Interactions of the zinc-regulated factor (ZiRF1) with the mouse metallothionein Ia promoter

Paolo REMONDELLI*†§ and Arturo LEONE†‡

*Department of Pharmacology and Center for Molecular Genetics, UCSD School of Medicine, La Jolla, CA 92093-0636, U.S.A., †Dipartimento di Biochimica e Biotecnologie Mediche, Facoltà di Medicina e Chirurgia, Università degli Studi di Napoli 'Federico II', 80131 Napoli, Italy, and ‡Dipartimento di Scienze Farmaceutiche, Facoltà di Farmacia, Università degli Studi di Salerno, Salerno, Italy

A mouse cDNA clone, M96, encoding a metal-regulating-element (MRE)-binding protein, was analysed for its ability to act as a metal-regulated transcription factor. The metal depletion of a glutathione S-transferase (GST)–M96 fusion protein showed that Zn^{2+} ions modulate the MRE-binding activity, suggesting that the M96-encoded protein is a Zn^{2+}-regulated factor (ZiRF1). The methylation interference assay showed the specific interactions of ZiRF1 with the MRE, $MRE_{d/c}$, present on the mouse metallothionein Ia promoter. Point mutations of the $MRE_{d/c}$ nullified the metal-regulatory properties of this region. In mouse L-cell nuclear extracts, mobility-shift assays revealed a Zn^{2+}-dependent MRE-binding complex (MBC) with DNA-recognition properties similar to those of ZiRF1. Antibodies raised against purified GST–ZiRF1 were able to specifically recognize MBC in Western-blot analyses. Competition analysis of MRE-binding proteins from mouse NIH3T3 cells with oligonucleotide matching the binding sites for SP1 and MTF1 confirmed that both the basal SP1 and the metal-regulated MBC/ZiRF1 interact with the $MRE_{d/c}$ region. The significance of mutual interactions with the metal-responsive promoter regions of either metal-regulated or basal transcription factors is discussed.

INTRODUCTION

The metallothionein (MT) gene family encodes low-molecular-mass cysteine-rich metal-binding proteins expressed in a wide variety of organisms, from yeast to man [1,2]. Although other stimuli such as steroid hormones, interleukins, cAMP, phorbol esters and interferon are able to activate MT expression, the function of MT has been suggested to be the regulation of both metal homoeostasis and metal detoxification [3,4]. Activation of mammalian MT genes after metal induction is mediated by short DNA repeats, the metal regulatory elements (MREs) [5–7]. These are present in multiple imperfect copies in all the mammalian MT promoter regions analysed so far [8,9]. MREs are able to confer metal inducibility on heterologous promoters that are normally unable to respond to heavy metals [10,11]. Although a variable number of MRE copies are present in mammalian MT promoters, a minimum of two is required for optimal metal inducibility [12].

There is evidence that the metal-regulated transcription factors interact with the MRE, thus acting as transcriptional activators and not as repressors [13]. Genomic footprinting performed on nuclei from metal-treated cells revealed that the protein interactions with the MRE were enhanced after Zn^{2+} treatment, suggesting that such proteins may act as positive metal-dependent transactivators [14].

Several MRE-binding proteins of various size have been detected in nuclear extracts [15–21], and the effects of various metal ions (e.g. Zn^{2+}, Cd^{2+} and Cu^{2+}) on MRE-binding activity have been studied [22–24]. The cDNA clone encoding a zinc finger protein called mMTF-1 has been isolated from a cDNA expression library using synthetic MRE as probe [25]. The overexpression of mMTF-1 leads to an increased constitutive expression of an MRE-driven reporter gene, whereas knocking out the mMTF-1 gene prevented induction by all the metals in mouse embryonic stem cells [26].

A distinct factor, MafY, has been cloned by *in vivo* screening of cDNA expression libraries based on genetic complementation in yeast [27], but neither direct DNA binding nor metal-dependence has been demonstrated for MafY.

