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Specific Inhibitors of the Three RNA Polymerases from the Aquatic Fungus *Blastocladiella emersonii*

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ABSTRACT **Specific inhibitors of each of the three RNA polymerases of *Blastocladiella emersonii* have been found. Cycloheximide specifically inhibited the *in vitro* activity of the DEAE-fraction I enzyme, alpha-amanitin specifically inhibited the DEAE-fraction II enzyme, and rifampicin specifically inhibited the fraction III enzyme. DNA stimulation and dependency on the four riboside triphosphates were shown to be characteristic of each of the three fractions. Optimum concentrations of magnesium ions required were shown to differ among the three fractions and to be somewhat higher than optimum concentrations of manganese ions. The effect of pH on activity was essentially identical for each of the three fractions. Kinetic experiments and nuclease assays indicated the presence of some interfering substances in the partially purified RNA polymerase fractions.**

A rapidly expanding literature currently deals with multiple forms of RNA polymerase in eukaryotic organisms (1–4). Multiple forms have been demonstrated in higher plants (4) and in higher animals (1–3), but as yet have not been studied in primitive eukaryotes. In all of the systems examined at least two forms have been isolated from nuclear preparations. The notable exception is developing sea urchin, where three fractions of RNA polymerase have been reported (1).

Alpha-amanitin, a toxin from the basidiomycete, *Amanita phalloides*, has been shown to specifically inhibit the DEAE fraction II enzyme (2–5) that is found in the nonnucleolar portion of the nucleus. It has been suggested that the fraction II enzyme functions in the synthesis of DNA-like RNA. The fraction I enzyme's cellular location is in the nucleolus, and it is reported to serve specifically in the synthesis of ribosomal RNA (1, 2, 6). At present there is no evidence or suggested function for the fraction III enzyme, which is also apparently extra-nucleolar (5, 6). Although it has been suggested that the polymerases have different cellular functions, for more conclusive investigations specific inhibitors would be very useful.

While it is generally accepted that cycloheximide interrupts protein synthesis in eukaryotes, there have also been reports that it has some effect on the synthesis of RNA (7–10). The antibiotic rifampicin and rifamycins in general have been shown to specifically inhibit procaryotic RNA polymerases (11–13).

The purpose of this study was to examine the RNA synthesis machinery in the primitive water mold *Blastocladiella emersonii*. Since the results indicated the presence of multiple RNA polymerases, another objective was to observe the effects of cycloheximide, alpha-amanitin, and rifampicin on *in vitro* RNA synthesis to determine whether specific inhibitors may exist for the three polymerases.

MATERIALS AND METHODS

Growth, harvesting, and disruption of cells

Single-generation cultures of *B. emersonii* (Strain 49-1) were grown in aerated carboys at 23°C according to Horgen and Griffin (14). Vegetative, ordinary colorless thalli were harvested by filtration and were washed with glass distilled water. The thalli were suspended in homogenizing fluid (15) and were disrupted with a French pressure cell. The cultures and the enzyme preparations were monitored for microbial contamination by plating on peptone–yeast extract–glucose agar (PYG) with negative results.

Chemicals

Medium components were purchased from Difco Laboratories, Detroit, Mich. The unlabeled nucleoside triphosphates and dithiothreitol were purchased from Sigma Chemical Co., St. Louis. The [^{3}H]ATP was obtained from New England Nuclear Corp., Boston. Salmon sperm DNA and enzyme grade ammonium sulfate were obtained from Mann Research, N.Y. Alpha-amanitin was purchased from Henley and Co., N.Y., rifampicin and cycloheximide (actidione) from Calbiochem, Los Angeles. Other chemicals used were reagent grade.

Isolation of crude nuclear pellet

Cell wall debris was removed by filtration through Miracloth and the homogenate was centrifuged for 20 min at 6000 $\times$ *g*. The resulting crude nuclear pellet was used for enzyme isolation. Further nuclear purification by centrifugation through 4 molal sucrose (15) did not increase the purity of the enzyme preparations.

