

Lead Inhibition of DNA-Binding Mechanism of Cys₂His₂ Zinc Finger Proteins

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ABSTRACT

The association of lead with chromatin in cells suggests that deleterious metal effects may in part be mediated through alterations in gene function. To elucidate if and how lead may alter DNA binding of cysteine-rich zinc finger proteins, lead ions were analyzed for their ability to alter the DNA binding mechanism of the Cys₂His₂ zinc finger protein transcription factor IIIA (TFIIIA). As assayed by DNase I protection, the interaction of TFIIIA with the 50-bp internal control region of the 5S ribosomal gene was partially inhibited by 5 μM lead ions and completely inhibited by 10 to 20 μM lead ions. Preincubation of free TFIIIA with lead resulted in DNA-binding inhibition, whereas preincubation of a TFIIIA/5S RNA complex with lead did not result in DNA-binding inhibition. Because 5S RNA binds TFIIIA zinc fingers, this result is consistent with an inhibition mechanism

via lead binding to zinc fingers. The complete loss of DNase I protection on the 5S gene indicates the mechanism of inhibition minimally involves the N-terminal fingers of TFIIIA. Inhibition was not readily reversible and occurred in the presence of an excess of β-mercaptoethanol. Inhibition kinetics were fast, progressing to completion in ~5 min. Millimolar concentrations of sulfhydryl-specific arsenic ions were not inhibitory for TFIIIA binding. Micromolar concentrations of lead inhibited DNA binding by Sp1, another Cys₂His₂ zinc finger protein, but not by the nonfinger protein AP2. Inhibition of Cys₂His₂ zinc finger transcription factors by lead ions at concentrations near those known to have deleterious physiological effects points to new molecular mechanisms for lead toxicity in promoting disease.

Xenobiotic metals such as cadmium, arsenic, and lead can induce a variety of adverse physiological responses in rodents and humans, including carcinogenesis, reproductive and developmental defects, nephropathies, and neuropathies (Goyer, 1996). These effects are thought to be mediated through metal ion-protein interactions of a variety of cellular targets; in some cases, the metal ions are complexed with certain proteins in detoxification mechanisms (Goyer, 1983, 1984). To aid in risk assessment and to help elucidate the biochemical mechanisms for adverse effects of metal ions on biological systems, it is important to identify potential protein targets for their toxic action and elucidate underlying inhibitory mechanisms, including concentration dependence and kinetics. Because cysteine amino acids in proteins are highly reactive to electrophilic metal ions, proteins containing such residues are proposed to be primary targets for metal ions, especially those in the heavy-element category (Thomas and Wofford, 1983). An important class of cysteine-rich proteins is the regulatory factors that contain Cys₂His₂

zinc-binding domains first identified in transcription factor IIIA (TFIIIA) and referred to as "zinc fingers" (Hanas et al., 1983; Miller et al., 1985). TFIIIA binds the internal control region (ICR) of the 5S ribosomal RNA gene and activates 5S RNA synthesis by RNA polymerase III (Engelke et al., 1980). Cysteine-rich zinc finger proteins are proposed to be cellular targets for many xenobiotics, including metal ions, and are possibly responsible for carcinogenic effects of metal ions (Sunderman and Barber, 1988).

Mechanistic effects of arsenic and cadmium ions on zinc finger structure were previously examined in two structurally and functionally distinct proteins, the steroid hormone receptor and transcription factor IIIA (Simons et al., 1990; Predki and Sarkar, 1992; Hanas and Gunn, 1996). Both of these proteins are prototypes of transcription factor superfamilies. Members of the hormone receptor family contain two Cys₂Cys₂ zinc-binding domains and members of the TFIIIA superfamily contain various numbers of Cys₂His₂ zinc-binding domains. Divalent metal ions such as cadmium and arsenic ions display increased avidity for two closely spaced thiols in a vicinal orientation (Joshi and Hughes, 1981). Arsenic(III) was found to inhibit both hormone and DNA-binding functions of the estrogen receptor (Simons et

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ABBREVIATIONS: TFIIIA, transcription factor A for RNA polymerase III; Cys₂, two cysteine amino acids; His₂, two histidine amino acids; ICR, internal control region; S, sedimentation constant; DNase I, deoxyribonuclease I; RNase A, ribonuclease A; SV40, simian virus 40; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol.

al., 1990). Significantly, micromolar amounts of arsenic inhibited hormone binding and millimolar amounts inhibited DNA binding. The micromolar sensitivity to arsenic for hormone binding is indicative of the disruption and necessity of a vicinal thiol arrangement for this function. Cadmium ions at micromolar amounts were found to be nondetrimental to the DNA binding function of the steroid hormone receptor and could replace zinc in the finger structure with no loss of function (Predki and Sarkar, 1992). Micromolar concentrations of cadmium ions were found to inhibit the DNA binding mechanism of the prototypical Cys₂His₂ zinc finger protein, TFIIIA (Hanas and Gunn, 1996).

