Zinc Deficiency and the *Euglena gracilis* Chromatin: Formation of an α-Amanitin-Resistant RNA Polymerase II†

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ABSTRACT: Both the single DNA-dependent RNA polymerase found in zinc-deficient (−Zn) *Euglena gracilis* and the RNA polymerase III from zinc-sufficient (+Zn) cells have been isolated by methods previously used to purify polymerases I and II [Falchuk, K. H., Mazus, B., Ulpino, L., & Vallee, B. L. (1976) *Biochemistry 15*, 4468; Falchuk, K. H., Mazus, B., Ulpino, L., & Vallee, B. L. (1977) *Biochem. Biophys. Res. Commun. 74*, 1206]. Like class II polymerases, the enzyme from −Zn organisms elutes from DNA–cellulose and phosphocellulose with 0.6 M NaCl and 0.35 M NH_4Cl, respectively. It is inhibited by 8-hydroxyquinoline, 8-hydroxyquinoline-5-sulfonic acid, α,α′-bipyridyl, dipicolinic acid, and 1,10-phenanthroline (OP); 4,7-phenanthroline, the nonchelating analogue, does not inhibit. The $pK_{I(OP)}$ of this enzyme is identical with that of polymerase II but distinct from those of polymerases I and III. Elemental analysis confirms that zinc is the functional metal while copper, manganese, iron, and magnesium are absent. However, the −Zn enzyme is at least 4 orders of magnitude more resistant to α-amanitin (α-A) than the class II polymerase. Further, its response to α-A is unlike that of either polymerase I or polymerase III. Thus, −Zn cells contain a single, α-amanitin-resistant (α-A^r) RNA polymerase, whose behavior otherwise resembles that of the α-amanitin-sensitive polymerase II.

Zinc deficiency affects *Euglena gracilis* DNA, histone, and non-histone protein metabolism (Wacker, 1962; Falchuk et al., 1975a; Stankiewicz et al., 1983; Mazus et al., 1984); it represses transcription of the genome and alters the composition and/or function of the RNAs which are transcribed (Schneider & Price, 1962; Prask & Plocke, 1971; Falchuk et al., 1975b, 1978; Crossley et al., 1982; Vallee & Falchuk, 1981, 1983; Falchuk & Vallee, 1984). This study examines its effects on the enzymes transcribing DNA.

In dark-grown −Zn *E. gracilis* a single zinc RNA polymerase is found compared with the three RNA polymerases typical of +Zn eukaryotic cells (Chambon, 1975). While its chromatographic behavior and response to chelating agents are identical with that of class II enzymes, it exhibits a very low sensitivity to α-amanitin. Moreover, both the responses to this toxin and that to OP differ from those of the class I or III polymerases.

The present findings demonstrate that zinc profoundly affects the metabolism of the major enzymatic components of *E. gracilis* chromatin involved in transcription. The significance of these effects on gene expression and cellular metabolism is discussed.

Materials and Methods

Euglena gracilis, strain Z, was incubated in the dark at 22 °C in +Zn and −Zn media (Falchuk et al., 1975b). The cultures were grown to early stationary phase and harvested by centrifugation at 150*g* for 10 min. The +Zn and −Zn cell pellets were washed with ice-cold buffer, 0.05 M tris(hydroxymethyl)aminomethanehydrochloride (Tris-HCl), pH 8, and stored at −20 °C.

