, obtained as described in Materials and Methods, was first bound to a DEAE-cellulose column and subsequently eluted with a potassium phosphate gradient. Figure 3A shows the DNA polymerase activity profile obtained by using activated DNA as template in the DNA polymerase assay. Two major peaks of activity, which we will show later to be DNA polymerase α species, could be observed at phosphate concentrations of 0.12 and 0.16 M. A similar pattern of DNA polymerases from *Xenopus* ovaries, after separation on DEAE-cellulose, was reported by Grippo et al. (10). Using $(rA)_n \cdot (dT)_{12-18}$ as a template in an assay for DNA polymerase α activity, we detected a small peak eluting at a phosphate concentration of 0.08 M which did not coincide with the other activities found with activated DNA as the template (Fig. 3A). Each DNA polymerase peak was pooled, applied individually to phosphocellulose columns, and eluted with a potassium phosphate gradient. The DNA polymerase that could use $(rA)_n \cdot (dT)_{12-18}$ as a template eluted at 0.29 M potassium phosphate (Fig. 3B). The two DNA polymerase α activities which eluted at 0.12 M (α_1) and 0.16 M (α_2) in the DEAE-cellulose chromatography (Fig. 3A) were applied separately to a phosphocellulose column. Figure 4 shows that these peaks were eluted at 0.21 M (α_1) and 0.19 M (α_2) potassium phosphate, respectively.

In addition, we noted that about 1% of the total DNA polymerase activity, measured on an activated DNA template, did not bind to DEAE-cellulose and passed through the column. This flow-through of the DEAE-cellulose column, a fraction in which DNA polymerase β is ordinarily found (21), was also chromatographed on phosphocellulose. The low DNA polymerase activity, which was detected in an assay with activated DNA as a template, eluted from phosphocellulose at 0.19 and 0.21 M potassium phosphate, at the same positions as the α polymer-

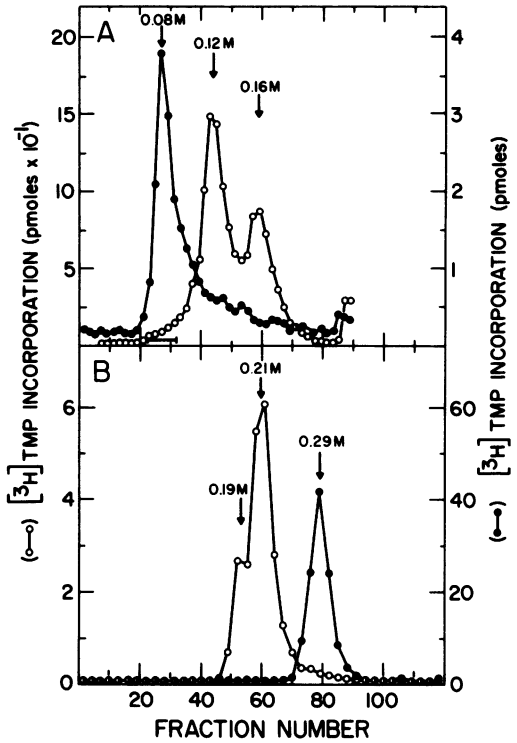

FIG. 3. *DEAE-cellulose and phosphocellulose column chromatography of extracts from X. laevis ovaries. The preparation of the ovary extract and ion-exchange chromatography are described in the text. (A) DEAE-cellulose chromatography of the ovarian extract. Fractions 23 through 31 were pooled and applied to a phosphocellulose column whose elution pattern is shown in (B). Five-microliter portions of the indicated fractions from phosphocellulose (0.5 ml) or DEAE-cellulose (6 ml) were assayed for DNA polymerase α (○) and DNA polymerase γ (●). The DNA polymerase peak eluting at 0.12 M potassium phosphate is designated α_1; the peak eluting at 0.16 M potassium phosphate is designated α_2.*

ases. These DNA polymerase peaks were inactive in a DNA polymerase β assay using $(rA)_n \cdot (dT)_{12-18}$ as a template. No evidence was found for the presence of DNA polymerase β in the ovarian extract.