Lead is the most common metal in the environment that has known adverse effects on biological systems. Normal blood lead levels in children and adults measure in the 0.1 to 0.2 μ M range (Pirkle et al., 1994). Although this level is still high, it has decreased ~5-fold from the mid-1970s. Children and neonates are especially sensitive to lead effects because they absorb significantly more of ingested metal than adults. Blood lead levels in the 0.5 to 5 μ M range are known to have deleterious effects on nervous, renal, and reproductive tissue and the metal is a known carcinogen in rodents (Goyer, 1996). At the cellular level, lead accumulates in cell nuclei and associates with nuclear proteins and chromatin (Hitzfeld and Taylor, 1989). The presence of lead in the nucleus could result in adverse effects on gene function if lead ions at low concentrations are capable of having deleterious effects on gene regulatory proteins. Alterations in gene expression could be manifested in developmental, reproductive, and carcinogenic effects of the metal, which are known to occur in animals (Johnson, 1998). It is therefore important to mechanistically determine if lead ions can have deleterious effects on gene expression in general and on gene regulatory proteins in particular and at what concentrations. In the present study, mechanistic effects of metal ions on zinc finger proteins are extended by investigating lead interactions with TFIIIA and transcription factor Sp1, another Cys₂His₂ zinc finger protein that binds GC-rich regions in RNA polymerase II promoters (Kadonaga et al., 1987).

Materials and Methods

Isolation of TFIIIA. Immature ovarian tissue was removed from anesthetized 4- to 5-cm female *Xenopus laevis* frogs (Nasco Biologicals, Fort Atkinson, WI) and homogenized briefly in buffer A (50 mM Tris-HCl, pH 7.6, 50 mM KCl, 5 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride). This homogenate was centrifuged for 20 min at 10,000g and aliquots were layered onto 15 to 30% v/v glycerol gradients in the buffer A. These gradients were centrifuged for 24 h at 34,000 rpm in a Beckman SW41 rotor; all manipulations were performed at 0–4°C. The 7S particle complex containing TFIIIA and 5S ribosomal RNA sedimented slightly faster than the 5S hemoglobin molecule and was identified by UV absorption. The ribonucleoprotein particles were further purified to 90% homogeneity by diethylaminoethyl cellulose ion exchange chromatography as described previously (Hanas et al., 1983). 5S RNA was removed from TFIIIA by digestion of the 7S particle (20 μ g/ml) with RNase A (10 μ g/ml) in buffer B (20 mM Tris-HCl, pH 7.6, 320 mM KCl, 2 mM MgCl₂, 0.4 mM DTT, 0.1% v/v Nonidet P-40 detergent (Sigma Chemical Company, St. Louis, MO) for 30 min at room temperature and then placed on ice. Protein concentration was determined by the method of Bradford with bovine serum albumin as a standard (Bradford, 1976).

Transcription Factor-DNA-Binding Reactions. A 303-bp DNA insert containing the 120-bp *Xenopus borealis* somatic 5S ribosomal RNA gene was ³²P end-labeled on the coding strand by digesting a pT7 plasmid containing the insert with *Bam*HI followed by alkaline phosphatase removal of the 5' phosphates. After removal of alkaline phosphatase by phenol-chloroform extraction, the 5' ends were rephosphorylated with polynucleotide kinase and [γ -³²P]ATP. The end-labeled plasmid was then ethanol precipitated, digested with *Eco*RI to excise the insert, and the 303-bp end-labeled fragment was purified on a 6% w/v polyacrylamide gel. The specific activity of the DNA insert was determined by absorbency at 260 nm and Cerenkov counting. To study the effects of xenobiotic metal ions on TFIIIA function, TFIIIA in buffer B is diluted 5-fold in buffer C (20 mM Tris-HCl, pH. 7.6, 70 mM NH₄Cl, 7 mM MgCl₂, 0.2 mM β -mercaptoethanol, 0.1% v/v nonionic detergent Nonidet P-40) and incubated at room temperature with lead chloride (Aldrich Chemical Co. Wilwaukee, WI), sodium arsenate, or sodium arsenite (both purchased from Sigma Chemical Co., St. Louis, MO) at the concentrations and times indicated in the figure legends. TFIIIA was then diluted (20-fold) to 10 mM in the same buffer minus the lead, end-labeled 5S gene was added to a final concentration of 1 nM (~10⁴ cpm), and the binding reaction (20 μ l) took place for 15 min. DNA-binding reactions with transcription factors Sp1 and AP2 (obtained from Promega Life Sciences, Madison, WI) were performed in similar fashion with the template simian virus 40 (SV40) promoter DNA end-labeled according to the vendor's instructions.

DNase I Protection Assays. DNase footprint analyses to identify specific DNA-protein interactions were performed in accordance with a previously described procedure (Galas and Schmitz, 1978). Briefly, DNase I was added to the binding reactions to a final concentration of 1 to 2 μ g/ml and incubated for an additional minute at room temperature. The digestion was terminated by addition of 100 μ l of stop buffer (20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.1% SDS, 30- μ g sonicated salmon sperm DNA/ml). The DNA was ethanol-precipitated and resuspended in 4 μ l of formamide solution (20 mM Tris-HCl, pH 7.6, 95% deionized formamide, 1 mM EDTA, 0.01% xylene cyanol and bromphenol blue), heated at 95°C for 5 min, and then electrophoresed through a 7 M urea-7% w/v polyacrylamide gel until the xylene cyanol marker migrated two-thirds down the gel. The gel was then transferred to blotting paper, dried, and subjected to autoradiography overnight at –70°C exposed to Kodak XAR-5 film.

