E. GRACILIS RNA POLYMERASE I: A ZINC METALLOENZYME

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SUMMARY

E. gracilis DNA dependent RNA polymerase I has been purified to homogeneity. α-amanitin, over the concentration range 0.05 to 200 μg/ml, does not affect its activity, consistent with its being classified as an RNA polymerase I. Based on a molecular weight of 624,000 daltons the enzyme contains 2.2 g atom of Zn but no Mn, Cu, Fe, as determined by microwave excitation emission spectrometry. Zinc is essential for activity since the chelating agent, 1,10-phenanthroline, inhibits enzymatic function but its non-chelating analogue, 4,7-phenanthroline is ineffective. Thus, like the RNA polymerase II, zinc is a catalytically essential component of E. gracilis RNA polymerase I (1).

INTRODUCTION

Zinc is an essential component of multiple processes involved in transcription and translation, and its deficiency results in growth arrest (2,3). Studies with the eukaryote, E. gracilis, implicate derangements in RNA metabolism in zinc deficiency (1, 4-7). Hence, a general study regarding the role of zinc in the RNA metabolism of this organism has been initiated, thus far centering both on the role of this metal in the RNA polymerases and RNA function. We have reported recently that DNA dependent RNA polymerase II from zinc sufficient E. gracilis is a zinc metalloenzyme (1). The RNA polymerase I from the zinc sufficient organism has now been purified to homogeneity. The enzyme contains, on the average, 2.2 g atom of zinc/mole and is inhibited by 1,10-phenanthroline. The results provide further evidence for the involvement of zinc in eukaryotic RNA synthesis.

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METHODS AND MATERIALS

The DNA dependent RNA polymerases isolated from E. gracilis strain Z were assayed as described previously (1). One unit of activity represents the incorporation of 1 nmole of [^{3}H] UMP into RNA in 15 min. at 30°. All chemicals were reagent grade. Metal-free water was used throughout. All procedures utilized to isolate RNA polymerase I were carried out between 0 and 4°. 20 grams of cells were suspended in 40 ml of cold buffer A, 50 mM Tris HCl, pH 8, 6 mM $MgCl_2$, 10 mM mercaptoethanol. One gram of glass beads (Superbright, 3M Corp., Minneapolis, Minn.) was added per each gram of cells to aid in their disruption. The cells were broken manually, for 10-15 minutes, with a mortar and pestle which had been pre-cooled overnight to -20° C. Thereafter, 10 ml of 2.5 M ammonium sulfate, pH 7.6, and an additional 40 ml of buffer A were added. The resultant viscous solution was twice homogenized for 15 seconds in a VirTis homogenizer at 40,000 rpm. The homogenized solution was centrifuged for 20 minutes at 48,000 x g and the supernatant (Fraction I) recovered. The pellet was resuspended in 30 ml of buffer A and 10 ml of 2.5 M ammonium sulfate, pH 7.6. The resultant homogenate was sonicated in 20 second bursts, for a total of 1 minute, with a Bromwill Biosonic sonicator (Bromwill Scientific, Rochester, N.Y.). The sonicate was centrifuged at 48,000 x g and the supernatant (Fraction II) combined with Fraction I. The polymerase activity in the combined supernatants was precipitated by adding 30 gm of solid ammonium sulfate per 100 ml. After 1 hour of stirring the precipitated RNA polymerases were collected by centrifugation at 48,000 x g for 10 minutes. The pellet, (Fraction III), was dissolved in 10 ml of buffer B, 50 mM Tris HCl pH 8.0, 6 mM $MgCl_2$, 10 mM mercaptoethanol, 0.1% Triton-X 100, 10% glycerol, loaded onto a Sephadex G-25 column, 40 x 2.5 cm, and eluted with buffer B. The fractions containing RNA polymerase activity were pooled (Fraction IV) and loaded onto a 25 x 2 cm DEAE Sephadex A-25 column which had been equilibrated previously with buffer C, 50 mM Tris-HCl pH 8, 6 mM $MgCl_2$, 20% glycerol, 10 mM mercaptoethanol. The proteins were eluted by step gradients with 0.05 M, 0.15 M, 0.35 M and 0.45 M NH_4Cl, in buffer C (Fig. 1). Each active fraction was assayed in the presence and absence of α-amanitin, 0.05-200 μg/ml, to identify the nature of the various polymerases. Fraction V, eluted with 0.15 M NH_4Cl, contained RNA polymerase I. This enzyme was dialyzed against buffer C for 4 hours and then loaded onto a phosphocellulose column (12 x 0.5 cm) which had been equilibrated with buffer C, The proteins bound to the column were eluted by step gradients of 0.15 M, 0.25 M, 0.35 M, 0.45 M NH_4Cl, in buffer C (Fig. 2). The homogeneity and subunit composition of the enzyme eluted from the phosphocellulose column was determined according to Laemmli (8).

