Articles

A *Euglena gracilis* Zinc Endonuclease

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ABSTRACT: A 26-kDa endonuclease has been purified to homogeneity from zinc-sufficient *Euglena gracilis*. The protein binds to single-stranded DNA with a higher affinity than to double-stranded DNA, but it exhibits nucleolytic activity toward both. Thus, it converts supercoiled plasmid pBR322 DNA into the linear form, a property characteristic of endonucleases, and it continues to act on the linearized DNA until it is completely degraded. It also hydrolyzes heat-denatured, single-stranded calf thymus DNA. Moreover, at amounts below 1 μg, it enhances RNA synthesis by RNA polymerase II, a characteristic observed with other DNases. Its addition to an in vitro transcription assay increases RNA synthesis up to 3-fold. The nuclease requires two metal components to carry out its enzymatic activities. It hydrolyzes DNA only in the presence of millimolar amounts of magnesium or micromolar quantities of other activating metal ions, such as manganese, zinc, or cobalt. However, even when optimal concentrations of Mg^{2+} are present, micromolar amounts of the metal-chelating agents OP and HQSA completely inhibit pBR322 digestion. Transcription enhancement is also inhibited completely by both chelators at concentrations that do not affect the intrinsic polymerase II activity. By atomic absorption spectrometry, the enzyme contains 1 g-atom of Zn/mol, which is the likely target of chelator action. The nuclease protein can also be isolated from zinc-deficient *E. gracilis*, but remarkably it then contains 1 mol of Cu/g-atom and no zinc. The copper–protein does not bind to DNA, is unable to hydrolyze pBR322 (even in the presence of the activating magnesium ion), and does not enhance transcription, further confirming the essentiality of zinc in this enzyme.

Approximately one-third of the zinc in eukaryotic cells is contained in the nucleus (Fujii et al., 1954; Thiers & Vallee, 1957). Efforts to understand the biochemical roles of this metal fraction have focused on zinc-binding, nuclear macromolecules which led to the discovery of a number of proteins and enzymes that contain stoichiometric amounts of zinc and bind to single- or double-stranded DNA [see Vallee and Falchuk (1981, 1993)]. Typically, all of these zinc-containing, DNA-binding molecules from the nucleus participate in a variety of chemical reactions involving the hydrolysis, structural rearrangement, repair, replication, or transcription of this nucleic acid. Many of the corresponding DNA-binding zinc enzymes from prokaryotes and viruses have also been found. Eukaryotic zinc metalloenzymes that bind DNA and have been isolated thus far include RNA polymerases I, II, and III (Falchuk et al., 1976, 1977, 1985; Auld et al., 1976; Wandzylak et al., 1977; Petranyi et al., 1977), *Penicillium* (P1) nuclease (Fujimoto et al., 1974a,b, 1975; Volbeda et al., 1991), and snake venom phosphodiesterases (Pollack et al., 1983; Mori et al., 1987). Those metalloenzymes that have been described from prokaryotic and viral organisms include *Eco*RI restriction endonuclease that hydrolyzes site-specific regions of DNA (Barton et al., 1982), the *Escherichia coli* topoisomerase I that cleaves and rejoins DNA phosphodiester bonds (Tse-Dinh & Beran-Steed, 1988), the *E. coli* RNA polymerase (Scrutton et al., 1971), and the reverse transcriptases (Auld et al., 1984).

Four regulatory proteins that activate transcription in different organisms are also among the zinc macromolecules from the eukaryotic nucleus. These include TFIIIA from *Xenopus* oocytes (Hanas et al., 1983; Miller et al., 1985), the mammalian glucocorticoid and estrogen receptors (Freedman et al., 1988; Schwabe et al., 1990), and GAL4 yeast protein (Pan & Coleman, 1989). A number of proteins that are involved in either replication or transcription in prokaryotes and visuses have now been found, specifically g32P in T4 phage (Giedroc et al., 1986), HV-1 protein from herpes virus (Gupta et al., 1991), and the Ada protein from *E. coli* (Myers et al., 1992). In nearly all of these regulatory proteins, zinc is assumed to generate the protein structure necessary to recognize and bind to specific DNA sequences.

In efforts to define the roles of zinc in chromatin structure and function, in particular, and, generally, in developmental biology, we have identified zinc macromolecules in the nuclei of *Euglena gracilis* and determined how zinc deficiency affects them. We have demonstrated that in zinc-deficient cells a single polymerase II replaces the three zinc RNA polymerases of (+Zn)[1] cells (Falchuk et al., 1976, 1977, 1985). This latter enzyme contains zinc and is active, but it is unusually resistant to α-amanitin (Falchuk et al., 1985). Its properties, like those

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[1] Abbreviations: (+Zn), zinc sufficient; (−Zn), zinc deficient; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; OP, 1,10-phenanthroline; HQSA, 8-hydroxyquinoline-5-sulfonic acid; HPLC, high-performance liquid chromatography; ZnP, zinc nuclear protein; CuP, copper nuclear protein.

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of other α-amanitin-resistant RNA polymerases II, have been attributed either to an alteration in or the absence of the 40-kDa α-amanitin-binding subunit (Ingles et al., 1976; Brodner & Wieland, 1976; Falchuk et al., 1985).

The present paper describes the purification and characterization of yet another zinc protein from the *E. gracilis* nucleus. It binds to DNA, is nucleolytic, and enhances the transcriptional rate of zinc RNA polymerase. Remarkably, (−Zn) organisms synthesize the same protein but containing copper not zinc. As a result of the change of the metal, the resultant molecule does not bind to or cleave DNA nor does it enhance transcription. To our knowledge, this is the first instance that copper replacement of zinc as a consequence of the deficiency of that metal alters the function of a zinc enzyme.

