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ZINC IN DNA REPLICATION AND TRANSCRIPTION

Felicia Y.-H. Wu and Cheng-Wen Wu

Department of Pharmacological Sciences, School of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794

CONTENTS

INTRODUCTION

Zinc (Zn) is an essential nutrient for most organisms (see reviews 64–67). In 1869, Raulin first found that *Aspergillus niger,* the common bread mold, requires Zn for growth, as was shown subsequently for other microorganisms, plants, and animals. It is generally recognized now that Zn is indispensible for human growth and development. Zinc deficiency in human and animals results in arrest of normal growth processes and causes teratological abnormalities.

Although the essentiality of Zn for cell growth, development, and differentiation has been well established, the biochemical mechanisms by which Zn exerts its effects are still unknown. Within the last two decades, Zn has been shown to be a functionally essential component of more than 200

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enzymes (67). Zn enzymes encompass all known classes of enzymes, i.e. oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases, which participate in a wide variety of metabolic processes such as synthesis and/or degradation of carbohydrates, lipids, proteins, and nucleic acids. Alteration in the function of any one, or a combination of many known Zn enzymes, however, cannot account for the manifestations of Zn deficiency. Hence, the metabolic basis for the clinical disorders induced by Zn deficiency requires further definition. Recent investigation on the potential role of Zn in the regulation of structure, composition, and function of the genome may provide a promising clue in this regard.

Histochemical observations in 1954 (23) indicated that the cell nucleus, nucleolus, and chromosomes contain Zn. Shortly afterward, Zn was shown to be associated with, and to stabilize, the native structure of RNA (68), DNA (54), and ribosomes (46). The effects of Zn deficiency on the synthesis of DNA and RNA in various tissues and organisms (18, 50, 62) indicated an extensive involvement of Zn in nucleic acid metabolism. In 1971, *Escherichia coli* RNA polymerase (RPase) (52) and DNA polymerase I (DNA Pol I) (57) were the first nucleotidyl transferases shown to be Zn metalloenzymes. Since then, a variety of enzymes from both prokaryotic and eukaryotic sources involved in DNA and RNA synthesis have been shown to contain stoichiometric amounts of Zn (Tables 1 and 2). Recently, the finding that the eukaryotic transcription factor TFIIIA (Factor A) from *Xenopus* is a Zn metalloenzyme has created a wide interest in how Zn may regulate the expression of specific genes. This article reviews the role of Zn in the transfer of genetic information. Emphasis is placed on the role of the intrinsic Zn ions in polymerases and transcription factors, as well as on the effects of Zn deficiency in DNA replication and transcription.

ZINC IN DNA REPLICATION

Effect of Zinc on DNA Synthesis

The cellular and biochemical characterizations of Zn deficiency in the single-cell eukaryote, *Euglena gracilis,* have been studied systematically by Vallee and his coworkers (see reviews 65, 66). This heterotroph grows readily in a defined synthetic medium whose content of Zn and other metals can be controlled. In Zn-sufficient (+Zn) media, growth plateaus after 7 days; approximately 14 days are required in Zn-deficient (−Zn) culture, and the maximum cell number is about 20 times less. Raising Zn content of the medium reverses the decreased growth rate. In the −Zn cells, cell proliferation arrests and DNA content of the cells doubles, whereas RNA content does not change. The arrest in cell proliferation suggests that a critical Zn-dependent step in the cell cycle is blocked by Zn deficiency. The physical size

Table 1 Zinc in DNA polymerases

Source	Zn content (mol/mol)	Molecular weight	No. of subunits	OP[a] for 50% inhibition (mM)	Ref.
Bacteriophage					
T4 phage	1.0	112,000	1	+[b]	61
Eukaryotes					
Sea urchin nuclei	4.2	150,000	[c]	0.4	57
RNA Tumor Viruses					
(Reverse transcriptases)					
Avian myeloblastosis	1.3	175,000	2	0.4	45
Avian myeloblastosis	1.8 ~ 2.0	170,000	2	0.1	4
Murine leukemia	1,4	[c]	[c]	0.01	5
Simian leukemia	1.0	[c]	[c]	0.01	5
Woolly monkey	1.0	[c]	[c]	0.01	5

[a] OP = Ortho- or 1,10-phenanthroline.
[b] Inhibited by OP, but the concentration for 50% inhibition is unavailable.
[c] Data unavailable.

of +Zn cells decreases during log phase, while that of −Zn cells increases because of an accumulation of cytoplasmic paramylon. Other cytoplasmic organelles and nuclear morphology are normal in −Zn cells.

By using alternating periods of dark and light, researchers can synchronize *Euglena* cells and thereby analyze the histograms for relative DNA contents of the cells at various phases of the cell cycle. While (+Zn) *Euglena* cells in the stationary phase show a histogram typical of cells in the G-1 phase, −Zn cells show a histogram typical of the DNA content of cells in the S/G-2 phase. This suggests that as Zn is exhausted, cells in S do not continue to G-2, while those in G-2 do not proceed to mitosis. Although this observation may be indicative of Zn primarily affecting steps following DNA replication, the presence of a fraction of cells in the G-1 phase for growth-arrested −Zn cultures suggest that there may also be a block in the processes preceding DNA replication. All these results provide circumstantial evidence implicating Zn as a required factor in the fundamental processes of DNA replication, transcription, and possibly protein synthesis.

