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Regulation of ZiRF1 and basal SP1 transcription factor MRE-binding activity by transition metals

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Abstract The metal-dependent activation of metallothionein (MT) genes requires the interaction of positive *trans*-activators (MRFs) with metal-regulatory (MRE) regions of MT promoters. In this report, we examined the role of transition metals in modulating the MRE-binding activities of two different MRE-binding proteins: the metal-regulated factor ZiRF1 and the basal factor SP1. We showed the ability of both proteins to interact with a similar sequence specificity with the cognate target site (MRE-S) of another known MRE-binding protein, mMTF1. We next evaluated the role of metal ions in modulating the MRE-binding activity of recombinant ZiRF1 and basal SP1 proteins by measuring the effect of different metal chelators on DNA interaction. We observed a dose-dependent inhibition of the GST-ZiRF1/MRE-binding activity using three different metal chelators: EDTA, 1,10 PHE and TPEN. Interestingly, EDTA treatment failed to inhibit the recombinant SP1 MRE-binding activity while the effect of 1,10 PHE was comparable to that obtained analyzing 1,10 PHE-treated GST-ZiRF1. The MRE-binding complexes detected in cell extracts showed a response to metal chelator treatment very similar to that displayed by the recombinant ZiRF1 and SP1 proteins. The hypothesis of mutual interactions of both basal and metal-regulated transcription factors with the same metal-regulatory regions is discussed.

Key words: Metallothionein; Transcription factor; Transition metal; SP1; Zinc-regulated factor 1

1. Introduction

Metallothioneins (MTs) are small, cysteine-rich proteins which bind metal ions through thiol clusters. MTs are found in all eukaryotes and they are supposed to play an essential role in metal ion homeostasis and/or detoxification (for reviews see [1–3]). Expression of the MT gene is transcriptionally up-regulated upon cell exposure to various agents (glucocorticoids, polypeptide hormones, UV and others) and metal ions belonging to the IB and IIB groups [4,5]. Metal induction of MTs is conferred by conserved, short, 12-bp sequences known as metal-regulating elements (MREs). MREs are present in multiple imperfect copies in all the promoter regions of MT genes [6,7]. Functional analysis of these regions and in vivo genomic footprinting experiments have suggested the metal-dependent interaction of putative *trans*-acting metal-responsive factors (MRFs) with the MREs and that these factors could act as activators of gene transcription and not as repressors [8,9].

Various putative MRFs have so far been identified but the mechanisms which trigger the metal-dependent MT gene expression are still unclear [10–14]. All of these factors bind in a metal-dependent manner and could be responsible for the metal-induced transcription of MTs. The mouse MTF-I [15] is the most extensively studied MRE-binding protein. mMTF1 is a zinc-finger protein that was cloned for its ability to bind the synthetic MRE-S oligonucleotide. Interestingly, the inactivation of the MTF1 gene in mouse ES cells abolished both basal and inducible MT gene expression suggesting a functional role of this factor in the regulation of MT gene expression [16]. We isolated a distinct mouse factor, the zinc-regulated factor ZiRF1, for its ability to activate MRE-driven reporter genes in yeast cells [17]. ZiRF1 has the ability to exhibit zinc-dependent binding in vitro to MREs which are functional in vivo. Recently, we reported that the basal SP1 displays MRE-binding properties similar to ZiRF1 [18]. These results indicated that both metal-regulated and basal factors could interact with the same metal-regulatory sequences. In this report, we examined the role of metal ions in modulating the MRE interactions of both the metal-regulated factor ZiRF1 and the basal transcription activator SP1, and their ability to interact with the MTF1 site, MRE-S.