Purification of DNA-Dependent RNA Polymerases. All preparative work was carried out from 0 to 4 °C (Falchuk et al., 1976, 1977). A homogenate of either +Zn or −Zn cells containing 0.5 M $(NH_4)_2SO_4$ in buffer A, 50 mM Tris-HCl, pH 7.8, 6 mM $MgCl_2$, and 10 mM mercaptoethanol, was centrifuged at 48000*g* for 15 min. The supernatant (I) was saved and the pellet resuspended and homogenized in buffer A also containing 0.5 M $(NH_4)_2SO_4$. The homogenized pellet was sonicated in 20-s bursts for a total of 2 min with a Bronwell Biosonic sonicator (Bronwell Scientific, Rochester, NY). The sonicate was centrifuged at 48000*g* for 15 min. The resultant supernatant (II) was combined with supernatant I and then diluted 1:1 with buffer B, 50 mM Tris-HCl, pH 7.8, 10 mM mercaptoethanol, 6 mM $MgCl_2$, and 20% glycerol. The preparation was allowed to precipitate for 30 min with protamine sulfate (final concentration 0.1%). The protamine sulfate fraction was centrifuged at 48000*g* for 20 min, and the supernatant, containing the RNA polymerase activity, was precipitated with 65% $(NH_4)_2SO_4$ for 2 h. The precipitate was collected after centrifugation at 48000*g* for 30 min and dissolved in and dialyzed against buffer B until the conductivity was 2 $m\Omega^{-1}$. The sample was loaded onto a DEAE-Sephadex A-25 column and washed with 0.05 M NH_4Cl in buffer B. The polymerase activity was then eluted as a single fraction with 0.4 M NH_4Cl. The enzymatically active fractions were concentrated by using an Amicon ultrafiltration cell with a P30 filter, and the resultant concentrate was equilibrated with buffer C, 50 mM Tris-HCl, pH 7.8, 10 mM mercaptoethanol, 0.10 M NaCl, 6 mM $MgCl_2$, and 20% glycerol. The fraction was loaded onto a DNA–cellulose column, 4 mL, washed with 0.10 M NaCl buffer, and the DNA binding proteins were eluted with 0.6 and 0.8 M NaCl. The conductivity of the fractions from DNA–cellulose which contained RNA polymerase activity was brought to 2 $m\Omega^{-1}$, and the sample was then loaded on a phosphocellulose column, 10 mL. The column was washed with buffer B, and the enzymes were eluted with step gradients utilizing 0.15 and 0.35 M NH_4Cl.

Zinc, 10^{-5} M, was added to a homogenate of −Zn cells to study the effect of adding this metal on the purification of RNA polymerase activity. Following addition of zinc, RNA

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polymerase isolation was carried out as described above. RNA polymerases were stored in 50% glycerol in 2-mL vials at −170 °C in a liquid nitrogen tank (Cryogenics East, Incorp.) *Escherichia coli* RNA polymerase was obtained commercially (Boehringer). All assays for polymerase activity were performed as described previously (Falchuk et al., 1976, 1977).

Effect of α-Amanitin on RNA Polymerase Activity. The sensitivity to α-amanitin of all of the RNA polymerases isolated from +Zn and −Zn cells was examined at concentrations ranging from 0.01 to 200 μg/mL of this inhibitor. Each assay mixture contained 10 μg of protein.

Inhibition by Chelating Agents. The effects of a number of chelating agents and the nonchelating analogue 4,7-phenanthroline on the activities of the RNA polymerases from −Zn cells, the class II enzyme from +Zn cells, and the polymerase from *E. coli* were tested. A stock solution of 1,10-phenanthroline (OP), 10^{-2} M, pH 7.8, was diluted with 50 mM Tris-HCl, pH 7.8, to obtain a range of inhibitor concentrations from 10^{-6} to 5×10^{-3} M OP. Dipicolinic acid, 8-hydroxyquinoline, 8-hydroxyquinoline-5-sulfonic acid, and α,α′-bipyridyl were all used at 1 and 5 mM. The concentrations of template, nucleotide, and other components were standard in all assays. Mg^{2+} was the only activating cation throughout. In all assays in which OP was used in conjunction with the RNA polymerases from *E. gracilis*, the chelator was added last to the reaction mixture. In all other assays, the enzyme and chelators were preincubated at 37 °C for 20 min before they were added to the reaction mixture.

Metal Content of DNA-Dependent RNA Polymerase from −Zn Cells. All glassware used in the preparation of the enzymes for metal analysis was soaked for 24 h in a 50:50 mixture of nitric and sulfuric acid and washed extensively in metal-free distilled water in order to avoid external metal contamination. A 50-μg aliquot of RNA polymerase was chromatographed on a Sephadex G-75 column which had been rendered metal free (Auld et al., 1974; Falchuk et al., 1976). Forty-microliter fractions were collected, and the content of the Zn, Mg, Fe, Mn, and Cu and protein was measured in each fraction by microwave-induced emmision spectroscopy and micro-Lowry method, respectively, as previously described (Auld et al., 1974, 1976; Kawaguchi & Vallee, 1975; Kawaguchi & Auld, 1975; Falchuk et al., 1976).