The zinc, manganese, copper and iron content of three different preparations of the purified enzyme were determined by microwave induced emission spectrometry and utilizing previously described methods (1, 9-11). Before analysis, 50-100 μg of RNA polymerase were dialyzed for 24 hours against four changes of 100-fold excess of metal free 0.05 M Tris HCl, pH 8.0, at 4° C. Alternatively, 100 μg aliquots of enzyme were chromatographed on Sephadex G-75 and the metal content of the active fractions was then determined (9).

RESULTS AND DISCUSSION

The procedure utilized previously to isolate E. gracilis RNA polymerase II (1) results in only small amounts of polymerase I, and polymerase III is not obtained. The present procedure maximizes the yield of polymerase I. RNA polymerases which remain bound to the cellular material following manual

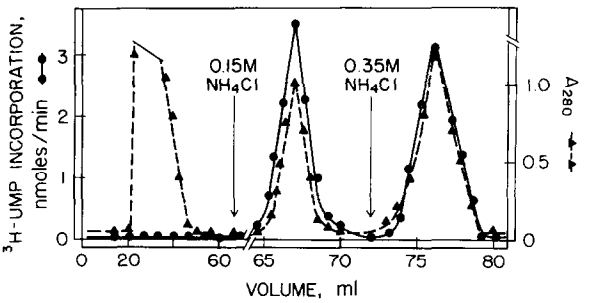

Fig. 1. DEAE Sephadex A-25 chromatography of DNA dependent RNA polymerases from zinc sufficient organisms.
45 ml fraction IV was loaded onto a 25 x 2 cm DEAE Sephadex A-25 column at a flow rate of 20 ml/hr and 0.5 ml fractions were collected. Aliquots were assayed for polymerase activity (●) and A_{280} (▲). Step gradients with 0.15 M and 0.35 M ammonium chloride eluted two fractions containing RNA polymerase. No enzymatic activity was eluted from the column with 0.45 M NH_4Cl.

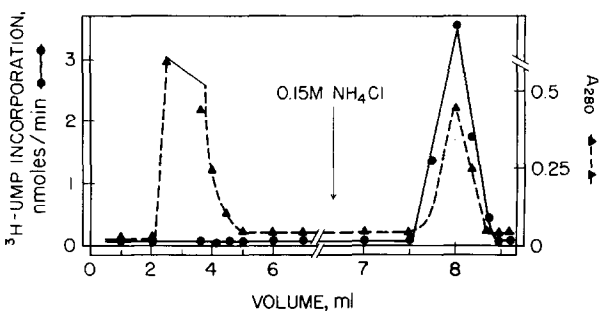

Fig. 2. Phosphocellulose chromatography of RNA polymerase I.
5 ml of the fraction eluted from DEAE SEphadex A-25 with 0.15 M NHCl was dialyzed against buffer C and then was loaded onto a 12 x 0.5 cm phosphocellulose column at a flow rate of 8-10 ml/hr. and 0.5 ml fractions were collected. Aliquots were assayed for polymerase activity (●) and A_{280} (▲). The enzyme was eluted with 0.15 M NH_4Cl.