MATERIALS AND METHODS

Cell Culture. *E. gracilis*, Klebs, strain Z, was grown in the dark in the presence of either 10 μM Zn^{2+} [(+Zn) cells] or 0.1 μM Zn^{2+} [(−Zn) cells], metal concentrations previously shown to achieve sufficiency or deficiency states, respectively (Falchuk et al., 1975). The concentrations of all media constituents and other metals were identical in both culture conditions.

Metalloprotein Isolation. All buffers were extracted with 0.001% dithizone prior to use, and all glassware was rendered metal-free by standard procedures (Falchuk et al., 1988). Nuclei were obtained as described (Czupryn et al., 1987). The nuclear pellet from 30–40 g of cells—equivalent to about 30 mg of DNA—was extracted with 10 mM Tris-HCl, pH 7.8, and 0.75 M NH_4HCO_3 for 2 h, and the extract was chromatographed on Sephadex G-50 (1.6 × 100 cm) equilibrated with 50 mM Tris-HCl, pH 7.6. The zinc-enriched fraction was collected and resolved further by reversed-phase HPLC on a Synchropak C_8 column (250 × 4.1 mm) equilibrated with buffer A (10 mM ammonium acetate/acetonitrile, 5:1). Elution was carried out at room temperature with a linear gradient of buffer B (7 mM ammonium acetate/2-propanol, 2:3) at a flow rate of 0.7 mL/min for 1 h. Fractions containing nuclear metalloprotein were pooled and rechromatographed under the same conditions. For preparative scale isolation, the gel filtration step was omitted, and instead, nuclear extract proteins were fractionated on a SEP-PAK C_{18} cartridge (Millipore, Bedford, MA). Proteins retained on the cartridge were recovered by elution with 2–3 mL of buffer B, lyophilized, dissolved in buffer A, and separated by reversed-phase HPLC as described above.

Aliquots of the HPLC-purified material were subjected to SDS–PAGE, followed by electroblotting of the resolved proteins onto a PVDF membrane (Immobilon P; Millipore) in 10 mM CAPS, pH 11.0, and 10% methanol (Matsudaira, 1987). The membrane was stained with 0.5% Ponceau S in 5% acetic acid to visualize proteins, and the strip of membrane containing the nuclear metalloprotein was excised. The protein was extracted with 50 mM Tris-HCl, pH 8.8, 1% Triton X-100, and 2% SDS and precipitated with 4 volumes of acetone at −70 °C (Szewczyk & Summers, 1988). Aliquots were subjected to SDS–PAGE or to enzymatic assays as described below.

To isolate the corresponding metalloprotein from (−Zn) cells, the nuclear extract was passed through a Bio-Rex 70 (Bio-Rad, Richmond, CA) column (1.6 × 24 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.5, to remove the basic peptides uniquely associated with the nuclei of these cells (Stankiewicz et al., 1983; Mazus et al., 1984; Czupryn et al., 1987). The flow-through material was collected and further fractionated as described above.

Protein concentration was estimated from amino acid analysis performed with the Pico-Tag method (Bidlingmeyer et al., 1984).

Metal Analyses. Zinc, copper, iron, cobalt, nickel, manganese, cadmium, magnesium, and calcium in chromatographic fractions were measured by atomic absorption spectrophotometry using a graphite furnace (Perkin Elmer, Model AA5000). The zinc and copper contents of nuclear pellets were analyzed after ashing on a hot block for 1 h with spectrally pure 40% nitric acid at 105–110 °C. The DNA content of aliquots of the material ashed was estimated as described (Burton, 1956).

Electrophoresis. SDS–PAGE was carried out as described by Laemmli (1970). Proteins were visualized by staining with silver (Wray et al., 1981).

Isoelectric focusing was performed with the PhastSystem (Pharmacia LKB) on PhastGels IEF 3–9. DNA electrophoresis was carried out in 0.7% agarose gels in TBE buffer (0.089 M Tris–borate, 0.089 M boric acid, and 0.002 M EDTA). The gels were stained with ethidium bromide.

DNA Binding. Purified protein of known metal content was chromatographed at 4 °C on either native (ds) or denatured (ss) DNA–cellulose columns (Pharmacia LKB Biotechnology Inc.). The protein was applied at a flow rate of 0.1 mL/min onto the column with 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, and 5% glycerol as the binding buffer. The bound protein was then step-eluted with 0.1, 0.3, 0.6, and 1.0 M NaCl in the binding buffer.

Endonuclease Assays. Nuclear protein (up to 1 μg) or bovine pancreatic DNase I (up to 4 ng) was incubated with 0.5 μg of plasmid pBR322 DNA in 40 mM Tris-HCl, pH 7.9 (final volume 10 μL), containing 10 mM $MgCl_2$ as the activating ion. Incubation was carried out for 30 min at 37 °C and terminated by addition of 10 mM EDTA. The requirement for activating ions was tested by incubating the reaction mixture without any added metal or with 1–20 mM $MgCl_2$, 0.1 mM $ZnSO_4$, 0.1 mM $MnSO_4$, 0.1 mM $CoCl_2$, or 0.1 mM $CuSO_4$. Reaction products were analyzed by agarose gel electrophoresis. Some of the assays were carried out in the presence of OP and HQSA, at concentrations ranging from 10^{-6} to 10^{-3} M with 10 mM Mg^{2+} as the activating ion. The nucleolytic activity of the protein also was tested directly in the SDS–polyacrylamide gel by copolymerizing the gel with 20 μg/mL calf thymus DNA. After electrophoresis, the gel was washed in three changes of 40 mM Tris-HCl, pH 7.6, and 2 mM $MgCl_2$ in 6 h, stained with ethidium bromide (0.5 μg/mL), and then incubated in 40 mM Tris-HCl, pH 7.6, and 10 mM $MgCl_2$ until DNA hydrolysis was apparent from the lack of DNA fluorescence (usually 7–8 days).