2. Materials and methods

2.1. Reagents

Chemicals were obtained from E. Merck (Darmstadt, Germany) and Sigma Chemicals (St. Louis, MO, USA). Tissue culture reagents were supplied by Gibco BRL (Inchinan, UK). TPEN was obtained from Molecular Probes Inc. (Eugene, OR, USA). γ-[^{32}P]ATP (specific activity 3000 Ci/mmol) was from Du Pont-New England Nuclear (Boston, MA, USA). Restriction enzymes were purchased from New England Bio Labs (Beverly, MA, USA). Plasmid vectors were obtained from Invitrogen (La Jolla, CA, USA) and Stratagene (La Jolla, CA, USA). Glutathione Sepharose 4B was from Pharmacia (Uppsala, Sweden). Oligonucleotides were synthesized by Primm (Milan, Italy). The human recombinant SP1 was purchased from Promega (Madison, WI, USA).

2.2. Cell culture and media

Mouse L fibroblasts were cultured in MEM supplemented with 10% FBS, 100 U/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine.

2.3. Protein expression and metal chelator treatment

Escherichia coli strain Xl1 blue was transformed with pGEX-ZiRF1 expression vector [18], grown at 37°C in liquid broth (LB) and collected at a density range of OD_{600} = 0.6–0.8. Expression of the GST-

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Abbreviations: CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; GSH, glutathione; GST, glutathione *S*-transferase; IPTG, isopropyl β-D-thiogalactopyranoside; MBC, MRE-binding complex; MEM, minimal essential medium; MRE, metal-regulating element; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; 1,7 PHE, 1,7 phenanthroline; 1,10 PHE, 1,10 phenanthroline; TPEN, tetrakis(2-pyridylmethyl)ethylenediamine

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ZiRF1 fusion protein was obtained by adding 0.1 mM IPTG for 4 h at 30°C to the cell cultures; cells were harvested with 1/20 volume of PBS containing 2 mM DTT and protease inhibitors (2 μg/ml aprotinin, pepstatin A, leupeptin, chymostatin and 1 mM PMSF), sonicated 4×20 s and spun at 4°C, 30 000 rpm for 30 min. The supernatant was then adjusted to 1% Triton and incubated with GSH-Sepharose 4B beads for 1 h at 4°C. Proteins bound to the beads were eluted with a buffer containing 5 mM GSH/50 mM Tris-Cl pH 8.0/100 mM NaCl/2 mM DTT. Further dialysis was used to remove free GSH.

Metal depletion by 1,10 PHE, EDTA and TPEN was obtained by dialysis of proteins in PBS buffer containing metal chelators at the indicated doses for 3 h at 4°C. Chemicals were subsequently removed by a second dialysis with PBS.

2.4. Mobility shift assays

Synthetic oligonucleotides corresponding to the $MRE_{3/4}$ and MRE-S regions (for sequences see Fig. 1) were ^{32}P end-labeled with T4 polynucleotide kinase, annealed and incubated with 0.5–1.0 ng of partially purified recombinant GST-ZiRF1 fusion protein, in binding buffer containing 12% glycerol/12 mM HEPES-NaOH pH 7.9/50 mM KCl/5 mM Tris-HCl pH 7.9/100 ng bovine serum albumin (BSA)/0.1 μg poly(dI·dT)/1 mM DTT in a volume of 20 μl [17]. Binding reactions were incubated for 45 min on ice and samples were layered on low-ionic-strength 6% polyacrylamide gel (acrylamide/bis-acrylamide, 28:2). Gels were pre-electrophoresed for 1 h at 15 mA in 22.5 mM Tris-borate pH 8.3/0.5 mM EDTA and samples were electrophoresed for 3 h at 4°C. Competition experiments were performed by adding unlabeled oligonucleotides before the addition of the protein.

Analysis of the human recombinant SP1 [19] was performed by assaying 1 footprinting unit (fpu) of human recombinant SP1 (hrSP1) per binding reaction.

Nuclear extracts were prepared as previously described [20] and binding reactions were performed under the same conditions as described for the recombinant proteins, except that 4 μg of nuclear proteins were assayed in a reaction buffer containing 1 μg of poly-(dI·dC)/poly(dA·dT), 1:1 mix. All figures display representative experiments that were repeated at least three times.

