Histone Formation, Gene Expression, and Zinc Deficiency in *Euglena gracilis*†

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ABSTRACT: Histones, and other basic proteins, have been isolated from zinc-sufficient (+Zn) *Euglena gracilis* by standard chromatographic methods. These cells contain 2.46 μg of histones and 1.96 μg of DNA per 10^6 organisms. Each of the histones, H1, H3, H2A, H2B, and H4, is present in both log- and stationary-phase +Zn cells and has been characterized according to its electrophoretic mobility and molecular weight. H1 has been further identified on the basis of its amino acid composition and its cross-reactivity with calf thymus histone H1 antibodies. Similarly, H3 has been recognized as well by its specific reaction with an H3 antibody. In contrast, log-phase zinc-deficient (−Zn) cells contain H1 and H3 while H2A, H2B, and H4 are absent. All of the histones vanish in stationary-phase −Zn organisms. The DNA content increases as the −Zn cells progress from log to stationary phase, reaching a value of 4.40 μg/10^6 cells, double that of comparable stationary-phase +Zn organisms. A 2000–3000-dalton polypeptide whose electrophoretic behavior differs from that of the known histones constitutes over 90% of the total basic proteins of −Zn cells. On addition of zinc to stationary −Zn cells, cell division resumes, and all the histones and other basic proteins reappear. Together with previous results, the data demonstrate that zinc significantly affects the metabolism of *all* major chromatin components, i.e., the RNA polymerases, DNA, and histones of *E. gracilis* [Vallee, B. L., & Falchuk, K. H. (1981) *Philos. Trans. R. Soc. London, Ser. B 294*, 185–197]. The implications of these effects of zinc on chromatin structure and function are discussed.

Zinc deprivation arrests the growth of *Euglena gracilis* and alters the metabolism of its nucleic acids, proteins, and carbohydrates (Vallee & Falchuk, 1981). Previous studies have examined the RNA polymerases and the amounts of DNA synthesized, the rate of RNA synthesis, the composition and function of the mRNAs, and the types of proteins generated (Wacker, 1962; Falchuk et al., 1975a,b, 1976, 1977, 1978; Vallee & Falchuk, 1981, 1983; Crossley et al., 1982). The results led to the conclusion that the genome of zinc-deficient (−Zn) cells is markedly repressed, while, concurrently, a discrete number of genes are selectively activated. We now find that zinc deficiency profoundly alters the types and amounts of chromatin proteins, including the histones, which are known to regulate gene expression. The data suggest that zinc affects gene repression and/or activation through these chromatin constituents.

Materials and Methods

Dark-cultured *E. gracilis* grown in media containing 10 and 0.1 μM Zn^{2+}, henceforth called +Zn or −Zn, respectively (Falchuk et al., 1975a), were harvested at mid-log phase, late-log phase, or at the stationary phase of growth. In addition, aliquots of −Zn cells were allowed to reach stationary phase; at that time, their culture medium was replenished with Zn^{2+} to a final concentration of 10 μM followed by harvesting 2 and 5 days later. Basic proteins were isolated from homogenates or from the partially purified chromatin of each of these cells collected at different stages of growth. Chromatin isolated from nuclei of stationary-phase +Zn and −Zn cells was purified (Jardine & Leaver, 1977) by using a nitrogen bomb (2500 psi for 20 min) instead of a French press to disrupt cells.

The chromatin proteins were extracted by suspending the nuclear pellets in 20 volumes of 0.4 N H_2SO_4 followed by sonication for a total of 90 s with a 10-kHz Branson sonic power and mixing the suspension for 24 h at 4 °C. The suspension was centrifuged for 10 min at 12000*g*, and acid-soluble proteins in the supernatant were precipitated with 5 volumes of absolute ethanol at −20 °C for 48 h. Precipitates were dissolved in 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 6.8, 2.5 mM 2-mercaptoethanol, and 0.1% sodium dodecyl sulfate ($NaDodSO_4$). They were dialyzed 3 times at 4 °C for 6 h, each against 100 volumes of buffer composed of 0.5 M Tris-HCl, pH 6.8, 0.1% $NaDodSO_4$, 1.25 mM 2-mercaptoethanol, and 10% glycerol.

The basic proteins from whole cells were isolated by first suspending the organisms in 10 volumes of 0.075 M NaCl, 0.024 M ethylenediaminetetraacetic acid (EDTA), and 0.05 M bisulfite buffer. The cell suspension was then frozen and thawed 3 times to damage the cell pellicles. Subsequently, 4 N H_2SO_4 was added slowly to obtain a final concentration of 0.4 N. The suspension was sonicated as described above and stirred for 4 h, and the acid-soluble material was collected by centrifugation at 12000*g* for 15 min.