In Vitro Transcription. Transcription assays were performed as described Falchuk et al. (1976). A partially purified RNA polymerase II preparation from (+Zn) *E. gracilis* was the source of endogenous enzyme. Heat-denatured *E. gracilis* DNA served as the template. Purified metalloprotein was added to the assay mixture, always as the last component, in amounts ranging from 50 to 1000 ng. The chelating agents OP and HQSA and the nonchelating analogue of OP, 1,7-phenanthroline, were used at concentrations ranging from 10^{-9} to 5 × 10^{-2} M, at pH 7.8. DNA was preincubated with RNA polymerase to obviate hydrolytic effects of OP (Mazus et al., 1986). In order to examine the reversibility of inhibition, Zn^{2+} was added to the assay mixture in concentrations equimolar to those of the chelating agents.

Table I: Effect of Zinc Deficiency on Nuclear Metal Content

cell type	Zn[a]	Cu[a]
(+Zn)	82	<1
(–Zn)	16	64

[a] Units of fentograms per nucleus.

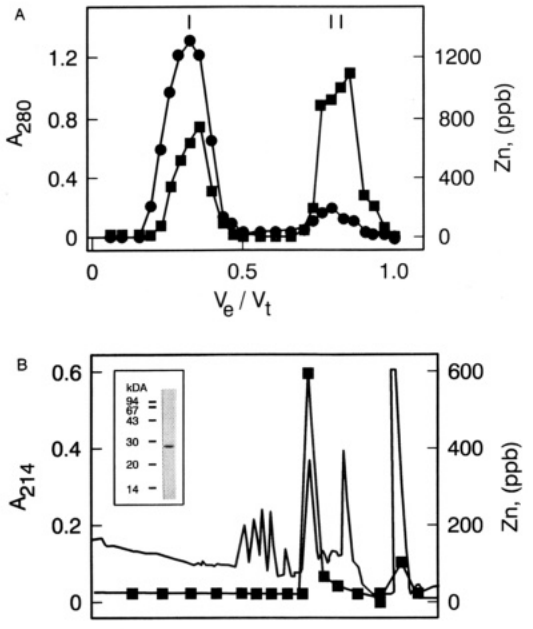

FIGURE 1: Purification of zinc nuclear protein, ZnP. (A) Sephadex G-50 chromatography of salt-extracted nuclear proteins from (+Zn) cells. (●) A_{280}; (■) Zn. (B) Reversed-phase HPLC of the Sephadex G-50 fraction II; (■) Zn. Inset: SDS–PAGE of the zinc-containing peak.

RESULTS

One-third of the 240 fg of zinc found in each (+Zn) *E. gracilis* cell is present in the nucleus (Table I). When isolated nuclei are lysed in 0.75 M NH_4HCO_3, many proteins, mostly of the non-histone type, are solubilized together with approximately half of the zinc present in the starting material. These proteins are separated into two zinc-containing fractions by gel filtration on Sephadex G-50 (Figure 1A). The second, lower molecular weight fraction (M_r <30 000) contains 65% of the zinc but only 11% of the total A_{280} applied; it was chosen for further purification. On reversed-phase HPLC, it resolves into a number of fractions, two of which contain zinc. The first of these comprises a heterogeneous population of proteins which do not bind to the column at all; they appear in the breakthrough (not shown). The second fraction (Figure 1B) binds tightly and is eluted with approximately 36% 2-propanol. The predominant protein in this fraction migrates on SDS–polyacrylamide gels as a 26-kDa species, and its repeated chromatography yields the 26-kDa protein which is devoid of appreciable impurities (Figure 1B, inset). Metal analysis reveals 1 g-atom of tightly bound zinc/mol of protein (Table II). Copper, iron, cobalt, nickel, manganese, cadmium, calcium, and magnesium are not detected.

Essentially the same procedure has been employed to purify a corresponding protein from (–Zn) *E. gracilis* nuclei. The basic peptides that are uniquely associated with the nuclei of (–Zn) cells (Staniewciz et al., 1983; Mazus et al., 1984; Czupryn et al., 1987; Falchuk et al., 1986) were first removed by chromatography on a BioRex column. Subsequent steps followed the identical protocol developed to isolate the protein from (+Zn) cells. In addition to zinc, copper was also measured throughout the isolation procedure. Copper analysis

Table II: Properties of ZnP and CuP

property	ZnP	CuP
molecular weight	26000	26000
isoelectric point	6.85	6.85
zinc (g-atom/mol)	1.0	ND
copper (g-atom/mol)	ND[a]	1.0
Fe, Mg, Ca, Cd, Co, Ni, Mn	ND	ND

[a] ND, not detected.

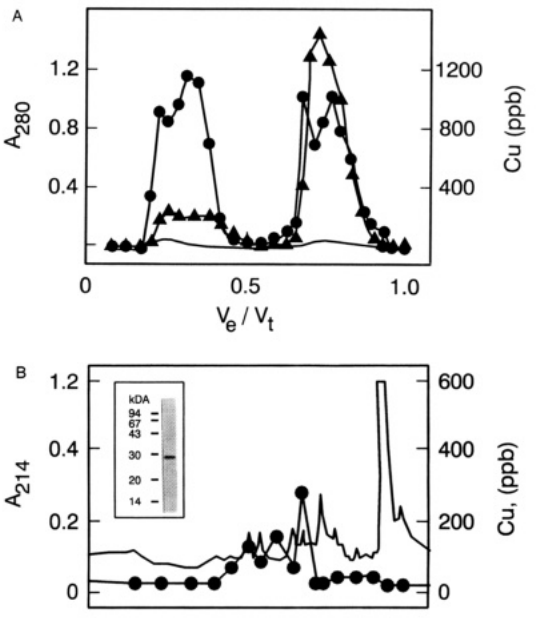

FIGURE 2: Purification of copper nuclear protein, CuP. (A) Sephadex G-50 chromatography of nuclear proteins extracted from (–Zn) cells. (●) A_{280}; (▲) Cu. Zn baseline in ppb is shown without symbols. (B) Reversed-phase HPLC of the Sephadex fraction II; (●) Cu. Inset: SDS–PAGE of the copper-containing peak.