3. Results

3.1. ZiRF1 and SP1 factors bind the high-affinity MTF1 site, MRE-S

We investigated the possibility that metal-regulated ZiRF1 and basal SP1 factors could interact with different MRE sequences, including the high-affinity mMTF1-binding site, MRE-S (Fig. 1). Mobility shift assays showed binding of

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MRE_d/c   5'- AATTCTCTGCACTCCGCCCGAAAAGTGCGCTCGG - 3'
                          SP1
MRE_3/4   5'- AATTCGGTGCGCCCGGCCCAGTGCGCGCGGCCG -3'
                          SP1
MRE-S     5'- CGAGGGAGCTCTGCACACGGCCCGAAAAGT -3'
                          SP1
mutG2     5'- AATTCTCTGCACTCCGTCTGAAAAGTGCGCTCGG -3'
mutMRE    5'- AATTCGGAATGTCAGGCCCAGAATGTGAGGCCG -3'
SP-HSV    5'- CCGGCCCCGCCCATCCCCGGCCCCGCCCATCC -3'
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Fig. 1. Sequences of the synthetic oligonucleotides. $MRE_{d/c}$ was synthesized according to the mouse MTI promoter sequence spanning from position −150 to −123 [5]; $MRE_{3/4}$ was obtained by the synthesis of the 5′ region of the hMTIIa promoter spanning from nucleotides −147 to −112 [9]. In mutG2 mutated nucleotides are indicated in bold. The MRE-S sequence was designed as reported by Radtke et al. [15]. The multiple SP1-binding site, SP-HSV, was obtained by synthesizing the SP1-binding sites present in the promoter of the herpes simplex virus immediate-early 3 (HSV IE-3) gene [15,19]. Synthetic MREs were synthesized with an *Eco*RI site at both ends. Overlines correspond to MRE core elements; underlined sequences correspond to SP1-like sites.

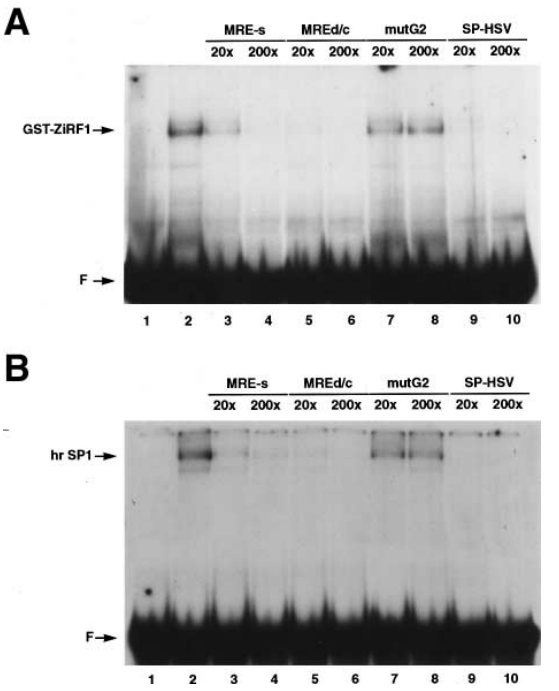

Fig. 2. Recombinant GST-ZiRF1 and SP1 factors bind the mMTF1 site MRE-S. A: Mobility shift assays were performed using the recombinant GST-ZiRF1 fusion protein and ^{32}P-labeled MRE-S as probe; lane 1, no added protein; lanes 2–10, 10 ng of bacterial expressed GST-ZiRF1. 20- and 200-fold molar excess of the cold competitors MRE-S (lanes 3–4), $MRE_{d/c}$ (lanes 5–6), mutG2 (lanes 7–8) and SP1-HSV (lanes 9–10). B: Binding of the human recombinant SP1 to the ^{32}P-labeled MRE-S oligonucleotide. Lane 1, no added protein; lanes 2–10, 1 fpu (footprinting unit) of hrSP1 protein; lanes 3–10, same amounts of competitors as in A.

