RNA Polymerase II from Wheat Germ Contains Tightly Bound Zinc

Paul Petranyi, Jerome J. Jendrisak, and Richard R. Burgess

McArdle Laboratory for Cancer Research

University of Wisconsin, Madison, Wisconsin 53706

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Summary: Atomic absorption studies indicate that the DNA-dependent RNA polymerase II from wheat germ contains about 7 tightly bound zinc atoms per enzyme molecule. This value has been repeatedly obtained with a number of enzyme preparations subjected to varying conditions of purification and dialysis. However, prolonged dialysis of the enzyme with the metal chelator o-phenanthroline results in the loss of enzyme activity and extraction of the bound zinc. Other metals including copper, cobalt, manganese, magnesium, chromium, nickel and iron were not present in significant amounts.

Wheat germ has been shown to be a rich source of the α-amanitin-sensitive DNA-dependent RNA polymerase II (1,2,20). This enzyme is composed of two large polypeptides and a number of low molecular weight polypeptides (2,20), and is similar to the analogous RNA polymerases isolated from other eukaryotic sources (3,4,5).

Nucleotidyl transferases from a number of viral (6,7,8), bacterial (9,10) and eukaryotic (6,11,17,18) sources have been shown to contain zinc (see Table 2). Zinc is required for polymerase activity in the avian myeloblastosis virus RNA-dependent DNA polymerase (6,7) and in Escherichia coli DNA polymerase I (9). Virtually all of the nucleotidyl transferases which have been studied are inhibited by the metal chelator o-phenanthroline. It has been suggested that the eukaryotic RNA polymerases from rat liver and sea urchin may also contain zinc on the basis of inhibition by o-phenanthroline (12). Recently zinc has been directly detected in yeast RNA polymerases I (17) and II (18).

The work presented here indicates that the purified wheat germ RNA polymerase II contains a complement of tightly bound zinc. A preliminary report of part of this work has been published (20).

Materials and Methods

Materials: EDTA, Tris base and o-phenanthroline were obtained from Sigma. Enzyme grade ammonium sulfate was obtained from Schwarz Mann, glycerol from Fischer, ethylene glycol from Aldrich, concentrated nitric acid from Du Pont, bovine serum albumin from Miles and E. coli alkaline phosphatase from Worthington. All other chemicals were of reagent grade. Glass double distilled water was used in all solutions.

Buffers and Zinc Contamination: Dialysis buffer contained 50 mM Tris-HCl (pH 7.9 at 25°C), 0.1 mM EDTA, 1 mM dithiothreitol and 25% (v/v) glycerol and is referred to as TEDG. Chromatography buffer contained 25% (v/v) ethylene glycol instead of glycerol to decrease buffer viscosity and is referred to as TEDL. The contributions of the various components to the total zinc content of these buffers were measured and found to be 0.005 µM, 0.002 µM, 0.002µM, 0.05 µM, 1.5 µM, and 0.03 µM for 50 mM Tris, 0.1 mM EDTA, 1 mM dithiothreitol, 25% glycerol, 25% ethylene glycol and 0.1 M $(NH_4)_2SO_4$, respectively.

All glassware was soaked for several hours in 1 M HNO_3 and rinsed with double distilled water prior to use. Dialysis tubing was boiled several times in 0.1 M EDTA (pH 10) and carefully rinsed in double distilled water.

Metal Analysis: A Perkin-Elmer Model 503 atomic absorption spectrometer with a graphite furnace attachment was used for the determination of zinc. Enzyme samples were diluted with 0.01 N HNO_3 to a final concentration of about 10 µg/ml prior to zinc analysis. 1-10 µl volumes were assayed for zinc. The samples were heated at 120°C, 500°C and 2000°C for 15 seconds each in a programmed sequence. Absorption was measured at 213.9 nm. 50 ng of enzyme gave a strong zinc absorption corresponding to about 35 picogram zinc. The zinc response was linear in the range 0-35 picogram. A standard zinc solution in 0.01 N HNO_3 was prepared prior to each zinc analysis by dissolving granular zinc metal in 6 N HNO_3 and diluting appropriately. Zinc absorbance was recorded on chart paper and converted to picogram zinc by reference to a standard curve. Zinc determinations by atomic absorption were not effected by the presence in the sample of a variety of materials such as o-phenanthroline, EDTA or protein. Iron was assayed for by atomic absorption spectrometry in a similar manner.