was included in the isolation protocol since the nuclei of these cells contain 4-fold less zinc and 60-fold more copper than (+Zn) cells (Table I). Sephadex G-50 chromatography of proteins solubilized from (–Zn) nuclei with 0.75 M NH_4HCO_3 separates two protein fractions (Figure 2A), much as noted with (+Zn) nuclei (Figure 1A). However, copper, not zinc, is the major metal associated with the two peaks. Reversed-phase HPLC of the low molecular weight material separates a protein fraction, eluting from the column at the same retention time as the zinc protein from (+Zn) cells but associated with copper instead of zinc (Figure 2B). Rechromatography of this fraction purifies a single protein that migrates as a 26-kDa polypeptide on SDS–PAGE (Figure 2B, inset). It contains 1 g-atom of Cu/mol but no zinc, in contrast to the protein isolated from (+Zn) cells (Table II).

The amino acid compositions (Table III) and isoelectric points (Table II) of the zinc protein isolated from (+Zn) cells and that containing copper from (–Zn) cells are nearly identical. Both proteins are composed of more than 50% hydrophobic residues, likely accounting for their tight binding to the C_8 column. The corresponding amino acid sequences of both proteins could not be obtained since their N-terminii are blocked.

Since the metalloprotein is purified from the nucleus, its interaction with DNA was studied by chromatography on DNA–cellulose. The buffers used in the binding steps do not contain magnesium. The zinc-containing protein isolated from (+Zn) cells which is free of magnesium binds preferentially to ssDNA. The major part of the retained protein fraction (50%) binds tightly to DNA, since it is eluted by 1.0 M NaCl

Table III: Amino Acid Composition (Residues per Mole) of ZnP and CuP[a]

	ZnP	CuP
Asx	16.7	17.9
Glx	32.1	34.7
Ser	12.5	14.4
Gly	23.3	24.5
His	4.0	4.2
Arg	11.4	11.3
Thr	11.8	11.4
Ala	21.6	21.7
Pro	18.5	16.2
Tyr	6.0	5.3
Val	13.6	12.9
Met	2.9	3.1
Ile	8.9	7.7
Leu	17.0	16.8
Phe	6.3	6.3
Lys	13.4	11.6
Cys[b]	3.0	3.0
Trp	ND[c]	ND
	total 223	223

[a] Values represent the average of five analyses. [b] Determined as cysteic acid. [c] ND, not determined.

Table IV: Binding of ZnP and CuP to DNA–Cellulose[a]

	eluted protein (%)			eluted protein (%)	
[NaCl] (M)	ZnP	CuP	[NaCl] (M)	ZnP	CuP
0.05	39	59	0.6	3	0
0.1	4	41	1.0	50	0
0.3	4	0			

[a] Binding buffers as well as the enzyme preparations used do not contain magnesium.

(Table IV), a salt concentration that is required to elute histones from DNA–cellulose affinity columns. When applied to dsDNA–cellulose using the same conditions, nearly all of the protein elutes in the flow-through fraction; only a minor amount binds to the column (not shown). In contrast to the zinc-containing form, the copper-containing protein from (–Zn) cells binds very weakly to ssDNA and is eluted with 10-fold less salt, 0.1 M NaCl (Table IV).

The zinc protein exhibits nucleolytic activity toward DNA. This activity was assayed with negatively supercoiled DNA of plasmid pBR322 as a substrate (Shishido & Ando, 1982). At the concentrations used, the enzyme converts supercoiled plasmid DNA into a linear form through a nicked circular intermediate, indicative of the action of an endonuclease (Figure 3A). However, it continues to digest the DNA beyond the initial cleavage step, resulting in more extensive DNA hydrolysis. Both hydrolytic activities toward DNA require the presence of divalent cations. Mg^{2+} is required at a minimum of 10 mM, while Zn^{2+}, Mn^{2+}, and Co^{2+} can activate the protein at submillimolar concentrations. Cu^{2+}, 0.1 mM, does not activate DNA hydrolysis at all (Figure 3B). The nucleolytic activity is approximately 5000-fold less than that observed with equivalent molar amounts of pancreatic DNase I (not shown).

Endonucleolytic activity is an intrinsic catalytic property of the zinc enzyme and is not due to the presence of minute amounts of a highly active contaminant as confirmed by a HPLC-purified enzyme preparation electroblotted onto a PVDF membrane (Matsudaira, 1987) after SDS–PAGE. This procedure should separate the 26-kDa band from any potential contaminant(s) that differ(s) in molecular mass by as little as 1 kDa. The enzyme preparation obtained by this method remains homogeneous on SDS–PAGE combined with silver

