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Inhibition of zinc finger protein–DNA interactions by sodium selenite

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Abstract

Sodium selenite and sodium selenate were analyzed for their ability to alter the DNA binding mechanisms of the Cys_2His_2 zinc finger proteins, transcription factor IIIA (TFIIIA) and Sp1. TFIIIA is a positive regulator of 5S ribosomal RNA synthesis, and Sp1 is involved in cell proliferation and invasiveness. As assayed by DNase I protection, the interaction of the DNA binding domain of TFIIIA with the 5S ribosomal gene was inhibited by 25 μM selenite ions but not by 250 μM selenate ions. Selenite inhibition kinetics of TFIIIA progressed to completion in about 5 min. Preincubation of free TFIIIA with selenite resulted in DNA binding inhibition, whereas preincubation of a TFIIIA/5S RNA complex with selenite did not. Since 5S RNA binds to the TFIIIA DNA binding domain, this result is consistent with an inhibition mechanism via selenite binding to that region of this protein. Inhibition was not readily reversible and occurred in the presence of an excess of β-mercaptoethanol; elevated amounts of dithiothreitol mitigated the inhibitory effect. Significantly less selenite (2.5–5 μM) inhibited the specific DNA binding of transcription factor Sp1 to the simian virus 40 (SV40) early promoter/enhancer. The selenite inhibition kinetics of Sp1 were fast, going to completion in about 1 min. SV40 DNA binding by the non-zinc finger transcription factor AP-2 was not inhibited by selenite. Inhibition of Cys_2His_2 zinc finger proteins by micromolar amounts of selenite points to additional mechanisms for selenite-induced diminution of cell growth and anticancer activity.

Keywords: Selenium; Transcription factor–DNA interactions; Zinc finger proteins; Small molecule inhibitors; Cancer

1. Introduction

Small molecules have proven useful as probes for the mechanism of action and structure/function of DNA binding proteins involved in the regulation of gene transcription [1–3]. Such studies have added significance when the small molecules have physiological consequences on animal cells, e.g. retinoids or xenobiotic metal ions [4–6]. The essential trace element selenium affects the transcription machinery in cells [7,8]. Selenite, an oxyanion form of selenium, was shown previously to inhibit the DNA binding mechanisms of both AP1 and NF-κB transcription factors *in vitro* [9–12]. Micromolar amounts of selenite inhibit the growth of, and induce apoptosis in, human colonic carcinoma cells [13], brain tumor cells [14], and breast cancer cells [15]. Sodium selenite inhibits 7,12-dimethylbenz(a)anthracene (DMBA)-induced rat mammary tumors [16], human fibrosarcoma tumor cell invasiveness [12], and pulmonary metastasis of melanoma cells in mice [17]. Selenium in the form of selenocysteine is found in a variety of mammalian enzymes *in vivo* and is essential for their activity [18].

With respect to the molecular mechanisms of selenite action, the general hypothesis is that selenite and other selenium compounds are involved in thiol reactions with protein sulfhydryl groups and that these interactions inhibit protein activity [19]. This inhibition, in turn, is likely responsible for the physiological effects observed: inhibition of cell growth and induction of apoptosis. Other proteins whose activity was shown previously to be inhibited by selenite include c-Jun N-terminal kinase/stress-activated protein kinase [20], and caspase-3 [21]. A major mechanism for the anticancer effects of selenium is hypothesized to be inhibition of transcription factors and downstream growth regulatory pathways [9–12]. The largest transcription factor superfamily in vertebrates is composed of Cys_2His_2 zinc binding proteins typified by

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Abbreviations: TFIIIA, transcription factor A for RNA polymerase III; Cys_2His_2, two cysteine and two histidine amino acids; ICR, internal control region; S, sedimentation constant; DNase I, deoxyribonuclease I, DEAE, diethylaminoethyl; RNase A, ribonuclease A; DTT, dithiothreitol; SV40, simian virus 40.

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TFIIIA [22–24]. TFIIIA is a positive regulator of 5S ribosomal RNA synthesis and ribosome biogenesis in eukaryotic cells [25]. Active ribosome synthesis is essential for cell growth. TFIIIA binds specifically to the ICR of the 5S RNA gene and initiates RNA polymerase III transcription complex formation on the gene [26]. The structure and function of TFIIIA were shown previously to be sensitive to cadmium and lead ions, which display affinity for sulfhydryl groups [5,27]. Sp1 is an RNA polymerase II-specific transcription factor that has three Cys_2His_2 zinc binding domains in its C-terminal region, which bind to GC-rich regions in polymerase II promoters [28]. This protein plays a prominent role in stimulating cell growth and invasion [29]. Members of the cysteine- and histidine-rich TFIIIA superfamily are hypothesized to be potential targets for selenite and other selenium compounds. The purpose of the present study was to elucidate the effects of selenium oxyanions on the function of Cys_2His_2 zinc finger proteins.