Electrophoretic Mobility Shift Assays (EMSAs). For EMSAs (Yoshinaga et al., 1989), TFIIIA treated with RNase A in buffer B was diluted 5-fold in buffer D (10 mM Tris-HCl, pH 7.6, 75 mM KCl, 2.5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 0.1% v/v Nonidet P-40, 5% glycerol), and incubated at room temperature with lead chloride or zinc chloride at the concentrations indicated in the figure legend. The TFIIIA was then diluted to 10 mM in the same buffer minus the metals, end-labeled 5S gene and poly dI-dC (Sigma Chemical Co.) were added to a final concentration of 1 nM and 1 μ g/ml, respectively, and the binding reaction (20 μ l) took place for 15 min. The samples were then electrophoresed for 1 h at 250 V on a pre-electrophoresed 6% w/v polyacrylamide gel (60:1 w/w acrylamide/bis-acrylamide) in buffer E (90 mM Tris-HCl, 90 mM boric acid, pH 7.9). The gel was transferred to blotting paper and subjected to autoradiography overnight at –70°C exposed to Kodak XAR-5 film.

Results

Inhibition of TFIIIA-DNA Interactions by Lead Ions.

In a protein-dependent DNA EMSA, exposing TFIIIA to increasing concentrations of lead ions (10–30 μ M Pb²⁺) inhibited the ability of the protein to bind to and retard the mobility of the ³²P end-labeled 5S gene fragment as evidenced by the autoradiogram in Fig. 1A. The faster mobility of the unbound DNA bands in lanes 3 to 5 is evident com-

pared with that in lane 2 (the positive control) where TFIIIA binding to the 5S gene has slowed the electrophoretic mobility of the end-labeled DNA; the negative control reaction containing end-labeled DNA but no TFIIIA was electrophoresed in lane 1. Significantly, inhibition of the TFIIIA-dependent mobility shift was not observed with treatment of the factor with the same concentrations of zinc ions (Zn²⁺, lanes 3–5, Fig. 1B). To examine the concentration dependence of the lead inhibition more closely, the DNase I protection assay was used, which allows a “visualization” of the specificity and location of the TFIIIA binding event on the 5S gene (Fig. 2). TFIIIA in this assay binds to and protects from DNase digestion a large surface on the 5S gene, from nucleotides +43 to +96, relative to the +1 start site of transcription (lane 2); in the absence of TFIIIA binding, DNase I readily nicks this area of the gene as evidenced by the large number of [³²P]DNA fragments in this region (lane 1). Exposing TFIIIA to concentrations of lead ions <10 μM results in significant loss of DNase I protection (lane 4, 5 μM); lower concentrations of lead ions result in slight inhibition (lane 3, 2.5 μM). The inhibition of TFIIIA-dependent DNase I protection occurs over the entire factor-binding region, from +43 to +96 on the 5S gene where all nine zinc fingers of TFIIIA interact. It is noted that the DNase I protection and EMSA DNA-binding reactions contain 0.2 mM β-mercaptoethanol and 0.5 mM DTT respectively; these reagents are necessary for TFIIIA activity. These excess thiol groups may bind lead ions, thus increasing the lead concentration necessary for TFIIIA inhibition. We have observed that it takes higher lead concentrations to see complete TFIIIA inhibition in the EMSA than in the DNase I protection assay. This may be due in part to the use of 0.5 mM DTT in the EMSA.

Time Course of Lead Inhibition of TFIIIA Binding.

The inhibition of TFIIIA function by lead ions is taking place before the DNA binding assay as TFIIIA is first exposed to

the lead ions and then diluted ~20-fold into the DNA binding assay. In addition, the inhibition is taking place in the presence of an excess of thiol reagent. These results indicate that the inhibitory effect of the lead ions on TFIIIA structure is not readily reversible. In the experiments depicted in Figs. 1 and 2, TFIIIA was exposed to the various concentrations of lead ions for 20 min at room temperature before assaying for DNA binding, which takes place in an additional 15-min incubation. To elucidate the time course of this inhibition, a kinetic analysis of lead exposure was performed to gain additional information about the inhibition mechanism; a rapid inhibition would suggest a direct and specific interaction with lead on TFIIIA. TFIIIA was pretreated with lead ions for various times before addition to the DNA binding reaction, which was shortened to 1 min. Figure 3 is an autoradiogram that illustrates this kinetic analysis of the DNA-binding inhibition as assayed by DNase I protection. Very slight lead inhibition of TFIIIA-dependent DNase I protection is observed at the 1-min lead exposure time point (lane 4) and substantial inhibition is observed at the 2.5-min and 5-min time points (lanes 5 and 6). The loss of DNase I protection at 5 min is about the same as at 20 min (lane 8). This rapid inhibition is consistent with a direct binding mechanism between lead ions and TFIIIA.