Neutron activation analysis was used for the determination of Cu, Co, Cr, Ni, Mg and Mn. The enzyme (3.1 mg/ml) was dialyzed for 4 days at 4°C against TEDG buffer prior to analysis. Duplicate aliquots (0.4 mls) of RNA polymerase and dialysis buffer were placed in plastic vials and were irradiated in a TRIGA reactor at a neutron flux rate of 5×10^{12} neutrons/cm^2/ second for 3 hours. The energies and intensities of gamma radiation from the decaying isotopes were monitored for 2 hours on a lithium drifted germanium detector coupled to a Tracor-Northern NS 710 multichannel analyzer. A standard solution containing 0.1 µg/ml of the indicated metals was also irradiated and the metal content of the RNA polymerase was determined by reference to this standard.

RNA Polymerase Purification and Assay: Wheat germ RNA polymerase II was purified and assayed as described by Jendrisak and Burgess (2). This assay employs denatured calf thymus DNA as template and limiting ^{3}H-UTP as the labeled substrate. An active preparation of enzyme has a specific activity of 250-350 activity units per mg, where an activity unit is defined as

Table 1: Zinc Content of RNA Polymerase after Gel Filtration Chromatography

Column Material and Chromatography Buffer	Fraction Number	Protein mg/ml	Gram-atom Zinc per Mole Enzyme	Specific Activity (percent of peak fraction)
a) BioGel A-1.5m (Fig. 2) TEDL + 0.1 M $(NH_4)_2SO_4$	14	.38	7.3	110%
	15	.52	6.4	100%
	16	.48	6.2	91%
	17	.36	6.2	87%
	18	.21	6.6	77%
b) BioGel A-1.5m TEDL + 0.1 M $(NH_4)_2SO_4$ without 0.1 mM EDTA	16	.22	6.6	83%
	17	.33	6.6	100%
	18	.32	7.5	105%
	19	.20	7.4	112%
c) Ultrogel AcA 22 TEDL + 0.1 M $(NH_4)_2SO_4$	20	.20	7.0	94%
	21	.34	7.2	100%
	22	.27	6.3	109%
	23	.09	7.7	128%

Three separate columns were run as described in the legend of Fig. 2. The zinc concentrations in the chromatography buffers of columns a, b, and c were 1.4 μM, 1.2 μM, 0.5 μM, respectively. Column C contained only 12.5% ethylene glycol. The protein concentration of each fraction was determined spectrophotometrically as described in Methods.

Additional chromatography of the purified RNA polymerase reveals only a slight variation in the zinc content about a mean of 7 gram-atom/mole enzyme. Analysis of the zinc contents of 12 separate enzyme preparations yielded an average of 7.0 ± 0.7 gram-atom zinc/mole enzyme. The error represents one standard deviation. The values ranged from 5.8 to 8.6.

The Zinc is Tightly Bound: In each of the enzyme preparations analyzed, the enzyme, purified as described previously (2), was either dialyzed against dialysis buffer for 2-7 days or subjected to gel filtration prior to zinc analysis. No correlation was observed between the zinc level in the buffers and the amount of zinc bound to the enzyme. During gel filtration the buffer contained approximately one zinc per polymerase molecule and during dialysis the buffer contained only 0.01 to 0.1 zinc per polymerase.