The pellet was reextracted 3 more times with 5 volumes of 0.4 N H_2SO_4—the last extraction lasting 24 h—and then with 2 M NaCl. The supernatants were then combined and clarified by centrifugation for 30 min at 12000*g*. Proteins were precipitated from the clarified extract by the addition of 5 volumes of 95% ethanol at −20 °C. After storage for 48 h, the protein pellet was collected by centrifugation at 12000*g* for 15 min, washed 4 times with cold 95% ethanol, and dissolved in 0.02 N H_2SO_4. The solution was dialyzed against distilled water containing 0.5 mM phenylmethanesulfonyl fluoride (PMSF) and the dialysate clarified by centrifugation at 12000*g* for 15 min and then lyophilized.

The individual proteins in this material were separated on the basis of their electrophoretic mobilities on 15% polyacrylamide gels by using a slab gel technique (Laemmli, 1970; Panyim & Chalkley, 1969) and including the modifications needed to detect *E. gracilis* histone H1 proposed by Jardine

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Table I: Acid Extraction of Basic Proteins from +Zn and −Zn *E. gracilis*[a]

extraction ($0.4\ N\ H_2SO_4$)	mg of basic proteins/g of cells	
	+Zn	−Zn
I	3.19	2.95
II	0.86	0.53
III	0.78	0.44
IV	0.14	0.19
	4.97[b]	4.11[b]

[a] Protein concentration determined by A_{230}. [b] Total.

& Leaver (1978). Calf thymus histones and commercially available low molecular weight standards served as the references. The gels were fixed by the procedure described by Steck et al. (1980) prior to staining either with Coomassie brilliant blue or with silver (Switzer et al., 1979).

The histones as a group were separated further from the acid-solubilized ethanol precipitate by chromatography first on Bio-Rex-70 (Luck et al., 1958; Bonner et al., 1968) and, subsequently, by chromatography on Bio-Gel P-6 (Kaye et al., 1979). H1 was separated from the other histones by chromatography on a Bio-Gel P-10 column of the samples from the Bio-Gel P-6 column. Dr. Michael Bustin, Laboratory of Molecular Carcinogenesis, NCI, NIH, provided two different rabbit sera containing antibodies against calf thymus H3 and H4, respectively. Each also contained small amounts of antibodies to H1 which were separated and concentrated by selective adsorption onto a column containing 5 mg of calf thymus H1 (Sigma) coupled to 3 mL of prewashed Affi-Gel 10 slurry in 0.1 M 3-(*N*-morpholino)propanesulfonic acid (Mops), pH 6.8. The antibody bound to the H1 ligand was eluted with 1 M NaSCN in PBS buffer, dialyzed, and lyophilized. Each of the antibodies was incubated with standard calf thymus histones, the histone fraction from the Bio-Gel P-6 column, the purified H1 fraction, or the crude ethanol precipitate from +Zn, −Zn, and −Zn → +Zn cells. The antigen–antibody reaction was analyzed by using the Elisa method (Towbin et al., 1979). The histone fractions were first subjected to electrophoresis as described above and then transferred from the gel onto nitrocellulose paper. Histone transfer was confirmed by staining one of the sheets with amido black. A separate, unstained sheet was used for the solid-phase immunoassay reaction. The paper was first saturated with bovine serum albumin to block any binding sites not occupied by histones and was then treated with rabbit anti-calf thymus histone antibody. Horseradish peroxidase conjugated to goat anti-rabbit immunoglobulin G (IgG) antibody was used to visualize the histone antibody. Nonimmune rabbit serum served as a control for the nonspecific interaction between antibodies and histones.

The DNA content of the cell homogenates was determined subsequent to the removal of acid-soluble and lipoidal compounds (Schneider, 1968; Burton, 1968). An aliquot was then diluted with water to obtain a range of 25–100 μg of DNA/mL. Calf thymus DNA treated in the same manner was used as the standard.

The concentration of histones and other basic proteins was measured either by the Bio-Rad protein assay method or from A_{230} (3.5 units mg^{-1} mL^{-1}). Bovine plasma albumin (Bio-Rad) and calf thymus histones (Worthington) were used as standards. The amino acid composition of the purified H1 histone fraction was analyzed after hydrolysis with constant-boiling HCl for 20 h at 110 °C under vacuum. The hydrolysate was analyzed on a Durrum D-500 amino acid analyzer, employing either ninhydrin or *o*-phthalaldehyde.

Table II: Content of Total Basic Proteins, Histones, and DNA in Stationary-Phase +Zn and −Zn *E. gracilis* Cells[a]

cell type	total basic proteins	histones	DNA	histone/ DNA ratio
+Zn	13.9	2.46	1.93	1.27
−Zn	12.4		4.40	

[a] Protein and DNA contents are expressed in micrograms per 10^6 cells. In both the stationary and log phases of growth, the total amount of histone in +Zn cells is the same.

