Messenger Ribonucleic Acid Function and Protein Synthesis in Zinc-Deficient *Euglena gracilis*†

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ABSTRACT: Protein metabolism is disturbed in zinc-deficient (−Zn) cells [Vallee, B. L., & Falchuk, K. H. (1981) *Philos. Trans. R. Soc. London, Ser. B 294*, 185–197; Vallee, B. L., & Falchuk, K. H. (1982) *Biological Aspects of Metals and Metal Related Diseases* (in press)]. To delineate both the level at which the process is disrupted and its consequences, we have elected to study the functional properties of mRNAs and their translation products and the capability of rRNAs to form monosomes. These two RNA species were chosen because the base composition of the total poly(A+) mRNA and the stability of the polysomes formed by rRNA are both altered in −Zn cells [Prask, J. A., & Plocke, D. (1971) *Plant Physiol. 48*, 150–155; Falchuk, K. H., Hardy, C., Ulpino, L., & Vallee, B. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4175]. The poly(A+) mRNAs from both +Zn and −Zn cells are translated equally well in reticulocyte lysates. Their rate of incorporation of [^{35}S]methionine, the amount required for optimal incorporation (0.4 μg), and the requirements for K^+ (80 mM) and Mg^{2+} (0.5 mM) are the same. Comparison of the translation products of the mRNAs from both cell types, produced either by polysomes from reticulocytes or in intact *Euglena gracilis* cells, shows that a number of proteins are synthesized only by −Zn cells and some, which are formed by +Zn organisms, are not made at all by −Zn *E. gracilis*. The rRNAs from −Zn *E. gracilis* form 89S subunits which dissociate into 39S and 60S subunits and reassociate in the presence of 0.5 and 0.12 M KCl, respectively. Thus, the disturbances in protein metabolism of −Zn organisms are not the consequence of an inability of their mRNA to be translated or of their rRNA to form stable monosomes. The effects on the types of polypeptides made indicate that zinc is critical to the activation and repression of the synthesis of selected proteins, suggesting a role for this metal in the regulation of gene function.

The biosynthesis of proteins is now known to require zinc at virtually every important metabolic step, and its deficiency markedly disturbs protein metabolism. Major alterations in zinc-deficient (−Zn) *Euglena gracilis* have been recognized for nearly 20 years (Price & Vallee, 1962; Vallee & Falchuk, 1981, 1982). However, the primary lesion, its mode of induction, and its significance to the arrest of growth and differentiation characteristic of zinc deficiency are still unknown.

It is established that the composition of −Zn *E. gracilis* mRNA is altered (Falchuk et al., 1978) and that the stability of the polysomes on which these mRNAs are translated into proteins is reduced relative to that of +Zn cells (Prask & Plocke, 1971). The present work was undertaken to define the effects of zinc deficiency on the functional properties of mRNAs from *E. gracilis*, to examine the proteins for which they code and translate, and to investigate one possible basis for the reduced stability of the polysomes of these cells, i.e., the capacity of its rRNA to form monosomes.

The results demonstrate that the mRNAs from −Zn cells are completely competent and functional, and the rRNAs form subunits that can associate into monosomes with sedimentation properties similar to those from +Zn cells. However, the mRNA translation products from +Zn and −Zn organisms differ; some proteins are made *only* by −Zn cells, including some that are associated with polysomes, while others are synthesized only in +Zn *E. gracilis*. The data suggest that protein metabolism in −Zn cells is altered selectively, apparently by concurrent activation and repression of specific segments of the genome, implicating zinc in the regulation of gene expression of *E. gracilis*.

Materials and Methods

Euglena gracilis, strain Z, was grown in the dark at 22 °C as described previously (Falchuk et al., 1975). +Zn and −Zn media differed only in that they contained 10 and 0.1 μM Zn^{2+}, respectively. The cells were harvested on reaching early stationary phase by centrifugation at 2000*g* for 5 min and washed with ice-cold 0.1 M Tris-HCl, pH 7.6. Washed cell pellets were stored at −20 °C.