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disruption of E. gracilis are solubilized by sonication in high salt buffer. Subsequently polymerases are separated from the DNA complexes by ion exchange chromatography, obviating the loss of nearly 70% of the total activity incurred when protamine sulfate precipitation is employed (1). As expected, the resultant enzymes are solely dependent on exogenous DNA for activity. Figure 1 shows a typical chromatogram of fraction IV on DEAE Sephadex A-25. Two

TABLE I: Subunit composition and molecular weight of E. gracilis RNA Polymerase I.

Polypeptide	Mol Weight x 10^{-3} g	Molar Ratio
1	162	1.10
2	148	1.10
3	74	1.08
4	46	1.10
5	36	1.08
6	34	1.20
7	28	2.03
8	24	1.00
9	18	2.2
10	8	1.13

200 µg of purified enzyme shown to be homogeneous on 5% polyacrylamide gel, were separated on 0.1% sodium dodecyl sulfate gel. Standards of reference included 10 µg of highly purified, homogeneous E. coli RNA polymerase (Boehringer Mannheim) and 10 µg of albumin. Electrophoresis was carried out for 4 hours at 4° C with a constant current of 4 mA/tube. RNA polymerase I separates into 10 distinct bands. Their densitometry in stained gels was performed in a Gilford spectrophotometer 240 at 500 nm. The proportional amount of each band was established by determining the area under the densitometric profile.

peaks of activity are eluted with 0.15 M and 0.35 M NH_4Cl but none at higher salt concentrations. RNA polymerase II is completely inhibited by 0.1 µg/ml α-amanitin while polymerase I is unaffected by up to 200 µg/ml α-amanitin (1). α-amanitin at a concentration of 200 µg/ml does not inhibit the RNA polymerase activity of the 0.15 M NH_4Cl fraction, (Fig. 1), indicating that this enzyme is an RNA polymerase I. In contrast, α-amanitin at a concentration of 200 µg/ml reduces the activity of the 0.35 M fraction to 50% that of the control, suggesting that this fraction may constitute a mixture of classes II and III polymerases.

The 0.15 M NH_4Cl fraction was purified further on phosphocellulose. A

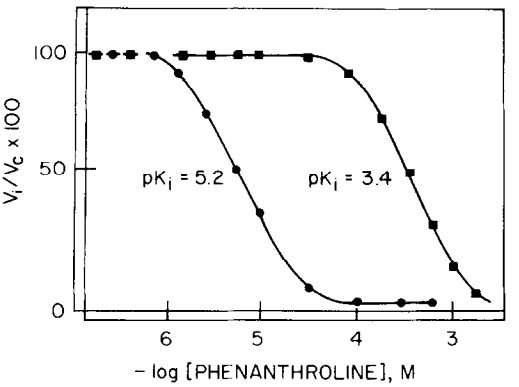

Fig. 3. The effect on enzyme activity of incubating RNA polymerases I and II with 1,10-phenanthroline (OP), at concentrations between 10^{-6} and 5 x 10^{-3} M. V_i is the velocity in the presence of inhibitor and V_c that in its absence. Ten micrograms of each enzyme were preincubated with OP and assays were performed at 30°C for 20 min. Mg^{2+} (10 mM) served as the activating metal.

single peak of activity is eluted with 0.15 M NH_4Cl, while all proteins eluting at higher or lower salt concentrations are inactive (Fig. 2).

This phosphocellulose fraction is homogeneous based on polyacrylamide gel electrophoresis which detects only a single band. On SDS gels, the enzyme dissociates into 10 subunits, and Table I shows their average molecular weights and ratios. The molecular weight of this homogeneous enzyme, calculated by summing the individual weights of the subunits, is approximately 624,000 daltons, a value consistent with those of other RNA polymerase I from eukaryotic cells (12-14). As recently discussed for RNA polymerase II, the molecular weight of polymerase I estimated by these methods must remain provisional until more accurate determinations are obtained by e.g. the ultracentrifuge.