By the use of a similar approach, the cDNA clone, M96, was isolated for its ability to activate an MRE_d-driven reporter gene in yeast cells. Sequence analyses revealed that M96 encodes a protein rich in cysteine and histidine residues, which contains potential metal-binding regions, some of them similar to the trithorax zinc finger type and is able to interact specifically with functional MREs [28]. Data reported here show that Zn^{2+} ions could act as positive modulators of the MRE-binding activity encoded by M96 which is therefore a Zn^{2+}-regulated factor (ZiRF1). Our evidence suggests that ZiRF1 may compete with a number of different factors for the interaction with the metal-responsive mouse MT-I promoter region, $MRE_{d/c}$. The presence of different MRE-binding factors in mouse nuclear extracts may reflect the different activation mechanisms required by the MT gene for basal or metal-regulated expression.

EXPERIMENTAL

Materials

All culture reagents were supplied by Gibco (Inchinann, Scotland, U.K.). Solid chemicals and liquid reagents were obtained from E. Merck (Darmastadt, Germany), Farmitalia Carlo Erba (Milan, Italy), Serva Feinbiochemica (Heidelberg, Germany) and Sigma Chemicals (St. Louis, MO, U.S.A.). [γ-^{32}P]ATP (specific radioactivity 3000 Ci/mmol) was obtained from DuPont–New England Nuclear (Boston, MA, U.S.A.). Re-

Abbreviations used: CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; FBS, fetal bovine serum; GST, glutathione S-transferase; hmMBC, higher-molecular-mass MRE-binding complexes; HRP, horseradish peroxidase; MBC, MRE-binding complexes; MRE, metal regulating element; MT, metallothionein; 1,10 PHE, 1,10-phenanthroline; TPEN, tetrakis-(2-pyridylmethyl)ethylenediamine; ZiRF1, Zn^{2+}-regulated factor.

§ To whom correspondence should be addressed.

striction enzymes were purchased from New England BioLabs (Beverley, MA, U.S.A.). Plasmid vectors were obtained from Invitrogen (San Diego, CA, U.S.A.) and Stratagene (La Jolla, CA, U.S.A.). Sepharose 4B was from Pharmacia (Uppsala, Sweden). Oligonucleotides were synthesized by Primm (Milan, Italy). The chemiluminescent assay to detect horseradish peroxidase (HRP) activity was carried out with the Amersham ECL kit from Amersham International (Amersham, Bucks., U.K.). The human recombinant SP1 was purchased from Promega (Madison, WI, U.S.A.).

Oligonucleotides and construction of expression vectors and plasmid reporters

A plasmid encoding the glutathione S-transferase (GST)–ZiRF1 fusion protein was obtained by ligating the M96 cDNA excised from the *Bgl*II site of a pACT-M96 expression vector [28] in the *Bgl*II site of *Bam*HI-digested pGEX-3X.

The $MRE_{d/c}$ oligomer derived from the mouse MT-I promoter sequence spanning from position -150 to position -123 was synthesized: 5′-AATTCTCTGCACTCCGCCCGAAAAGTG^{-123}-CGCTCGG-3′. The $MRE_{3/4}$ was obtained by synthesis of the 5′-region of the hMTIIa promoter spanning from nucleotides -147 to -112: 5′-AATTCGGTGCGCCCGGCCCAGTGCGCGC^{-112}-GGCCG-3′. The mutated $MRE_{3/4}$, mutMRE, was obtained by synthesizing a $MRE_{3/4}$ in which the MRE core sequences were randomly mutagenized (mutated nucleotides are represented in bold: 5′-AATTCGG**AATGTCA**GGCCCAG**AATGTGA**GGC-CG-3′. The mutated $MRE_{d/c}$, mutG2, was obtained by synthesizing a similar oligomer in which the guanine residues were replaced by adenines at positions -140 and -138 (mutated nucleotides are represented in bold): 5′-AATTCTCTGCACT-CCG**TCT**GAAAAGTGCGCTCGG-3′. The MRE-s sequence was designed as reported by Radtke et al. [25]: 5′-CGAGGG-AGCTCTGCACACGGCCCGAAAAGT-3′. The multiple SP1-binding site, SP-HSV, was obtained by synthesizing the SP1-binding site present in the promoter of the herpes simplex virus immediate-early 3 (HSV IE-3) gene [25,29]: 5′-CCGGCCCCG-CCCATCCCCGGCCCCGCCCATCC-3′.

