YEAST RNA POLYMERASE I: A EUKARYOTIC ZINC METALLOENZYME

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SUMMARY: Microwave excitation spectrometry and metal binding inhibition studies show that zinc is a catlytically essential component of the highly purified RNA polymerase I from yeast, the first eukaryotic RNA polymerase I available in quantities sufficient for such studies. It contains 2.4 g-atom of zinc based on a molecular weight of 6.5×10^5 (8). Copper, iron, manganese and magnesium are absent, i.e., below the limits of detection, 10^{-13} to 10^{-14} g-atoms. A number of derivatives of 1,10-phenanthroline reversibly inhibit the polymerase catalyzed reaction, apparently by forming a ternary polymerase·Zn·OP complex while the nonchelating isomer, 1,7-phenanthroline, is ineffective.

INTRODUCTION: A number of RNA- and DNA-dependent polymerases from prokaryotes and viral sources (1-6) have recently been shown to be zinc metalloenzymes. Indirect evidence suggests that eukaryotic DNA-dependent RNA polymerase from rat liver are metalloenzymes (7). However, investigation of the metalloenzyme nature of eukaryotic polymerases in general and of RNA polymerase I in particular have been hindered due to lack of suitable purification procedures, a problem which has recently been overcome for studies of yeast RNA polymerases (8). Microwave-induced emission spectrometry (3,5,6,9) and inhibition of the enzyme by chelating agents demonstrate that yeast RNA polymerase I is a zinc metalloenzyme.

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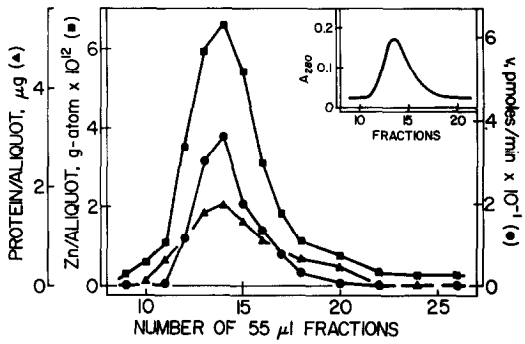

Figure 1: Distribution of polymerase activity, zinc and protein in fractions from a G-75 Sephadex column. Metal contaminants were removed from an Altex microbore column (0.3 cm x 25 cm) by washing with 3 ml of 1,10-phenanthroline, 0.01 M, pH 7.8 Tris·HCl, 0.01 M, followed by 30 ml of Tris buffer. The column was equilibrated at 4° with pH 7.8 Tris·HCl, 0.01 M; KCl, 0.01 M; DTT, 1 mM, and Triton X-100, 0.001%. Approximately 50 μg of protein in 50 μl was placed on the column and eluted with the above buffer at a flow rate of 0.05 ml/min. Droplet fractions, 55 μl, were collected for assays of enzyme activity (●), duplicate zinc analysis (■) and protein content (▲). The zinc content was calculated by reference to the emission of standard zinc solutions. Zinc and protein content are expressed as g-atoms and μg per 5 μl aliquot, respectively, and the velocity for 5 μl of enzyme added to the standard assay. The insert is the optical density recorded at 280 nm of the effluent of the column, measured in an 8 μl flow through cell.

MATERIALS AND METHODS: Yeast RNA polymerase I was purified as described (8). The stock enzyme was stored at -90° in Tris-HCl, pH 7.9, 20mM; glycerol, 25%; EDTA, 0.5mM; β-mercaptoethanol, 10mM and sucrose, 10%.

Nucleotides were obtained from P.L. Biochemicals and [^{3}H]UTP, 23 Ci/mM, from New England Nuclear. All chemicals were reagent grade. Optical density at 280nm was measured using an Altex 153 UV detector containing an 8 μl flow through cell and an Altex 210 scale expanded chart recorder. Droplet fractions were monitored by a 10 μl Gilson DCTH Drop Counter-Timer and collected in Kontes Microflex Tubes on a Gilson Escargot 5C-15 Fractionator base. Microgram quantities of enzyme, obtained by gel exclusion chromatography, were analyzed for protein on 15 μl aliquots (3) and for zinc, manganese, magnesium, copper and iron on 5 μl aliquots according to previously published procedures (3,5,6,9), using a microwave-induced emission spectrometer designed by Dr. George Wooten, Monsanto Research Corporation, Dayton Laboratories, Dayton, Ohio.

RESULTS AND DISCUSSION: Microwave-induced emission spectrometry combined with gel-exclusion chromatography (3,5,6,9) was employed to examine the eukaryotic DNA dependent RNA polymerase I from yeast. Figure 1 shows the results of the analysis of 55 μl droplet fractions. Fraction 14 of the chromatogram contains the highest enzyme activity, zinc and protein

TABLE I

Analysis of Gel Exclusion Chromatography
Fraction (55 µl) Exhibiting Maximal Activity*

Fraction No.	Zn g at x 10^{12}	Protein µg	Zn/Protein g at/mole	Cu	Fe	Mn	Mg
				g at/mole			
13	5.5	1.5	2.4	0.05	<0.14	--	<0.07
14	6.1	1.6	2.4	0.03	<0.13	<0.03	<0.06
15	5.0	1.3	2.4	--	<0.16	<0.03	--

*Calculations are based on a molecular weight of 650,000 for this polymerase (8). Zinc and protein are expressed as g-atoms or µg per 5 µl aliquot, respectively. Zinc analyses were performed in duplicate while all other metal analyses were measured only once.

concentrations. For the most active fractions, 13, 14 and 15, ratios of Zn/activity and Zn/protein and, hence, the specific activity (10) remain constant indicating purified enzyme fractions with specific activities of about 250 nmoles of nucleotide incorporated per 10 min per mg of protein, closely similar to the most active preparation of this enzyme reported so far (8). The absorbance at 280nm of the effluent of the column, measured directly in an 8 µl flow through cell, closely follows the activity, zinc and protein profiles (Figure 1, insert). This elution pattern has been very reproducible. In addition, analyses of two different preparations of this enzyme have yielded completely analogous results.