2. Materials and methods

2.1. Isolation of TFIIIA

Immature ovarian tissue obtained from 4 to 5 cm female *Xenopus laevis* (Nasco) was homogenized in buffer A [50 mM Tris–HCl (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride]. The homogenate was centrifuged for 20 min at 10,000 *g*, and aliquots were layered onto 15–30% (v/v) glycerol gradients in buffer A. Gradients were centrifuged for 24 hr at 34,000 rpm in a Beckman SW41 rotor; all manipulations were performed at 0–4°. The 7S particle complex containing TFIIIA and 5S ribosomal RNA sedimented faster than the 5S hemoglobin molecule and was identified by UV absorption of gradient fractions. The 7S particles were further purified to 90% homogeneity by DEAE ion exchange chromatography as described previously [22]. 5S RNA was removed from TFIIIA by digestion of the 7S particle (20 μg/mL) with RNase A (10 μg/mL) in buffer B [20 mM Tris–HCl (pH 7.6), 320 mM KCl, 2 mM $MgCl_2$, 0.2 mM β-mercaptoethanol, 0.1% (v/v) Nonidet P-40 (NP-40) detergent] for 30 min at room temperature and then placed on ice.

2.2. Transcription factor–DNA binding reactions

A 303 bp DNA insert containing the 120 bp *Xenopus borealis* somatic 5S ribosomal RNA gene was ^{32}P 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 phenol–chloroform extraction, the 5′ ends were rephosphorylated with polynucleotide kinase and [γ-^{32}P]ATP. The end-labeled plasmid was ethanol precipitated, digested with *Eco*RI, 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 selenium oxyanions on TFIIIA function, TFIIIA in buffer B was diluted 5-fold in buffer C [20 mM Tris–HCl (pH 7.6), 70 mM NH_4Cl, 7 mM $MgCl_2$, 0.2 mM β-mercaptoethanol, 0.1% (v/v) non-ionic detergent NP-40] and incubated at room temperature with sodium selenite or sodium selenate (Sigma Chemical Co.) at the concentrations and times indicated in the figure legends. TFIIIA was then diluted (20-fold) to 10 nM in the same buffer minus the oxyanions, end-labeled 5S gene was added to a final concentration of 1 nM (about 10^4 cpm), and the binding reaction (20 μL) took place for 15 min. DNA binding reactions with transcription factors Sp1 and AP-2 (obtained from Promega Life Sciences) were performed in similar fashion using as a template the SV40 promoter DNA obtained from Promega and end-labeled according to the instructions of the vendor.

2.3. DNase I protection assay

DNase footprint analyses to identify specific DNA–protein interactions were performed using a previously described procedure [30]. Briefly, DNase I was added to the binding reactions to a final concentration of 1–2 μg/mL and incubated for an additional minute at room temperature. The digestion was terminated by the addition of 100 μL of stop buffer [20 mM Tris–HCl (pH 7.6), 1 mM EDTA, 0.1% SDS, 30 μg sonicated salmon sperm DNA/mL]. The DNA was ethanol precipitated and resuspended in 4 μL 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° for 5 min, and then electrophoresed through a 7 M urea–7% (w/v) polyacrylamide gel until the xylene cyanol marker migrated 2/3 down the gel. Then the gel was transferred to blotting paper, dried, and subjected to autoradiography overnight at −70° using Kodak XAR-5 film.

2.4. Transcription assays

In vitro transcription of the human adenovirus VA RNA gene [31] and the cytomegalovirus immediate early gene were performed using the phosphocellulose 0.8 M KCl fraction of the human nuclear extract as a source of RNA polymerase II and III and associated factors [32]. Transcription assays (25 μL) contained 10 mM HEPES (pH 7.9), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM β-mercaptoethanol, 0.1 mM EDTA, 10% glycerol, 400 μM (GTP, ATP, UTP), 1.6 μM CTP, 10 μCi [α-^{32}P] CTP, 3 μg phosphocellulose fraction; sodium selenite and template DNA additions are described in the figure legends. The phosphocellulose fraction and sodium selenite were incubated in the transcription buffer for 30 min at 30° in the absence of nucleotides and DNA and then for an additional hour in

the presence of nucleotides and DNA. The reactions were quenched with 100 μL of SDS buffer [0.5% SDS, 0.3 M Tris–HCl (pH 7.4), 0.3 M sodium acetate, 2 mM EDTA, 3 μg/mL of yeast tRNA], phenol–chloroform extracted, and ethanol precipitated. Samples were resuspended in 10 mM EDTA (pH 8.0), 98% deionized formamide, 0.04% xylene cyanol/bromophenol blue and heated at 65°. Samples were electrophoresed through a 7% polyacrylamide gel containing 7 M urea and 89 mM Tris, 110 mM boric acid, 1 mM EDTA, pH 8.0. Gels were subjected to autoradiography overnight using Kodak XAR-5 film.

3. Results

3.1. Inhibition of TFIIIA–DNA interactions by micromolar amounts of selenite oxyanions

To elucidate whether sodium selenite or sodium selenate has the ability to inhibit the specific binding of TFIIIA to the 5S RNA gene, we used the DNase I protection assay, which allows a ''visualization'' of the specificity and location of the TFIIIA binding event on the 5S gene (Fig. 1). TFIIIA in this assay bound to and protected 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 nicked this area of the gene as evidenced by the large number of ^{32}P DNA fragments in this region (lane 1). Exposing TFIIIA to 10 μM selenite ions ($[SeO_3]^{2-}$) resulted in some loss of DNase I protection (lane 3); 25 μM selenite ions inhibited specific TFIIIA binding (lane 4). The inhibition of TFIIIA-dependent DNase I protection occurred over the entire factor binding region, from +43 to +96 on the 5S gene where all nine zinc fingers of TFIIIA interact.