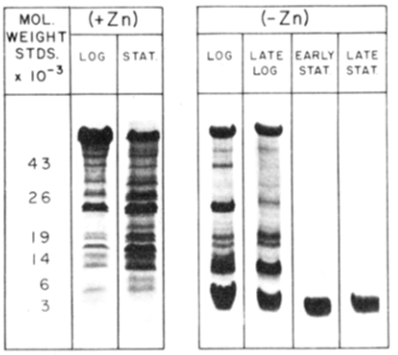

FIGURE 1: +Zn and −Zn *E. gracilis* basic proteins at different growth stages. Twenty micrograms of acid-solubilized ethanol precipitate obtained from +Zn and −Zn cells at the log phase and at various times during the stationary phase was electrophoresed on 15% polyacrylamide gels containing 0.1% $NaDodSO_4$ and Tris–glycine buffer, pH 8.5.

Results

Successive extractions with $0.4\ N\ H_2SO_4$ solubilize progressively less of the total basic protein from either +Zn or −Zn cells so that >60% of the total appears in the supernatant on the first extraction and <5% on the fourth extraction (Table I). These procedures, however, result in nearly the same amount of basic proteins per 10^6 cells for both +Zn and −Zn organisms (Table II).

The amount of acid-solubilized, ethanol-precipitated protein per/10^6 cells remains relatively constant in the course of the growth of both types of organisms. However, during each of the different growth stages, the individual protein constituents of this fraction vary. Thus, polyacrylamide gel electrophoresis of the ethanol precipitate from +Zn cells separates the proteins into bands ranging from 3000 to greater than 43 000 daltons (Figure 1). The electrophoretic patterns of proteins corresponding to log- and stationary-phase +Zn organisms differ characteristically (Figure 1). Two or three bands of molecular weights of approximately 35 000, 21 000, and 7000 can be visualized in material from the stationary phase which are either completely absent or present only in very small amounts in that from log-phase cells.

In contrast to this discrete but perhaps relatively minor difference in proteins from log- and stationary-phase +Zn cells, both differ significantly from log-phase −Zn cell gels where many proteins are absent. Moreover, on reaching the stationary phase, −Zn cells principally contain a single acid-soluble, ethanol-precipitable band which is, however, present only in trace amounts in stationary +Zn cells. It migrates to the most cathodic region of the $NaDodSO_4$ gel, consistent with an apparent molecular weight of 2000–3000. Thus, as the −Zn cells progress from the log to the stationary phase, the acid-soluble proteins vanish, except for this single, low molecular weight species.

Chromatography on Bio-Rex 70 and Bio-Gel P-6 separates histones from other proteins in the ethanol-precipitable ma-

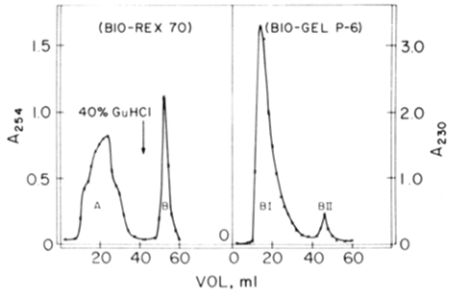

FIGURE 2: Chromatography of +Zn *E. gracilis* basic proteins on Bio-Rex 70 and Bio-Gel P-6. Forty to fifty milligrams of lyophilized ethanol precipitate was dissolved in 10 mL of 8% guanidine hydrochloride in buffer composed of 0.1 M sodium phosphate, pH 6.8 (conductivity 30 $m\Omega^{-1}$). The sample was applied to a 18 cm × 1.8 cm Bio-Rex 70 column (200–400 mesh) previously equilibrated with the same buffer. Adsorbed material, peak B, was eluted with 40% guanidine hydrochloride buffer (conductivity 82 $m\Omega^{-1}$). Ten milligrams of the eluted fraction, B, was dialyzed against distilled water with 0.5 mM PMSF, lyophilized, and then applied on a 50 cm × 1 cm Bio-Gel P-6 column, equilibrated with 0.02 N HCl. One-milliliter fractions were collected at a flow rate of 3 mL/h. The eluant was monitored by A_{230}. Two fractions (BI and BII) were pooled, separately dialyzed, and lyophilized.

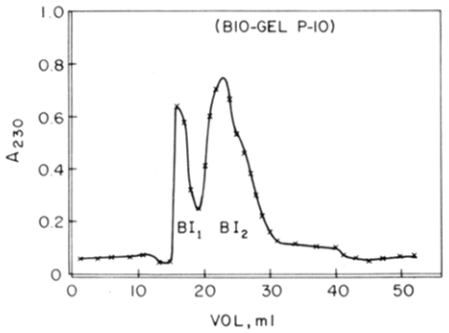

FIGURE 3: Chromatography of fraction BI on Bio-Gel P-10. The Bio-Gel P-6 fraction BI was rechromatographed on a long Bio-Gel P-10 column (130 × 0.6 cm) at a flow rate of 1.5 mL/h (Kaye et al., 1979).