Zinc is a stoichiometric component of the purified enzyme, while Mn, Mg, Cu and Fe are present only close to or at the limits of detection, 10^{-13} to 10^{-14} g-atom (Table I). Based on a molecular weight for the protein of 650,000 (8) the fractions which are enzymatically most active contain 2.4 g-atoms of zinc per mole of protein, a stoichiometry closely similar to that reported for the RNA polymerase from E. coli (2) and for RNA polymerase II from E. gracilis (14). Zinc is tightly bound, and inhibition

TABLE II

Effect of Phenanthrolines
On the Activity of Yeast RNA Polymerase I*

Metal Binding Agent	Concentration x 10^4, M	V/V_C** %
1,10-phenanthroline	3.3	31
	6.0	17
1,7-phenanthroline	3.3	100
	6.0	98
4,7-diphenyl-1,10-phenanthroline (BP)	3.3	38
4,7-diphenyl-2,9-dimethyl-1,10-phenanthroline (BC)	3.3	6

*All rates measured at 30°. See Figure 2 caption for other conditions.

**Enzyme activities in the absence, V_C, and in the presence, V, of inhibitor.

by metal binding agents suggest that it serves a catalytic role. In contrast, the enzyme does not contain Mn and Mg, again similar to previous results for the E. coli RNA polymerase (2) and the reverse transcriptase from avian myeloblastosis virus (3).

A number of 1,10-phenanthroline derivatives instantaneously inhibit yeast RNA polymerase when assayed at 30° (Table II). The metal complexing properties of the 1,10-phenanthrolines account for their inhibition, since the 1,7-isomer of 1,10-phenanthroline binds metals weakly and does not inhibit the nucleotide polymerization reaction under conditions where 1,10-phenanthroline inhibits it completely.

The values of pK_I for 1,10-phenanthroline (OP) and its analog bathocuproin (BC) are 3.8 and 4.25, respectively (Figure 2). For both of these inhibitors, $\bar{n}$, the number of moles of inhibitor bound to the metal (10) is 1.1, close to that observed for a number of the reverse transcriptases

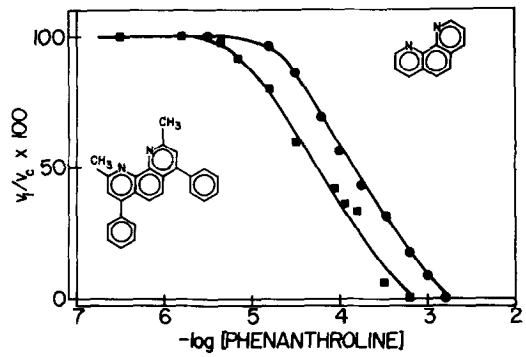

Figure 2: Instantaneous inhibition of the RNA polymerase I from yeast by 1,10-phenanthroline (●) and 4,7-diphenyl-2,9-dimethyl-1,10-phenanthroline (■) at 30°. The agents are added only to the assay system. Concentrations of agents in the standard 60 µl assay are $MnCl_2$, 2 mM; calf thymus DNA, 20 µg; ATP, 0.6 mM; GTP, 0.6 mM; CTP, 0.6 mM; UTP, 10 µM; [^{3}H]-UTP, 0.34 µM; NaF, 6 mM; KCl, 32 mM; β-mercaptoethanol, 3 mM; BSA, 120 µg; Tris, pH 7.9, 50 mM. Enzymes, 5 µl, was added to initiate the reaction. Assays were quenched and analyzed as described (8). V_i is the velocity in the presence of the inhibitor, and V_c that in the absence.

(5). The fact that the value of $\bar{n}$ is close to 1.0 may indicate that the phenanthroline derivatives inhibit the enzyme by binding to the E·Zn complex and forming a ternary complex, E·Zn·OP, much as has been demonstrated for LADH (11,12). The fact that for bathocuproin (BC) inhibition, the K_I value is about 3-fold lower than that for OP is further consistent with the formation of a mixed complex since in the absence of enzyme BC binds slightly less firmly to zinc than does OP (13). If OP and BC inhibit by forming a ternary complex with the enzyme, E·Zn·I, properties other than the chelating nitrogens at positions 1 and 10 may also contribute to their capacity to bind to the enzyme. Thus, the 2,9-dimethyl groups might be important, since the 4,7-diphenyl derivative (BP, Table II) is slightly less effective than either BC or OP.

Additional kinetic studies will be needed in order to determine if the 1,10-phenanthrolines compete with the binding of template or nucleotide or act noncompetitively, blocking the polymerization step. The results might reveal whether zinc plays a specific role in binding substrates, in

polymerization, or both. Thus, zinc could orient the substrates for catalysis or serve as a Lewis acid catalyst and assist in the nucleophilic attack of the 3'-OH group of the terminal ribose on the α-phosphate group of the nucleotide to be attached to the growing chain.

The results obtained here strongly resemble those obtained with the RNA polymerase II from another eukaryote, E. gracilis (14). Microwave emission spectrometry again shows the presence of 2 g-atoms of zinc in this polymerase, and metal binding agents reversibly inhibit it. Yeast and E. gracilis are both suitable sources for large-scale purification of eukaryotic RNA polymerases. The ready availability of a pure eukaryotic polymerase should allow direct inspection of the details of metal interactions with enzyme·nucleotide complexes, and hence the yeast RNA polymerase should be very useful in defining the structural and/or functional role of metals in nucleotide polyerases.

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