E. gracilis mRNA. The mRNA bound to free cytoplasmic polysomes was prepared according to the method of Swan et al. (1972) by using glassware that was acid washed and heated at 200 °C for at least 2 h before use. mRNA was also prepared from membrane-bound polysomes from −Zn and +Zn cells; however, since all results on the mRNA from both membrane-bound and free cytoplasmic polysomes are comparable, only the data on the latter are shown.

Frozen cells (20 g) were suspended in 50 mL of buffer A [0.1 M Tris-HCl, pH 7.6, 20 mM KCl, 5 mM $Mg(CH_3CO\text{-}OH)_2$, and 5 mM 2-mercaptoethanol containing 0.88 M sucrose] and disrupted by manual grinding with glass beads. The resultant homogenate was centrifuged at 20000*g* for 20 min and the supernatant removed, avoiding the lipid layer on the surface. The supernatant was diluted with buffer A to a final sucrose concentration of 0.62 M, and 10-mL aliquots were layered on discontinuous sucrose gradients composed of 5 and 7 mL of buffer A containing 2.0 and 1.5 M sucrose, respectively. On centrifugation at 110000*g* for 5 h in a Beckman Ti60 rotor, the free cytoplasmic polysomes sediment through the 2.0 M sucrose layer. This free cytoplasmic polysomal fraction was collected and diluted with buffer B (0.1 M Tris-HCl, pH 9, and 0.1 M NaCl) to a concentration of 20 A_{260} units/mL. The solution was made 1% in sodium dodecyl sulfate. The mRNA associated with this fraction then was

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extracted by the method of Aviv & Leder (1972). An equal volume of phenol–chloroform–isoamyl alcohol (50:50:1) was added and the mixture stirred for 10 min at 22 °C. After the mixture was chilled in an ice bath for 5 min, the phases were separated by centrifugation at 12000*g* for 10 min. The upper, aqueous phase was removed, reextracted as described above, and made 2% in CH_3COOH (pH 5.5). The RNA in the aqueous fraction was precipitated by adding 2 volumes of ethanol and allowing the mixture to stand at −20 °C overnight. The RNA precipitate was collected by centrifugation at 12000*g* for 20 min and washed twice with 0.2 M NaCl–ethanol (1:2). It was then dissolved in 5–10 mL of buffer C (10 mM Tris-HCl, pH 7.6, 0.5 M KCl, and 10% glycerol), dialyzed overnight, and clarified by centrifugation (12000*g*, 10 min). The poly(A)-containing RNA in this fraction was purified by affinity chromatography on oligo(dT)–cellulose (Collaborative Research) as previously described (Falchuk & Hardy, 1978). The purified poly(A)-containing RNA was precipitated by the addition of CH_3COOH and ethanol, as above, and stored overnight at −20 °C. The RNA precipitate was collected, washed twice with 0.2 M NaCl–ethanol, and dissolved in water. The concentration of the RNA solution was determined (approximately 24 A_{260} units/mL = 1 mg) prior to storage in small aliquots in the vapor phase of a liquid nitrogen Dewar flask.