GST-ZiRF1 fusion protein to the ^{32}P-labeled MRE-S (Fig. 2A, lane 2). Therefore, we performed competition experiments to determine if the DNA-protein complexes were specific. Interaction of GST-ZiRF1 with MRE-S was inhibited more efficiently by the naturally occurring $MRE_{d/c}$ (Fig. 2A, lanes 5–6) than by self-competition with the synthetic MRE-S (Fig. 2A, lanes 3–4). Sequences of competitor DNAs are reported in Fig. 1. Conversely, incubation with 20- and 200-fold molar excess of the point-mutated $MRE_{d/c}$, mutG2, did not affect the ZiRF1/MRE-S interaction (Fig. 2A, lanes 7–8). Furthermore, we observed that the multiple SP1-binding site, SP-HSV, was able to displace the complex as well as the wild-type $MRE_{d/c}$ (Fig. 2A, compare lanes 5–6 with lanes 9–10).

We next compared the MRE-binding activity of the metal-regulated ZiRF1 and the basal SP1 factor by performing a similar analysis with hrSP1. As measured by mobility shift analysis, hrSP1 was also able to bind MRE-S (Fig. 2B, lanes 2–4). The results of competition assays were similar to those displayed by GST-ZiRF1 (compare Fig. 2B with Fig. 2A, lanes 3–10)). The SP1/MRE-S complex was displaced by self-competition with MRE-S (Fig. 2B, lanes 3–4) and by the wild-type metal-regulating $MRE_{d/c}$ (Fig. 2B, lanes 5–6). The effects of incubation with cold SP-HSV and mutG2 also overlapped with those obtained in ZiRF1-MRE-S analysis (compare Figs. 2A and 2B, lanes 7–10). These results confirmed that the basal SP1 transcription factor exhibits MRE recognition properties very similar to those evidenced for the metal-regulated ZiRF1 and mMTF1.

3.2. Dose-dependent inhibition of ZiRF1/MRE binding by metal chelator treatment

We next tested the possibility that the interaction of GST-ZiRF1 with the MREs was metal-dependent. We therefore performed mobility shift assays to measure the effect of 1,10 phenanthroline (1,10 PHE) and of its isomer 1,7 phenanthroline (1,7 PHE) which lacks the ability to bind divalent cations. By treating purified GST-ZiRF1 fusion protein with 1,10 PHE we obtained inhibition of its binding to $MRE_{3/4}$ (Fig. 3A, lanes 7–10). This effect was dose-dependent and started at metal chelator concentrations of 200 μM. The higher inhibitory effects were observed at doses of 1,10 PHE ranging between 1 (Fig. 3A, lane 10) and 2 mM (data not shown). Conversely, we did not find inhibition of GST-ZiRF1 protein-binding activity using the non-metal-binding 1,7 PHE as a control (Fig. 3A, lanes 2–6).

Analyses of the metal requirement of the GST-ZiRF1/MRE complexes were confirmed by binding assays of the recombinant protein dialyzed in the presence of increasing concentrations of EDTA or the high-affinity zinc chelator TPEN. GST-ZiRF1/MRE binding was inactivated in a dose-dependent manner by both EDTA (Fig. 3B, lanes 2–4) and TPEN (Fig. 3B, lanes 5–7); in all the experiments we performed, we observed a stronger inhibition of TPEN compared with EDTA.