Significantly, selenate ions ($[SeO_4]^{2-}$) were not inhibitory for TFIIIA binding to the 5S RNA gene ICR as concentrations from 10 to 250 μM (lanes 3–7, Fig. 1B) did not lead to the loss of TFIIIA-dependent DNase I protection exhibited in lane 2. It was noted that the DNase I protection reactions contain excess thiol reagent, which is necessary for TFIIIA activity. These excess thiol groups may interfere with the selenite ion inhibition, thus increasing the selenite concentration necessary for TFIIIA inhibition.

3.2. Time course of selenite inhibition of TFIIIA binding to the 5S RNA gene

In the experiments depicted in Fig. 1, TFIIIA was exposed to various concentrations of selenite ions for 20 min at room temperature prior to assaying for DNA binding, which takes place in an additional 15-min incubation. To elucidate the time course of this inhibition, a

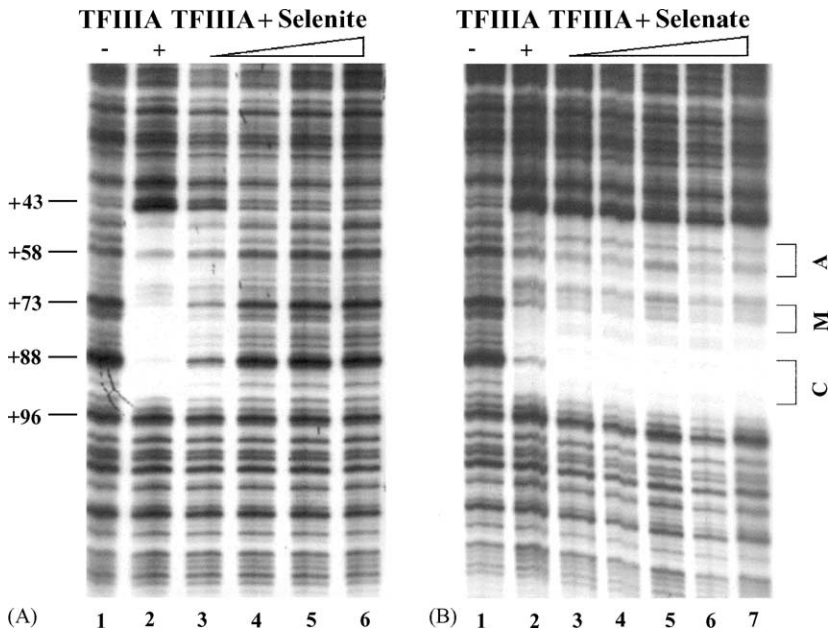

Fig. 1. DNase I protection autoradiogram of selenite inhibition of TFIIIA binding to the 5S RNA gene. TFIIIA isolation, 5S gene end-labeling, metal incubations, DNA binding, DNase I protection reactions, polyacrylamide gel electrophoresis, and autoradiography are described in Section 2. The nucleotide positions marked in 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 in the right margin. TFIIIA–DNA binding and DNase I 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 3–6, panel A, was preincubated with 10, 25, 50, or 100 μM selenite; reactions involving TFIIIA incubated with 10, 25, 50, 100, or 250 μM selenate are exhibited in lanes 3–7, panel B.

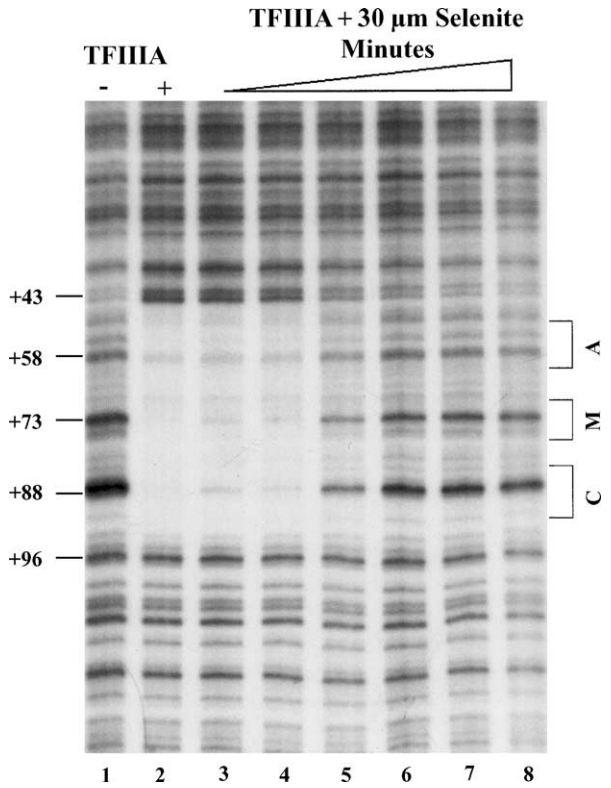

Fig. 2. Time-dependence of selenite inhibition of TFIIIA interaction with the 5S gene. Experimental components, TFIIIA reactions, analyses, and 5S gene structural designations are described in Section 2 and the legend of Fig. 1. TFIIIA (200 nM) used in reactions electrophoresed in lanes 3–8 was preincubated with 30 µM selenite ions for 1, 2.5, 5, 10, 20, or 30 min, respectively. The DNase I protection reactions electrophoresed in lanes 3–8 took place with 1 nM end-labeled 5S gene and 10 nM selenite-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 untreated TFIIIA. All TFIIIA–DNA binding reactions took place for 1 min prior to the addition of DNase I.