Cell-Free Translation of E. gracilis mRNA. A number of different cell-free lysates were tested for their suitability to accept the *E. gracilis* mRNA species as messengers capable of directing the synthesis of proteins. An *E. gracilis* S_{30} lysate was tried first, but its capability to accept exogenous messenger was poor, resulting in less than a 2-fold activation of protein synthesis compared to the endogenous activity of the S_{30} lysate. On the other hand, both the reticulocyte (New England Nuclear) and wheat germ S_{30} (Bethesda Research Laboratories) lysates, known to accept viral, plant, and mammalian mRNAs, proved suitable and effective to both detect mRNA activity and perform comparative studies of messengers obtained from +Zn and −Zn cells. The functional integrity of the lysates was tested with known, viable globin mRNAs. Each assay contained a final volume of 30 μL of which 10 μL was reticulocyte lysate (New England Nuclear) composed of 8.5 μL of L-[^{35}S]methionine (500 Ci/mmol) and 2 μL of a standard mixture of all amino acids except methionine with the remainder comprising optimal amounts of K^+, Mg^{2+}, and varying amounts of mRNA. The reaction mixture were incubated at 37 °C for 45 min. For each assay of translation, triplicate aliquots of 2 μL were precipitated on Whatman 3MM paper with trichloroacetic acid as described by Roberts & Patterson (1973). Radioactivity was determined by liquid scintillation counting with 10 mL of the toluene scintillant Econoflor (New England Nuclear).

Aliquots of translation assays of the reticulocyte lysates were mixed with 2 volumes of 0.15 M Tris-HCl, pH 8.8, 1% sodium dodecyl sulfate ($NaDodSO_4$), 1% 2-mercaptoethanol, 10% glycerol, and 0.001% phenol red and immersed for 2 min in boiling water. The samples were electrophoresed in 12.5% polyacrylamide gel slabs for 7 h at 15 mA (Laemmli, 1970). Gels were stained with Coomassie brilliant blue, destained, and treated with 20% 2,5-diphenyloxazole in dimethyl sulfoxide according to the method of Laskey & Mills (1975). After the gels were dried in a Bio-Rad gel drier, radioactive proteins were located by exposing Kodak RP/2 Royal X-Omat film at 70 °C for several days.

E. gracilis Products of Translation. The products of translation of intact +Zn and −Zn cells were compared by using proteins isolated from a high-speed supernatant of cell homogenates or those found in an enriched ribosomal fraction. The latter fraction was used to study the sedimentation values of the monosomes.

The enriched ribosomal fraction was obtained by disrupting cells (30 g) in 0.1 M Tris-HCl, pH 7.6, 30 mM KCl, 2 mM $MgCl_2$, 0.25 mM $MnCl_2$,, 5 mM 2-mercaptoethanol, 27% sucrose, and 2% Triton X-100. The homogenate was centrifuged at 30000*g* for 30 min.

This supernatant was layered over 3 mL of buffer D (10 mM Tris-HCl, pH 7.6, 120 mM KCl, 5 mM $MgCl_2$, and 5 mM 2-mercaptoethanol) containing 40% sucrose per tube and centrifuged in a Beckman Ti60 rotor at 110000*g* for 2.5 h. The pellet from +Zn cells was resuspended in 1 mL while that from −Zn cells was resuspended in 0.5 mL of buffer D. Aliquots were first frozen in dry ice–acetone and then stored in the vapor phase of a liquid nitrogen Dewar flask. The sedimentation coefficient values of ribosomes in this material were determined by analytical centrifugation in a Spinco Model E ultracentrifuge. The material was diluted with buffer D to approximately 8–16 mg of ribosomes/mL, assuming 12 A_{260} units/mL = 1 mg/mL, and dialyzed against buffer D for 1 h at 4 °C prior to ultracentrifugation analysis. The proteins found in this ribosome-rich fraction were studied by using aliquots stored in the liquid nitrogen Dewar flask. Samples were brought to 4 °C, diluted to 1 mL with buffer D, and spun at 10000*g* for 10 min, and the Mg^{2+} concentration was raised to 0.2 M. Two volumes of glacial acetic acid was added to each sample which was then stirred for 1 h at 0 °C. Following a 10-min centrifugation at 10000*g*, the clarified supernatant was dialyzed against 200 mL of 1 M acetic acid at 4 °C overnight and lyophilized. The material was subjected to electrophoresis in 10% polyacrylamide gels in Tris–glycine buffer, pH 6.3, 0.1% $NaDodSO_4$, and 0.1% mercaptoethanol.