terial of +Zn cells (Figure 2). While fraction A from Bio-Rex 70, which accounts for most of the initial material, is not adsorbed, fraction B binds to it. It comprises <20% of the proteins applied which are separated further into two peaks on Bio-Gel P-6 (Figure 2). Peak BI from Bio-Gel P-6 contains nearly 95% and BII the remainder of the A_{230}-absorbing material. Chromatography on Bio-Gel P-10 resolves fraction BI into distinct components (Figure 3). Electrophoretic analysis on 15% $NaDodSO_4$–polyacrylamide gels documents progressive purification of a group of proteins comprising the majority of the material in Bio-Gel P-6 peak BI (Figure 4). Their migration patterns and estimated individual molecular weights (Figure 5) are similar to those of calf thymus histones (Elgin & Weintraub, 1975) and acid-soluble proteins isolated directly from purified *E. gracilis* chromatin. The Bio-Gel P-10 peak which elutes ahead of the other proteins in peak BI (Figure 3) is purified to near-homogeneity with an apparent molecular weight of 20 500, comparable to that reported for histone H1 (Elgin & Weintraub, 1975). Its high lysine/arginine ratio and the amounts of other amino acids are typical of H1 from other sources (DeLange & Smith, 1979), including *E. gracilis* (Jardine & Leaver, 1978). This material also reacts specifically with purified anti-H1 antibodies, identifying it as histone H1 (Figure 5).

The electrophoretic properties of the protein with an apparent molecular weight of 15 300 correspond to those reported for H3 (Elgin & Weintraub, 1975; Jardine & Leaver, 1978),

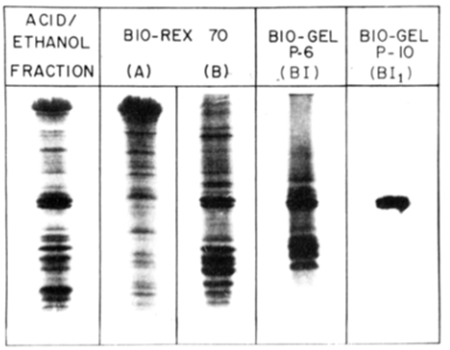

FIGURE 4: Electrophoretic analysis of the proteins from +Zn cells. Acid-solubilized ethanol precipitate, Bio-Rex 70 fractions A and B, Bio-Gel P-6 fraction BI, and Bio-Gel P-10 fraction BI_1 were electrophoresed on 15% polyacrylamide gels. Twenty micrograms of protein was used for each sample except for the Bio-Gel P-10 fraction for which 3 μg was utilized. The latter was stained by the silver method (Switzer et al., 1975).

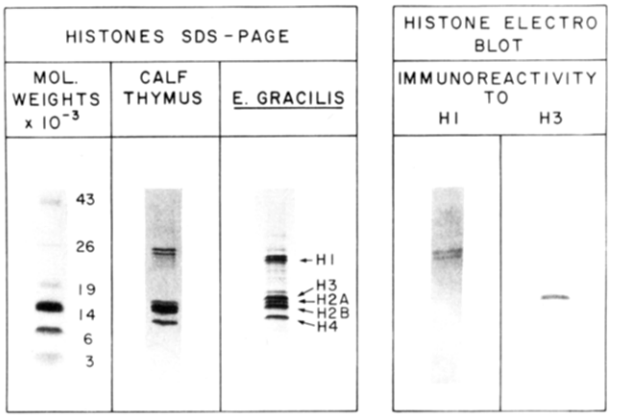

FIGURE 5: Electrophoretic and immunologic properties of +Zn *E. gracilis* histones. The major constituents found in Bio-Gel P-6 fraction BI separate on 15% polyacrylamide gels into five distinct proteins. Their molecular weights are 20 500, 15 300, 14 200, 13 200, and 11 000, respectively, similar to those of calf thymus histones, and have been assigned as H1, H3, H2A, H2B, and H4, respectively.

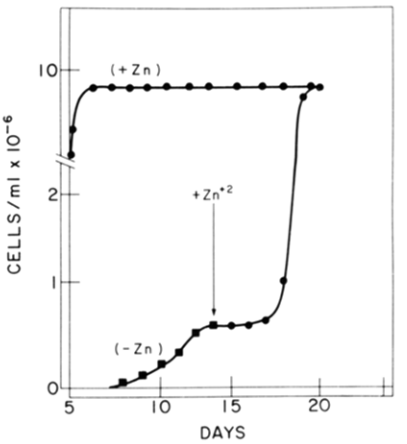

FIGURE 6: Time course for the recovery of stationary-phase −Zn cells following Zn^{2+} repletion.

and its specific reaction with the rabbit antiserum against histone H3 confirms its identity. The antiserum against calf thymus H4 does not cross-react with any of the *E. gracilis* histone fractions. H2A, H2B, and H4 are assigned on the basis of their characteristic electrophoretic behavior relative to that of H1 and H3 (Panyim & Chalkley, 1969; Jardine & Leaver, 1978). All five histones are present in all stages of growth of +Zn cells (Figure 1).