Lead Ions Inhibit TFIIIA Zinc Finger Structure. Although zinc fingers comprise three fourths of the TFIIIA structure (30 kDa out of 40-kDa total mass), nonfinger regions could possibly be involved in this lead inhibition. TFIIIA zinc fingers bind 5S RNA in a mechanism competitive with 5S gene DNA (Hanas et al., 1984). The TFIIIA-5S RNA complex is referred to as the 7S ribonucleoprotein particle. Previously, cadmium ions were shown to inhibit TFIIIA only upon direct exposure to the free protein but not when the protein was bound to 5S RNA in the 7S particle. This result

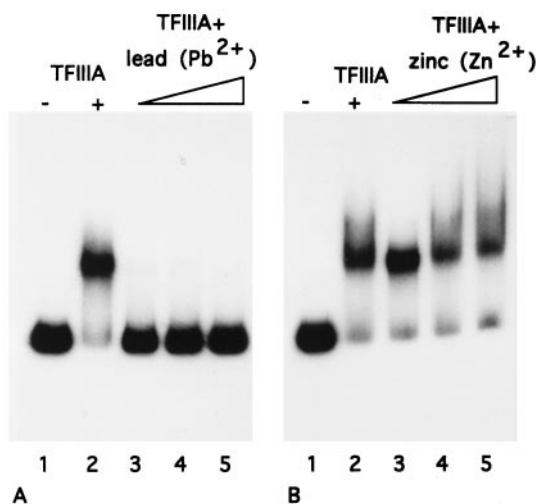


Fig. 1. Gel shift analysis of lead and zinc ion effects on TFIIIA-DNA interaction. TFIIIA isolation from immature *X. laevis* ovarian tissue, ³²P end-labeling of the *X. borealis* somatic 5S RNA gene, metal incubations, DNA-binding reactions, polyacrylamide gel electrophoresis, and autoradiography were performed as described in *Materials and Methods*. Reactions electrophoresed in lanes 1 and 2 were performed with 1 nM end-labeled 5S gene incubated in the absence or presence of 10 nM TFIIIA, respectively. TFIIIA samples (200 nM) were preincubated 20 min with 10, 20, or 30 μM lead ions (Pb²⁺) (A) or 10, 20, or 30 μM zinc ions (Zn²⁺) (B); the final TFIIIA and DNA concentrations used in the 5S gene-binding reactions electrophoresed in lanes 3 to 5 were 10 and 1 nM, respectively.

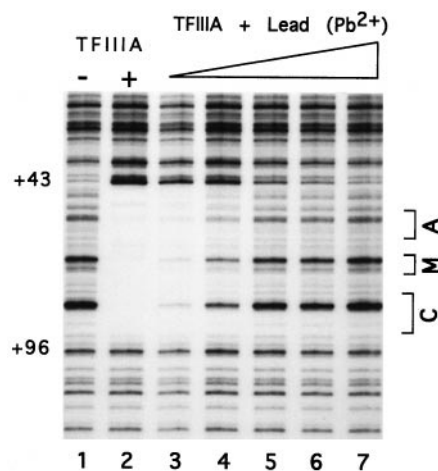


Fig. 2. DNase I protection autoradiogram of lead inhibition of TFIIIA binding to the 5S RNA gene. TFIIIA isolation, 5S gene end-labeling, metal incubations, DNA binding, DNase I protection reactions, polyacrylamide gel electrophoresis, and autoradiography were described in *Materials and Methods*. The nucleotide positions marked on the left margin (+43 and +96) are on the coding strand of the 5S gene and are relative to the +1 transcription start site. The locations of the transcriptional promoter C-box, intermediate element (M), and A-box on the 5S gene ICR are indicated on the right margin. TFIIIA-DNA binding and DNase protection reactions electrophoresed in lanes 1 and 2 were performed with 1 nM end-labeled 5S gene in the absence or presence of 10 nM TFIIIA. TFIIIA (200 nM) used in the reactions electrophoresed in lanes 3 to 7 was preincubated with 2.5, 5, 10, 20, or 30 μM lead ions (Pb²⁺).

is consistent with an inhibitory mechanism in which the cadmium ions were directly binding the TFIIIA zinc fingers (Hanas and Gunn, 1996). A similar experiment was performed with lead ion inhibition of TFIIIA in the present study, which is exhibited in the autoradiogram in Fig. 4. Lanes 4 and 5 are control TFIIIA-dependent DNase I protection patterns in the presence and absence of 7S particle-specific 5S RNA, respectively; note the lack of TFIIIA binding is observed in the presence of 5S RNA (no DNase I protection from nucleotides +43 to +96, lane 4) but not in the absence of 5S RNA (lane 5). This result indicates the 5S RNA is preventing the TFIIIA zinc fingers from binding the 5S gene. Lanes 6 and 7 exhibit the same experiment although with initial exposure of the 7S particle to 30 μ M lead ions; the same result is observed as in lanes 4 and 5. This demonstrates that the 7S particle-specific 5S RNA was able to protect the TFIIIA from lead ion inhibition as evidenced by DNA binding after removal of the 5S RNA by RNase digestion (lane 7). The positive controls for TFIIIA binding and lead inhibition of binding are in lanes 2 and 3, respectively.

The results in Fig. 4 indicate lead ions are inhibiting the zinc finger-DNA binding function of TFIIIA. To see if zinc ions in solution can competitively block this finger inhibition, increasing concentrations of zinc ions were added with constant amounts of lead ions in a TFIIIA incubation reaction. The TFIIIA was then assayed for DNA binding and the results are exhibited in the autoradiogram in Fig. 5. The lead ions are still able to inhibit TFIIIA zinc fingers in the presence of increasing amounts of zinc ions as evidenced by the loss of TFIIIA-dependent DNase I protection on the 5S gene ICR (lanes 4–6). This result suggests that the lead ions are not simply competing with and replacing the zinc ions in the finger metal coordination sphere but rather inhibiting finger structure by another mechanism.