On the basis of their chromatographic behavior and template requirement, the DNA polymerases eluting from phosphocellulose at 0.21 and 0.19 M phosphate are of the α polymerase type (α_1 and α_2). The polymerase eluting at 0.29 M from phosphocellulose is a γ polymerase (29). To further characterize the DNA polymerases, their activities on different templates were quantitated, and their sensitivities towards different DNA polymerase inhibitors were determined (Table 1). DNA polymerases α_1 and α_2 were inactive on $(rA)_n$ with a $(dT)_{12-18}$ primer, whereas DNA polymerase γ was more active on the ribohomopolymer template than on activated DNA. All three polymerases were completely inhibited by 5 mM *N*-ethylmaleimide (Table 1); therefore, none of the isolated DNA polymerases belongs to the β class (29). In addition, the two α DNA polymerases are relatively insensitive to dideoxy-TTP, a selective inhibitor of DNA polymerase β and γ (8, 16). Fifty percent inhibition

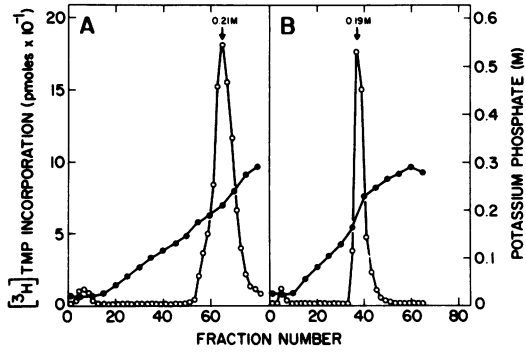

FIG. 4. *Phosphocellulose chromatography of DNA polymerases α_1 and α_2. Fractions containing DNA polymerases α_1 and α_2, which had been separated by DEAE-cellulose chromatography similar to the experiment shown in Fig. 3, were pooled separately and chromatographed on phosphocellulose as described in the text. Five microliters of each alternate fraction (0.6 ml) was assayed for DNA polymerase activity on an activated DNA template in a DNA polymerase α assay. ○, [3H]thymidine monophosphate incorporated; ●, potassium phosphate concentration. (A) α_1; (B) α_2.*

TABLE 1. *Template specificity and N-ethylmaleimide sensitivity of X. laevis DNA polymerases*[a]

Template	Deoxynucleotide incorporation (pmol) by DNA polymerase:		
	α_1	α_2	γ
Activated DNA	346	354	13.9
Activated DNA + 5 mM *N*-ethylmaleimide	2	1	0.2
$(rA)_n \cdot (dT)_{12-18}$	0.2	0.3	24.2

[a] DNA polymerase α_1, α_2, and γ activities were assayed on activated DNA and $(rA)_n \cdot (dT)_{12-18}$ as templates as described in the text. The concentration of [3H]TTP was 100 μM (250 cpm/pmol). DNA polymerase γ activity on an activated DNA template was measured in the presence of 0.25 M KCl and 10 μM [3H]TTP (2,500 cpm/pmol). Mixtures were incubated for 30 min at 37°C. For determination of *N*-ethylmaleimide inhibition, the enzymes were incubated for 30 min at 0°C in a complete assay mixture with the inhibitor and then transferred to 37°C. Incorporation is expressed as picomoles of nucleotides incorporated into acid-precipitable material. The DNA polymerase activities using activated DNA templates are corrected to show total deoxynucleotide incorporation.

was observed at inhibitor-to-substrate ratios (dideoxy-TTP/TTP) of 5 and 4 for DNA polymerases α_1 and α_2 respectively (Fig. 5). A similar high concentration of dideoxy-TTP has been reported by Krokan et al. (16) to be needed for 50% inhibition of DNA polymerase α from CV-1 cells. In contrast, DNA polymerase γ is 100-fold more sensitive to dideoxy-TTP since 50% of the enzyme activity is inhibited at an inhibitor-to-substrate ratio of ~0.04 (Fig. 5). This is in agreement with published data for DNA polymerase γ isolated from CV-1 cells (8, 16).