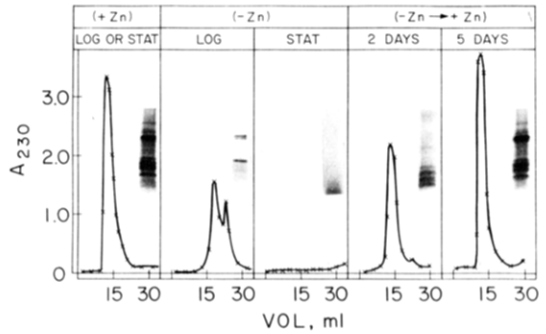

FIGURE 7: Effect of zinc deficiency on *E. gracilis* histones. The histones present in the Bio-Gel P-6 fraction BI in +Zn cells vanish as −Zn organisms progress into the stationary phase; they reappear in −Zn → +Zn cells within 5 days of Zn^{2+} replenishment.

The types and amounts of histones vary, however, according to the growth stage of −Zn organisms. The log phase of growth of these cells is slower than that of +Zn cells, but ultimately they also reach stationary phase (Figure 6) (Price & Vallee, 1962). A 2-day lag phase follows the addition of Zn^{2+} to the growth-arrested −Zn cells; thereafter, they promptly resume division until the cell density equals that of a normal +Zn culture.

As the cells in −Zn media progress through log phase, the total amount of protein found in peak BI, the region in which histones elute from a Bio-Gel P-6 column (Figures 2, 4, and 5), decreases, becoming close to zero on reaching the stationary phase (Figure 7). On recovery from zinc deficiency, the histones reappear to become virtually identical with those of +Zn cells after 5 days following zinc repletion. In the early part of that process, during the log phase, histones H1 and H3 are discrete while H2A, H2B, and H4 either are absent or comigrate with one of the other bands. Histone-like bands are virtually missing from stationary-phase −Zn cells; only traces of these proteins can be detected after staining with silver. In the course of the 2-day lag period subsequent to replenishment with zinc, several discrete bands appear, most of them migrating faster than histones, but faint bands corresponding to H3 and H4 can be detected. The full complement of histones is restored within 5 days when the stationary phase is attained. The electrophoretic pattern is typical of histones from normal +Zn cells (Figures 5 and 7). Histone H1 or H3 antiserum reacts with crude ethanol fractions from zinc-replenished cells but not those from stationary-phase −Zn cells, confirming their absence in the former.

Stationary-phase histone-free −Zn cells contain nearly twice the amount of DNA in +Zn cells at the same stage of growth (Table II). Thus, the histone/DNA ratio in −Zn and +Zn cells differs dramatically. In +Zn cells, the ratio is slightly higher than 1.0 in both the log and the stationary phases (Table II).

Discussion

The basic proteins of +Zn and −Zn *E. gracilis* have been purified and characterized by using standard chromatographic, electrophoretic, and immunologic methods and criteria (Figures 1–5). All of the histones, H1, H3, H2A, H2B, an H4, whether isolated directly from chromatin or from whole cells, have molecular weights and migrate on 15% polyacrylamide gel electrophoresis (PAGE) with characteristics virtually identical with those reported earlier (Panyim & Chalkley, 1969; DeLange & Smith, 1979; Elgin & Weintraub, 1975; Jardine & Leaver, 1978) (Figure 5). The histones are present in both log- and stationary-phase +Zn *E. gracilis* cells, as would be expected for all eukaryotes and from their known metabolism in other cell lines (Bradbury & Matthews, 1982; Chai & Sandberg, 1982; Stein & Stein, 1982). In contrast, these and other basic proteins are altered profoundly in −Zn *E. gracilis* cells, from which nearly all of them have vanished when growth ceases as confirmed by means of a number of different approaches (Figures 5–7). Histones could not be demonstrated chromatographically, electrophoretically, or immunologically in acid extracts of the −Zn cellular homogenate.

This remarkable effect on histone metabolism can be ascribed directly to zinc deficiency. Repletion with zinc completely reverses the lesion, promptly restoring the histone pattern to that of +Zn-grown cells (Figures 5–7). It is not related to shifts in the normal +Zn growth pattern. These changes in histones do not take place in +Zn cells as they progress through their growth stages or when they cease dividing in the stationary phase (Figures 5–7). Effects of zinc on the metabolism of histones have not been reported previously. Both the basis and significance of this observation, therefore, deserve further discussion and exploration.