To investigate further the effect of Zn on DNA replication, activities of DNA polymerases were measured in cells grown under +Zn or −Zn conditions. For example, the specific activity of DNA polymerase in developing rat embryos is significantly lower in embryos taken from dams fed a −Zn diet than in those from ad-libitum-fed and restricted-intake controls (15). Addition of 0.01–0.05-mM Zn to −Zn extracts has little effect on restoration of the

Table 2 Zinc in DNA polymerases

Source	Zn content (mol/mol)	Molecular weight	No. of subunits	OP[b] for 50% inhibition (mM)	Ref.
Bacteria					
E. coli	2	493,000	5	0.8 ~ 0.9	52
E. subtilis	2	451,000	5	1.2	27
Bacteriophages					
T3 phage	1	105,000	1	+[c]	[d]
N4 phage	1	200,000	4	0.5	8
Eukaryotes					
E. gracilis					
RPase[a] I	2.2	634,000	10	0.01	21
RPase II	2.2	700,000	5	0.3	20
RPase III	2.2	650,000	9 ~ 10	+[c]	65
Yeast					
RPase I	2.4	650,000	11 ~ 12	0.3	3
RPase II	1	460,000	9	0.6	37
RPase III	2	380,000	11 ~ 12	0.1	69
Wheat germ					
RPase II	7	550,000	10	+[c]	44
HeLa					
RPase II	2	500,000	12	0.5	[e]

[a] RPase = RNA polymerase.
[b] OP = Ortho- or 1,10-phenanthroline.
[c] Inhibited by OP, but the concentration for 50% inhibition is unavailable.
[d] F. Wu, unpublished information.
[e] F. Wu, S. Tyagi, D. Reinberg, unpublished information.

DNA polymerase activity; Zn (but not other metal ions) added at 0.2 mM inhibits the same enzyme activity. These findings suggest the hypothesis that the teratogenic effects of Zn deficiency are associated with the enzymes involved in DNA synthesis. Results obtained from similar studies with bacteria are less conclusive. The specific activity of DNA polymerase of *Salmonella typhimurium* grown in Zn-supplemented media is 3–8-fold higher than in Zn-free media (80). On the other hand, the specific activity of DNA polymerase of *Mycobacterium smegmatis* is not affected by Zn depletion in the media (72). These discrepancies are probably due to the very low concentrations of Zn required for bacterial growth, thus rendering the usual metal extraction techniques insufficient to make the bacterial cells Zn deficient.

Metal ions have a profound effect on the fidelity of DNA replication, which has been reviewed elsewhere (38). The divalent Zn ion acts poorly as an

extrinsic metal activator for DNA polymerases. DNA synthesis observed in the presence of Zn is less than 1% of that obtained with Mg ion. In the in vitro fidelity assay using poly(dA-dT) as template, Zn, like other nonmutagenic and noncarcinogenic metals, does not alter the fidelity of DNA synthesis.

Intrinsic Zinc in DNA Polymerases

A number of DNA polymerases from various sources were reported to contain stoichiometric amounts of Zn that are required for the enzyme activity (Table 1). The role of Zn in DNA polymerases is reviewed in (14, 41, 78). Some DNA polymerases were inferred to be Zn metalloenzymes by virtue of their inhibition by the metal chelator, *o*-phenanthroline (OP), but not by its nonchelating analog, *m*-phenanthroline (MP). Such an inference may be misleading because OP, but not MP, may form a divalent cation-mediated complex with the DNA template-primer. DNA polymerase bound to this complex is unable to catalyze the polymerization reaction (2). In addition, the inhibition of DNA polymerase by OP may be due to the formation of the OP-Cu^{+1} complex, which results in the cleavage of DNA (55).

Investigations relating Zn to DNA polymerase structure or function were most extensively carried out with *E. coli* DNA Pol I. Although this enzyme is primarily involved in DNA repair rather than replication, it is the most abundant DNA polymerase in *E. coli* and thus the first to be purified and extensively studied. The enzyme was reported in 1971 (57) to contain two moles of Zn per mole of protein; this was later revised to one mole of Zn per mole of protein (61). That Zn is essential for the enzymatic activity was demonstrated by inactivation of the enzyme upon removal of Zn and reactivation upon its readdition (61). Furthermore, a series of nuclear magnetic resonance (NMR) and kinetic studies have been performed to elucidate the mechanistic role of Zn in this enzyme (see review 40).

In 1982, Walton et al (70) found that DNA Pol I, purified from an overproducing strain of *E. coli* lysogenized with λ pol A phage, has full catalytic activity but contains only 0.13 mole of Zn per mole of protein. This is contrary to the previous investigations (61), which stated that Zn binds to the enzyme in a stoichiometric amount and is necessary for polymerase activity. Ferrin et al (22) confirmed the above observation using the polymerases isolated from the same overproducing strain and from wild-type *E. coli*. It was therefore concluded that *E. coli* DNA Pol I is not a Zn metalloenzyme and the stoichiometric Zn previously found was tightly bound contaminant not easily removed by dialysis. Slow reactivation of the enzyme by Zn ions after dialysis against OP might have resulted from removal of the inhibitory ligand OP by the added metal. A similar conclusion was reached by

Graham & Sigman (26), who also examined the Zn contents of *E. coli* DNA Pol I and its Klenow fragment.

Another DNA polymerase shown not to be a Zn metalloenzyme is bacteriophage T7 DNA polymerase (56). The holoenzyme can be resolved into its active subunit, gene 5 protein, and *E. coli* thioredoxin. It was found that the holoenzyme does not contain Zn. Also, experiments showed that both the holoenzyme and the gene 5 protein in assay mixtures containing enzyme concentration in excess of the Zn concentration had full enzymatic activity. Furthermore, the addition of Zn resulted in no stimulation and the enzyme was completely inhibited by 0.1-mM Zn ions.