Results

The initial purification steps solubilize and remove endogenous DNA associated with the RNA polymerases. The resultant DNA-free enzymes require exogenous template for activity and bind to and are purified further on DNA–cellulose which separates RNA polymerase activity from +Zn cells into two peaks (Figure 1). The first elutes with 0.6 M NaCl. It has been shown previously to contain a mixture of class I and II enzymes which separate on phosphocellulose by elution with 0.15 and 0.35 M NH_4Cl, respectively (Falchuk et al., 1976, 1977). The second peak elutes with 0.8 M NaCl and has not been characterized previously (Figure 1). This 0.8 M NaCl fraction contains an RNA polymerase activity that elutes as a single peak from phosphocellulose with 0.35 M NH_4Cl. Buffers containing lower or higher salt concentrations applied to the phosphocellulose column do not elute any other activity (not shown).

The sensitivity of this RNA polymerase fraction to α-amanitin (α-A), $pK_{I(\alpha\text{-A})} = 4.8$, differs from that of class I and II and identifies it as a class III enzyme (Figure 2). Typically, RNA polymerase I is insensitive to α-A at concentrations as high as 200 μg/mL. In contrast, polymerase II is very sensitive to α-A ($pK_{I(\alpha\text{-A})} = 7.5$).

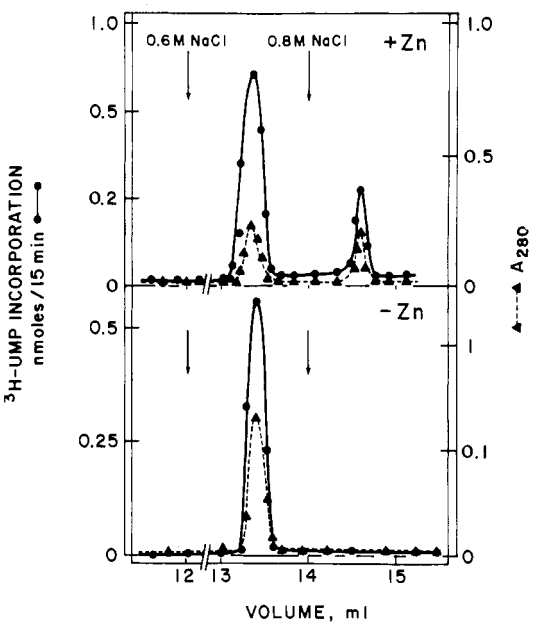

FIGURE 1: +Zn and −Zn *E. gracilis* RNA polymerases: DNA–cellulose chromatography. The pooled active fractions eluted from a DEAE-Sephadex column were loaded onto a DNA–cellulose column (4 mL). From the +Zn preparation, top, two fractions are eluted with 0.6 and 0.8 M NaCl, respectively. The 0.6 M NaCl fraction contains a mixture of RNA polymerase I and polymerase II which are readily separated by chromatography on phosphocellulose (Falchuk et al., 1976, 1977). The 0.8 M NaCl contains only one RNA polymerase which elutes as a single peak from phosphocellulose with 0.35 M NaCl (not shown). From the −Zn preparation, bottom, only a single fraction is eluted with 0.6 M NaCl.

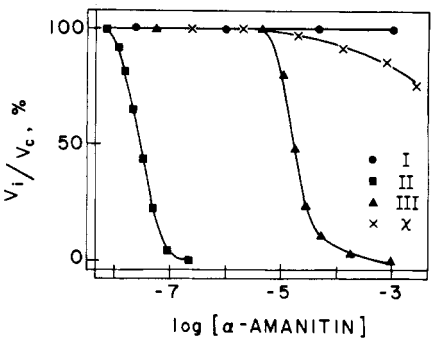

FIGURE 2: α-Amanitin sensitivity of the RNA polymerases from +Zn and −Zn *E. gracilis*. V_i is the velocity in the presence of inhibitor, and V_c is the velocity in its absence. The inhibition by α-amanitin of the 0.8 M NaCl DNA–cellulose fraction is typical of an RNA polymerase III (▲). The effect of α-amanitin on the single RNA polymerase from −Zn cells (×) differs from that of this as well as the other two enzymes from +Zn organisms: I (●) and II (■).