A high-speed cellular homogenate was prepared by disrupting cells in 0.1 M Tris-HCl, pH 7.6. The homogenates were centrifuged at 100000*g* for 1 h. The supernatant was dialyzed extensively against 50 mM NaH_2PO_4, pH 6.0, for 2 days. The dialyzate was centrifuged at 10000*g* for 10 min in a JA20 rotor in a Beckman Model J-21B centrifuge. The supernatant was washed with 50 mM NaH_2PO_4, pH 6.0, buffer and concentrated in a Diaflo chamber with a PM10 membrane. Equal amounts of material (1.0 OD unit at 280 nm) in a 2-mL volume were chromatographed on a CM-Sephadex A-50 column (1.6 × 3.5 cm). The proteins which did not bind and those which were eluted with 1 M NaCl from this column were subjected to electrophoresis on 12% polyacrylamide gels in the presence of 0.1% $NaDodSO_4$. The gels were stained with silver according to Switzer et al. (1979).

Results

The mRNA from −Zn cells serves as an exogenous messenger for both the reticulocyte and wheat germ lysates. In the reticulocyte system, the mRNA requires 0.5 mM Mg^{2+} and 80 mM K^+ for optimal incorporation of [^{35}S]methionine into proteins. The rate of activation, the time course for incorporation, and the relationship of [mRNA] to protein synthesis are shown in Figure 1A,B. While the reticulocyte system has a measurable, endogenous rate of protein synthesis, increasing the amount of −Zn mRNA added to this lysate from 0 to 0.4 μg/mL activates protein synthesis more than 20-fold (Figure 1A). Further increases in [mRNA] up to 2 μg/mL have no additional effect. In assays using 0.4 μg of mRNA, incorporation increases as a function of time and becomes maximal between 45 and 60 min. The conditions, rate, and overall incorporation for the mRNA from −Zn cells are

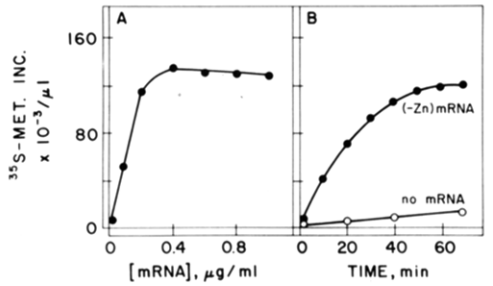

FIGURE 1: (A) Cell-free translation of −Zn *E. gracilis* mRNA isolated from free cytoplasmic polysomes. The effect of increasing the amount of mRNA in the assays was examined. (B) Cell-free translations of −Zn *E. gracilis* mRNA as a function of time. Assay conditions are identical with those in (A) except that 0.4 μg of mRNA was used, and aliquots to determine incorporation were removed at the times indicated.

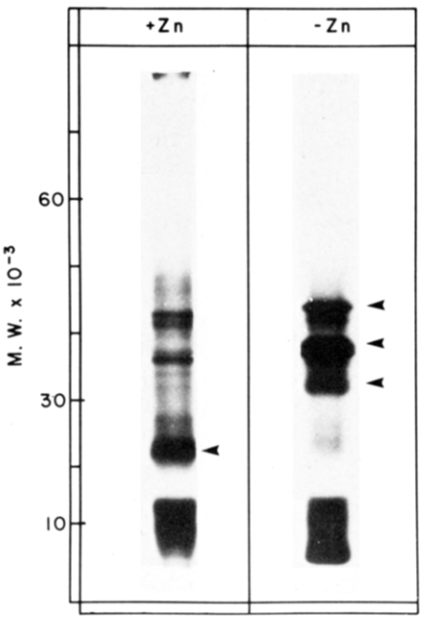

FIGURE 2: Cell-free translation products of +Zn and −Zn mRNAs. Aliquots of the reaction mixtures used for translation assays (Figure 1) containing an equal number of cpm due to [^{35}S]methionine-labeled polypeptides were electrophoresed in 12.5% polyacrylamide. The three arrows in the fluorographs of the translation products generated by the −Zn mRNAs point to three broad bands which are not produced by +Zn mRNAs, while the arrow in the other fluorograph points to one which is formed by +Zn but not −Zn messengers.

identical with those from +Zn *E. gracilis*.