So far, DNA polymerases from sea urchin (57) and phage T4 (51, 61) are still candidates as Zn metalloenzymes (in addition to reverse transcriptases discussed below, see Table 1). However, in view of the negative results described above for *E. coli* DNA Pol I and phage T7 DNA polymerase, careful reevaluation is warranted regarding the necessity of Zn for catalytic activity of those other enzymes.

Zinc in Reverse Transcriptases

Type C oncogenic RNA viruses contain an RNA-dependent DNA polymerase that synthesizes the DNA complement of the RNA genome, hence the name reverse transcriptase. Reserve transcriptases from avian myeloblastosis virus (AMV) (4, 45), murine leukemia (5), and woolly monkey (5) type C viruses were shown to be Zn enzymes (see Table 1). The obligatory requirement of Zn for AMV reverse transcriptase activity was demonstrated by experiments on removing and replacing Zn (45). The activities of all the above enzymes can be reversibly inhibited by OP. For the AMV reverse transcriptase, the rate of inactivation by OP is much faster than for animal or bacterial DNA polymerases (45). It may therefore be possible selectively to inactivate viral enzyme by exposing it briefly to the appropriate metal chelators to remove its intrinsic Zn ions. The quantitative difference in the rate of removal of Zn from viral reverse transcriptase and cellular DNA polymerase may allow one to use the enzyme-bound Zn as a chemotheraputic target.

It has long been known that Zn plays a significant role in the metabolism of leukocytes and that type C oncogenic RNA viruses are associated with leukemias and lymphomas (4, 5). The Zn-modulated differences in metabolism between normal and leukemic leukocytes have led to the postulate that the disturbance of a Zn-dependent enzyme system is critical in the pathophysiology of leukemia (4). The presence of Zn in both viral reverse transcriptase and cellular DNA polymerase has argued against a simple relationship between altered Zn metabolism and leukemia. With the recent findings that certain DNA polymerases are deprived of Zn, the above postulate may deserve further investigation.

ZINC IN GENE TRANSCRIPTION

Effect of Zinc on RNA Synthesis

As described earlier, the biochemical and morphological characterizations of the cells deficient in Zn have been examined most thoroughly with *E. gracilis* (65, 66). While Zn deficiency doubles the DNA content, it does not alter the total RNA content of the cell. However, there is a striking difference in the G+C/A+U ratio of mRNA: 1.6 for +Zn and 2.9 for −Zn cells. In addition, mRNA in −Zn cells contains three major and several minor unknown bases not found in mRNA from +Zn cells.

Although Zn deficiency changes the composition of mRNA, it does not alter its functional capacity as expressed by translation. The +Zn mRNA system produces a number of proteins that are absent from the −Zn mRNA system. Conversely, the −Zn system contains proteins found either in much lesser amounts or not at all in the +Zn system. The most interesting findings concern the compositions of RPases and histones. Like all other eukaryotic organisms, normal *E. gracilis* cells contain three types of RPase: I, II, and III. In contrast, there is only one RPase in −Zn cells and it differs from all three +Zn RPases (19). While all forms of histones are present in +Zn cells, −Zn cells contain only histone H1 and H3, lacking H2A, H2B, and H4 (19). In addition, a family of arginine- and asparagine-rich polypeptides, which are characteristic of −Zn cells, vanish when Zn sufficiency is restored. These dramatic changes induced by Zn deficiency suggest a selective effect of Zn in gene expression, a process in which RPases and histones play crucial roles.

The effects of Zn deficiency on RNA synthesis have also been studied, but less extensively, in other organisms, including *Candidas* (34), sea urchin (16), and rats (33, 71).

Intrinsic Zinc in RNA Polymerases

DNA-dependent RPase catalyzes the sequential assembly of four ribonucleoside triphosphates into RNA in the presence of DNA template and an extrinsic divalent metal ion, Mg (see review 39). This reaction can be divided into four steps: promoter binding, RNA chain initiation, elongation, and termination. In addition to the requirement of extrinsic metal, most RPases also possess intrinsic metal. In 1971, RPase from *E. coli* was the first shown to be a Zn metalloenzyme containing two moles of Zn per mole of enzyme (57). More than a dozen RPases isolated from both prokaryotic and eukaryotic sources have since been reported to contain constant and stoichiometric amounts of Zn (Table 2). We discuss below our current knowledge about the role of the intrinsic Zn in RPases, with an emphasis on *E. coli* RPase, which has been studied most extensively. This topic is the subject of several previous reviews (14, 41, 76–78).

E. COLI RPASE *E. coli* RPase is an oligomeric enzyme consisting of five subunits ($\alpha_2\beta\beta'\sigma$) (39). The two intrinsic Zn ions are located in the β and β' subunits (43, 75), which have been implicated in substrate and template binding, respectively (39). Several approaches have been used to investigate the roles of these intrinsic metal ions and to employ them as probes in the structural and mechanistic studies of RPases.