kinetic analysis of selenite exposure was performed to gain additional information about the inhibition mechanism; a rapid inhibition would suggest a direct and specific interaction with selenite on TFIIIA. TFIIIA was pretreated with selenite ions for various times before addition to the DNA binding reaction, which was shortened to 1 min. Fig. 2 is an autoradiogram that illustrates this kinetic analysis of the DNA binding inhibition as assayed by DNase I protection. Very little selenite inhibition of TFIIIA-dependent DNase I protection was observed at the 1- and 2.5-min exposure time points (lanes 3 and 4), whereas substantial inhibition (loss of protection from nucleotides +43 to +96) was observed at the 5-min time point (lane 5).

3.3. Selenite inhibition of the DNA binding domain of TFIIIA

Although the zinc finger DNA binding domain of TFIIIA comprises 3/4 of the structure (30 kDa out of 40 kDa total mass), non-finger regions could possibly be involved in this selenite inhibition. The TFIIIA DNA binding domain binds 5S RNA in a mechanism competitive with 5S gene DNA [33]. Previously, cadmium and lead 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 is consistent with an inhibitory mechanism in which the cadmium and lead ions were binding directly to the TFIIIA zinc finger DNA binding domain [5,27]. A similar experiment was performed with selenite ion inhibition of TFIIIA in the present study, which is exhibited in the autoradiogram in Fig. 3. Lanes 4 and 5 are control TFIIIA-dependent DNase I protection patterns in the presence and absence of 7S particle-specific 5S RNA, respectively; a lack of TFIIIA binding was 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 that the 5S RNA is preventing the TFIIIA DNA binding domain from binding the 5S gene. Lanes 6 and 7

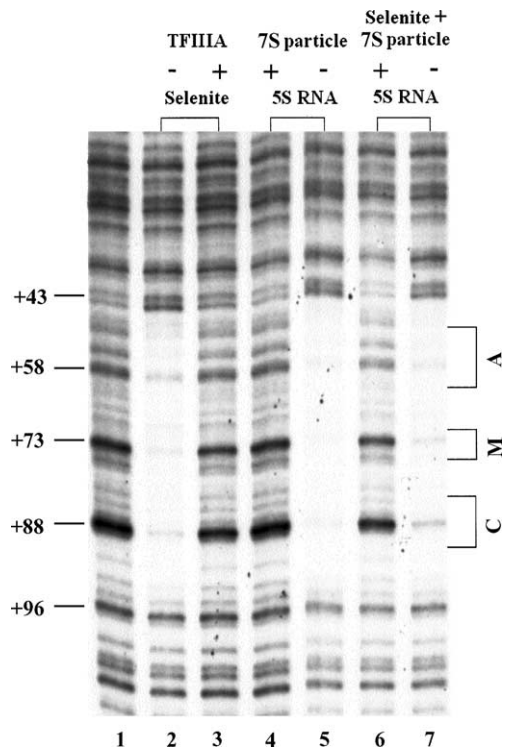

Fig. 3. Selenite inhibition of the TFIIIA DNA binding domain. Isolation of TFIIIA, ^{32}P end-labeling of the 5S gene, DNase I digestions, gel electrophoresis, autoradiography, and 5S gene structure designations are described in Section 2 and the legend of Fig. 1. Lanes 2 and 3 exhibit DNase I digestion protection patterns for TFIIIA (200 nM) incubated in the absence or presence of 30 µM selenite ions for 20 min prior to 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 was removed in the DNA binding reaction in lane 5 by addition of RNase A (10 µg/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 µM selenite ions for 20 min prior to 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.

exhibit the same experiment although with initial exposure of the 7S particle to 30 μM selenite ions; the same result was observed as in lanes 4 and 5, demonstrating that the 7S particle-specific 5S RNA was able to protect the TFIIIA from selenite 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 selenite inhibition of binding are in lanes 2 and 3, respectively. The DNase I protection observed in lane 7 by the 30 μM selenite-treated 7S particle followed by 20-fold dilution and RNase removal of 5S RNA in the footprint reaction indicates that the diluted selenite is not inhibiting binding at the DNA level and that the inhibition at the protein level is not readily reversible. To see if the addition of excess thiol groups to the selenite inhibition reaction mitigates the inhibitory effects on TFIIIA, the inhibition reaction was performed in 0.1 or 1 mM dithiothreitol (Fig. 4). Overall, greater inhibition was observed in the 20–100 μM range in the selenite plus 0.1 mM DTT (lanes 3–7) than in the same concentration range of selenite plus 1 mM DTT (lanes 10–14). It was noted that selenite inhibition of TFIIIA in 0.1 mM DTT was less than in 0.2 mM β-mercaptoethanol (compare Fig. 4 with Fig. 1).