Purification of RNA polymerase

RNA polymerase was partially purified by ammonium sulfate fractionation followed by DEAE-cellulose column chromatography after Mertelsmann and Matthaei (16), except that the column used was 1 $\times$ 8 cm, and the volumes were scaled down accordingly. Activity was measured by the incorporation of [^{3}H]ATP into trichloroacetic acid-insoluble polynucleotides (17). Protein determinations were made by A_{280}/A_{260} ratios.

RESULTS

The elution pattern from the column of 17-hr ordinary colorless thalli (Fig. 1*A*) showed three peak fractions of activity.

Rechromatography of each of the three activities under identical conditions (Fig. 1*B*) suggested no evidence of dissociation of the individual complexes or interconvertibility among the three forms. These data are very similar to the profiles reported by Roeder and Rutter (1) for developing sea urchin embryos. Each of the three polymerases showed a marked stimulation with added salmon sperm DNA and dependency on the four riboside triphosphates (Table 1).

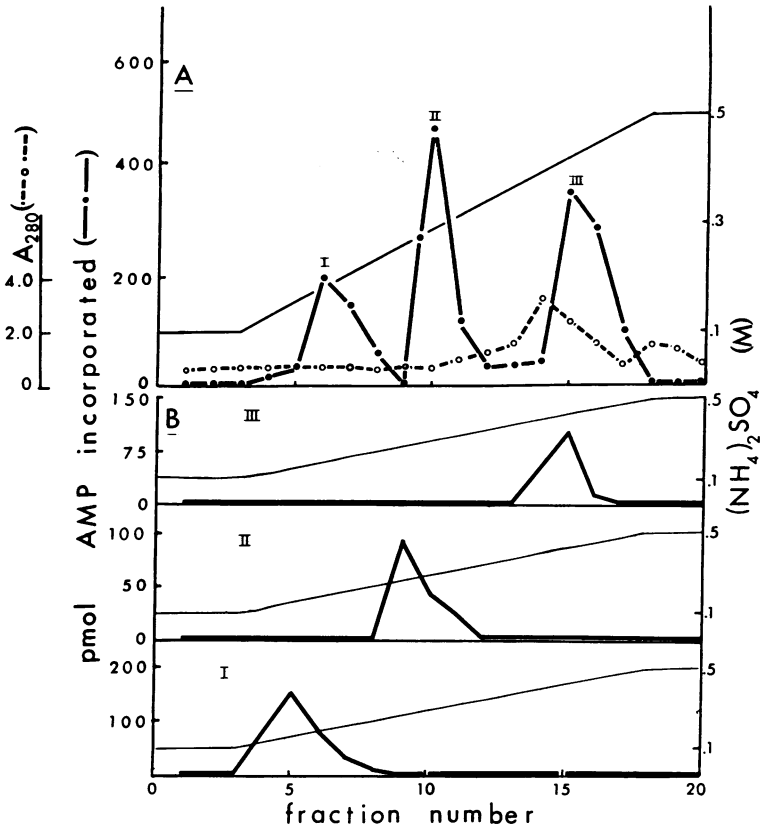
The optimum conditions for Mn^{++} ion were 1.0, 1.0, and 4.0 mM for polymerases I, II, and III respectively (Fig. 2). Mg^{++} ion optima were higher: 75, 50, and 25 mM for species I, II, and III respectively (Fig. 2). The pH activity curves plateaued between 8.1 and 8.4 for all three polymerase species.

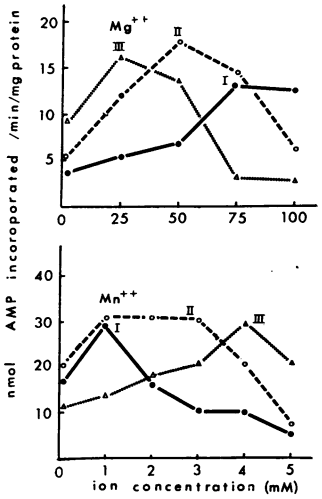
The kinetics of activity are shown in Fig. 3. The incorporation of AMP increased linearly for 30 sec and then began to decrease. Meisler and Tropp (18) observed a similar pattern for polymerase activity from rat liver, which they attributed to ribonuclease activity. When the polymerases from *B. emersonii* were examined for ribonuclease (19), considerable activity could be measured in each of the three fractions. The use of several RNase inhibitors and adsorbing agents did not significantly alleviate the nuclease problem.