The major fraction of proteins applied to the DNA–cellulose from preparations of −Zn cells either appears in the initial eluate or appears while washing the column with 0.10 M NaCl but does not contain activity either way. All RNA polymerase activity is contained in a single fraction which elutes with 0.6 M NaCl, and none is obtained when eluting with 0.8 M NaCl (Figure 1). Subsequent chromatography of the 0.6 M NaCl fraction on phosphocellulose further purifies this RNA polymerase (Figure 3). A peak of protein containing polymerase activity elutes with 0.35 M NH_4Cl, but none appears with 0.15 M or salt concentrations higher than 0.35 M. The addition of zinc to cellular homogenates neither induces the appearance of additional RNA polymerases nor increases the specific activity of the enzyme purified in this manner.

The chromatographic behavior on both ion exchange gels and DNA affinity resins of this single enzyme from −Zn cells

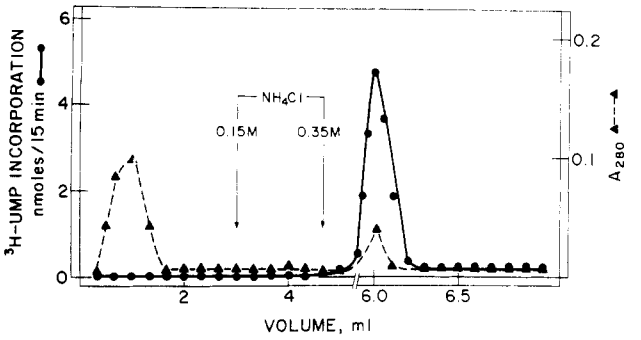

FIGURE 3: −Zn *E. gracilis* RNA polymerase: phosphocellulose chromatography. The 0.6 M NaCl fraction from DNA cellulose was dialyzed against buffer B until its conductivity was brought to 2 $m\Omega^{-1}$. Twenty milliliters was loaded onto a phosphocellulose column (10 mL). A single activity elutes with 0.35 M NH_4Cl. No activity elutes with 0.15 or 0.6 M NH_4Cl.

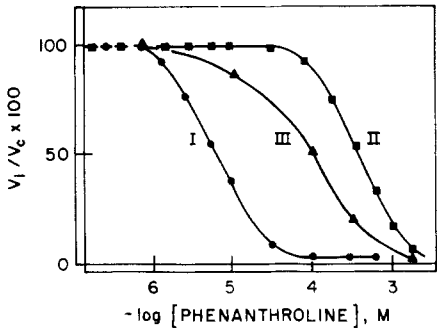

FIGURE 4: OP inhibition of RNA polymerases I (●), II (■), and III (▲) from +Zn *E. gracilis*.

Table I: Effect of Chelating Agents and of a Nonchelating Analogue on RNA Polymerase Activity

agent	concn (mM)	% inhibition of the RNA polymerase class α-A^{r}II	II	*E. coli*
8-hydroxyquinoline	1	37	40	77
	5	79		89
8-hydroxyquinoline-5-sulfonic acid	1	23	22	29
	5	49	40	57
α,α′-bipyridyl	1	13		22
	5	48	43	45
dipicolinic acid	1	35		34
	5	68		41
1,10-phenanthroline	1	71	70	19
	5	100	100	76
4,7-phenanthroline	1	0	0	0
	5	0	2	4

coincides with that of class II polymerases.

Its behavior toward α-amanitin, $pK_{I(\alpha\text{-}A)} < 3.0$, however, differs significantly from that of all three classes of polymerases from +Zn cells (Figure 2). Therefore, this enzyme has been categorized as an α-amanitin-resistant (α-A^r) RNA polymerase II.

This α-A^r RNA polymerase II contains a metal essential for activity. A number of chelating agents of differing structures inhibit +Zn and −Zn *E. gracilis* RNA polymerases II as well as that of *E. coli* (Table I).