3.4. Selenite ion inhibition of transcription factor Sp1 but not transcription factor AP-2

Fig. 5A is an autoradiogram of the effects of increasing micromolar selenite ion concentration on the ability of the three zinc finger region of recombinant Sp1 to bind the SV40 viral DNA promoter region, as assayed by DNase I protection. Significant inhibition of Sp1 DNA binding (loss of DNase I protection on the SV40 early promoter) was observed at 10 and 25 μM selenite ion concentrations (lanes 3 and 4); sodium selenate up to 100 μM had no effect on Sp1-dependent DNase I protection (not shown). As a control, the ability of selenite ions to inhibit the DNA binding ability of a non-zinc finger protein, AP-2, is shown in Fig. 5B. AP-2 is an RNA polymerase II enhancer binding protein that regulates differential gene expression [34]. No inhibition of AP-2 binding to either demarcated binding site on the SV40 promoter region was observed at the 10–25 μM selenite ion level (lanes 3 and 4) or at higher selenite concentrations in the 50–100 μM range (lanes 5 and 6). Fig. 6 exhibits a lower range concentration–response experiment of selenite inhibition of Sp1 binding (panel A) and an inhibition kinetics experiment (panel B). Selenite ions strongly inhibited Sp1 binding in the 2.5–5 μM range (lanes 5 and 6). With respect to inhibition kinetics, at 5 μM selenite ions, inhibition of Sp1 binding to the SV40 early promoter occurred within 1 min (lane 3, panel B). The concentration and time dependence of selenite inhibition of Sp1 were significantly lower and faster than for TFIIIA.

3.5. Selenite ion inhibition of transcript synthesis by RNA polymerase II and III in vitro

The inhibition of the TFIIIA DNA binding mechanism by micromolar amounts of selenite would be expected to

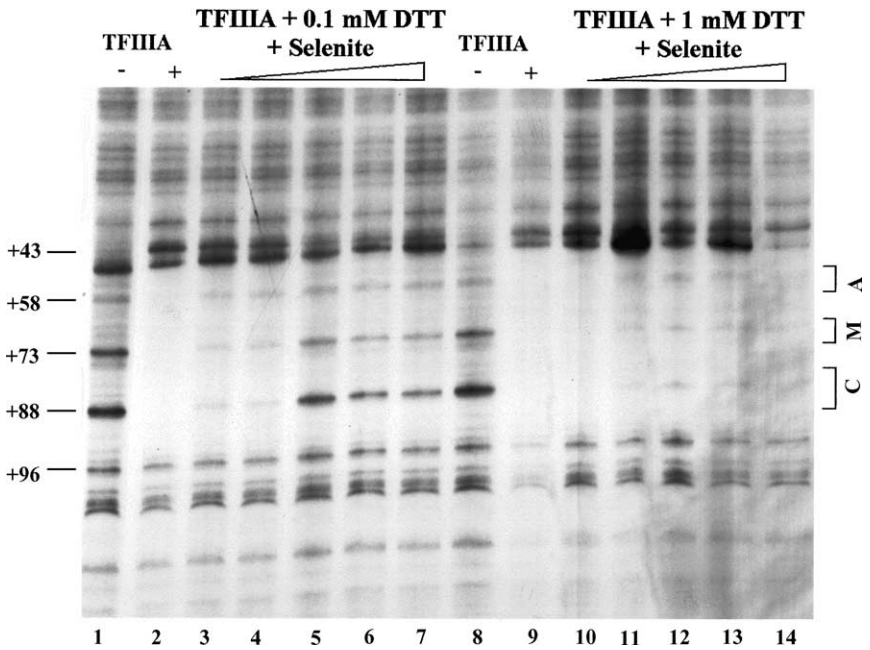

Fig. 4. Mitigation of selenite inhibition of TFIIIA DNA binding by DTT. Isolation of TFIIIA, ^{32}P end-labeling of the 5S gene, DNA binding reactions, DNase I digestions, gel electrophoresis, autoradiography, and 5S gene structure designations are described in Section 2 and the legend of Fig. 1. DNase I digestions electrophoresed in lanes 1, 2 and 8, 9 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 3–7 and 10–14 was preincubated for 20 min with 20, 40, 60, 80, or 100 μM selenite ions plus 0.1 mM DTT (lanes 3–7) or 1 mM DTT (lanes 10–14).