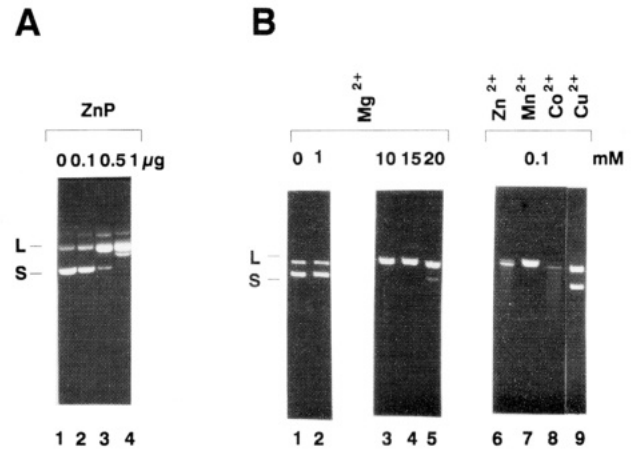

FIGURE 3: Nucleolytic activity of zinc nuclear protein, ZnP. (A) Effect of increasing amounts of nuclease, ZnP, on hydrolysis of pBR322 DNA. The supercoiled (S) and linear (L) forms of the plasmid are indicated. Note that the untreated plasmid DNA (lane 1) contains some of the linear form. 0 (lane 1), 0.1 (lane 2), 0.5 (lane 3), and 1.0 μg (lane 4) of ZnP was incubated with DNA in the presence of 10 mM $MgCl_2$. (B) Divalent cation requirement for nuclease activity. pBR322 DNA (lane 1) was incubated with 1 μg of ZnP in the absence of added cations or in the presence of 1 mM $MgCl_2$ (lane 2), 10 mM $MgCl_2$ (lane 3), 15 mM $MgCl_2$ (lane 4), 20 mM $MgCl_2$ (lane 5), 0.1 mM $ZnSO_4$ (lane 6), 0.1 mM $MnSO_4$ (lane 7), 0.1 mM $CoCl_2$ (lane 8), or 0.1 mM $CuSO_4$ (lane 9).

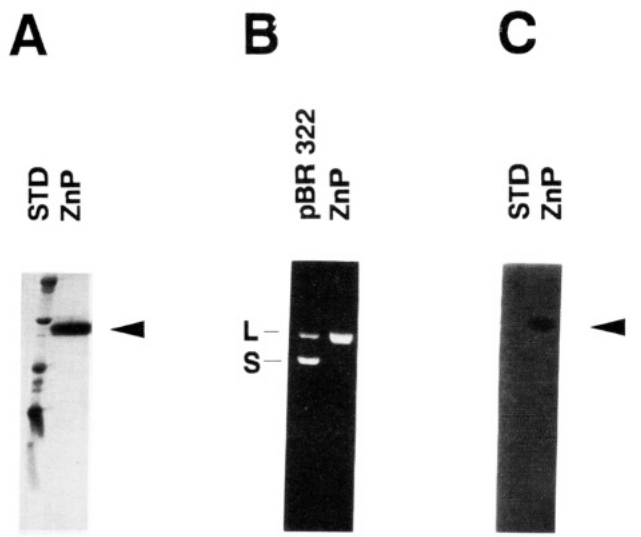

FIGURE 4: Nucleolytic activity is associated with the 26-kDa zinc-containing protein, ZnP. (A) SDS–PAGE of ZnP purified by elution from a PVDF membrane after protein electroblotting. (B) Activity assay using purified ZnP and pBR322 DNA. (C) Detection of the nucleolytic activity of ZnP in a SDS gel containing the DNA substrate. The arrowhead denotes the electrophoretic mobility of ZnP.

staining (Figure 4A). The preparation is active whether the nucleolytic assay is performed in solution, as above (Figure 4B), or directly in the SDS–polyacrylamide gel, in which the substrate, calf thymus DNA, was copolymerized within the gel (Figure 4C). In the latter case, DNA hydrolysis is observed only in the position around 26 kDa, which corresponds to the electrophoretic mobility of the zinc enzyme (Figure 4C).

Zinc, the only stoichiometric metal found to be associated with the enzyme, is essential to the endonucleolytic activity, as shown by two separate experiments. First, under conditions where the effective activating metal concentration is not affected, structurally distinct metal chelators, OP and HQSA, inhibit pBR322 DNA hydrolysis (Figure 5). Second, the copper protein obtained from zinc-deficient cells is inactive, despite the addition of extraneous activating metal ions such as Mg^{2+} or even Zn^{2+}. The copper species exhibits no

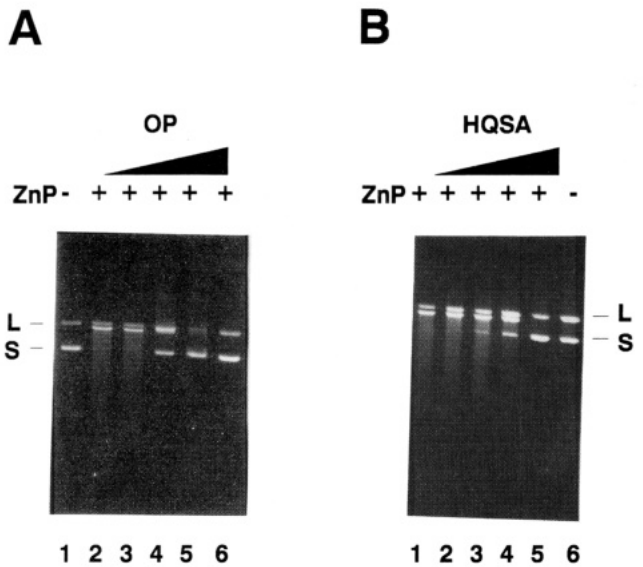

FIGURE 5: Effect of preincubating ZnP, 1 μg, with the chelating agents OP or HQSA on pBR322 DNA hydrolysis. (A) No protein added (lane 1). No OP (lane 2); OP, 10^{-6} M (lane 3); OP, 10^{-5} M (lane 4); OP, 10^{-4} M (lane 5); OP, 10^{-3} M (lane 6). (B) No HQSA (lane 1); HQSA, 10^{-6} M (lane 2); HQSA, 10^{-5} M (lane 3); HQSA, 10^{-4} M (lane 4); HQSA, 10^{-3} M (lane 5); pBR322 DNA, no protein (lane 6).