The influence of aphidicolin on DNA polymerases α_1, α_2, and γ was also analyzed. Aphidicolin inhibits both forms of DNA polymerase α from *X. laevis* ovaries to the same extent. Fifty percent reduction of DNA polymerase α_1 and α_2 activity was observed at 2 μg of aphidicolin per ml under standard assay conditions (Fig. 6). The mode of inhibition of both polymerases by aphidicolin is competitive with deoxycytidine triphosphate (data not shown), as previously demonstrated for DNA polymerase α from sea urchin embryos (19), mouse myeloma (19), HeLa (18), and KB cells (12), and cultured rice cells (24). K_i values of approximately 0.2 and 0.6 μM were determined for DNA polymerases α_1 and α_2, respectively (data not shown). On the other hand, DNA polymerase γ was completely resistant towards aphidicolin, even at 50 μg/ml, the highest concentration tested (Fig. 6).

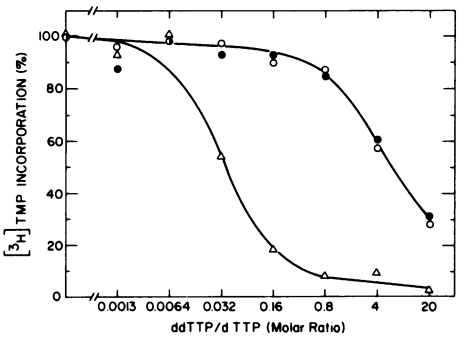

FIG. 5. *Effect of dideoxy-TTP on the activity of X. laevis DNA polymerases. DNA polymerases α_1 and α_2 (after 10-fold dilution with 0.2 M potassium phosphate, pH 7.4–5 mM 2-mercaptoethanol–25% glycerol) and DNA polymerase γ were assayed on activated DNA templates at different dideoxy-TTP/TTP ratios as indicated. The enzyme activities obtained after phosphocellulose chromatography were used in this experiment. When DNA polymerase γ activity was determined, 0.25 M KCl was included in the assay mixture and the concentration of [^{3}H]TTP was 10 μM (2,500 cpm/pmol). Samples were incubated for 30 min at 37°C. Results are expressed as a percentage of the amount of [^{3}H]thymidine monophosphate incorporated into acid-insoluble material in the absence of inhibitor. One hundred percent incorporation was 4.5 pmol for DNA polymerase α_1 (○), 4.5 pmol for DNA polymerase α_2 (●), and 2.8 pmol for DNA polymerase γ (△).*

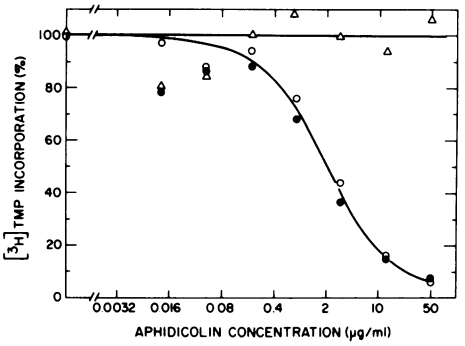

FIG. 6. *Effect of aphidicolin on the activity of X. laevis DNA polymerases. DNA polymerases α_1, α_2, and γ were assayed on activated DNA as described in the legend to Fig. 5, in the presence of different concentrations of aphidicolin. Control samples were given an equal volume of DMSO (final concentration, 5%, vol/vol). This concentration of DMSO inhibits the DNA polymerase α and γ activities about 25%. Results are expressed as a percentage of the amount of [^{3}H]thymidine monophosphate incorporated into acid-insoluble material in the absence of inhibitor. One hundred percent incorporation was 3.4 pmol for DNA polymerase α_1 (○), 3.6 pmol for DNA polymerase α_2 (●), and 2.5 pmol for DNA polymerase γ (△).*