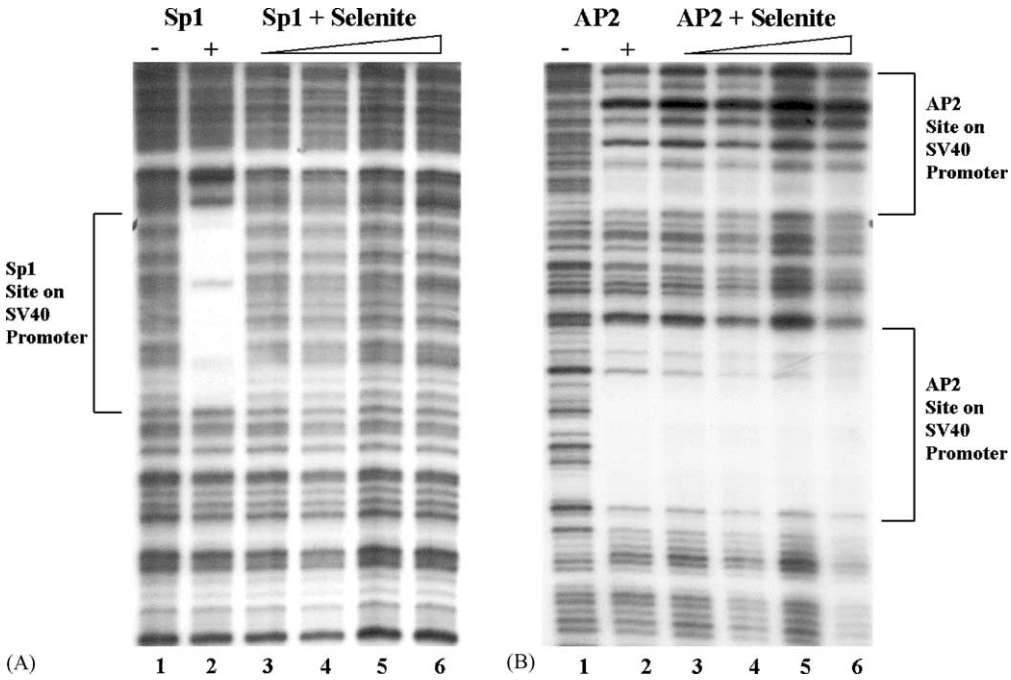

Fig. 5. Selenite ion inhibition of the Cys_2His_2 zinc finger protein Sp1. DNase I protection experiments with transcription factors Sp1, AP-2, and the SV40 viral DNA promoter were performed as described in Section 2. Binding sites for Sp1 and AP-2 on the SV40 early promoter fragment (all obtained from Promega Life Sciences) are designated in the figure margins. Electrophoresed in lane 1 is the DNase I digestion pattern of the SV40 ^{32}P end-labeled promoter DNA; electrophoresed in lanes 2–6, panel A, are the DNase I digestion patterns from Sp1–DNA binding reactions in which two footprinting units of Sp1 were incubated for 20 min with 0, 10, 25, 50, or 100 μM selenite ions, respectively, prior to DNA addition (10,000 cpm); binding reactions with SV40 DNA were incubated for 15 min followed by DNase I digestion. Lanes 2–6, panel B, are similar reactions except that the AP-2 transcription factor was assayed.

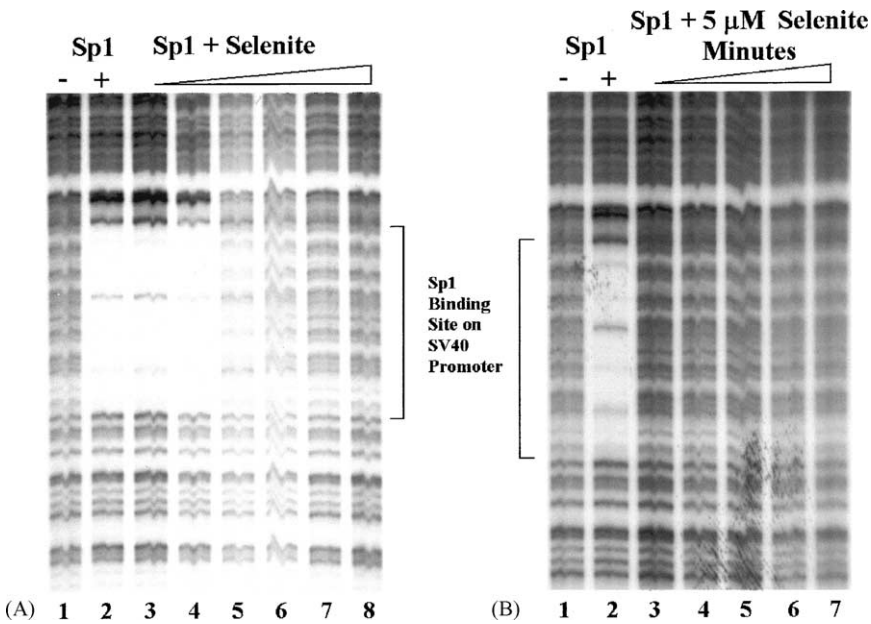

Fig. 6. Concentration–response and kinetic experiments of selenite inhibition of transcription factor Sp1 binding to DNA. DNase I protection reactions with transcription factor Sp1 and the SV40 viral DNA early promoter fragment (Promega Life Sciences), gel electrophoresis, and autoradiography were performed as described in Section 2. Electrophoresed in lanes 3–8, panel A, are DNase I digestion patterns from Sp1–DNA binding reactions in which two footprinting units of Sp1 were incubated for 20 min with 0.5, 1, 2.5, 5, 10, or 20 μM selenite ions, respectively, prior to DNA addition (10,000 cpm); binding reactions with SV40 DNA were incubated for 15 min followed by DNase I digestion. Sp1 reactions electrophoresed in lanes 3–7, panel B, were preincubated with 5 μM selenite ions for 1, 2.5, 5, 10, or 20 min, respectively, prior to a 1-min DNA binding reaction followed by DNase I digestion. DNase I digestion reactions electrophoresed in lanes 1 or 2 in both panels are of the end-labeled SV40 DNA in the absence or presence of Sp1.