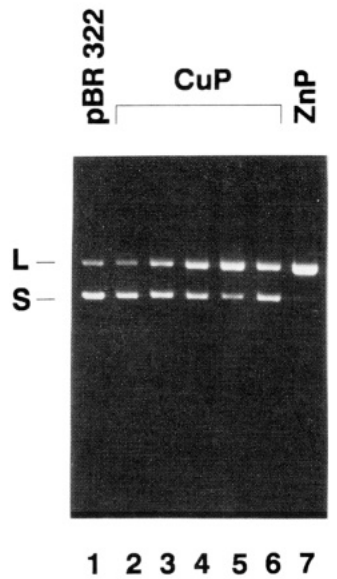

FIGURE 6: Lack of nucleolytic activity of CuP. pBR322 DNA alone (lane 1) or incubated with different amounts of CuP (0.5–1 μg of protein) in the presence of 10 mM $MgCl_2$ (lanes 2–4) or with 0.1 mM $ZnSO_4$ (lanes 5 and 6). For comparison, an assay using ZnP is shown (lane 7).

nucleolytic activity toward pBR332 DNA (Figure 6), even at a concentration where the zinc-containing molecule would hydrolyze the DNA completely.

The zinc-containing endonuclease enhances the rate of transcription in an in vitro system that contains partially purified *E. gracilis* RNA polymerase II and with denatured DNA as template. The amount of RNA synthesized by such a system increases linearly as a function of concentration to reach 300% of the control in the presence of 1 μg of the protein (Figure 7). The enhancement could be due to nicking of the DNA by nuclease, which would generate a template that is transcribed more efficiently. It also could be the result of enzyme-induced stabilization of the DNA–polymerase complex, among other possibilities. Independent of the mechanism, however, the enhancement provides an additional readout that

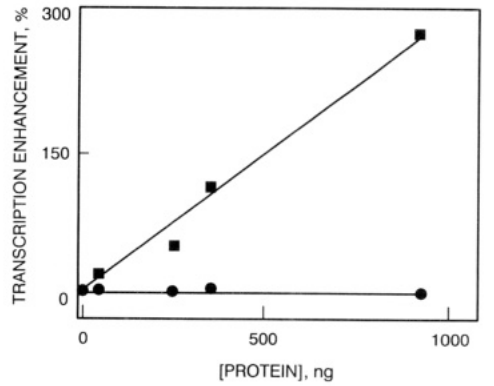

FIGURE 7: Effect of ZnP and CuP on the transcription rate by RNA polymerase II. (■) ZnP; (●) CuP.

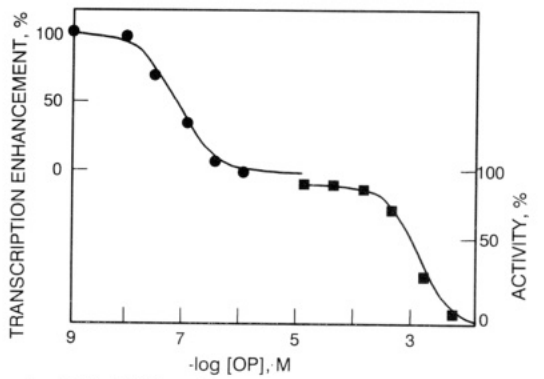

FIGURE 8: OP inhibition of transcription enhancement by ZnP (●) and of transcription by RNA polymerase II (■).

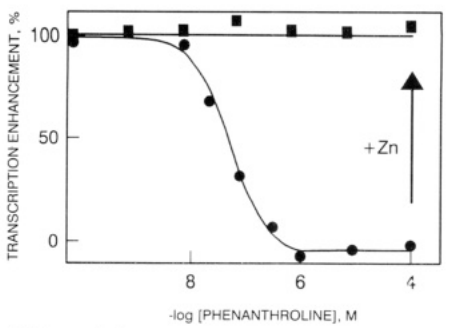

FIGURE 9: Effect of the nonchelating analogue 1,7-phenanthroline on transcription enhancement and reversal of the inhibition by OP by $ZnSO_4$. (●) OP; (■) 1,7-phenanthroline.

establishes the functional importance of the zinc atom of the endonuclease. Thus, the replacement of zinc in the enzyme by copper abolishes the transcriptional rate enhancement (Figure 7). OP also inhibits the enhancement in a concentration-dependent manner (Figure 8). The chelator is ineffective at 10^{-8} M but completely abolishes enhancement at 10^{-6} M. The reduction in transcription enhancement over this concentration range is due to inhibition of the nuclease and not to chelation of Mg^{2+}, the activating metal which is present at 1000-fold excess over that of the chelator. Moreover, it is not due to inhibition of the RNA polymerase, another zinc enzyme known to be inhibited by OP, since the base-line transcription rate is not affected at all, even up to 10^{-4} M OP (Figure 8). Only at concentrations higher than 10^{-4} M is the RNA polymerase activity inhibited, as previously reported (Falchuk et al., 1985). The nonchelating analog of OP, 1,7-phenanthroline, has no effect on transcription enhancement, and there is a complete reversal of the inhibition by addition of Zn^{2+} (Figure 9). Thus, OP inhibition occurs through chelation of zinc, the only metal intrinsic to the protein and the only one that could be affected in the reaction. Similar

inhibitory effects on transcription enhancement by the nuclease have been observed with HQSA (not shown).

DISCUSSION

The enzyme that has been isolated from (+Zn) *E. gracilis* nuclei is classified as an endonuclease since it converts the supercoiled DNA of plasmid pBR322 into the linear form. Its requirement for an activating metal (Mg^{2+}, Mn^{2+}, Co^{2+}, or Zn^{2+}) and the concentrations needed, millimolar for Mg^{2+} and micromolar for the rest, are all characteristic of other nucleases (Basile & Barton, 1989). The failure of Cu^{2+} to serve as an activator is not unusual; as best as can be ascertained, among all of the known nucleases, only that of *Chlamydomonas* endonuclease A can be activated by this metal (Small & Sparks, 1972; Basile & Barton, 1989).