Removal and replacement of Zn A functional role for the metal in a metalloenzyme can be directly proven by demonstrating that the enzyme is inactivated upon removal of the metal and reactivated upon its subsequent addition. Early attempts with this approach were not successful, because the Zn bound to the enzyme was extremely tight and could not be removed by dialysis against chelating agents under neutral, undenaturating conditions (60). A sequential denaturation-reconstitution method was later developed (58, 59) to remove the two intrinsic ions in the presence of 7-M urea and 10-mM EDTA. With or without subsequent low pH (2.2) treatment, the inactive apoRPase was then reconstituted with the added Zn ions to form, respectively, half active Zn_1-RPase (58) and fully active Zn_2-RPase (59). The latter enzyme (rec-Zn_2-RPase) is fully active as native enzyme in binding DNA and catalyzing the DNA-dependent dinucleotide formation (abortive initiation) as well as total RNA synthesis. Analysis by sucrose density gradient sedimentation showed that apoRPase consists of randomly folded protein species (5–18S), while rec-Zn_2-RPase forms a single 13S peak as native enzyme. These studies demonstrated that the Zn in *E. coli* RPase is essential for its activity, probably by maintaining the proper conformation of the enzyme.

Substitution of Zn with other metals Zinc is a silent metal in a biophysical sense: it is diamagnetic and exhibits no visible absorption spectrum. By substituting Zn in RPase with other metals, which possess paramagnetic and spectroscopic properties, one may obtain useful information from physical studies of the metal-substituted enzymes.

The substitution of intrinsic Zn ions with other metals was first achieved by an in vivo method (60). RPase purified from *E. coli* cells grown in Zn-depleted, Co-enriched media contains two Co instead of two Zn ions. The Co_2-RPase is identical to native Zn_2-RPase in many physical properties except for their metal content and absorption spectra. Whereas the Zn_2 enzyme has no visible absorption spectrum, the Co_2-RPase exhibits two major peaks at 584 and 703 nm, which suggests an irregular tetrahedral coordination of Co in RPase. Addition of a DNA template reduces the amplitude of both peaks, while substrate ATP or GTP selectively perturbs the 703-nm peak in the absence of DNA. These observations suggest that the intrinsic metal may be

involved in substrate or template binding. Also, these two types of RPase are very similar in their biochemical properties (60). The fidelity of transcription by Co_2-RPase is the same as that by native Zn_2-RPase. However, Co_2-RPase initiates RNA chains less efficiently at the A_2 promoter of T7 DNA template and is less sensitive than the Zn_2 enzyme to cyclic AMP and cyclic AMP receptor stimulation in the in vitro transcription of *lac* operon. These results imply that the intrinsic metal ions in RPase play a role in specific promoter recognition and RNA chain initiation.

Recently, Giedroc & Coleman (24) employed the same in vivo metal substitution method to obtain Co_2- and Cd_2-RPase that gave 50 and 27% activity, respectively, relative to native enzyme. The Co_2 enzyme exhibited three discrete d-d electronic transitions in its absorption spectrum corresponding approximately to those described above. In addition, a broad, charge-transfer band centered at about 484 nm was observed. The position and intensities of both the visible and charge-transfer bands were attributed to a possible tetrathionate-Co(II) coordination complex, consistent with an earlier finding that sulfhydryl groups are involved in the metal binding by RPase (58).

Three in vitro methods have been developed to substitute selectively one of the two intrinsic Zn ions to yield a series of metal hybrid enzymes. The first method involves a sequential denaturation-reconstitution process (11), as described before but omitting the low pH treatment that destroys the metal binding site in the β' subunit (58). Thus the method selectively removes the Zn ion from the β subunit, which can then be replaced by dialysis against buffer containing another metal. The second method (63) involves the treatment of RPase with NaCl-$NaNO_3$-diaminocyclohexane tetraacetate followed by reconstitution with another metal. This method also offers a selective substitution of the Zn ion in the β subunit. With these two methods, we have obtained a number of the β-substituted metal hybrid enzymes, Co(β)Zn(β')-, Mn(β)Zn(β')-, Cd(β)Zn(β')-, Ni(β)Zn(β')-, and Cu(β)Zn(β')-RPases, having 100, 100, 60, 60, and 40%, respectively, of the enzyme activity as compared to rec-Zn_2-enzyme. These enzymes also decrease in the same order in their abilities to catalyze the abortive initiation reaction, which suggests that the variation in the enzymatic activity resides at the level of RNA chain initiation. Moreover, Co(β)Zn(β')-, Ni(β)Zn(β')-, and Cu(β)Zn(β')-RPases also possess characteristic absorption spectra, which can be perturbed by ATP and GTP but not by CTP and UTP. Since the initiation of RNA occurs primarily with purine nucleotides, these spectral changes suggest that the intrinsic metal in the β subunit may be located at or near the initation site of the enzyme.

The third method of metal substitution (24) utilizes the sulfhydryl reagent, *p*-(hydroxymercuri)benzene sulfonate (PMPS), which releases one Zn ion

from RPase (B site). Thus, incubation of the enzyme-PMPS complex with Cd yields Zn(A)Cd(B)RPase. If EDTA is present, an RPase containing only one Zn ion in the A site [Zn_1(A)-RPase] is formed, and retains full transcriptional activity. Thus the metal in the B site is dispensable for transcription. Treatment of Co_2- and Cd_2-RPases with PMPS/EDTA results in Co_1(A)- and Cd_1(A)-enzymes. Comparison of the absorption spectra of Co_1(A)- and Co_2-enzyme suggests that the Co at site A is tetrahedral with intense d-d transitions, while the Co at site B, having no such band, is apparently in octahedral symmetry. Since the absorption spectrum of Co_1(A)-RPase corresponds approximately to that observed for Co(β)Zn(β)-enzyme (60), it appears that site A is located in the β subunit, and hence site B is in the β' subunit.