Histones are highly conserved, and in all eukaryotic cells, the histone/DNA ratio is maintained constant, $\simeq 1$, through regulation of synthesis, principally during the S phase of the cell cycle (Kornberg, 1974). This always makes newly formed histones for nascent DNA (DeLange & Smith, 1979). In contrast to stationary-phase +Zn cells, which are mostly in G0/G1, stationary-phase −Zn cells are blocked in late S or G2; hence, they contain nearly *twice* the amount of DNA found in +Zn organisms (Table II) (Wacker, 1962; Falchuk et al., 1975a,b). The absence of histones in such cells (Figures 1 and 7), therefore, is contrary to findings expected from the known relationship between DNA and histone synthesis described above. At this time, the metabolic basis for this change is quite obscure and can only be speculated upon, as in the following surmises:

(1) −Zn cells synthesize DNA but not histones. This possibility is compatible with the effects of zinc and its deficiency on the presence or absence of a number of other proteins in +Zn and −Zn cells (Crossley et al., 1982). One explanation is that zinc activates and/or represses the genes which code for the proteins affected in this manner (Vallee & Falchuk, 1981; Crossley et al., 1982). The expression of histone genes could then be repressed when zinc becomes limiting.

(2) −Zn cells synthesize histones, but they are displaced by another protein formed as a consequence of zinc deficiency. At least one such basic protein fraction has, indeed, been found to accumulate in −Zn cells (Figure 1). This protein is now known to associate with DNA and might occupy the histone DNA binding sites and successful compete with and displace histones (Stankiewicz et al., 1983). The displaced histones might then be degraded. Alternatively, the protein might bind to chromatin, forming a ternary DNA–histone–protein complex which could prevent the dissociation of histone by acid treatment of chromatin. This latter possibility could account for the failure to detect histones in the acid-soluble fractions; in this case, histone genes need not be repressed.

(3) −Zn cells could form or activate histone proteases. Activation of existent chromatin proteases or induction of additional chromatin proteases could destroy histones in −Zn cells. This view requires that such proteases be either repressed or inhibited in the normal zinc-sufficient state. As much as zinc and other metals play major roles in the function of many proteolytic enzymes, their effects on the synthesis and/or activity of histone proteases does not seem to have been studied (Vallee & Wacker, 1970).

(4) Zinc deficiency could alter the capacity of histones to be bound to DNA. A decreased capacity to bind to DNA both has been thought to signal the accessibility of histones to proteolytic digestion and has been cited specifically as the mechanism underlying their disappearance in sperm (Marushige & Dixon, 1971). This process is believed to be dependent, in part, on the extent to which the histones are phosphorylated, methylated, and/or acetylated (Sperling & Wachtel, 1981), all chemical modifications which are metal dependent. The altered metal content of −Zn cells (Wacker, 1962; Falchuk et al., 1975a) could affect both the activity of constituent proteases and the capacity of histones to bind to DNA and, hence, their accessibility to these enzymes. The above postulated mechanisms are not meant to be mutally exclusive. Any combination of these effects could pertain and would require zinc to be important at more than one stage of histone synthesis, degradation, or function.

The significance of the present observation can best be understood in terms of the known role of the histones and the effects of zinc on chromatin function. The histone–DNA interaction is crucial to the formation, maintenance, and function of the nucleosome, the basic unit of chromatin (Kornberg, 1977). When complexed to histones, the template function of DNA is markedly reduced (Spelsberg & Hnilica, 1971). Consequently, to initiate gene transcription, histones, and H1 in particular, are partially or totally replaced by other proteins which facilitate the template function of DNA (Sonnenbiehler, 1979). Changes in physicochemical properties of histones induced mainly by chemical modification, and variation in the association of different types of proteins with DNA, therefore, are thought to provide one important aspect of the mechanisms which regulate transcription (Bradbury & Matthews, 1982). On this basis, it would be expected that the transcription of histone-depleted DNA in −Zn cells would be increased markedly, an expectation which experimental observations do not confirm. In effect, the overall rate of RNA synthesis in −Zn cells is reduced (Falchuk et al., 1975a) and leads to the postulate that non-histone proteins replace histones in the chromatin of these organisms, likely to repress DNA transcription to a greater extent than that achieved by histones. Electron microscopic studies of −Zn cells, indeed, show that their chromatin is arranged in clusters that more nearly resemble nucleoprotein complexes than dispersed, protein free DNA (Falchuk et al., 1975a). A protein involved in this complex has now been shown to have a molecular weight of 3000. It accumulates in −Zn cells (Figure 1) and induces an organization of the chromatin of −Zn cells relative to that of +Zn cells that is more compact (Stankiewicz et al., 1983), typical of nontranscribing chromatin.

The consequences of histone depletion in −Zn *E. gracilis*, therefore, would seem to keep the cell viable but at a greatly reduced metabolic rate until replenished with zinc.