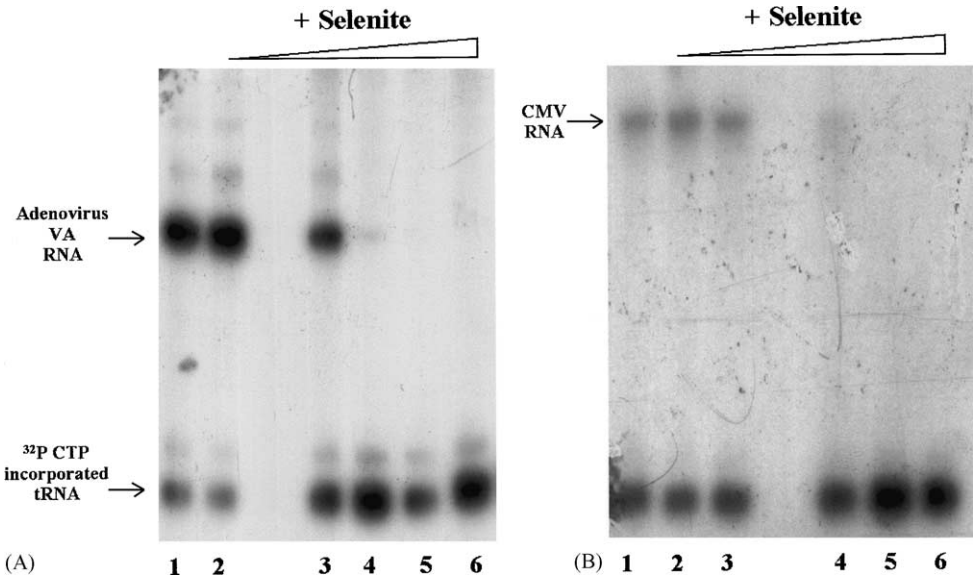

Fig. 7. Selenite inhibition of RNA polymerase III and II transcription *in vitro*. Transcription assays, gel electrophoresis, and autoradiography were performed as described in Section 2. Transcription reactions electrophoresed in lanes 1–6 in both panels contained phosphocellulose column fractions incubated for 30 min at 30° with 0, 2.5, 5, 10, 25, and 50 μM selenite ions, respectively, prior to the addition of nucleotides and transcription templates. Transcription templates used were the adenovirus VA RNA gene-containing plasmid (0.5 μg, panel A) or the cytomegalovirus (CMV) early gene product-containing fragment plasmid (Promega Life Sciences, 0.2 μg, panel B).

result in selenite-dependent inhibition of RNA polymerase III-dependent transcription of the 5S RNA gene. As a possible negative control for this experiment, we examined the ability of selenite to inhibit RNA polymerase III transcription of a gene that did not require TFIIIA; we chose the adenovirus VA RNA gene which only requires TFIIIB and TFIIIC plus RNA polymerase III but no TFIIIA for transcription. We found that synthesis of VA RNA gene product was sensitive to selenite inhibition at about 10 μM (Fig. 7A, lane 4). This concentration also inhibited transcription of the cytomegalovirus early gene product *in vitro*, which is transcribed by RNA polymerase II (Fig. 7B, lane 4).

4. Discussion

Suppression of transcription factor function is hypothesized to be a major biochemical mechanism by which selenite ions inhibit tumor cell growth [9–12]. These protein inhibitions are likely the result of selenite-thiol redox reactions. Selenite ions pass through membrane phosphate or sulfate transporter systems and once in the cell this oxyanion can become involved in sulfuryl transfer reactions with protein thiol groups [35]. A major class of cysteine-rich regulatory proteins are the TFIIIA-type Cys_2His_2 zinc binding proteins [22,23]. These proteins comprise the largest protein superfamily, and members have roles in a wide variety of cellular functions including embryogenesis and development, growth control, and homeostasis [24]. In the present study, we demonstrated that Cys_2His_2 zinc finger proteins (TFIIIA and Sp1) are sensitive to micromolar levels of selenite oxyanions *in vitro*, the first such observation for this important class of transcription factors. These proteins are not sensitive to the oxyanion selenate ($[SeO_4]^{2-}$). Other cell culture and biochemical studies have also observed inhibitory effects with the selenite but not the selenate oxyanions [7,12,36]. The selenate ion is not as strong an oxidizing agent as selenite and does not react with thiol groups [37].