TABLE 1. *Analysis of RNA polymerase from Blastocladiella emersonii*

Enzymes	Conditions	nmol AMP/min per mg protein
Homogenate of ordinary colorless thalli	Complete	5.8
Fraction I	Complete	56.7
	−DNA	11.4
	−GTP, UTP, CTP	2.4
Fraction II	Complete	58.1
	−DNA	14.7
	−GTP, UTP, CTP	1.4
Fraction III	Complete	72.0
	−DNA	23.2
	−GTP, UTP, CTP	3.1

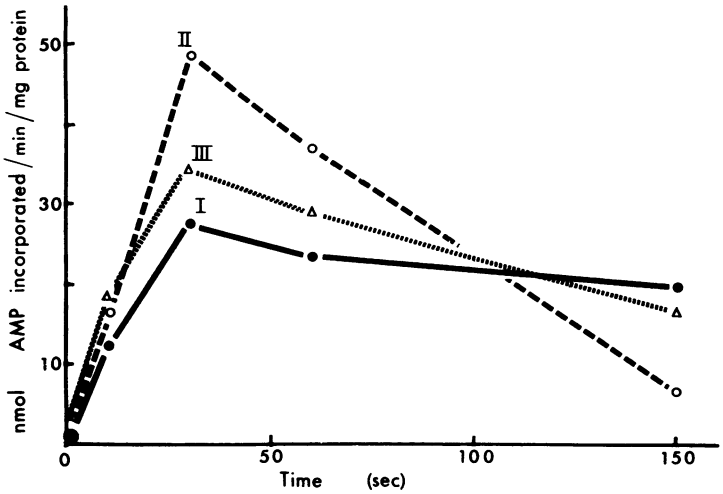
A complete reaction mixture contained Tris·HCl, magnesium acetate, dithiothreitol, ammonium sulfate, and DNA according to Stout and Mans (17). UTP, GTP, and CTP (5 μmol/reaction mixture), 10 μl of enzyme preparation (3–10 mg of protein/ml as determined by A_{280}/A_{260}), 4.5 μmol of ATP and [^{3}H]ATP (2.5 μCi/reaction mixture, 15.7 Ci/mmol) were also added.

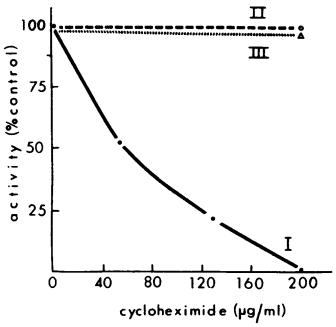




(*Left*) FIG. 1. (*A*) DEAE-cellulose chromatography of *B. emersonii* RNA polymerases from 17-hr vegetative, ordinary colorless thalli. A 4-ml sample of soluble nuclear polymerase isolated according to Mertelsmann and Matthaei (16) was chromatographed on a 1 × 8 cm column. The column was washed with 150 ml of buffer as described (16) and eluted with a linear gradient of buffer from 100 to 500 mM ammonium sulfate. Fractions were collected in 1-ml aliquots. Reaction mixtures as in Table 1. The temperature during chromatography was maintained at 4 ± 1°C. (*B*) Rechromatography of the three species of polymerase. Approximately 1.5 ml of either fraction I, II, or III (3–5 mg protein) was chromatographed and assayed under the same conditions as above.

(*Right*) FIG. 2. Effects of divalent metal ion concentration on RNA polymerases. For these studies the enzyme preparations were dialyzed against buffer A minus Mg^{++} (16). The dialyzed preparations were assayed and protein was measured as outlined in Table 1.