The *E. gracilis* endonuclease has not been described previously. It differs from the *light*-inducible endonuclease L, the only other one isolated from this organism, in the culture conditions of the cells from which it has been purified, its activating metal ion requirements, and its cellular localization. Thus, the present enzyme derives from the nuclei of dark-grown cells and is activated by Mg^{2+}. Endonuclease L is present primarily in the particulate material of light-grown cells and is not activated by Mg^{2+} (Carrell et al., 1970; Small & Sturgen, 1976).

The endonuclease is a zinc metalloenzyme. In other organisms, zinc has been shown to be involved in the function of enzymes that cleave phosphodiester bonds of DNA and RNA as well as the hydrolysis of phosphate monoesters (Basile & Barton, 1989). A comparison of these enzymes with the *E. gracilis* zinc endonuclease, therefore, might serve to further characterize the latter.

Zinc *activates* nucleases isolated from *Neurospora* (Linn, 1967), *mung bean* (Kowalski et al., 1976; Laskowski, 1980), *Aspergillus* (S1) (Vogt, 1980), *Physarum* (Waterborg & Kuyper, 1979, 1982), wheat seedling (Hanson & Fairley, 1969), *Ustilago* (Fraser & Cohen, 1983; Holloman & Holliday, 1973), and spinach (Doetsch et al., 1989). However, in none of them has zinc been shown to be an intrinsic component by direct atomic absorption spectrometry analysis. Thus, so far, the *E. gracilis* zinc endonuclease differs from all of them.

On the other hand, three nucleases from different sources are known to contain stoichiometric amounts of zinc: *Penicillium* (P1) nuclease; phosphodiesterases from the venom of *Crotalus ruber ruber* and *adamanteus*, both of which cleave DNA at nonspecific sites; and the site-specific *Eco*RI restriction endonuclease (Fujimoto et al., 1974a,b, 1975; Volbeda et al., 1991; Pollack et al., 1983; Mori et al., 1987; Barton et al., 1982). A fourth enzyme, S1 nuclease, has been claimed to contain 3 mol of Zn/mol (Shishido & Habuka, 1986) but without any reference to the means of method by which this was determined. The *E. gracilis* enzyme can be distinguished from the *Penicillium* (P1) nuclease on the basis of zinc content—the latter contains 3 mol of Zn/mol (Fujimoto et al., 1974a,b, 1975; Volbeda et al., 1991) versus 1 for the former (Table II). The venom phosphodiesterases from *Crotalus ruber ruber* and *adamanteus* contain 1 mol of Zn/mol (Mori et al., 1987), but the molecular mass (98 kDa) and p*I* (10.5) for the *ruber ruber* (Mori et al., 1987) and the *adamanteus* enzyme (115–120 kDa) are higher than for the present *E. gracilis* enzyme. *Eco*RI also contains 1 mol of Zn/mol (Barton et al., 1982), but it is a prokaryotic enzyme whose amino acid composition differs markedly from the *E. gracilis* endonuclease described here; the restriction enzyme is active as a dimer of 62 kDa (Modrich & Zabel, 1976; Jen-Jacobson et al., 1983).

The *E. gracilis* zinc metallonuclease requires two metals for function, the intrinsic zinc and the activating magnesium ion. The specific biochemical roles of each of the metals in DNA hydrolysis and in the associated enhancement of the transcriptional rate of RNA polymerase II have not been identified in this enzyme though the available data allow for a number of conclusions to be made in this regard. Chelating agents inhibit both of these activities which cannot be due to chelation of magnesium since the concentration of that metal is several thousandfold higher than that of the chelator both in DNA hydrolysis (Figure 5) and in the transcription assays (Figure 8). Moreover, the chelating agents used have a low affinity for magnesium. Therefore, inhibition must be attributed to chelation of the intrinsic zinc atom of the endonuclease.

It was not possible to establish whether the chelating agent inhibits nucleolytic activity and transcriptional enhancement by forming an enzyme–zinc–chelate complex or by removing the metal from the enzyme altogether, resulting in an apoenzyme. In either case, the enzyme is not modified since addition of Zn^{2+} reverses the inhibition (Figure 9). The copper-containing molecule does not exhibit either one of the two activities, confirming the indispensability of zinc for function.

Nuclease-catalyzed hydrolysis of DNA requires two steps: binding to the nucleic acid and phosphodiester bond cleavage. Minimally the zinc atom of the enzyme may be involved in the DNA-binding step, as confirmed by the observation that the zinc-containing, magnesium-free *E. gracilis* endonuclease, but not its copper-containing counterpart (Table II), binds to DNA (Table IV). A role for zinc in the cleavage of DNA itself has not been ruled in or out, though it can be concluded that the presence of zinc alone, in the absence of an activating metal, is not sufficient to enable the nuclease to digest the nucleic acid. In the other three known zinc nucleases, the role of zinc has not been defined either. The structure of the zinc P1 nuclease has been reported recently (Lahm et al., 1990; Volbeda et al., 1991), but the functions of the three zinc atoms remain an open question. Two of the zinc atoms are found in sites that are apparently inaccessible to the nucleic acid while the third zinc is associated with ligands characteristic of enzyme catalytic sites (Vallee & Auld, 1990). Hence, two of the zinc atoms have been proposed to serve a structural role and the third, a catalytic role (Volbeda et al., 1991).

Similarly, the specific mechanism by which the activating metal participates in the hydrolytic process itself is not defined. For *Eco*RI, as for the *E. gracilis* enzyme, magnesium is not required for specific DNA binding (Barton et al., 1982). The formation of an initial *Eco*RI–nucleic acid complex is believed to induce a conformational transition in the DNA that facilitates a stronger association between the protein and nucleic acid through a bridging magnesium atom (Barton & Basile, 1989). Once achieved, the hydrolysis occurs. In the *E. gracilis* enzyme, the presence of magnesium alone, however, is insufficient to cleave DNA, as demonstrated by the inability of the copper-containing protein to hydrolyze the nucleic acid (Figure 6).