In all instances, the higher the concentration of chelator, the greater the inhibition. Among all these agents only OP is sufficiently soluble to induce complete inhibition of the −Zn *E. gracilis* RNA polymerase α-A^{r}II as well as the class I, class II, and class III enzymes from +Zn cells (Figures 4 and 5). Their $pK_{I(OP)}$ differentiates among the three standard classes of polymerases (Figure 4) but are identical for polymerases

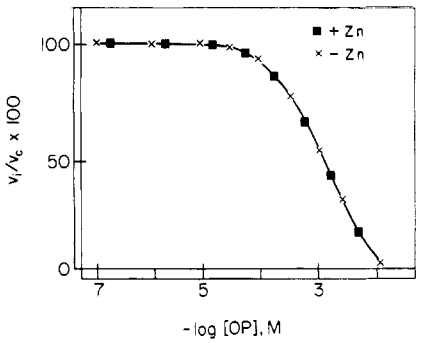

FIGURE 5: Comparison of OP inhibition of the single RNA polymerase from −Zn *E. gracilis* (×) and RNA polymerase II (■).

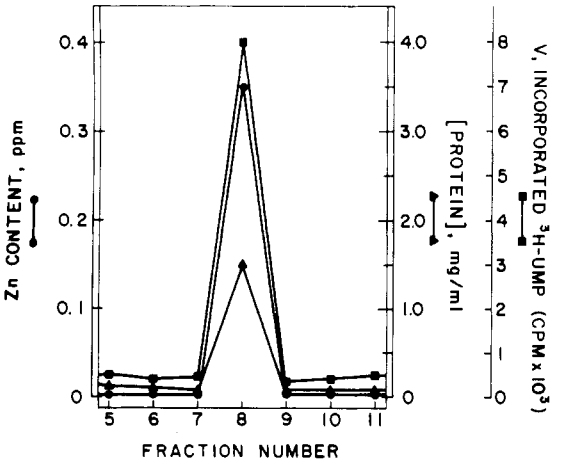

FIGURE 6: Zinc content of RNA polymerase from −Zn cells. A single peak of protein, activity, and zinc is observed when the RNA polymerase from −Zn cells is chromatographed on a metal-free Sephadex G-75 column. Fe, Mn, Mg, and Cu are not present in this fraction.

α-A^r II and II (Figure 5). A nonchelating analogue, 4,7-phenanthroline, does not affect the activities of RNA polymerases (Table I). Metal analyses of the enzyme reveals the presence of zinc. The enzyme preparation elutes as a single peak from a metal-free Sephadex G-75 column and contains 0.23 μg of zinc/mg of protein (Figure 6). Mn, Mg, Fe, or Cu is not detected. Since the molecular weight of the enzyme remains to be determined, stoichiometry cannot be calculated with certainty at present. The value for that of other RNA polymerases from eukaryotic cells, including *E. gracilis*, ranges from 650000 to 700000 daltons (Chambon 1975; Roeder, 1976; Falchuk et al., 1976, 1977; Sebastiani, 1977, Pauli, 1981). Assuming the enzyme from −Zn cells to be in this range, the stoichiometry would be expected to be similar to that of the corresponding enzymes from *E. gracilis*, i.e., ~2.0 mol of zinc/mol (Falchuk et al., 1976, 1977).

Discussion

The purification procedures used previously for RNA polymerases I and II from +Zn cells (Falchuk et al., 1976, 1977) have been modified to isolate RNA polymerase III from +Zn cells as well as the single polymerase from −Zn *E. gracilis* (Figures 1–3). A number of comparative studies of these enzymes from both cell types have been performed.

The chromatographic properties and sensitivity to α-amanitin of eukaryotic RNA polymerases are characteristic, and their differences have served to classify them (Chambon, 1975; Roeder, 1976). The RNA polymerases from +Zn *E. gracilis* are recognized on this basis (Figures 1 and 2). The chelating agent 1,10-phenanthroline inhibits all three enzymes from +Zn

E. gracilis, with a pK_I characteristic of each (Figure 4).

The single RNA polymerase from −Zn *E. gracilis* is a class II enzyme in all ways, save its responses to α-amanitin, which makes it an enzyme novel for this organism: an α-amanitin-resistant (α-A^r) RNA polymerase II (Figures 1–5).