(*Left*) FIG. 3. Time course of AMP incorporation for *B. emersonii* RNA polymerases. Reaction mixture and measurement of protein as in Table 1.

(*Right*) FIG. 4. Comparative effect of cycloheximide on *B. emersonii* RNA polymerases. Assay procedure as described in Table 1.

All reactions reported were run for 30 sec at 20°C to minimize interference.

Alpha-amanitin at 0.25 μg/ml completely inhibited the activity of fraction II, whereas it had no effect on either fraction I or fraction III. Rifampicin (150 μg/ml), the prokaryotic polymerase inhibitor, had no effect on fractions I and II, but completely inhibited fraction III.

Cycloheximide at a concentration of 200 μg/ml in the reaction mixture had no effect on the activity of fractions II and III, but completely inhibited the incorporation of [^{3}H]AMP into polynucleotides by fraction I. The effect of concentration of cycloheximide on fraction I activity is shown in Fig. 4.

DISCUSSION

The present studies demonstrate that the vegetative ordinary colorless thalli of *B. emersonii* possess three chromatographically distinct species of RNA polymerase activity. These three activities are apparently similar to those observed for developing sea urchin embryo (1, 6). Stimulation by exogenously added DNA and dependency on the presence of the four nucleoside triphosphates indicate that the activities measured were all DNA-directed RNA-synthesizing enzymes. The effects of Mn^{++} ions on enzymatic activity were similar to those previously reported for other multiple RNA polymerases (1, 2), whereas Mg^{++} ion optima were somewhat higher.

Since considerable nuclease activity could be measured in each of the three polymerase fractions, nucleases probably caused the decrease in incorporation seen after 30 sec. Nucleoside triphosphate hydrolases may also interfere but have not been examined.

The specific inhibition of fraction II by alpha-amanitin suggests that the enzyme in *B. emersonii* is similar to the fraction II enzyme found in all organisms studied to date. This enzyme is reported to be located in the nonnucleolar nucleoplasm and is specifically involved in the synthesis of DNA-like RNA (2, 3, 5).

The specific inhibition of fraction III polymerase by rifampicin suggests that the fraction III enzyme of *B. emersonii* is similar to prokaryotic RNA polymerases. This observation may be explained by either of the following hypotheses. Our nuclear preparations may be contaminated with mitochondria and the possibility exists that the fraction III enzyme may be soluble mitochondrial RNA polymerase that is sensitive to rifampicin. Since it has been reported that the fraction III enzyme of sea urchin is located in the nucleus (1, 5, 20), it is possible that *B. emersonii*'s fraction III is in fact a nuclear enzyme. Its prokaryotic nature may therefore suggest that certain eukaryotic organisms, such as *B. emersonii*, have vestigial prokaryotic enzyme systems representing transitions in evolution from earlier forms.

Cycloheximide, a known protein synthesis inhibitor of eukaryotes, has been reported to specifically inhibit the synthesis of ribosomal RNA when added to growing cultures of certain fungi (7, 8). Cycloheximide was also shown to affect RNA synthesis at much higher concentrations than needed to affect protein synthesis (9, 21). The DEAE fraction I enzyme has been identified as being nucleolar and specifically involved in the synthesis of ribosomal RNA (2, 6, 20). Since the fraction I enzyme of *B. emersonii* is analogous to those previously studied, it appears that cycloheximide specifically inhibits *in vitro* ribosomal RNA synthesis. The concentrations needed to effectively inhibit *in vitro* synthesis are very similar to the concentrations needed for *in vivo* inhibition (7).

At the present time multiple RNA polymerases have been reported from calf thymus (3), rat liver (1, 2), sea urchin (1), coconut (4), and now the fungus, *B. emersonii*. In all of these systems at least two forms, having a specific cellular location and function, have been identified. This suggests that similar RNA-synthesizing enzyme systems exist in a broad sampling of eukaryotic organisms. In *B. emersonii* there are specific inhibitors that affect each of the three different enzyme fractions. The existence of such inhibitors may prove to be valuable in discerning the precise transcriptive functions of the multiple polymerases.

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