CYCLOHEXIMIDE INHIBITION OF THE DNA-DEPENDENT RNA-POLYMERASE I OF ACHLYA BISEXUALIS

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SUMMARY

Achlya bisexualis has multiple forms of DNA-dependent RNA-polymerase similar to those of other eukaryotes. Cycloheximide acts as a specific inhibitor of DEAE-fraction I enzyme at low concentrations with 50% inhibition at 0.47 μM cycloheximide.

INTRODUCTION

Specific inhibitors have been reported for each of the three forms of RNA-polymerase isolated from eukaryotes. Alpha-amanitin inhibits the DEAE-fraction II enzyme at very low concentrations (1, 2). Rifampicin, an inhibitor of bacterial RNA-polymerase, inhibits polymerase III which may originate in the mitochondria (3-7). In one case cycloheximide was shown to inhibit polymerase I specifically, but at relatively high concentrations (6). Cycloheximide has also been implicated as an in vivo inhibitor of ribosomal RNA synthesis in several organisms including both animals and fungi (8-10). The availability of inhibitors of all three eukaryotic polymerases would greatly aid in the elucidation of their function in cellular RNA synthesis.

Since cycloheximide inhibition of RNA-polymerase I isolated from the aquatic fungus Blastocladiella emersonii occurred at high concentrations (6), it is possible that a minor impurity of the chemical was actually responsible. The purpose of this report is to show that the polymerase I of the water mold Achlya bisexualis is specifically inhibited by very low concentrations of cycloheximide, and that the inhibition is most likely not due to a contaminant of the cycloheximide.

EXPERIMENTAL PROCEDURE

Chemicals were obtained as follows: sodium deoxycholate (DOC) (enzyme grade), $(NH_4)_2SO_4$ (enzyme grade), (^{3}H)ATP (22.3 Ci/mMole), Schwarz-Mann; unlabeled ATP, GTP, CTP, and UTP, 2-mercaptoethanol (BME), Tris, Sigma; bovine serum albumin (BSA) (Pentex), Miles Laboratories; dithiothreitol, NBC; cycloheximide, Calbiochem (Lot No. 62343) and NBC (Lot No. 6999). All other chemicals were reagent grade.

Achlya bisexualis (Strain 65-1) was grown using medium and inoculation as described by Griffin and Breuker (11). Cultures in 1 liter of PYG in 2 liter Erlenmeyer flasks were incubated for 22 hrs at 24°C on a rotary shaker, collected by filtration and washed with 0.5 mM $CaCl_2$. The mycelia were then suspended in PVP buffer (8% polyvinylpyrrolidone, 0.5 mM $MgCl_2$, 20 mM potassium phosphate, pH 6.5, 50 mM BME and 0.3 M sucrose), and homogenized in an Omni-Mixer (Ivan Sorvall, Inc., Norwalk, Conn.) with glass beads. A crude nuclear pellet was prepared by filtering the homogenate through Miracloth and centrifuging the filtrate for 10 min at 1500 X g. The pellet was suspended in solubilization buffer (20 mM Tris-HCl, pH 7.8, 30% glycerol, 6 mM $MgCl_2$, 1 mM Na-EDTA, 50 mM BME, 50 mM $(NH_4)_2SO_4$, 10 mg/ml BSA and 0.5% DOC) and chromatographed on DEAE-cellulose as described by Horgen and Griffin (6). One ml fractions were collected and RNA-polymerase activity was measured by the incorporation of (^{3}H)ATP into trichloroacetic acid-insoluble polynucleotides.

RESULTS

The elution profile of RNA-polymerase activity on chromatography of the extract from the crude nuclear pellet was similar to those obtained from other eukaryotes (1, 6, 12). Polymerase I (nucleolar enzyme) was eluted from the column at 0.13 - 0.16 M $(NH_4)_2SO_4$, polymerase II (nucleoplasmic enzyme) at 0.22 - 0.25 M, and polymerase III (mitochondrial enzyme) at 0.29 - 0.32 M. Fig. 1 shows that maximum activity of polymerase I was obtained only when the column fraction was diluted four-fold (25% column fraction). Activity then decreased linearly with further dilutions. In

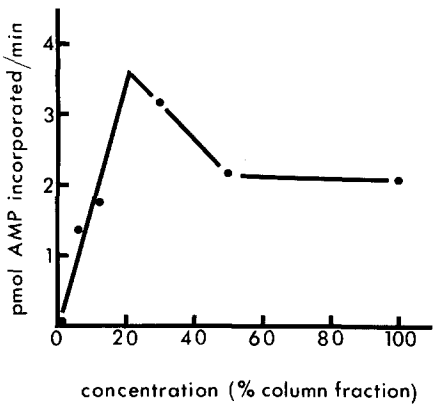

Fig. 1. Dilution of RNA-polymerase I column fraction. The enzyme fraction from the column was diluted with an appropriate volume of solubilization buffer, and 10 µl was added to each reaction mix. The reaction mix consisted of 0.2 M Tris-HCl, pH 8.1, 25 mM magnesium acetate, 25 mM dithiothreitol, 2.5 mM each of CTP, UTP, and GTP, 2.2 mM ATP, specific activity 6.25 µCi/µMole, 0.25% DOC, 35 µg of T-4 phage DNA in 0.21 ml. The reaction was stopped by the addition of 5 ml of cold trichloroacetic acid. Precipitates were collected on glass fiber filters, washed with 30 ml of trichloroacetic acid and counted in Bray's solution (13). Assays were for one minute at 20°C.