The RNA polymerase II from +Zn cells is a zinc metalloenzyme, based both on inhibition by chelating agents and on metal analysis (Falchuk et al., 1976). Inhibition of the α-A^r RNA polymerase II with both OP and other, structurally different chelating agents, together with atomic absorption analysis, show that it, too, contains functional zinc (Figures 4–6; Table I). Studies considered beyond the scope of the present investigations are required to define the role and mechanism of zinc in the function of this enzyme.

The enzyme does not contain Mg, Mn, Cu, or Fe, a possibility which had to be explored (Falchuk et al., 1976) considering their accumulation, and that of yet other metals in −Zn organisms (Wacker, 1962; Falchuk et al., 1975b). Zinc addition fails to increase RNA polymerase activity either of −Zn *E. gracilis* homogenates or of the purified α-A^r enzyme; these organisms apparently do not synthesize apoRNA polymerases which can then be rendered functional by post-translational addition of zinc.

The striking findings reported here call for an examination of the basis for the presence of a single, zinc enzyme in zinc deficiency compared with the three standard ones found in zinc-sufficient cells and the eventual consequences to RNA metabolism. The formation of the three polymerases in normal eukaryotic cells is believed to be dependent on three distinct gene loci (Thonart et al., 1976; Sebastian, 1977). The expression of these different polymerase genes varies during growth and differentiation. Thus, during cell proliferation the specific activities of the products of both polymerase I and polymerase III genes increase; when growth ceases or during impairment of protein synthesis, the activities decrease but do not disappear (Roeder 1976; Muramatsu et al., 1970; Yu et al., 1972). In contrast, the activity of the product of the polymerase II gene remains relatively constant throughout all growth stages (Roeder & Rutter, 1970).

Thus, changes in rates of growth, proliferation, and/or protein synthesis represent a number of pathways for the normal regulation or alteration of RNA polymerases. The consequences of zinc deficiency on *E. gracilis* polymerases may be analogous. Polymerase I and polymerase III genes could be repressed during the growth arrest accompanying zinc deficiency, either indirectly owing to metabolic changes associated with the characteristic blocked proliferation or reduced protein synthesis of these cells or directly as the result of effects of the metal on transcription itself. A role for zinc in the repression (or activation) of specific *E. gracilis* genes has been postulated previously to account for effects of its deficiency on the absence (or presence) of other proteins (Crossley et al., 1982; Vallee & Falchuk, 1981; Mazus et al., 1984), though such roles for zinc in the *regulation* of transcription of specific genes have not been confirmed experimentally.

The failure to detect polymerases I and III in −Zn cells could also be the result of enhanced degradation. Both enzymes are turned over rapidly, with a short half-life (Muramatsu et al., 1972, Chambon, 1975). The accumulation of amino acids and that of small peptides (Wacker, 1962) are consistent with increased proteolysis in zinc deficiency.

The polymerase II gene likely is active in zinc deficiency, as in other growth-arrested cells, since its product, RNA polymerase II, is prominent (Figure 1–5). However, additional processes in either gene transcription, mRNA processing, or posttranslational events must be involved in the formation of the α-A^r polymerase II in −Zn but not in +Zn cells. α-A^r polymerases II are not unique to −Zn cells, and their induction in *other* organisms provides clues to the mechanism by which it could be formed in −Zn *E. gracilis*. Thus, when a variety of mammalian cells, including Chinese hamster ovaries, rat myoblasts, short-term cultures of human diploid fibroblast explants, and mouse lymphoblastoid cell lines are incubated first with ethylmethanesulfonate, a mutagenic agent, followed by the inhibitor, α-amanitin, an RNA polymerase is formed, which, in terms of its elution properties, behaves like a class II enzyme but, unlike it, requires 2–800-fold greater concentrations of α-amanitin for inhibition (Chan et al., 1972; Somers et al., 1975; Amati et al., 1975; Buchwald & Ingles, 1976; Ingles et al., 1976; Wolf & Bantz, 1971; Crorar et al., 1977; Bryant et al., 1977; Ingles, 1978). Moreover, both the electrophoretic properties of this variant polymerase II (Milman et al., 1976; Steinberg et al., 1977) and its DNA template preferences are altered (Bryant et al., 1977). The α-amanitin-resistant enzyme has been thought to be the result of a change in either the structure or composition of the 40 000-dalton α-amanitin binding subunit of polymerase II originating in a presumed alteration of the gene coding for this polymerase (Ingles et al., 1976; Brodner & Wieland, 1976). Under these circumstances polymerases I and III appear to be synthesized normally (Bryant et al., 1977), restricting the effects of ethylmethanesulfonate to class II enzymes. Therefore, the polymerase II gene, and particularly the gene segment involved in the formation of the subunit responsible for α-amanitin binding, appears to be susceptible to alterations by chemical mutagens. The present observations suggest that this susceptibility may be extended to include other environmental mutagens, e.g., metals. The latter have been shown to induce misreading of the DNA template by the *E. gracilis* RNA polymerase II which could lead to unusual gene products (Falchuk et al., 1978). In vivo the alterations in metal content and concentrations characteristic of −Zn cells (Wacker, 1962; Falchuk et al., 1975) could affect transcription of the polymerase II gene and account for the altered properties of the enzyme (Figure 2). Alternatively, alterations in the post-transcriptional processing of mRNA affecting the homologous α-amanitin binding segments or posttranslational changes such as, e.g., modification and/or *limited* proteolysis of the α-amanitin binding subunit itself could also generate a polymerase II such as described.