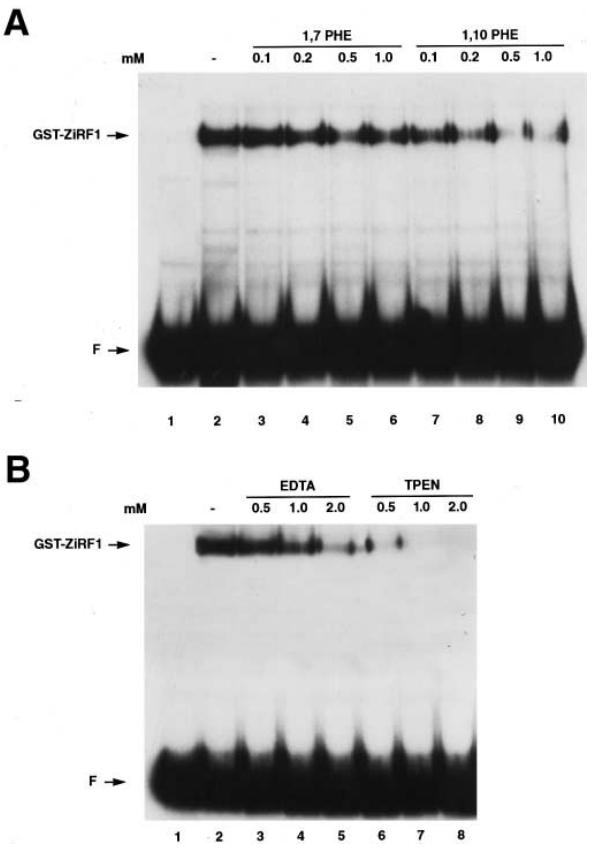

Fig. 3. Effect of metal depletion on GST-ZRF1/MRE interaction. A: Mobility shift assays of partially purified GST-ZiRF1 protein to ^{32}P-labeled $MRE_{3/4}$ following dialysis with 1,7 PHE or 1,10 PHE. Lane 1, no added protein; lane 2, untreated protein; lanes 3–6, GST-ZiRF1 protein treated with 1,7 PHE; lanes 7–10, GST-ZiRF1 protein treated with 1,10 PHE. B: Binding of GST-ZiRF1 protein to ^{32}P-labeled $MRE_{3/4}$ after metal depletion by EDTA or TPEN. Lane 1, untreated protein; lanes 2–4, EDTA-treated protein; lanes 5–7, TPEN-treated protein. Doses of metal chelators are indicated in mM. Arrows indicate GST-ZiRF1/MRE complex and the unbound probe, F.

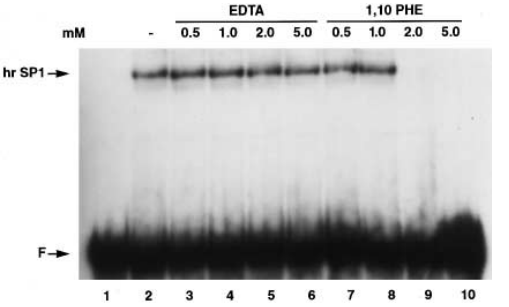

Fig. 4. Effect of metal depletion on hrSP1/MRE interaction. Binding of human recombinant SP1 to ^{32}P-labeled $MRE_{3/4}$ was measured by mobility shift assays following metal depletion with EDTA or 1,10 PHE. Lane 1, no added protein; lane 2, untreated protein; lanes 3–6, hrSP1 treated with EDTA for 1 h; lanes 7–10, 1,10 PHE-treated protein. Concentrations of metal chelators are expressed in mM. Arrows indicate hrSP1/MRE complexes and the unbound probe, F.

3.3. Metal-dependent MRE-binding activity of recombinant SP1

In order to evaluate the role of metal ions in modulating the binding of metal-regulated and/or basal SP1 transcription factor to the MREs, we extended the analysis of metal requirement to the complexes formed by the recombinant hrSP1 with the $MRE_{3/4}$ human MTIIa promoter region (Fig. 4, lane 2). The MRE-binding activity of hrSP1 was measured by mobility shift assay using the recombinant SP1 protein treated with different concentration of EDTA (Fig. 4, lanes 3–6). We found that EDTA treatment lacked the ability to interfere with the binding of hrSP1 to the human $MRE_{3/4}$ region when we used concentrations ranging between 0.2 and 2.0 mM (Fig. 4, lanes 3–5). Conversely, treatment of recombinant SP1 with 1,10 PHE decreased the intensity of the bands corresponding to the SP1/MRE complexes, giving a maximal effect at 2 mM (Fig. 4, lanes 7–10). Similar results were obtained with TPEN treatment, although in this case the inactivation was weaker than with 1,10 PHE treatment (data not shown).