The mRNA populations from −Zn and +Zn cells direct the synthesis of a number of polypeptides of molecular weights ranging from <10000 to >70000. The polypeptides for which the +Zn and −Zn mRNAs code in a cell-free system are separated by elctrophoresis on polyacrylamide gels. Multiple fluorographs of these gels show that with both mRNAs the majority of proteins are the same, though the intensity and, hence, the amounts of a number of these common bands differ. The segment of the fluorograph shown in Figure 2 illustrates that, in addition, the −Zn mRNAs form a number of polypeptides in the molecular weight range of 32000–45000 which are not made at all or in far lesser amounts by the +Zn messengers. In contrast, the mRNA from +Zn cells codes for a polypeptide of molecular weight 22000 which either is absent from or made in very small quantities by the mRNAs from −Zn organisms.

Differences between polypeptides made by +Zn and −Zn *E. gracilis* can be also demonstrated by comparing the types and/or quantities of other products of translation of intact cells. Thus, electrophoretic analysis of the polypeptides obtained by

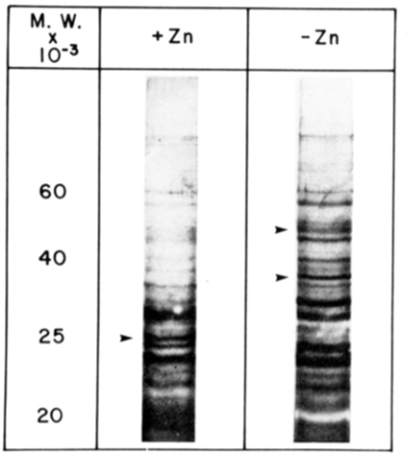

FIGURE 3: Comparison of products of translation from +Zn and −Zn *E. gracilis*. Cell homogenates were chromatographed on CM-Sephadex A-50 as described under Materials and Methods. 250 μg of material obtained from the −Zn and +Zn homogenates was analyzed, and the component polypeptides were separated by electrophoresis on 12.5% polyacrylamide gels. Differences in the polypeptides of the same gel sections from +Zn and −Zn samples are apparent as shown by the arrows.

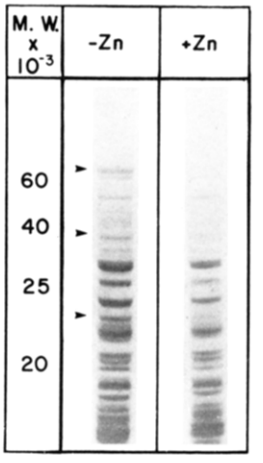

FIGURE 4: Comparison of products of translation from +Zn and −Zn *E. gracilis*. Polysomal-enriched fractions were obtained as described under Materials and Methods. Equal aliquots of proteins associated with either the +Zn or the −Zn fraction were separated by electrophoresis on 10% polyacrylamide gels. The intensity and migration of most of the bands are the same for both the samples. Arrows illustrate three polypeptides which either are unique to the −Zn material or are greatly reduced in amount in the +Zn material.

chromatography of cell homogenates on CM-Sephadex A-50 shows that the majority of the polypeptides from both +Zn and −Zn cells are the same, though again they differ in the relative amounts of several of the proteins which they share. In addition, some bands are present only in +Zn and −Zn cells, but not in both (Figure 3). Proteins associated with an enriched fraction of cytoplasmic ribosomes constitute a class affected by zinc deficiency. Their separation on 10% polyacrylamide gels demonstrates the presence of numerous bands with molecular weights in the range from 5000 to >45000 (Figure 4). The migration pattern of the −Zn material is closely similar to that of the +Zn extract, but in several instances, the staining intensity of a number of bands differs significantly. That of *three* of the −Zn band cells is reduced so greatly compared with that of the +Zn as to raise questions about their very presence in the latter (Figure 4, arrows).