While these and perhaps yet other possibilities have not been differentiated, the results of these and previous studies clearly show that in early stationary phase −Zn cells, the three standard polymerases are absent and only a single, α-A^r polymerase II is found (Figures 1–3). Yet these cells contain the three distinct RNA classes (Falchuk et al., 1978). In the limit, two distinct sets of biochemical events, among others, could account for the existence of only one polymerase and three types of RNA. In the first, each RNA type would be synthesized by its respective polymerase. On reaching stationary phase, the polymerases would fail to be generated and disappear but the RNAs that they have formed already would be preserved because they would be stored and/or not hydrolyzed. Given that this set of events would abolish two of the normal polymerases, de novo synthesis of the respective RNAs would be reduced. In the second, the formation of all three polymerases would be blocked, and all of them would vanish followed by de novo synthesis of the α-A^r polymerase II which then would synthesize each of the various RNA types. This would imply that a eukaryote, which normally depends

on three polymerases to form three RNA types, could also function in a manner typical of prokaryotes which are equipped with only one polymerase to achieve analogous objectives. Under these conditions, ongoing RNA synthesis would be the same as that of +Zn cells which are forming the three types of RNA and constitute an example of ontogeny reflecting phylogeny in lower forms.

The differentiation of the possibilities requires knowledge of the synthesis and degradation of the three RNA polymerases, of the α-A^r polymerase, and of their respective mRNAs. At present, the 4-fold *reduction* of de novo RNA synthesis in −Zn relative to +Zn organisms (Falchuk et al., 1975b) favors the first sequence described.

The phenotypic changes characteristic of the biology and biochemistry of −Zn *E. gracilis* (Vallee & Falchuk, 1981; Falchuk & Vallee, 1984) encompass at least each of the major components of chromatin: DNA template (Wacker, 1962; Falchuk et al., 1975b), the histone and non-histone proteins that determine the binding of enzymes to, and transcription of, DNA (Stankiewicz et al., 1983; Mazus et al., 1984), and the very enzymes that transcribe the DNA. Functionally, these various alterations in chromatin constituents decrease the accessibility of DNA to enzymes to which it binds, e.g., nucleases (Stankiewicz et al., 1983), and reduce its capability to serve as a template for RNA polymerases, in general (B. Mazus, K. H. Falchuk, and B. L. Vallee, unpublished observations). One of the major consequences of the phenotypic changes that accompany zinc deficiency is the alteration of the machinery for gene expression. Such alterations operate *both* at the level of template availability and at the level of the enzymes which use the template. We have presented a number of alternative mechanisms by which zinc deficiency induces such phenotypic changes and have called attention to hitherto unrecognized roles for zinc in the regulation of gene expression, and the induction of proteolytic enzymes, among others (Vallee & Falchuk, 1981; Stankiewicz et al., 1983; Crossley et al., 1982; Mazus et al., 1984). Jointly, these observations and considerations now give direction to future experimentation regarding the role of zinc in genomic function.

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