DISCUSSION

DNA polymerase α plays a major role in the amplification of ribosomal RNA genes during oogenesis in *X. laevis*. This conclusion is derived from the observation that ribosomal DNA replication in isolated ovaries is completely inhibited by aphidicolin, a specific inhibitor of eucaryotic α-type DNA polymerases (12, 16, 18, 19, 24). In addition, we could show that the two aphidicolin-sensitive DNA polymerases isolated from ovaries of adult frogs are α-type polymerases, based on their preference for activated DNA as template, their inability to read $(rA)_n \cdot (dT)_{12-18}$, their inhibition by *N*-ethylmaleimide, and their relative high resistance towards dideoxy-TTP. It is not known whether these two forms of DNA polymerase α have different functions in the cell. The presence of two forms of DNA polymerase α has been reported in HeLa cells (21), and the heterogeneity of the α polymerase in many species is well known (28). DNA polymerase γ, an activity which we find associated, in part, with a particulate fraction of the cell extract, is not inhibited by aphidicolin. Thus, aphidicolin shows the same specificity of inhibition towards the α-type polymerase from *Xenopus* sp. as in other eucaryotic systems (16, 19). Our assignment of the isolated polymerases from

Xenopus ovaries to the α and γ class differs from earlier reports by Joenje and Benbow (15) and Grippo et al. (10). We believe that the DNA polymerases described by these authors have been, in part, incorrectly classified. In particular, the assignment of a DNA polymerase β designation (15) to an enzyme which we find to be DNA polymerase γ seems contrary to our data.

From our results, we cannot definitely conclude whether DNA polymerase α is involved in copying one of the repeat units of the clustered chromosomal genes coding for ribosomal RNA (31) or in the final multiplication step of ribosomal DNA via a rolling-circle mechanism (13, 23, 31), or in both processes. It is, however, more likely that DNA polymerase α participates in the massive multiplication of the original extrachromosomal ribosomal DNA copy since aphidicolin abolishes ribosomal DNA synthesis completely. Other DNA polymerases might also play a role in this gene amplification process; if so, their function has to be tightly coupled with the action of DNA polymerase α.