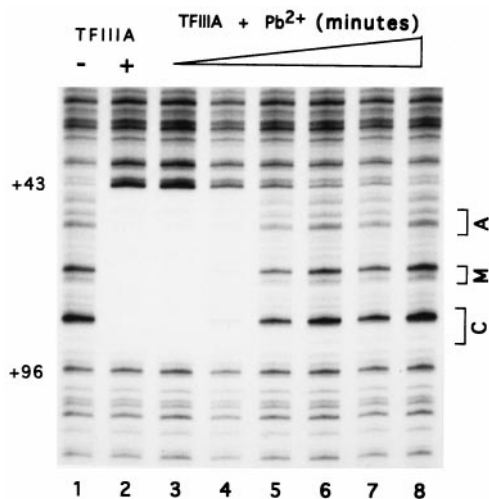


Fig. 3. Time dependence of lead inhibition of TFIIIA interaction with the 5S gene. Experimental components, TFIIIA reactions, analyses, and 5S gene structural designations are described in *Materials and Methods* and in the Fig. 1 legend. TFIIIA (200 nM) used in reactions electrophoresed in lanes 3 to 8 was preincubated with 30 μ M lead ions (Pb^{2+}) for 0.5, 1, 2.5, 5, 10, or 20 min, respectively. The DNase I protection reactions electrophoresed in lanes 3 to 8 took place with 1 nM end-labeled 5S gene and 10 nM lead-treated TFIIIA. The reactions electrophoresed in lanes 1 and 2 were performed with 1 nM end-labeled 5S gene in the absence or presence of 10 nM TFIIIA. All TFIIIA-DNA-binding reactions took place for 1 min before DNase I addition.

Millimolar Concentrations of Arsenic Do Not Inhibit TFIIIA Binding to 5S Gene. Arsenic, mercury, and zinc also exhibit specificity for binding thiols in proteins. Specific DNA binding by TFIIIA is inhibited by micromolar concentrations of cadmium (Hanas and Gunn, 1996) and lead but not by zinc (Fig. 1B). Micromolar amounts of mercury were previously found not to be inhibitory for TFIIIA (Hanas and Gunn, 1996). Because arsenite is highly selective for binding to protein vicinal thiols and was found to inhibit the estrogen receptor transcription factor (Simons et al., 1990), this compound was tested for inhibition of TFIIIA. Figure 6 exhibits the DNase I protection patterns in the presence of TFIIIA exposed to increasing concentrations of arsenate (A) or arsenite (B). In both cases, no inhibition of TFIIIA binding is observed even at very high arsenic concentrations (2 mM, lane 5). It is noted that these arsenic concentrations inhibit the DNase I enzyme as evidenced by the reduction in ^{32}P banding intensities in lanes 4 and 5 compared with the negative and positive controls (lanes 1 and 2). Although zinc ions in the 10- to 30- μ M range do not inhibit TFIIIA-dependent gel shift ability (Fig. 1), zinc ions in the concentration range used in this arsenic analysis do inhibit TFIIIA-dependent DNase I protection (data not shown).

Micromolar Concentrations of Lead Ions Inhibit DNA Binding by Transcription Factor Sp1 But Not

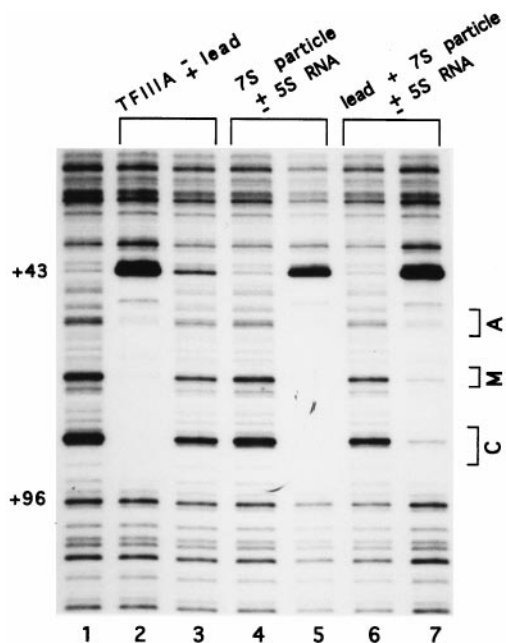


Fig. 4. Lead ion inhibition mechanism involves interaction with TFIIIA zinc finger structure. Isolation of TFIIIA, ^{32}P end-labeling of 5S gene, DNase I digestions, gel electrophoresis, autoradiography, and 5S gene structure designations are described in *Materials and Methods* and in the Fig. 1 legend. Lanes 2 and 3 exhibit DNase I digestion protection patterns for TFIIIA (200 nM) incubated in the absence or presence of 30 μ M lead ions (Pb^{2+}) for 20 min before 20-fold dilution into the DNA-binding reactions; lane 1 is the DNase I digestion pattern of the end-labeled 5S gene. DNase I protection patterns electrophoresed in lanes 4 and 5 are of the TFIIIA (10 nM) interaction with the 5S gene (1 nM) when the factor is bound (lane 4) or unbound (lane 5) to 5S RNA; the RNA is removed in the DNA-binding reaction in lane 5 by addition of RNase A (10 μ g/ml). DNase I patterns in lanes 6 and 7 are from binding reactions where the TFIIIA/5S RNA complex (200 nM) was initially incubated in 30 μ M lead ions (Pb^{2+}) for 20 min before 20-fold dilution into the DNA-binding reaction; 5S RNA was removed in the DNA-binding reaction electrophoresed in lane 7 but not lane 6.