all subsequent experiments, the column fraction containing the peak of polymerase I activity was diluted five-fold prior to use.

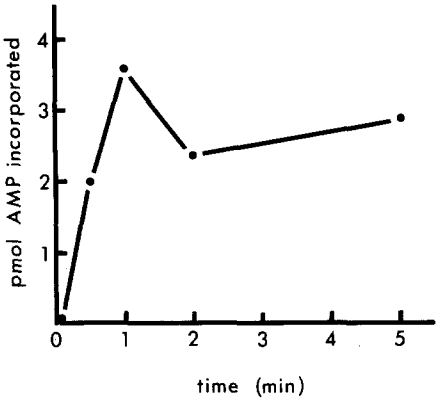

Fig. 2. Time course of RNA-polymerase I. The column fraction was diluted five-fold and assayed as described in Fig. 1.

Incorporation of AMP into RNA by polymerase I was linear for one minute (Fig. 2), then stopped. The decrease in acid-precipitable radioactivity after one minute suggests that the partially purified enzyme preparation was slightly contaminated by ribonuclease.

Polymerase I is further characterized in Table I. Synthesis of RNA

TABLE I

Treatment	pmol AMP incorporated/min
Complete	2.59
-DNA	0.09
-UTP	0.48
-CTP	0.95
-GTP	0.58

Nucleotide triphosphate and DNA dependency of polymerase I. Assays were as in Fig. 1 with the compound listed excluded from the reaction mix.

was stimulated by the addition of UTP, CTP, and GTP, and was dependent on the addition of DNA indicating that the activity was in fact DNA-dependent RNA-polymerase.

Cycloheximide inhibited polymerase I at all concentrations tested and

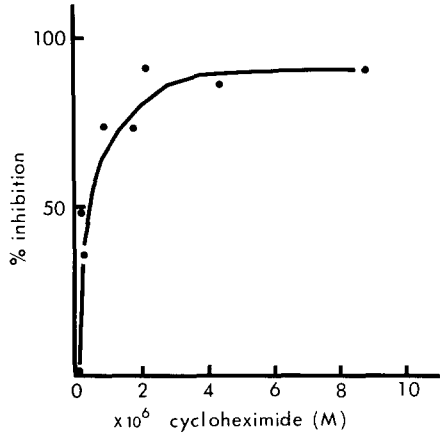

Fig. 3. Cycloheximide inhibition of RNA-polymerase I. Diluted enzyme was assayed in the presence of cycloheximide at the appropriate concentrations. The inhibitor was added in 10 µl of glass distilled water.

caused 50% inhibition at 4.7×10^{-7} M cycloheximide (Fig. 3). 100% inhibition was not obtained at 3.2×10^{-5} M cycloheximide, the highest concentration tested with polymerase I. Similar results were obtained with cycloheximide obtained from NBC. Cycloheximide inhibition was specific for RNA-polymerase I, showing no inhibition of polymerase II or III at 3.2×10^{-5} M.

DISCUSSION

The aquatic Oomycete Achlya bisexualis possesses three distinct RNA-polymerase activities chromatographically similar to those found in other eukaryotes. RNA-polymerase I from Achlya as obtained by column chromatography with DEAE-cellulose must be diluted several times in order to assure that reactions are enzyme-limited. This indicates that the quantitative interpretation of DEAE column profiles is difficult and must be approached with caution.

Cycloheximide, an inhibitor of protein synthesis in eukaryotes, has been reported to inhibit the in vivo ribosomal RNA synthesis of some fungi and animals (8-10). Furthermore, it is a specific in vitro inhibitor of the RNA-polymerase I of Blastocladiella emersonii, an aquatic fungus. We have now shown that cycloheximide is a specific in vitro inhibitor of polymerase I of Achlya, but is effective at concentrations about two orders of magnitude less than those reported for Blastocladiella (6). This distinct difference in sensitivities could be due to species differences, to non-enzyme-limiting conditions in the previous report, or to differences in the isolation or assay procedures. We have not completely ruled out the possibility that the inhibition is due to a contaminant of the cycloheximide preparations used, but this seems unlikely since nearly identical results were obtained with inhibitor purchased from two different vendors, and the concentration needed for 50% inhibition was less than 5.0×10^{-7} M.

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