Table 1 shows the zinc content of the peak RNA polymerase fractions

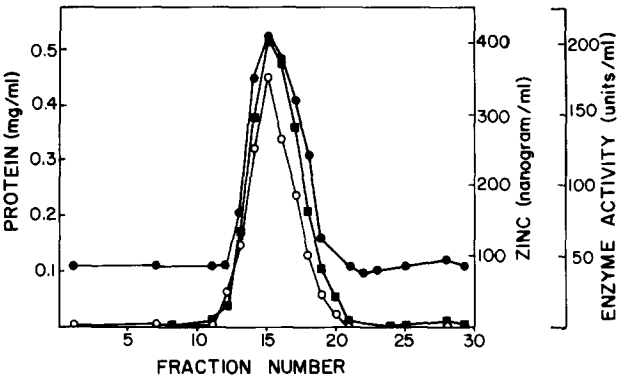

Figure 1. BioGel A-1.5 m chromatography of purified RNA polymerase showing copurification of zinc with RNA polymerase activity. A 2.6 x 29 cm column was packed with BioGel A-1.5 m agarose gel, washed with 10 mls of a 0.1 M EDTA pH 10 solution, and equilibrated for 24 hours with 250 mls of TEDL + 0.1 M $(NH_4)_2SO_4$ buffer. The enzyme was purified on DEAE-cellulose, phosphocellulose and DEAE-Sephadex prior to agarose gel filtration chromatography. The DEAE-Sephadex peak was ammonium sulfate precipitated and concentrated to about 2.5 mg/ml. A 5 ml polymerase sample was applied and run at a flow rate of 10 ml/hr at 4°C. 5 ml fractions were collected in acid washed tubes. Protein (—■—), zinc (—●—) and enzyme activity (—○—) were determined as described in Methods. Each zinc data point represents the average of 3-10 separate zinc assays.

obtained from the column shown in Fig. 1 and two other gel filtration columns. The presence of 0.1 mM EDTA during gel filtration did not alter the zinc content of the polymerase (compare experiments a and b of Table 1).

Samples of RNA polymerase at 100 µg/ml, dialyzed for 7 days against 1000 volumes of TEDG + 0.1 M $(NH_4)_2SO_4$ buffer at 4°C showed no loss of specific activity or bound zinc. Dialysis of *E. coli* RNA polymerase holoenzyme under these conditions and subsequent zinc analysis gave a value of 1.9 gram-atoms of zinc per mole of enzyme, in good agreement with the published value (10). However, dialysis of *E. coli* alkaline phosphatase under these conditions resulted in complete removal of the 4 weakly bound zinc atoms known to be present per molecule of this enzyme (16).

Nevertheless prolonged dialysis of 200 µg/ml wheat germ RNA polymerase II against TEDG + 0.1 M $(NH_4)_2SO_4$ buffer lacking EDTA but containing 2.5 mM *o*-phenanthroline at 25°C resulted in a loss of enzyme bound zinc and specific activity, as shown in Fig. 2. This indicates that although the zinc is

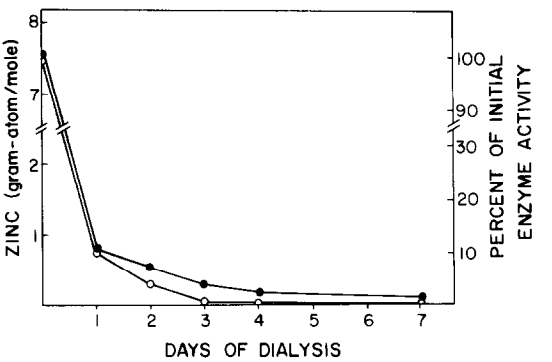

Figure 2. Dialysis of wheat germ RNA polymerase II against 2.5 mM o-phenanthroline at 25°C. Six 2 ml samples of 200 µg/ml RNA polymerase were dialyzed in separate dialysis bags in a common reservior of 2 liters of TEDG + 0.1 M $(NH_4)_2SO_4$ buffer containing no EDTA but containing 2.5 mM o-phenanthroline. At the indicated times of dialysis, the bags were opened and the protein concentration, enzyme activity, and zinc content were determined as described in Methods. Zinc content in gram-atom/mole enzyme (—●—) and percent initial specific activity (—○—) are indicated. Under these conditions, but lacking o-phenanthroline, the enzyme shows no loss of activity in several days.

tightly bound it can be removed if drastic dialysis conditions are employed. All attempts to restore activity to the zinc-depleted enzyme by addition of zinc were unsuccessful.