Coordination ligands of the intrinsic metal ions The metal ligands in a metalloenzyme may play important roles in enzyme catalysis and/or maintenance of proper enzyme conformation. As mentioned above, the intense d-d transitions observed in the absorption spectra of Co_1(A)- and Co_2-RPase suggest that the metal at site A in RPase may coordinate with four thiolate ligands. This agrees with our previous finding that potassium tetrathionate blocks reassociation of the Zn ions with apoRPase (58). Using a metal-directed affinity label for cysteine (Cys), α-bromo-β-(5-imidazolyl)propionic acid, we have determined that two Cys residues are labeled, one at each of the β and β' subunits (79). These results are in support of Cys residues coordinating with the intrinsic Zn in RPase. Another amino acid residue that has been implicated in Zn coordination is histidine (His). Chemical modifications of RPase with diethylpyrocarbonate (DEP) indicate that one His residue is critical for the enzyme activity and reacts 3 ~ 4 times faster than the other nonessential His residues (1). Substrate ATP but not DNA template protects the enzyme from DEP inactivation, which suggests that this highly reactive His is in close proximity to the substrate binding site.

Structural and mechanistic studies of RPase using the intrinsic metal as probe Taking advantage of the spectroscopic and paramagnetic properties of the substituted metals in *E. coli* RPase, we have performed NMR and fluorescence energy transfer experiments to obtain information on the enzyme's structure, which is relevant to its function.

The distance between the metal and substrate bound at the initiation site of Co(β)Zn(β')- or Mn(β)Zn(β')-RPase was determined by ^{1}H and ^{31}P NMR spectroscopy (9, 12). The results obtained with these two enzymes are in good agreement. There are two fast exchanging water molecules coordinated with Co(β), one of which can be replaced by ATP or initiator ApA but not by UTP, confirming that the Co ion is at the initiation site (12). Figure 1 illustrates the spatial relationship between the intrinsic Co(β) and the bound ATP in the

absence (12) and presence (9) of DNA template. The short distances from Co to H_2 and H_8 (4.1 and 3.6 Å) indicate a direct coordination of the metal to the base moiety of bound ATP, possibly by the stacking of Co with the aromatic ring to form a π complex. Such a stacking interaction may constitute the structural basis for the recognition of initiating nucleotide (purine versus pyrimidine) by the enzyme. In the absence of DNA, the triphosphate group of ATP is positioned more than 10 Å away from the metal center. In the presence of DNA, the triphosphate group moves > 3 Å closer to the metal, which means the conformation of the substrate was altered at the initiation site by the DNA template. Thus the intrinsic metal ion in the β subunit may play a role in the recognition and orientation of the initiating nucleotide in a stereospecific position for catalysis.

Fluorescence energy transfer was recently used to measure the distances from the Co ion in the β subunit to the initiation and elongation sites on the same subunit (63) as well as to a specific Cys on the σ subunit (10). In these experiments, the Cys-132 of σ was covalently labeled with a fluoresence sulfhydryl reagent, N-(1-pyrene)maleimide (PM), while the initiation and

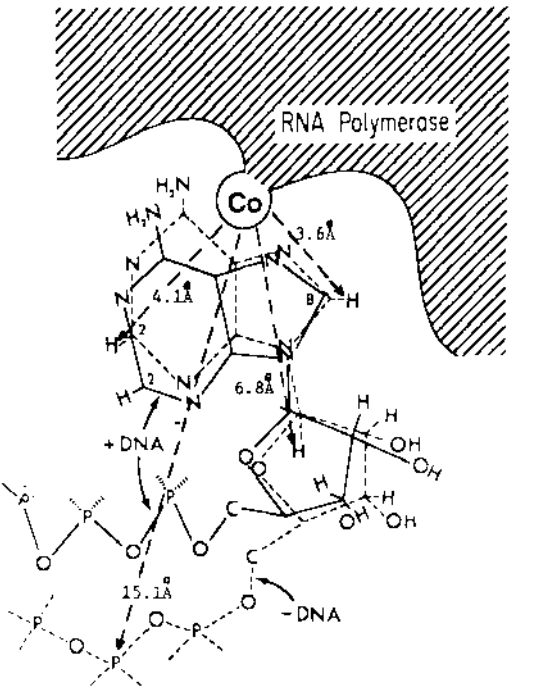

Figure 1 Distances from the intrinsic Co to various proton and phosphorus nuclei of ATP bound at the initiation site of Co(β)Zn(β') RNA polymerase in the absence (- - -) and presence (——) of DNA template.

elongation sites were specifically occupied by fluorescent analogs of initiator, (5'-AmNS)UpA, and substrate, (γ-AmNS)ATP or UTP, respectively. The fluorophore, 1-aminonaphthalene-5-sulfonate (AmNS), was attached to the 5'-OH of UpA and to the γ-P of ATP or UTP. The emission spectra of PM-σ, (5'-AmNS)UpA, (γ-AmNS)ATP, and (γ-AmNS)UTP overlap with the absorption spectrum of Co(β)Zn(β') RPase. From the measured energy transfer efficiencies, distances from Co(β) to the fluorophores at the initiation and elongation sites and Cys-132 of σ have been calculated to be 19, 21, and 22 Å, respectively. Information about these distance relationships and their changes induced by substrate or template binding as well as during enzyme catalysis will be very useful for elucidating the molecular mechanism of gene transcription.

OTHER RNA POLYMERASES

Bacterial RPases *B. subtilis* RPase resembles *E. coli* RPase with respect to molecular weight and subunit composition. The enzyme contains two moles of Zn per mole of protein and its activity is inhibited by OP (27). One Zn ion is bound more tightly than the other, similar to what is observed for *E. coli* RPase. However, the two Zn ions are located in β', the second largest subunit, which confers the rifampicin resistance and thus corresponds to the β subunit of *E. coli* RPase.