These findings suggest that zinc is essential to chromatin function by regulating DNA–protein interactions either as a Zn(II) ion or as a complex with a protein, nucleic acid, or another ligand. A reduction in the nuclear zinc concentration could well be limiting the metabolic functions of chromatin, setting in motion the extensive biochemical consequences characteristic of zinc deficiency (Vallee & Falchuk, 1981).

The manner in which sufficiency and deficiency of zinc affect the *E. gracilis* chromatin including its protein constituents would seem to constitute a simple and direct approach to probe the effect of this metal on the genome of this and other eukaryotic organisms.

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Registry No. Zinc, 7440-66-6.

References

Bonner, J., Chalkley, G. R., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R. C., Huberman, J., Jensen, R., Marushige, K., Ohlenbush, H., Olivera, B., & Wielholm, J. (1968) *Methods Enzymol. 12*, 3–65.

Bradbury, E. M., & Matthews, H. R. (1982) *Cell Growth* (Nicolini, C., Ed.) pp 411–452, Plenum Press, New York.

Burton, K. (1968) *Methods Enzymol. 12*, 163–166.

Chai, L. S., & Sandberg, A. A. (1982) *Genetic Expression in the Cell Cycle* (Padilla, G. M., & McCarthy, K. S., Eds.) p 3, Academic Press, New York.

Crossley, L. G., Falchuk, K. H., & Vallee, B. L. (1982) *Biochemistry 21*, 5351–5363.

DeLange, R. J., & Smith, E. L. (1979) *Proteins (3rd Ed.) 4*, 119–225.

Elgin, S. C. R., & Weintraub, H. (1975) *Annu. Rev. Biochem. 44*, 725–774.

Falchuk, K. H., Fawcett, D., & Vallee, B. L. (1975a) *J. Cell Sci. 17*, 57–58.

Falchuk, K. H., Krishan, A., & Vallee, B. L. (1975b) *Biochemistry 14*, 3439–3444.

Falchuk, K. H., Mazus, B., Ulpino, L., & Vallee, B. L. (1976) *Biochemistry 15*, 4468–4475.

Falchuk, K. H., Ulpino, L., Mazus, B., & Vallee, B. L. (1977) *Biochem. Biophys. Res. Commun. 74*, 1206–1212.

Falchuk, K. H., Hardy, C., Ulpino, L., & Vallee, B. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4175–4178.

Jardine, N. J., & Leaver, J. L. (1977) *Exp. Cell Res. 106*, 423–426.

Jardine, N. J., & Leaver, J. L. (1978) *Biochem. J. 69*, 103–111.

Kaye, J. S., Moss, S. B., & McMaster-Kaye, R. (1979) *Anal. Biochem. 94*, 220–227.

Kornberg, R. D. (1974) *Science (Washington, D.C.) 184*, 868–871.

Kornberg, R. D. (1977) *Annu. Rev. Biochem. 46*, 931–954.

Laemmli, U. K. (1970) *Nature (London) 277*, 680–685.

Luck, J. M., Ramusen, P. S., Satake, K., & Tsuetikov, A. N. (1958) *J. Biol. Chem. 223*, 1407–1415.

Marushige, K., & Dixon, G. H. (1971) *J. Biol. Chem. 246*, 5799–5805.

Panyim, S., & Chalkley, R. (1969) *Arch. Biochem. Biophys. 130*, 337–346.

Price, C., & Vallee, B. L. (1962) *Plant Physiol. 37*, 428–433.

Schneider, W. C. (1968) *Methods Enzymol. 12*, 680–684.

Sonnenbiehler, J. (1979) *Naturwissenschaften 66*, 244–250.

Spelsberg, T. C., & Hnilica, L. S. (1971) *Biochim. Biophys. Acta 228*, 202–211.

Sperling, R., & Wachtel, E. J. (1981) *Adv. Protein Chem. 34*, 1–60.

Stankiewicz, A. J., Falchuk, K. H., & Vallee, B. L. (1983) *Biochemistry 22*, 5150.

Steck, G., Leuthard, P., & Bürk, R. (1980) *Anal. Biochem. 107*, 21–24.

Stein, G. S., & Stein, J. L. (1982) *Cell Growth* (Nicolini, C., Ed.) pp 377–409, Plenum Press, New York.

Switzer, R. C., III, Merril, C. R., & Shifrin, S. (1979) *Anal. Biochem. 98*, 231–237.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Vallee, B. L., & Wacker, W. E. C. (1970) *Proteins (2nd Ed.) 5*, 1–192.
Vallee, B. L., & Falchuk, K. H. (1981) *Philos. Trans. R. Soc. London, Ser. B 294*, 185–197.
Vallee, B. L., & Falchuk, K. H. (1983) *Biological Aspects of Metals and Metal Related Diseases* (Arkar, B., Ed.) p 1, Raven Press, New York.
Wacker, W. E. C. (1962) *Biochemistry 1*, 858–865.
Watson, D. K., & Mandrianakis, E. N. (1982) *Biochemistry 21*, 249–256.