We therefore found that also the basal SP1 binding activity to the $MRE_{3/4}$ was metal-dependent, but that this complex was more resistant to EDTA treatment than that formed by the metal-regulated GST-ZiRF1 activator.

3.4. Metal chelators inhibit the formation of the MRE-binding complexes detected in nuclear extracts

Analysis of nuclear extracts derived from mouse L cells revealed MRE-binding complexes (MBC) with distinct molecular weights (Fig. 5, lane 2). Our previous studies suggested that higher-M_r complexes were more likely composed of SP1 or similar proteins, while the faster-migrating MBC was composed of ZiRF1 [18]. To further examine the role of metal ions in regulating DNA interaction of MRE-binding proteins, we treated cell extracts with increasing concentrations of different metal chelators and measured the effect of metal depletion on MBC formation by the use of mobility shift assays. Interestingly, only the faster MRE-binding complex (MBC_1) was inactivated by EDTA (Fig. 5, lane 5) and the inhibition

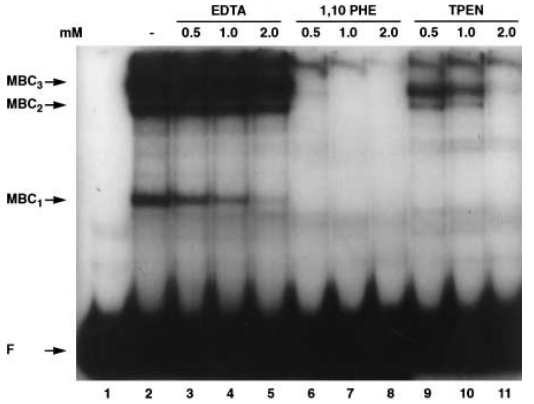

Fig. 5. Effect of metal depletion on nuclear cell extracts having MRE-binding activities. Nuclear proteins were treated with EDTA and analyzed in mobility shift assays for their ability to bind the ^{32}P-labeled $MRE_{3/4}$ oligomer. Lane 1, no added proteins; lane 2, untreated L cell nuclear extracts. Nuclear extracts dialyzed with: EDTA (lanes 3–5); 1,10 PHE (lanes 6–8); TPEN (lanes 9–11). The concentrations used for the metal chelating treatment are given in mM. Arrows indicate MRE-binding complexes (MBCs) and the unbound probe, F.

observed was dose-dependent (Fig. 5, lanes 2–5). Higher-M_r complexes (MBC_2 and MBC_3) were not affected by EDTA. Conversely, treatment with 1,10 PHE strongly interfered with all the MRE-binding complexes detected (Fig. 5A, lanes 6–8); TPEN treatment gave a similar effect, although inhibition of the higher-M_r complexes MBC_2 and MBC_3 occurred only at higher doses when compared to 1,10 PHE (Fig. 5A, lanes 9–11).

4. Discussion

We previously demonstrated the specific interactions of both the zinc-regulated factor ZiRF1 and the basal SP1 with the $MRE_{d/c}$ region of the mouse MTI promoter [18]. Our results showed very high similarities between ZiRF1 and SP1 in the ability to recognize functional MREs.

In this paper, we extend those observations to the characterization of DNA binding of the two purified transcription factors, ZiRF1 and SP1, to different MRE sequences and the role of metal ions in modulating their DNA interactions.