$MRE_{d/c}$ and mutG2 were inserted into the *Bam*HI–*Hin*dIII site of a pBL-CAT2 [30] reporter plasmid to obtain the pMRE$_{d/c}$–CAT and the pmutG2–CAT. Plasmid pBS-$MRE_{d/c}$ was obtained by inserting into the *Eco*RI site of a pBS-SK$^+$ a single copy of a double-stranded $MRE_{d/c}$ region synthesized with both 5′ and 3′ *Eco*RI-cleavable sites. Correct insertion of all oligonucleotides was determined by the Sanger DNA sequencing methodology [31].

Protein expression and metal chelator treatment

Escherichia coli strain Xl1-blue was used as recipient cells for pGEX-ZiRF1 expression vector. Cells were grown at 37 °C in liquid broth at a density ranging between $A_{600} = 0.6$ and 0.8. Cultures were incubated at 30 °C in the presence of 0.1 mM isopropyl β-D-thiogalactopyranoside [32]. After 4 h, cells were harvested and resuspended in 0.05 vol. of PBS containing 2 mM dithiothreitol (DTT) and protease inhibitors (2 μg/ml each aprotinin, pepstatin A, leupeptin and chymostatin and 1 mM PMSF), sonicated 4×20 s and spun at (8000 ***g***) for 30 min at 4 °C. The supernatant was then adjusted to 1% Triton and incubated with GSH-Sepharose 4B beads for 1 h at 4 °C. Bound proteins were eluted with a buffer containing 5 mM GSH, 50 mM Tris/HCl, pH 8.0, 100 mM NaCl and 2 mM DTT. Further dialysis was carried out to remove excess GSH. Metal depletion by the chelating agents 1,10-phenanthroline (1,10 PHE), EDTA and tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) was obtained by dialysis of the partially purified GST–ZiRF1 fusion protein in PBS buffer containing metal chelators at the indicated concentration for 3 h at 4 °C. Chelating agents were subsequently removed with a second dialysis buffer containing PBS only.

Cell cultures and DNA transfection experiments

L-cell fibroblasts and NIH3T3 mouse cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 50 mg/ml streptomycin and 2 mM L-glutamine. For optimal metal induction, cells were grown for 18 h in low-serum medium (0.1% FBS) followed by 6 h incubation in the same culture medium supplemented with 200 μM Zn^{2+} ions. These conditions were used because standard serum concentrations (10% FBS) greatly decreased metal induction. DNA transfections were performed by calcium phosphate co-precipitation as previously described [33]. Chloramphenicol acetyltransferase (CAT) reporter plasmids (5 μg/1×10^4 NIH3T3 mouse cells) were used in a 60 mm plate together with 0.5 μg of CMV–*LacZ* reporter plasmids as internal control. pBS-SK$^+$ was added to equalize the amount of DNA per plate to 10 μg. The medium was changed 5 h later, and serum starvation and metal induction were performed as described above. CAT activity was determined with butyryl-CoA [34] and c.p.m. values were normalized for total units of β-galactosidase activity/mg of protein.

Mobility-shift assays

Synthetic oligomers corresponding to the $MRE_{3/4}$ and $MRE_{d/c}$ were ^{32}P-end-labelled with T4 polynucleotide kinase, annealed and incubated with 0.5–1.0 ng of partially purified *E. coli*-expressed 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 of BSA, 0.1 μg of poly(dI·dT) and 1 mM DTT in a volume of 20 μl [35,36]. After 45 min of incubation on ice, the reaction mixtures were loaded on 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 unlabelled oligonucleotides before the addition of the protein. Analysis of the human recombinant SP1 interaction with MREs was performed under the above conditions, by assaying 1 footprint unit of human recombinant SP1 per binding reaction.