This preparation from 20 grams of cells yields approximately 2.2 mg of pure polymerase I with a specific activity of 80 units/mg, a yield of polymerase I five fold greater than that obtained previously by the method serving the isolation of polymerase II (1).

RNA polymerase I is inhibited completely by 1,10- but not by 4,7-phenanthroline showing that it is a zinc metalloenzyme, analogous to the

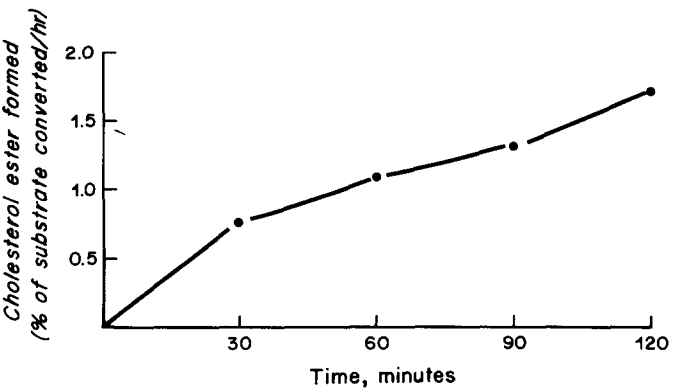

Fig. 1. Time course of cholesteryl ester synthetase activity in guinea pig gallbladder. Enzyme activity is expressed as percentage of added cholesterol converted to cholesteryl esters per mg protein in 1 hour.

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mCi/mmol), and [1-^{14}C]linoleic acid (specific activity 40 to 60 mCi/mmol) were purchased from New England Nuclear (Chicago, IL) and checked for purity by thin-layer chromatography (7) using the solvent system of heptane:isopropyl ether:acetic acid (65:40:4, vol/vol/vol).

Guinea pigs (Hartley strain) weighing 600 to 800 g were maintained on regular guinea pig chow during the study. The guinea pigs were sacrificed, and their gallbladders were quickly excised, opened, and rinsed completely free of bile with saline.

The method used to measure the activity of cholesteryl ester synthetase was that of Goodman et al. (8) with slight modification (9). The tissue was homogenized in 0.1 M phosphate buffer (pH 7.4) for 1 minute. The homogenate was centrifuged at 1,000 x g for 20 minutes to remove the cell debris, and the 1,000 x g supernatant was used for most studies. When further fractionation of the homogenates was desired, the supernatant was centrifuged at 12,000 x g for 15 minutes to remove the mitochondrial fraction. This supernatant was then centrifuged at 100,000 x g for 30 minutes using an ultracentrifuge (Beckman LK-75). The pelleted microsomes and mitochondrial fraction were suspended in 0.1 M phosphate buffer to give an approximate protein concentration of 0.5 to 1 mg/ml. Aliquots of the microsomal, mitochondrial, and supernatant fraction were then used for the enzyme studies. The protein concentrations were measured by the method of Lowry et al. (10).

The incubation consisted of 0.5 to 1 mg of the 1,000 x g supernatant fluid or appropriate cell fraction in phosphate buffer (pH 7.4), ATP 15 μmol, CoA 15 μmol, NaF 15 μmol, $MgCl_2$ 12 μmol, and [^{14}C]cholesterol 10 nmol in 25 μl of acetone. The incubation was carried out for 1 hour at 37°C. At the end of incubation, 20 ml of chloroform:methanol (2:1, vol/vol) were added to stop the reaction; 50 to 100 μg of the carrier cholesteryl oleate and cholesterol were then added to each tube. The organic layer was removed, evaporated to a small volume, and subjected to thin-layer chromatography on silica gel G using the solvent system of heptane:isopropyl ether:acetic acid (65:40:4, vol/vol/vol) to separate free and esterified cholesterol (7). The bands corresponding to the free and esterified sterols were then scraped from the

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