AP2. DNA binding by the Cys₂His₂ zinc finger transcription factor Sp1 in a crude cell extract was previously shown to be inhibited by millimolar levels of lead ions (Zawia et al., 1998). It was necessary therefore to determine what lead concentration range may inhibit a purified recombinant form of this protein. Figure 7A is an autoradiogram of the effects of increasing micromolar lead ion concentrations on the ability of the zinc finger protein Sp1 to bind the SV40 viral DNA promoter region as assayed by DNase I protection. Inhibition of Sp1 DNA binding (loss of DNase I protection) is observed

at 10 and 25 μM lead ion (lanes 4 and 5) but not at 5 μM (lane 3). Significant lead inhibition of TFIIIA binding was observed at 5 μM (Fig. 2A). As a control, the ability of lead ions to inhibit the DNA binding ability of a nonzinc finger protein, AP2, is exhibited in Fig. 7B. AP2 is an RNA polymerase II enhancer binding protein that regulates differential gene expression (Williams et al., 1988). No inhibition of AP2 binding to either demarcated binding site on the SV40 promoter region is observed at the 5 to 25 μM lead ion level in Fig. 7B (lanes 3–5) or at higher lead concentrations in the 50- to 100-μM range (not shown).

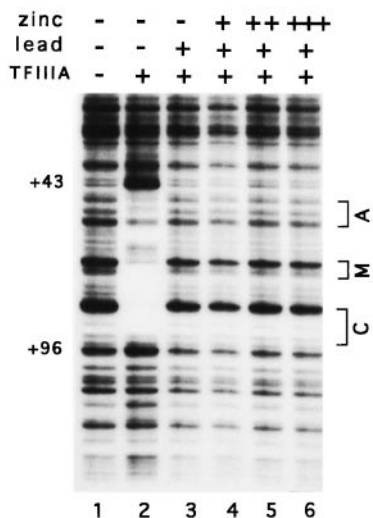


Fig. 5. Added zinc ions do not block lead inhibition of TFIIIA binding to the 5S RNA gene. Isolation of TFIIIA, ³²P end-labeling of the 5S gene, DNA-binding reactions, DNase I digestions, gel electrophoresis, autoradiography, and 5S gene structure designations are described in *Materials and Methods* and in the Fig. 1 legend. DNase I digestions electrophoresed in lanes 1 and 2 were of TFIIIA-DNA-binding reactions containing 1 nM end-labeled 5S gene in the absence or presence of 10 nM TFIIIA. TFIIIA (200 nM) used in the reactions electrophoresed in lanes 3 to 6 was preincubated 20 min with 20 μM lead chloride before 20-fold dilution into the DNA-binding reactions; TFIIIA used in lanes 4 to 6 also was preincubated with 20, 40, or 60 μM zinc chloride, respectively.

Discussion

The amino acid sequence of *Xenopus* TFIIIA revealed a structure of nine repetitive domains beginning near the amino terminus of the protein, each of ~30 amino acids with two cysteines and two histidines for zinc binding; these domains were termed “zinc fingers” (Ginsberg et al., 1984; Miller et al., 1985). Significantly, human TFIIIA has an identical overall zinc finger structure and function to frog TFIIIA (Drew et al., 1995; Ogilvie and Hanas, 1997). Mammalian genomes are estimated to contain at least 1000 distinct genes coding for TFIIIA-type Cys₂His₂ zinc finger proteins (Berg and Shi, 1996). The role of zinc in TFIIIA-type proteins is to hold the finger in the proper conformation for DNA interactions because removal of zinc by chelation, as was first demonstrated for TFIIIA, results in a protein conformational change and loss of specific DNA binding (Hanas et al., 1988). In addition, single amino-acid changes via site-directed mutagenesis in the zinc coordination sphere of TFIIIA fingers also led to loss of specific DNA binding (Smith et al., 1991). These two results demonstrated that structural alterations in the zinc coordination spheres of TFIIIA-type zinc fingers can significantly disrupt the DNA binding mechanism. Chemical agents such as electrophilic xenobiotics that interact with cysteine residues are potential disrupters of zinc finger structure and function. Previous work demon-