ACKNOWLEDGMENT

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LITERATURE CITED

1. **Bird, A. P., and M. L. Birnstiel.** 1971. A timing study of DNA amplification in *Xenopus laevis* oocytes. Chromosoma **35**:300–309.
2. **Birnstiel, M. L., J. Speirs, I. Purdom, K. Jones, and U. E. Loening.** 1968. Properties and composition of the isolated ribosomal DNA satellite of *Xenopus laevis*. Nature (London) **219**:454–463.
3. **Bollum, F. J.** 1959. Thermal conversion of nonpriming deoxyribonucleic acid to primer. J. Biol. Chem. **234**: 2733–2734.
4. **Brown, D. D., and I. B. Dawid.** 1968. Specific gene amplification in oocytes. Science **160**:272–280.
5. **Brown, D. D., and C. Weber.** 1968. Gene linkage by RNA-DNA hybridization. II. Arrangement of the redundant gene sequences for 28S and 18S ribosomal RNA. J. Mol. Biol. **34**:681–697.
6. **Coggins, L. W., and G. Gall.** 1972. The timing of meiosis and DNA synthesis during early oogenesis in the toad, *Xenopus laevis*. J. Cell Biol. **52**:569–576.
7. **Dawid, I. B., D. D. Brown, and R. A. Reeder.** 1970. Composition and structure of chromosomal and amplified ribosomal DNAs of *Xenopus laevis*. J. Mol. Biol. **51**:341–360.
8. **Edenberg, H. J., S. Anderson, and M. D. DePamphilis.** 1978. Involvement of DNA polymease α in simian virus 40 DNA replication. J. Biol. Chem. **253**: 3273–3280.
9. **Gall, J. G.** 1969. The genes for ribosomal RNA during oogenesis. Genetics **61**(Suppl.):121–132.
10. **Grippo, P., A. Caruso, G. Locorotondo, and T. Labella.** 1976. Multiple forms of DNA dependent DNA polymerase during early development and in somatic cells of *Xenopus laevis*. Cell Diff. **5**:121–128.
11. **Grippo, P., G. Locorotondo, and A. Caruso.** 1975. Characterization of the two major DNA polymerase activities in oocytes and eggs of *Xenopus laevis*. FEBS Lett. **51**:137–142.
12. **Habara, A., K. Kano, H. Nagano, Y. Mano, S. Ikegami, and T. Yamashita.** 1980. Inhibition of DNA synthesis in the adenovirus DNA replication complex by aphidicolin and 2',3'dideoxythymidine triphosphate. Biochem. Biophys. Res. Commun. **92**:8–12.
13. **Hourcade, D., D. Dressler, and J. Wolfson.** 1973. The amplification of ribosomal RNA genes involves a rolling circle intermediate. Proc. Natl. Acad. Sci. U.S.A. **70**: 2926–2930.
14. **Ikgami, S., T. Taguchi, M. Ohashi, M. Oguro, H. Nagano, and Y. Mano.** 1978. Aphidicolin prevents mitotic cell division by interfering with the activity of DNA polymerase α. Nature (London) **274**:458–460.
15. **Joenje, H., and R. M. Benbow.** 1978. A low molecular weight DNA polymerase from ovaries of the frog *Xenopus laevis*. J. Biol. Chem. **253**:2640–2649.
16. **Krokan, H., P. Schaffer, and M. L. DePamphilis.** 1979. Involvement of eukaryotic deoxyribonucleic acid polymerase α and γ in the replication of cellular and viral deoxyribonucleic acid. Biochemistry **18**:4431–4443.
17. **Loening, U. E., K. Jones, and M. L. Birnstiel.** 1969. Properties of the ribosomal RNA precursor in *Xenopus laevis*: comparison to the precursor in mammals and in plants. J. Mol. Biol. **45**:353–366.
18. **Oguro, M., M. Shioda, H. Nagano, and Y. Mano.** 1980. The mode of action of aphidicolin on DNA synthesis in isolated nuclei. Biochem. Biophys. Res. Commun. **92**: 13–19.
19. **Oguro, M., C. Suzuki-Hori, H. Nagano, Y. Mano, and S. Ikegami.** 1979. The mode of inhibitory action by aphidicolin on eukaryotic DNA polymerase α. Eur. J. Biochem. **97**:603–607.
20. **Pedrali Noy, G., and S. Spadari.** 1979. Effect of aphidicolin on viral and human DNA polymerases. Biochem. Biophys. Res. Commun. **88**:1194–1202.
21. **Pedrali Noy, G., and A. Weissbach.** 1977. HeLa cell DNA polymerases: the effect of cycloheximide *in vivo* and detection of a new form of DNA polymerase α. Biochim. Biophys. Acta **477**:70–83.
22. **Ritossa, F., and S. Spiegelman.** 1965. Localization of DNA complementary to ribosomal RNA in the nucleolus organizer region of *Drosophila melanogaster*. Proc. Natl. Acad. U.S.A. **53**:737–745.
23. **Rochaix, J.-D., A. Bird, and A. Bakken.** 1974. Ribosomal RNA gene amplification by rolling circles. J. Mol. Biol. **87**:473–487.
24. **Sala, F., B. Parisi, D. Burroni, A. R. Amileni, G. Pedrali Noy, and S. Spadari.** 1980. Specific and reversible inhibition by aphidicolin of the α-like DNA polymerase of plant cells. FEBS Lett. **117**:93–98.
25. **Sober, H. A. (ed.).** 1979. Handbook of molecular biology. The Clinical Rubber Co., Cleveland, Ohio.
26. **Speirs, J., and M. Birnstiel.** 1974. Arrangement of the 5.8 S RNA cistrons in the genome of *Xenopus laevis*. J. Mol. Biol. **87**:237–256.
27. **Suyama, Y., and J. Gibson.** 1966. Satellite DNA in photosynthetic bacteria. Biochem. Biophys. Res. Commun. **24**:549–553.
28. **Weissbach, A.** 1977. Eukaryotic DNA polymerases. Annu. Rev. Biochem. **46**:25–47.
29. **Weissbach, A., D. Baltimore, F. Bollum, and D. Korn.** 1975. Nomenclature of eukaryotic DNA polymerases. Eur. J. Biochem. **59**:1–2.
30. **Wellauer, P. K., I. B. Dawid, D. D. Brown, and R. H. Reeder.** 1976. The molecular basis for length heterogeneity in ribosomal DNA from *Xenopus laevis*. J. Mol. Biol. **105**:461–486.
31. **Wellauer, P. K., R. H. Reeder, I. B. Dawid, and D. D. Brown.** 1976. The arrangement of length heterogeneity in repeating units of amplified and chromosomal ribosomal DNA from *Xenopus laevis*. J. Mol. Biol. **105**: 487–505.