Evidence is presented in this study that the selenite ions are inhibiting the DNA binding domain of TFIIIA. This conclusion comes from the observation that 5S ribosomal RNA, when bound to the TFIIIA DNA binding domain forming the 7S ribonucleoprotein particle, protected the protein from selenite inhibition (Fig. 3, lane 7). Using this assay, a previous study demonstrated that mercuric ions inhibited TFIIIA in the presence of 5S RNA, which indicates mechanistic differences between selenite and mercuric ion action [38]. The kinetics of selenite inhibition of TFIIIA DNA binding go to completion in about 5 min (Fig. 2). The inhibition was not readily reversible, indicative of a strong interaction between the selenite and the DNA binding domain. This rapid inhibition kinetics is also indicative of a direct binding mechanism of selenite ions to the TFIIIA DNA binding domain. Elevated concentrations of DTT reduced the inhibitory effect of selenite ions on the TFIIIA DNA binding mechanism (Fig. 4). This result, as well as the lack of inhibition by selenate, is consistent with a thiol redox mechanism being responsible for the selenite inhibition of TFIIIA DNA binding. The reagent thiol concentration routinely present in these *in vitro* reactions was 0.2 mM, which is at least 10-fold higher than the inhibitory selenite concentration for TFIIIA and 40-fold

higher than for Sp1. This thiol concentration is in the concentration range for *in vivo* levels of glutathione, a major thiol agent available in cells [39]. Therefore, selenite inhibition of TFIIIA-type proteins as well as other thiol-containing proteins *in vivo* may be possible in the relatively high reducing environment present in cells.

Selenite ions were observed to inhibit the interaction of the TFIIIA DNA binding domain with the entire 5S gene ICR as evidenced by DNase I protection (from nucleotides +43 to +96, Figs. 1A, 2–4). The DNA binding mechanism of the RNA polymerase III-dependent factor TFIIIA was shown previously to involve three sub-domains of three fingers each, with the N-terminal sub-domain binding the C-box at the 3′ end of the ICR, the middle sub-domain binding the M-box or intermediate element in the middle of the ICR, and the C-terminal sub-domain binding the A box at the 5′ end of the ICR. [40]. *In vitro* mutagenesis experiments demonstrated that changing individual amino acids or deleting amino acids caused differences in DNA binding inhibition depending upon which sub-domain was mutated [30,41]. Mutation of the middle or C-terminal domains still allowed the N-terminal domain to afford DNase I protection of the C-box ICR region. However, mutation of the N-terminal domain resulted in complete inhibition of DNase I protection over the entire ICR (+43 to +96) by the middle and C-terminal domains as well as by the N-terminal domain. If selenite modification of an amino acid structurally mimics the changing or deletion of that amino acid, then the complete inhibition of TFIIIA-dependent DNase I protection by selenite ions along the entire ICR (Fig. 2) suggests that these oxyanions are minimally altering the structure of the N-terminal sub-domain. If just the middle or C-terminal domains were affected, some DNase I protection of the C-box region of the 5S gene ICR should be observed and was not. The nine-finger TFIIIA DNA binding domain contains cysteine residues other than those involved in zinc coordination; there is one in finger I, one in the linker region between fingers III and IV, one in finger VI, and one in finger IX [23]. These cysteines as well as those involved in zinc coordination are possible targets for the selenite inhibition observed in this study. An alternative explanation for the complete inhibition observed could be the interaction of selenite with any of these as well as other amino acids, resulting in substantial denaturation of the TFIIIA DNA binding domain.

Selenite oxyanions also inhibit the specific DNA binding of another Cys_2His_2 zinc finger protein, the RNA polymerase II-dependent factor Sp1 (Fig. 6A). The Sp1 inhibition occurred at significantly less selenite ion concentration than that found to be inhibitory for TFIIIA (5 μM vs. 25 μM). The kinetics of inhibition were also faster for Sp1 than for TFIIIA, inhibiting to completion in less than 1 min (Fig. 6B). The reasons for these inhibitory differences between Sp1 and TFIIIA are unclear but likely reflect differences in the secondary and tertiary structures in the zinc finger DNA binding domain of the two proteins. Selenite ions may prove useful as a probe to discern such structural differences among members of the Cys_2His_2 zinc finger protein superfamily. With respect to selenite inhibition of Sp1 binding, this protein has a three finger DNA binding domain that was inhibited completely as no partial DNase I footprint was apparent (Fig. 5A). In an important control experiment, transcription factor AP-2, which has no cysteine-rich DNA binding domains, was not inhibited by selenite oxyanions (Fig. 5B). Selenite ions were also shown to inhibit transcription of the adenovirus VA RNA gene (transcribed by RNA polymerase III) and the cytomegalovirus early gene (transcribed by RNA polymerase II) in a HeLa cell nuclear extract fractionated over phosphocellulose (Fig. 7). Potential cysteine-rich, zinc binding proteins in these transcription systems that could be responsible for this inhibition include the large subunit of the respective RNA polymerases [42] and/or the TFIIB/IIIB transcription factors [43].

Collectively, the results presented in this study are consistent with the hypothesis that transcriptional inhibitory mechanisms are involved in cancer chemoprevention by selenite [8,9,12]. The potential inhibition at varying sensitivities of members of the largest superfamily of transcription factors (Cys_2His_2 zinc binding proteins) opens up additional signal transduction pathways (some known, others unknown) for elucidating the mechanisms by which selenite inhibits tumor cell growth and metastasis and induces apoptosis. Inhibition of TFIIIA *in vivo* by selenite would be expected to reduce cell growth as ribosome synthesis is required for such growth. More significantly, the Sp1 inhibition observed in this study at the known tumor-inhibiting concentrations of 2.5–5 μM selenite is consistent with the reported roles of this factor in promoting carcinoma cell growth and invasiveness [29].

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