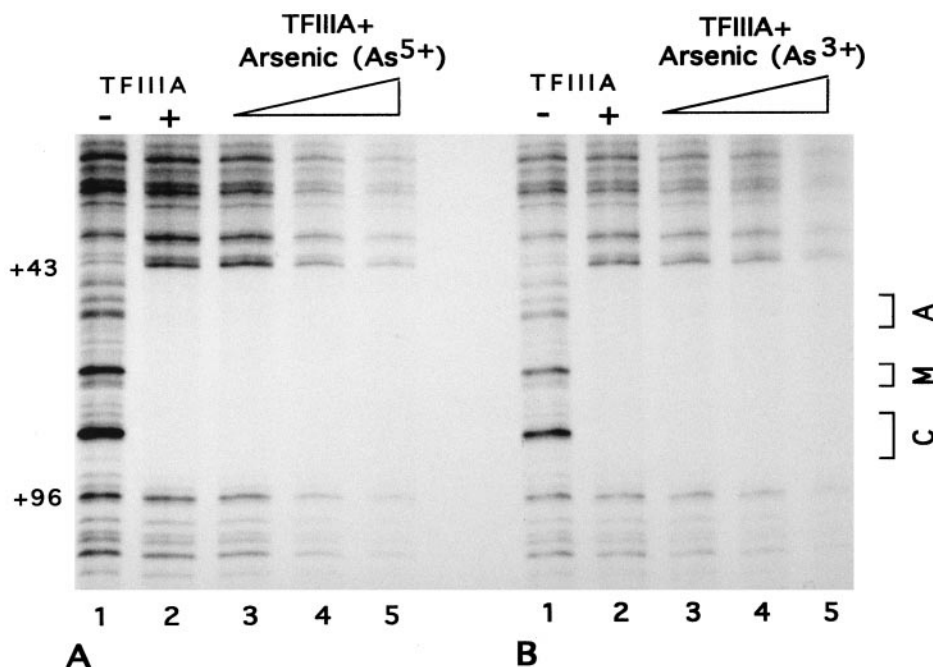


Fig. 6. Arsenic compounds do not inhibit TFIIIA binding to the 5S RNA gene. Isolation of TFIIIA, ³²P end-labeling of the 5S gene, DNA-binding reactions, DNase I digestions, gel electrophoresis, autoradiography, and 5S gene structure designations are described in *Materials and Methods* and in the Fig. 1 legend. DNase I digestions electrophoresed in lanes 1 and 2 were of TFIIIA-DNA-binding reactions containing 1 nM end-labeled 5S gene in the absence or presence of 10 nM TFIIIA. TFIIIA (200 nM) used in the reactions electrophoresed in lanes 3 to 5 was preincubated 20 min with 0.5, 1, or 2 mM arsenate ions (A) or arsenite ions (B) before DNA-binding reaction (15 min, 10 nM TFIIIA and 1 nM 5S gene) and DNase I digestion.

strated that micromolar amounts of cadmium were able to disrupt zinc finger function in TFIIIA (Hanas and Gunn, 1996).

Cadmium and arsenic ions have selective affinity for closely spaced thiol groups where they can bind one metal ion (Simons et al., 1990). This mechanism may exist for lead as well because at 5 to 10 μM , these ions were found to inhibit TFIIIA zinc finger structure (Fig. 2). The sensitivity and the speed of that inhibition (5 min, Fig. 3) may result from direct interaction with a vicinal orientation of thiols in the zinc finger structure. Such an orientation is known to exist in the crystallographic structure of TFIIIA-type zinc fingers (Pavletich and Pabo, 1993). However, excess exogenous zinc ions do not reverse the lead inhibition (Fig. 5) so a simple competitive inhibition model does not appear to hold. It is of interest that even millimolar concentrations of arsenite do not inhibit TFIIIA zinc fingers (Fig. 6). This surprising result suggests that the overall finger structure may determine which metal ions can gain access to its zinc coordination sphere. This differential access is likely to be true when comparing zinc finger proteins from different structural families such as TFIIIA and the steroid hormone receptors that have Cys₂Cys₂ metal coordination spheres. For example, arsenite inhibits steroid hormone receptor DNA binding (Simons et al., 1990) but not TFIIIA binding (Fig. 6). Within members of the TFIIIA superfamily, sensitivities to xenobiotic metals may be similar because lead ions were shown to inhibit the Cys₂His₂ transcription factor Sp1 in a similar although slightly higher micromolar concentration range (Fig. 7A). DNA binding by Sp1 also is sensitive to micromolar amounts of cadmium ions (data not shown). An important control experiment in these xenobiotic metal experiments is the observed lack of lead inhibition of the transcription factor AP2 (Fig. 7B). The amino acid sequence of this protein reveals no cysteine-rich finger motifs (Williams et al., 1988). In addition, AP2 DNA binding is not sensitive to cadmium ion inhibition at micromolar concentrations (data not shown).

The DNA binding mechanism of TFIIIA was previously shown to involve three groups of three fingers each, with the

N-terminal group binding the C-box at the 3' end of the ICR, the middle group binding the M-box or intermediate element in the middle of the ICR, and the C-terminal group binding the A box at the 5' end of the ICR. (Hanas et al., 1989). In vitro mutagenesis experiments demonstrated that disruption of the zinc coordination spheres either by changing individual amino acids or by amino acid deletions caused differences in DNA-binding inhibition, depending upon which group of fingers was mutated (Hanas et al., 1989; Smith et al., 1991). Mutation of the middle or C-terminal finger groups still allowed the N-terminal group to afford DNase I protection of the C-box region. However, mutation of the N-terminal group resulted in complete inhibition of DNase I protection over the entire ICR (+43 to +96) by the middle and C-terminal groups as well as by the N-terminal group. Therefore, the complete inhibition of TFIIIA-dependent DNase I protection by lead ions along the entire ICR (Fig. 2) indicates that the lead ions are minimally altering the structure of the N-terminal group of fingers. If just the middle or C-terminal fingers were affected, DNase I protection of the C-box region should have been observed and was not. With respect to lead inhibition of Sp1 binding, this protein has three Cys₂His₂ zinc fingers and all appear to be inhibited because no partial DNase I footprint is apparent (Fig. 7A).