Chelator Studies: The metal chelators EDTA and o-phenanthroline produce little or no immediate inhibition of wheat germ RNA polymerase II activity. The presence of 1 mM o-phenanthroline or 2.5 mM EDTA in the polymerase assay mixture (pH 7.9) containing 10 mM Mg^{++} produces less than a 10% inhibition in a 15 minute assay. Incubation of the purified polymerase in TEDG + 0.1 M $(NH_4)_2SO_4$ with 2.5 mM o-phenanthroline or 2.5 mM EDTA for periods up to 2 hours at 25° before addition to the assay mixture also produced less than a 10% inhibition of activity. Lowering the concentrations of Mg^{++} in the assay by chelation might account for the observed inhibition.

No Other Metals Detectible in RNA Polymerase: Atomic absorption was also used for the determination of iron in dialyzed RNA polymerase. No iron above the level in the dialysis buffer was detected in the enzyme indicating

Table 2: Zinc Contents of Various Nucleotidyl Transferases

Enzyme	Zinc (gram-atom/mole)	Reference
E. coli DNA Polymerase I	1	9
Sea Urchin DNA Polymerase	4	11
Phage T4 DNA Polymerase	1	9
Avian Myeloblastosis Virus DNA Polymerase	1-2	6,7
E. coli RNA Polymerase	2	10
Phage T7 RNA Polymerase	2-4	8
Yeast RNA Polymerase I	2.4	17
Yeast RNA Polymerase II	1	18
Wheat Germ RNA Polymerase II	7	This paper, 20

that iron was present at less than 0.1 gram atom/ mole in the RNA polymerase.

Neutron activation was used for the determination of Cu, Co, Mn, Mg, Cr and Ni. None of these metals were detected in the RNA polymerase at levels above that in the dialysis buffer. This data shows Mg, Ni, Cu, Co and Cr to be present at less than 0.1 gram-atom/mole enzyme and Mn to be present at less than 0.01 gram-atom/mole enzyme. Zinc at levels stoichiometric with the enzyme cannot be measured by neutron activation.

Discussion

The evidence presented here indicates that purified wheat germ RNA polymerase II contains about 7 atoms of zinc. This zinc content is not affected by chromatography of the purified enzyme on a number of ion exchange columns or by prolonged dialysis against low zinc buffers, and suggests that the zinc is tightly bound to the enzyme. This large amount of bound zinc was unexpected and is considerably higher than the zinc contents of E. coli RNA polymerase (10) and of two other eukaryotic RNA polymerases (17,18) as shown in Table 2.

The availability of large quantities of purified wheat germ RNA polymerase II has allowed precise zinc and protein determinations. Although neither of the recent reports on yeast RNA polymerases presented evidence of careful protein determinations, it is doubtful whether these values are in

error by more than two-fold and thus it appears that the wheat germ RNA polymerase II does contain considerably more zinc than the RNA polymerases I and II from yeast.

The function of the seven atoms of zinc per enzyme molecules is not presently known. It seems unlikely that all seven zinc atoms could play catalytic roles and more likely that most play structural roles. It is possible that the unusual stability of this enzyme compared to most eukaryotic RNA polymerases is due in part to the abundance of tightly bound zinc atoms. The example of T7 RNA polymerase with 2-4 zinc atoms bound to a single polypeptide of 107,000 daltons (see Table 2) makes it less surprising that wheat germ RNA polymerase II with over 10 polypeptides and a total molecular weight of 550,000 daltons might be able to bind seven zinc atoms. Those who attempt to reconstitute enzyme activity from separated subunits might take this high zinc content into account.

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