This difference in proteins does not affect the sedimentation properties of the −Zn monosomes or their ability to dissociate and reassociate. Thus, both the +Zn and −Zn fractions contain

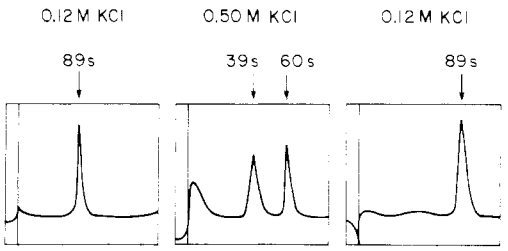

FIGURE 5: Sedimentation coefficients for monosomes and their subunits from −Zn *E. gracilis*. In 0.12 M KCl, 89S monosomes are observed. These dissociate into 39S and 60S subunits and reassociate into 89S particles in 0.5 and 0.12 M KCl, respectively. Identical values are obtained for the monosomes from +Zn cells.

ribosomes which sediment as monosomes of 89 S in buffer containing 0.12 M KCl (Figure 5). In the presence of 0.5 M KCl, both types of monosomes dissociate to subunits of 39 S and 60 S, which reassociate into 89S monosomes in 0.12 M KCl.

Discussion

−Zn *E. gracilis* mRNAs are functionally competent messengers which direct the synthesis of proteins when reticulocyte lysates serve as acceptors. Indeed, the amount of mRNA needed for maximal incorporation of [^{35}S]methionine into polypeptides, the absolute level achieved, and the rate of incorporation as well as the requirements for K^+ and Mg^{2+} are all identical for material from both −Zn and +Zn cells (Figure 1A,B). Similarly, a large number of the resultant proteins and polypeptides seem to be identical, at least as judged by their electrophoretic behavior (Figures 2–4). However, some of the polypeptides and proteins translated from −Zn and +Zn messengers clearly differ, as demonstrated by translating mRNAs both in cell-free systems (Figure 2) and in intact cells (Figures 3 and 4).

These differences affect a variety of categories of proteins, including enzymes such as RNA polymerases, for example (Vallee & Falchuk, 1982), and proteins associated with chromatin (Vallee & Falchuk, 1981) or with polysomal fractions (Figure 4) as well as cytosolic polypeptides (Figure 3).

Hence, the altered base composition of the −Zn mRNAs (Falchuk et al., 1978) does not interfere with binding to the polysomes of intact cells, from which they were isolated, and to those of the S_{30} lysates on which they can be translated (Figures 1 and 2). Further, polyadenylation must have occurred posttranscriptionally since they both bind to and can be purified on oligo(dT)–cellulose. Finally, the initiation codon must be present, since the mRNAs both initiate and elongate polypeptides (Figures 1 and 2). Therefore, zinc deficiency does not result in an mRNA processing disorder which precludes systematic translation or formation of the majority of polypeptides which −Zn and +Zn cells have in common. Therefore, the decrease in the synthesis of any particular protein, or all of the proteins, of −Zn cells (Wacker, 1962; Schneider & Price, 1962) cannot be attributed to their inability to produce viable, functional mRNAs or their failure to translate.

Analogous conclusions can be drawn regarding the RNA of monosomes, the components of polysomes. Following partial purification, the monosomes of −Zn cells remain stable. Importantly, they sediment as 89S particles, and their ribosomal subunits are identical with those from +Zn *E. gracilis* (Figure 5), both with sedimentation coefficients similar to those reported previously for the monosomes of this organism (Scott et al., 1970; Avadhani & Buetow, 1972). Thus, changes in the structural integrity of the polysomes of −Zn *E. gracilis*, if any, and potential consequences to protein synthesis could not be due to radical changes in rRNA composition, which we have previously demonstrated remains unchanged (Falchuk et al., 1978). Also, it would not likely be due to the capacity of this RNA to form different subunits capable of associating into monosomes.