The family of Cys₂His₂ zinc finger factors is the largest known protein superfamily in living systems (Henikoff et al., 1997). Different TFIIIA-type Cys₂His₂ zinc finger proteins vary in their finger number, structure, and DNA binding specificity (Berg and Shi, 1996). This family of proteins is only found in eukaryotes and their number and complexity have increased during evolution. Although the precise functions of most of these proteins are not known, their nucleic acid binding potential suggests they have roles in regulating gene expression, signal transduction, cell growth and differentiation, and/or chromosome structure. Lead ions are known to accumulate in cell nuclei where they interact with chromatin and other nuclear proteins (Hitzfeld and Taylor, 1989). The micromolar sensitivity to lead, the rapid inhibition kinetics, and other molecular information obtained from

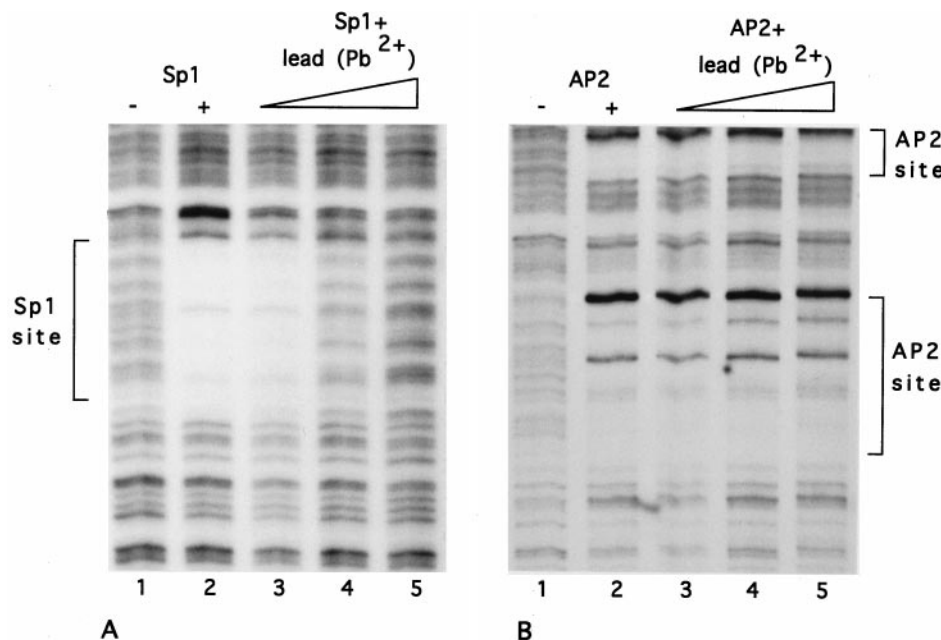


Fig. 7. Lead ion inhibition of Cys₂His₂ zinc finger protein Sp1. DNase I protection experiments with transcription factors Sp1, AP2, and the SV40 viral DNA promoter were performed as described in *Materials and Methods*. Binding sites for Sp1 and AP2 on the SV40 early promoter are designated in the figure margins. Electrophoresed in lane 1 is the DNase I digestion pattern of the SV40 ³²P end-labeled promoter DNA. Electrophoresed in lanes 2 to 5 (A) are the DNase I digestion patterns from Sp1-DNA-binding reactions in which two footprinting units of Sp1 (Promega Life Sciences) were incubated 20 min with 0, 5, 10, or 25 μM lead ions (Pb²⁺), respectively, before DNA addition (10,000 cpm); binding reactions with SV40 DNA were incubated 15 min followed by DNase I digestion. Lanes 2 to 5 (B) are identical reactions except with the AP2 transcription factor.

this present work on TFIIIA and Sp1 increase the likelihood that members of the Cys₂His₂ family of cellular zinc finger proteins are at risk for lead toxicity in cell nuclei in vivo. Higher organisms have evolved cysteine-rich metallothionein proteins to act as xenobiotic cellular "mops," possibly to protect critical cysteines in Cys₂His₂ regulatory factors as well as in other proteins. However, as the environmental load of such xenobiotics is increased above levels found in nature, cellular defense mechanisms may become limiting. This scenario could result in increased incidence of lead-related genetic diseases (Johnson, 1998). Our results with TFIIIA and Sp1 suggest that these as well as related cysteine- and histidine-rich regulatory proteins involved in transcription and signal transduction are potentially at risk in vivo, especially at low concentrations of lead that can be further concentrated in the cell nucleus. If such factors are involved in regulating transcription responsible for normal cell growth or differentiation for example, their inhibition by lead could result in abnormal cell growth. Lead is known to induce cancers in animal models (Goyer, 1996). Therefore, in these models, lead may be acting as an epigenetic tumor promoter inducing cell proliferation as opposed to acting simply as a DNA mutagen. Zinc fingers should be included as targets in possible mechanisms of lead-induced disease processes.

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