It is likely that the enzyme has two distinct binding sites, one for zinc and one for the activating metal. The identity of the amino acid ligands for any of the metals remains unknown. Cysteine and histidine, along with aspartic and glutamic acids that are present in the enzyme, all are potential metal ligands (Vallee & Auld, 1990). In the absence of

information on these metal-binding sites, it is not possible, of course, to ascertain which, if any, of the two putative sites is occupied by copper. One of the characteristics of ligands to zinc and copper is that they bind the metal tightly since neither one is removed by the various chromatographic steps used in purification. This suggests that in the protein from (–Zn) cells the zinc site might be occuppied by the copper atom.

The present results have implications for understanding of the basis for the pathological phenomena observed in zinc-deficient organisms. We have long considered that the abnormalities in proliferation, growth, and development of zinc-deprived systems would ultimately have to be explained on the basis of alterations in the function(s) of zinc-containing macromolecules. The data obtained with *E. gracilis* so far have yielded only two examples of the effects of zinc deficiency of known zinc enzymes that have been purified to homogeneity, the RNA polymerases and the present endonuclease.

When zinc is sufficient, *E. gracilis* cells synthesize three distinct zinc RNA polymerases (Falchuk et al., 1976, 1977, 1985). In the zinc-deficient state, only one, RNA polymerase type II, is present. The enzyme contains zinc and is active but exhibits an unusual resistance to inhibition by α-amanitin compared to its counterpart in (+Zn) cells (Falchuk et al., 1986). This type of α-amanitin-resistant polymerase II has also been isolated from cells exposed to mutagenic agents, conditions that result in the formation of a polymerase with an altered or missing α-amanitin-binding subunit (Ingles et al., 1976; Brodner & Weiland, 1976).

The molecular weight, chromatographic behavior, and p*I* of the protein isolated from (–Zn) cells are the same as those of the enzyme from (+Zn) organisms. Its amino acid composition is nearly identical, barring some minor differences. While these could easily reflect inherent variations in the analysis of multiple samples, the possibility that zinc deficiency changes a few of the amino acids cannot be ruled out at present. In a number of other zinc enzymes, leukotriene A_4 hydrolase and carboxypeptidase A, among others, changes in up to three amino acids by site-directed mutagenesis do not alter such properties as chromatographic behavior, molecular weight, and immunoreactivity much as they affect activity markedly (Medina et al., 1991; Le Moual et al., 1991). In the cases cited, the amino acids that have been changed are those that are responsible for zinc binding; consequently, the resultant mutant proteins do not bind zinc and are inactive. Therefore, while we believe the zinc endonuclease from (+Zn) cells and the inactive copper protein from (–Zn) cells are one and the same, the possibility remains that their amino acid sequences may differ somewhat. Such changes could occur if the fidelity of transcription by zinc-deficient cells were to be incorrect. Errors in transcriptional fidelity are known to be induced by variations in manganese and magnesium concentrations, and this condition may be particularly pertinent to zinc-deficient *E. gracilis* cells (Falchuk et al., 1978).

On the basis of these results and others reported elsewhere (Falchuk et al., 1976; Crossley et al., 1982), a number of conclusions regarding the role of zinc in gene expression, on the one hand, and the effects of zinc deficiency on that process, on the other, were drawn (Vallee & Falchuk, 1981). (–Zn) cells synthesize different types of proteins compared to sufficient ones, including histones, non-histone proteins, and RNA polymerases (Stankewicz et al., 1983; Mazus et al., 1984; Falchuk et al., 1985; Czupryn et al., 1987). It is now accepted generally that zinc participates in the function of transcription activators. This was established first by the demonstration that TFIIIA is a zinc metalloprotein (Hanas et al., 1983; Miller et al., 1985) and has been extended by the verification that other transcription and/or replication factors, including glucocorticoid and estrogen receptors (Freedman et al., 1988; Schwabe et al., 1990) and the GAL4 protein (Pan & Coleman, 1989) in eukaryotes, Ada protein in *E. coli* (Myers et al., 1992), *g32p* in T4 phage (Giedroc et al., 1986), and HV-1 from herpes virus (Gupta et al., 1991), all contain stoichiometric quantities of zinc. We previously suggested that the effects of zinc deficiency on the formation of proteins are mediated at the transcriptional level through alterations in the functions of zinc transcription factors. The alterations could be due to the lack of formation of transcription factors, or synthesis of inactive mutant proteins (Vallee & Falchuk, 1981). The present results demonstrate that in addition to a role in the regulation of protein synthesis at the level of transcription, zinc deficiency can alter the function of zinc enzymes by affecting posttranslational processes, such as those involved with the incorporation of metal into an apoprotein.

In the present case, a zinc enzyme is rendered inactive by the replacement of zinc by copper. It is unknown whether the replacement occurs in the cytoplasm or the nucleus. In both compartments, the concentration of copper is increased, by over 50-fold in the nucleus of (–Zn) organisms (Table I). This occurs despite the fact that the concentration of copper in the culture medium of (–Zn) cells is the same as in that of (+Zn) organisms. The generality of this metal replacement in other zinc proteins from the nucleus of *E. gracilis* and its pertinence to other organisms require examination. This is particularly relevant to proteins that are involved in developmental processes and believed to contain zinc. A number of putative zinc regulatory proteins have been suggested. Very few have been purified, and, hence, their metal content has not been analyzed. If these proteins were shown to be zinc macromolecules that are rendered inactive in zinc deficiency by virtue of replacement of zinc for another metal, it might be possible to account for major developmental abnormalities consequent to deprivation of this metal.

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