The basis for the reported instability of the polysomes isolated from −Zn cells (Prask & Plocke, 1971) must therefore be sought in terms of other variables which are known normally to lead to the aggregation of monosomes into polysomes. Differences in the *amounts* of the proteins in the polysomal fractions of +Zn and −Zn cells (Figure 4) could be one such parameter that would bear on polysomal stability and which would require further study.

Thus, the effects of zinc deficiency on protein metabolism are not due to an inability to form functional mRNAs or monosomes or to an overall decrease in the synthesis of *all* proteins. Instead, it appears that the lesion *selectively* decreases the synthesis of some proteins and concurrently increases that of others (Figures 2–4). In turn, the activation and transcription of a discrete number of gene and the repression of others can best account for such discriminative effects. Moreover, some of the genes activated in −Zn cells seemingly fail to be transcribed in +Zn cells and conversely.

We have proposed that the activation and concurrent repression of the genome by zinc may represent a hitherto unrecognized role of this metal in metabolic regulation (Vallee & Falchuk, 1981). The present findings must be considered in light of current knowledge of gene transcription and its control. This is accomplished, in part, through the interaction of DNA with RNA polymerases and specific chromatin proteins such as histones and nonhistone proteins (Delange & Smith, 1979) which can either facilitate or hinder binding of RNA polymerases and thereby transcription. Zinc could affect a number of these steps by two or three of its established biochemical roles. Thus, first a zinc-dependent catalytic process could be altered as the metal becomes limiting. The three zinc RNA polymerases present normally in +Zn cells are such an enzymological system involved in gene function. Zinc deficiency results in their replacement by a single, zinc-containing molecular species which appears to fulfill all enzymatic functions of the three normally present (Falchuk et al., 1978; Vallee & Falchuk, 1982; K. H. Falchuk et al., unpublished results). The reduction of three polymerases to one polymerase in going from +Zn to −Zn cells could profoundly affect the expression of different genes including those which each of the eukaryotic RNA polymerases selectively transcribes. On one hand, this could encompass complete repression due to the absence of a particular polymerase and on the other altered efficiency or fidelity in copying a gene owing to the single polymerase which is substituted.

Second, zinc could affect gene expression by direct binding to proteins (Vallee & Wacker, 1970), and/or nucleic acids (Fuwa et al., 1960; Shin & Eichhorn, 1968), involved in the transmission of the genetic message. For example, Zn ions or protein-bound zinc could modulate binding of regulatory proteins to their particular genes or directly affect gene function by interacting with, e.g., PO_4^{2-} groups of nucleic acids, thereby controlling the expression of messages. In the former case, gene activation or repression could be a function of the relative affinity of Zn in either form competing with the regulating molecules for a gene site. Third, zinc could affect the extent of phosphorylation, methylation, and acetylation of the activators or repressors, such as histones (Delange & Smith, 1979). The extent of some of the chemical modifi-

cations, e.g., phosphorylation of *individual* nuclear proteins, is known to be metal dependent and, hence, consistent with such an assumption. Thus, for example, two specific proteins of the nucleolus are modified by Zn^{2+}-activated kinases (Kang et al., 1974). These zinc-dependent modifications might provide the specificity needed for gene regulation by altering the binding of these nuclear proteins to DNA.

Presently available information is clearly inadequate to differentiate between these and yet other alternatives. In addition to the above, these include metabolic effects on histones whose qualitative and quantitative occurrence zinc deficiency alters drastically. Under these circumstances, moreover, low molecular weight arginine-rich peptides accumulate which can interact preferentially with DNA, affecting its template properties toward the RNA polymerases. The elucidation of this function for zinc in gene expression is important for the understanding of the pervasive role of this metal in growth, development, and differentiation.

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