Viral RPases Four RPases from *E. coli* phages—T7 (13), T3 (F. Wu, unpublished information), N4 (8), and the RPase (F. Wu, unpublished information) isolated from SP6 phage infected with *S. typhimurium*—were shown to contain stoichiometric amounts of Zn (Table 2). Recently, T7 RPase purified from an overproducing strain of *E. coli* containing cloned T7 RPase gene was found to have 0.01 mole of Zn per mole of protein, but it was highly active enzymatically (35). Furthermore, extrinsic Zn does not function as an activator. We have made a similar observation for phage SP6 RPase isolated from infected wild-type *E. coli* cells. The apoenzyme, obtained by dialysis against EDTA to remove the metal, still retains the same activity as native enzyme. It is apparent that the essentiality of Zn for other bacteriophage RPases may require further verification.

Eukaryotic RPases Fewer advances have been made in the studies of the intrinsic metal ions in eukaryotic RPases than in prokaryotic enzymes. This is mainly due to the difficulty in obtaining highly purified enzymes in sufficient quantities. Eukaryotic RPases from four sources [*E. gracilis* (20, 21, 65), yeast (3, 37, 69), wheat germ (44), and HeLa cells (F. Wu, S. Tyagi and D. Reinberg, unpublished information)] have now been reported to be Zn metal-

loenzymes. The Zn contents of RPase I, II, and III from these organisms are listed in Table 2.

Although the subunit location and the mechanistic aspect of the intrinsic Zn have not been elucidated, an essential role of Zn in eukaryotic RPases has been suggested mostly on the basis of their inhibition by OP but not by MP. As mentioned above, the inhibition of DNA or RNA polymerases by OP may be due to the formation of an OP-Cu^{+1} complex that cleaves DNA (55). Thus, the criterion for the presence of essential Zn in polymerases based on OP inhibition should be exercised with caution. All RPases (I, II, and III) from *E. gracilis* and yeast can be inhibited by OP but not by MP. A recent reinvestigation (40) indicates that millimolar concentrations of OP do not hydrolyze the DNA template even in the presence of Cu^{+2} (10^{-6} M), when DNA is complexed with *E. gracilis* or wheat germ RPase II. Under the same conditions, OP inhibits the activity of both enzymes. It is concluded therefore that OP inhibits the enzyme activity by chelation of metal essential for the function of RPases rather than by cleavage of the DNA template.

Another class of Zn-containing polymerase is poly(A) polymerase, an important enzyme for processing mRNA in eukaryotic cells. It is interesting to note that this enzyme from rapidly growing hepatoma, Morris hepatoma 3924A, contains 2 to 3 times as much Zn as the corresponding Zn enzyme in rat liver (47): Zn may also play a role in tumor growth.

Zinc in Transcription Factor A

An exciting new development concerning the role of Zn in gene transcription is the discovery that, in addition to RPases, some transcription factors from eukaryotic cells also contain intrinsic Zn ions that are essential for transcribing specific genes.

In eukaryotic cells, three different nuclear RPases (I, II, and III) transcribe different classes of cellular genes (39). For RPases III, the most abundant transcription products are transfer tRNAs and 5S rRNA. The enzyme itself has no specificity with regard to the recognition of the genes for these RNAs, but there exist several auxiliary transcription factors that are able to direct the polymerase to the target genes (53). Thus far only one of these factors has been isolated and characterized in detail (17, 28). We have found that this transcription factor (TFIIIA or factor A), isolated from *Xenopus* oocytes, contains two tightly bound Zn ions (29) that are involved in the multiple roles in the regulation of 5S RNA synthesis (74). This is the first example of a Zn metalloprotein controlling the transcription of a specific gene. Recent investigations from our laboratory have led to the discovery that factor A is also a Zn metalloenzyme (30).

FACTOR A PLAYS MULTIPLE ROLES IN 5S RNA SYNTHESIS The 5S RNA synthesis in *Xenopus* oocytes is under developmental control (see review 36). Immature *Xenopus* oocytes synthesize large amounts of 5S RNA prior to the synthesis of other rRNAs. After synthesis, the 5S RNA (120 nucleotides long) is bound to a 40-kilodalton protein forming a stable 1 : 1 nucleoprotein complex (called "7S storage particle") until other ribosomal precursors are available for ribosome assembly. The protein in this complex has been identified as factor A (17). Factor A activates the transcription of 5S RNA gene by specifically binding to a control region of approximately 50 base pairs (bp) in the center of these genes (7, 48). This step is followed by the sequential binding of factors TFIIIB and TFIIIC to form a functional transcription complex that directs RPase III to initiate the synthesis of 5S RNA faithfully at a site about 50 bp upstream from the intragenic control region (49). The ability of factor A to bind either the 5S RNA gene or its transcription product suggests an autoregulatory mechanism in which 5S RNA controls its own synthesis by sequestering factor A away from the gene after a high steady-state level of the gene product is reached.

In addition to specific binding to 5S RNA and genes, factor A has been shown to possess at least three other biochemical activities: it exhibits a high affinity for single-stranded DNA (31), promotes the reassociation of complementary single-stranded DNA to the duplex form (30), and is a DNA-activated ATPase (32). Although their precise functions are not known, it has been proposed that these biochemical activities may be involved in the generation and maintenance of the proper state of the 5S RNA gene throughout multiple rounds of transcription.