We found that both purified GST-ZiRF1 and SP1 proteins display similar binding properties for MRE and SP1 target sites in gel retardation assays. Our results show that ZiRF1 interacts more strongly with the naturally occurring $MRE_{d/c}$ than with the synthetic MRE-S, the recognition site for the previously cloned mouse factor MTF1 [15]. A role of mMTF1 in the regulation of MT gene expression was established by the finding that expression of MT genes was silent in ES cells where the mMTF1 gene was inactivated [16]. However, a number of lines of evidence suggest that different metal-regulated factors could interact with common binding sites [10–15,18]. Our results showed that ZiRF1 has the ability to interact specifically also with MRE-S and this indicates that metal-regulated ZiRF1 and mMTF1 factors could compete for the same regulatory sites. The ability of ZiRF1 protein to recognize the mMTF1 target site supports the hypothesis that the metal-dependent activation of MT genes could involve transcription factors having different responses or activation properties upon metal induction [21].

We also found that in addition to the metal-regulated mMTF1 and ZiRF1 factors the basal SP1 transcription activator was also able to bind MRE-S with a comparable sequence specificity (Fig. 2B). Our results confirm the finding that metal-regulated and basal factors could interact with the same metal-regulatory sequences [8,18] and suggest that the regulation of the expression of MT genes by metals might be modulated by competition between the two groups of transcription activators for the same cognate site.

We next analyzed the role of metals in the binding of recombinant ZiRF1 [18] and SP1 [19] proteins to MRE sequences.

The three metal chelators, EDTA, 1,10 PHE and TPEN, gave a dose-dependent inhibition of GST-ZiRF1 $MRE_{3/4}$-binding activity. The specificity of the effect of metal depletion is demonstrated by the inability of the non-functional analog, 1,7 PHE, to interfere with the DNA-protein interaction. We also observed that the ZiRF1 MRE-binding activity is more efficiently inhibited by TPEN as compared to EDTA. This could be due to the higher stability of the TPEN metal-bound complex and/or to the more efficient ability of the chelator to access the ZiRF1 metal-binding clusters. The metal dependence of ZiRF1 in binding to MRE sequences and our previous observations [18] propose for this nuclear factor a mechanism similar to the yeast transactivator ACE1/CUP1 [22,23] in which the metal (zinc in the case of ZiRF1) could directly modulate the interaction of the apoprotein with the metal-regulatory regions.

Previous studies have already shown that SP1 is a metal-bound protein that requires zinc ions for its interaction with DNA [24]. Our results showed the inability of EDTA to inhibit the SP1-$MRE_{3/4}$-binding activity, supporting the hypothesis that the metal depletion of SP1 is possible only in denaturing conditions, which allow the access of the metal chelator to the metal-binding domains present in the factor. Conversely, the chelating effect of 1,10 PHE was comparable on both SP1 and ZiRF1.

We previously showed the presence in mouse cell extracts of a zinc-dependent MRE binding activity (MBC) with DNA recognition properties similar to those displayed by ZiRF1 which cross-reacts with antibodies raised against purified GST-ZiRF1 [18]. In addition, we showed that higher-M_r MRE-binding complexes were presumably composed of SP1 or similar proteins. We now report that such detected MBCs give a different response to metal chelator treatment, similar to that observed assaying the purified SP1 and ZiRF1 proteins, i.e. EDTA inactivated only the faster MBC_1 complex in a dose-dependent manner, while all three MBC complexes, MBC_1, MBC_2 and MBC_3, were affected by 1,10 PHE and TPEN.

These results suggest a different molecular composition or chemical properties of the MBCs, possibly by mutual interactions of basal and metal-dependent transactivators.

We also observed down-regulation of the hMTTIIa promoter-CAT fusion plasmid in both non-induced and zinc-induced transfected cells expressing antisense ZiRF1 cDNA (P. Remondelli, unpublished results), although the effect was never complete. The consistent levels of hMTIIa promoter activity which are still detected upon antisense expression could be explained either by an incomplete inhibition of ZiRF1 endogenous expression or by the possibility that different factors may modulate the metal-dependent activation of MT promoters.

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