Iron–Bleomycin–Deoxyribonucleic Acid System. Evidence of Deoxyribonucleic Acid Interaction with the α-Amino Group of the β-Aminoalanine Moiety†

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ABSTRACT: The Fe(III) complex of bleomycin (BLM) is, at pH 4, in the high-spin form. At pH 7, the coordination of the α-amino group of the β-aminoalanine moiety of BLM converts it to a low-spin species: BLM·Fe(III)·αNH_2. The conversion of the high-spin species to the low-spin one can also take place at pH 4 (i) by addition of ligands L such as N_3^-, $S_2O_3^{2-}$, and SCN^- or (ii) through interaction with DNA. Moreover, the addition, at pH 7, of DNA to BLM·Fe(III) that has been previously complexed with one of these ligands L displaces this latter from its position. These results suggest that (i) the ligand L occupies the same site of coordination as the α-amino group and (ii) an interaction occurs between the β-aminoalanine moiety of BLM and DNA that lowers the pK_d of the α-amino group, promoting its coordination to iron.

Bleomycins (BLM) are a family of glycopeptide antibiotics clinically prescribed for the treatment of selected neoplastic diseases (Carter, 1978). The BLM have both deoxyribonucleic acid (DNA) interacting and metal-binding sites, and the biological activity is related to this bifunctionality. BLM induces a degradation of DNA in a reaction that has been shown to depend, in vitro, on the presence of ferrous ion and molecular oxygen (Sausville et al., 1976, 1978; Takita et al., 1978). BLM is capable of binding Fe(II) to yield an air-sensitive complex Fe(II)·BLM. It has been postulated that the oxidation of this complex by dioxygen yields Fe(III)·BLM and a radical that is responsible for DNA damage.

Up to now, the X-ray crystallographic analysis of Fe(II)·BLM and Fe(III)·BLM has not been reported; nevertheless, the structure of a copper complex of BLM was investigated by X-ray crystallographic analysis of a biosynthetic intermediate of BLM (P3A) (Itaka et al., 1978). It has thus been demonstrated that the secondary amine nitrogen, pyrimidine ring nitrogen, deprotonated peptide nitrogen of histidine residue, and histidine imidazole nitrogen coordinate as the basal planar donor and the α-amino nitrogen of β-aminoalanine coordinates as the axial donor; the metal site has fundamentally a square-pyramidal structure with four chelate rings of 5–5–5–6 ring members. Spectroscopic evidence taken together with titration results establishes the amino–pyrimidine–imidazole–sugar region of BLM as important for binding Fe(II) and Fe(III) [Dabrowiak et al. (1980) and references cited therein], but the detailed geometry and stereochemistry of the iron-binding site remain to be elucidated. Nevertheless a structure has been proposed for iron complexes of BLM involving the same coordination geometry as P3A·Cu(II), except for the carbamoyl group at the sixth coordination site (Itaka et al., 1978; Dabrowiak et al., 1978; Umezawa & Tomohisa, 1978). It should be emphasized that this is a proposed structure and that on grounds of nuclear magnetic resonance (NMR) measurements, other suggestions have been made (Oppenheimer et al., 1979a,b).

Sugiura has focused attention on the role of the α-amino nitrogen of the β-aminoalanine portion of BLM, showing that this ligand is necessary for effective molecular oxygen binding and efficient oxygen reduction by the iron complex of BLM (Sugiura, 1979, 1980). It has then been suggested that the molecular oxygen binds in a position trans to the α-amino nitrogen of the β-aminoalanine portion.

The BLM·Fe(III) complex obtained at pH 7 is in the low-spin form; a decrease of the pH down to 4 converts it in a high-spin species (Burger et al., 1979). The complex of Fe(III) with depyruvamide bleomycin (dep-BLM)—a derivative of BLM lacking the β-aminoalanine amide portion—does not exhibit this pH dependency of the spin state, being in the high-spin state even at pH 7 (Sugiura, 1980). It has thus been suggested that the spin-state change of the BLM-Fe(III) complex between pH 4 and 7 takes place through coordination of the α-amino nitrogen of the β-aminoalanine moieties (Burger et al., 1979; Sugiura, 1980).

In this paper, we report experiments showing that at pH 4 the high-spin BLM·Fe(III) species can be converted to a low-spin species (i) by addition of ligands L such as N_3^-, $S_2O_3^{2-}$, and SCN^- or (ii) by addition of DNA. On the other hand, the addition at pH 7 of DNA to BLM·Fe(III) that has been previously complexed with one of the above-cited ligands L displaces in fact this latter from its position. These results suggest that (i) the same position can be occupied either by the α-amino nitrogen or by a ligand L and (ii) an interaction occurs between the β-aminoalanine portion of BLM and DNA, which lowers the pK_d of the α-amino group promoting its

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