ZINC IS AN INTERGRAL PART OF FACTOR A IN 7S PARTICLE One unusual feature of the structure of factor A is the presence of 23 Cys residues (25). Since sulfhydryl groups are frequently involved in metal coordination, we have undertaken studies to identify the possible association of metal ions with factor A and to investigate the role of metal ions in factor A function (29). Atomic absorption analysis indicates that each mole of 7S particle contains two moles of Zn (Table 3) that cannot be removed by EDTA or OP. These Zn ions are associated with free factor A when 5S RNA in the 7S particle is removed by RNase treatment. Unlike the 7S particle, Zn is readily depleted from free factor A by metal chelators. Thus, it has been possible to obtain apo-factor A and compare its various properties with the native protein.

ZINC IS INVOLVED IN BINDING FACTOR A TO 5S RNA The Zn ions can be removed from free factor A by EDTA or OP but not from the 7S particle; the Zn ions may thus be located in the contact domain between 5S RNA and factor A, so that they are shielded by the RNA in the 7S particle. Alternative-

Table 3 Zn contents of 7S particles, Factor A, and its proteolytic fragments

Sample	Chelator	Zn/protein (mol/mol)
7S particle	3-mM EDTA	2.1
	5-mM EDTA	2.3
	0.5-mM OP[a]	2.3
Factor A	None	2.1
	3-mM EDTA	0.1
	5-mM EDTA	0.2
	0.5-mM OP	0.3
	1-mM OP	0.1
33-kilodalton factor A fragment–5S RNA complex	3-mM EDTA	1.9
28-kilodalton factor A fragment–5S RNA complex	3-mM EDTA	1.6

[a] OP = Ortho- or 1,10-phenanthroline.

ly, the Zn ions may be buried in factor A and become exposed to the chelater as a result of a conformational change of the protein induced by removing the 5S RNA from the particle.

That the Zn ions are involved in factor A binding to 5S RNA has been probed by limited proteolysis of the 7S particles. The 7S particles can be cleaved by papain or trypsin to yield polypeptide fragments of approximately 33 or 28 kilodaltons, respectively. These fragments, which remain associated with 5S RNA, still contain two Zn ions (Table 3). More direct evidence that Zn is necessary for the binding of factor A to 5S RNA derives from in vitro reconstitution experiments. 7S particles can be reconstituted from 5S RNA and the apo-factor A only when these molecules are incubated in the presence, but not in the absence, of exogenous Zn ions.

ZINC IS REQUIRED FOR THE SPECIFIC INTERACTION OF FACTOR A WITH 5S RNA GENE BUT NOT WITH NONSPECIFIC DOUBLE-STRANDED DNA Evidence that the Zn ions are required for the specific binding of factor A to the 5S RNA gene is provided by the observations that (*a*) factor A binds specifically to the 5S RNA gene as visualized by DNase I footprinting assay, whereas the apoprotein does not; and (*b*) the loss in specific DNA binding ability of the apoprotein can be restored only by the addition of exogenous Zn, but not by other divalent metal ions.

In addition to specific binding to the 5S RNA gene, factor A can also bind to nonspecific double-stranded DNA with an affinity about two orders of magnitude lower. Unlike specific binding, Zn is not involved in the nonspecific DNA binding of factor A, because neither EDTA nor OP has any inhibitory effect on nonspecific binding as measured by the nitrocellulose filter binding assay (31). These results suggest a possible role for Zn in the specific factor A–DNA interaction. If Zn were simply to provide a charge bridge between the protein and the negatively charged groups on the DNA, it would be involved in nonspecific DNA binding. The fact that Zn is not required for nonspecific DNA binding indicates a specific role for the metal in factor A binding to the 5S RNA gene. It is conceivable that the metal may be involved in the specific DNA sequence recognition by direct coordination with certain nucleotide residues (12) in the control region of the 5S RNA gene. Zn may also play a structural role in maintaining the proper protein conformation necessary for specific binding to the 5S RNA gene.

ZINC IS ESSENTIAL FOR 5S RNA GENE TRANSCRIPTION We have also studied the effect of Zn chelators on the function of factor A in the transcription of 5S RNA gene (29). In an in vitro transcription system synthesizing both 5S RNA and tRNA, a low concentration (0.25 mM) of OP specifically inhibits 5S RNA synthesis. Because factor A is not required for the transcription of tRNA gene, the selective inhibition of 5S RNA synthesis by OP is most likely due to Zn chelation resulting in the inability of factor A to bind the 5S RNA gene. At high chelator concentrations (5 mM) both 5S RNA and tRNA syntheses are abolished, an indication that Zn may be required for the enzymatic function of RPase III, which is responsible for both syntheses. This is not unexpected since the presence and requirement of Zn in other RPases have been well established, as described in the previous section.

Since factor A binding to the 5S RNA gene is essential for the transcription of this gene, the inhibition of 5S RNA synthesis by OP is likely due to Zn chelation resulting in the failure of factor A to bind the 5S RNA gene. This interpretation is confirmed by Wingender et al (73), who demonstrated a complete loss of 5S RNA synthesis in a Zn-depleted in vitro transcription system that was fully restored by the readdition of Zn ions. The Zn depletion in this study was achieved by chelation with EDTA and subsequent removal by dialysis from a cytoplasmic HeLa cell extract that contained a mammalian transcription factor A necessary for 5S RNA synthesis. These investigators also showed that removal of Zn from a partially purified fraction of the cytoplasmic extract containing mammalian transcription factor A precludes the subsequent assembly of active transcription complexes on the 5S RNA genes, while Zn depletion from fractions containing factors B and C does not affect the transcription capacity. This is in agreement with the conclusion that

recognizing 5S RNA gene by factor A is a prerequisite for the formation of active transcription complexes and that binding of factor A to the 5S RNA gene is Zn dependent (29).

FACTOR A IS A ZINC METALLOENZYME We have recently discovered (32) that factor A catalyzes the hydrolysis of ATP to ADP and P_i. This ATPase activity is stimulated by the presence of double-stranded DNA. The Zn in factor A is essential for its ATPase activity. Both EDTA and OP are effective inhibitors of the ATP hydrolysis. Moreover, the inhibition can be restored by the addition of exogenous Zn ions. The results presented above identify factor A as a Zn metalloenzyme; namely, Zn is an integral component of the protein and is essential for its ATPase activity. Although the role of ATPase activity of factor A in 5S RNA gene transcription still remains to be elucidated, the coincident observations that the ATPase activity can be stimulated by DNA and that the intrinsic Zn ions are essential for both the ATPase activity and the specific binding of factor A to the 5S RNA gene suggest that the enzyme activity may be intimately related to the transcription.

STRUCTURAL MODEL OF INTRINSIC ZINC IN FACTOR A *Xenopus* factor A is the first eukaryotic transcription factor to have been sequenced (25). Sequence analysis reveals nine copies of an imperfect repeated motif of 30 amino acids, extending from residues 13 to 276 of the protein (42). Miller et al (42) showed that in the absence of metal chelators the 7S particle contains between 7 and 11 Zn ions and that extensive proteolysis of factor A yields a progression of intermediates spaced at about 3-kilodalton intervals. Thus, they suggested that factor A is composed of nine flexibly linked, small, compact loop-like domains, each enclosing a Zn atom at its base by virture of the two invariant pairs of Cys and His (Figure 2). These loop-like domains or "fingers" are the proposed DNA binding regions. The subsequent findings that the intron-exon organization of the factor A genomic DNA reiterates the nine-finger structure (42) and that several other proteins that interact with DNA or RNA (6) possess similar motifs have generated a great deal of speculation about the evolutionary origin and function of these hypothesized nucleic acid binding structures, with a Zn binding site as a significant element of organization.

While the 7S particle may contain 7–11 Zn ions (42), only two of them are recalcitrant to extensive dialysis against metal chelators (29). The factor A derived from chelator-treated 7S particle containing only two Zn ions is fully active in binding 5S RNA gene and in stimulating 5S transcription; removal of these two Zn ions results in inactive apoprotein (29). Thus it remains a question whether the additional seven Zn ions are specifically associated with

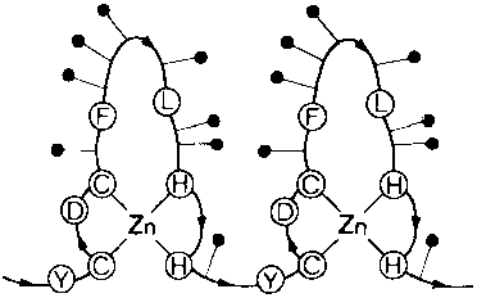

Figure 2 Folding scheme for a linear arrangement of repeated domains, each centered on a tetrahedral arrangement of Zn ligands, taken directly from Miller et al (42). Ringed residues are the conserved amino acids, which include the Cys and His zinc ligands, the negatively charged Asp, and the three hydrophobic groups that may form a structural core. Black circles mark the most probable DNA binding side chains.

the protein and required for the DNA binding activity, as has been hypothesized.

CONCLUDING REMARKS

Zinc is essential to the growth, development, and differentiation of all living species. Zn deficiency in human and animals causes teratological, genetic, and medical abnormalities. Although Zn is an essential component of more than 200 enzymes isolated from different sources, alterations in the functions of these enzymes alone cannot account for the manifestation of Zn deficiency. Studies of the function of Zn in *E. gracilis* have demonstrated that it is critical for replication, transcription, and translation. The deprivation of Zn from this eukaryotic single-cell organism alters the structure and function of DNA and RPases, as well as other regulatory proteins such as histones that play important roles in gene expression. These changes may lead to activation or repression of selective genes and result in synthesis or degradation of specific proteins. Such a role for Zn could be of overriding significance in its pervasive importance to cell metabolism. This hypothesis may provide a more general basis for interpreting the function of Zn in other organisms, including vertebrates.

The presence of Zn as an integral part of many DNA and RNA polymerases has been established, though two of them (*E. coli* DNA polymerase I and T7 RPase) were disqualified recently as Zn enzymes. Structural and functional studies of the intrinsic Zn in these polymerases, especially *E. coli* RPase, by direct Zn removal and replacement or by substitution with other divalent metals have revealed multiple roles of Zn in gene transcription. The intrinsic

Zn ions in RPases play (*a*) a regulatory role in specific promoter recognition and RNA chain initiation, (*b*) a role in the recognition and orientation of the initiation nucleotide for catalysis, and (*c*) a structural role in maintaining the proper enzyme conformation necessary for its function. These studies lay a foundation for our understanding of the mechanisms by which Zn can affect gene expression at molecular levels. In addition, the recent finding that *Xenopus* transcription factor A also contains functionally important Zn provides the first and exciting example of how Zn may directly participate in the selective activation of a specific gene. Further structural and mechanistic studies in the Zn on polymerases and regulatory proteins involved in gene expression will not only shed light on the role of Zn in cell growth and development, but also provide a rationale for medical disorders observed in Zn deficiency.

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