Metal treatment was performed by preincubating dialysed proteins with the indicated concentrations of metal ions for 1 h before the addition of the probes. Nuclear extracts were prepared as previously described [37], and binding assays were performed as above except that 4 μg of protein was used in binding buffer containing 1 μg of poly(dI·dC)/poly(dA·dT) (1:1, w/w).

Western-blot analysis

GST–ZiRF1 and L-cell nuclear extracts were incubated in binding buffer with ^{32}P-labelled oligomer as described for mobility-shift assays except that reactions were scaled up to 100 μl and contained 300000 c.p.m./reaction. Gels were exposed without drying to X-ray films. MRE-binding complexes (MBCs) were excised and eluted overnight at 4 °C in buffer containing 50 mM Tris/HCl, pH 8.0, 100 mM NaCl, 2 mM DTT and proteinase inhibitors as described. Proteins were precipitated with 9 vol. of acetone and separated by standard SDS/PAGE. Gels

were then transferred to nitrocellulose filters. Filters were saturated with 5% milk solution and incubated overnight at 4 °C with 1:500 (v/v) dilution of the anti-ZiRF1 polyclonal DE034 rabbit antibody. Filters were washed several times and incubated for 1 h at room temperature with HRP-conjugated antibody followed by chemiluminescent assays to detect HRP activity.

Methylation interference analysis

Plasmid pBS-$MRE_{d/c}$ was cut at the *Xho*I site, the 5′ ends were dephosphorylated with calf intestinal phosphatase and subsequently ^{32}P-labelled at the 5′-end with T4 polynucleotide kinase. Linearized ^{32}P-labelled plasmid was subsequently cut with *Xba*I and the 100 bp ^{32}P-labelled *Xho*I–*Xba*I fragment containing the $MRE_{d/c}$ region was gel purified and used as a probe to detect interactions with the coding-strand nucleotides. Conversely, the non-coding-strand probe was obtained by ^{32}P-end labelling the *Xba*-I end of pBS-$MRE_{d/c}$ and purifying the ^{32}P-labelled *Xho*I-*Xba*I fragment. End-labelled probes were treated with dimethyl sulphate as previously described [38].

For mobility-shift assays, partially methylated fragments were incubated with bacterially expressed and partially purified GST–ZiRF1 as described above, except that reactions were scaled up to 100 μl and contained approx. 300000 c.p.m./reaction. Free and bound DNA were separated by electrophoresis and electrotransferred from the gel to Whatman DE81 paper in 0.5 × Tris/borate/EDTA for 1 h at 500 mA; DE81 paper was exposed to X-ray film to locate the complexed and free DNA bands, which were excised and the DNA was eluted by incubation in 10 mM Tris/HCl, pH 7.5, containing 1 mM EDTA and 1 mM NaCl. DNA was phenol-extracted and ethanol-precipitated and cleaved with piperidine. Equal amounts of radioactive material were resuspended in buffer [80% (v/v) formamide, 50 mM Tris/borate, pH 8.3, and 1 mM EDTA], denatured at 95 °C for 2 min and applied to a standard 6% polyacrylamide sequencing gel.

RESULTS

Zn^{2+} ions are positive modulators of ZiRF1/MRE-binding activity

The requirement of a specific metal ion in the modulation of ZiRF1/MRE-binding activity was investigated. The MRE-binding activity of the bacterially expressed GST–ZiRF1 fusion protein was strongly decreased by dialysis in the presence of the metal chelator EDTA at a minimal concentration of 1 mM (Figure 1, compare lanes 2 and 3). Use of other metal chelators such as 1,10 PHE and the high-affinity Zn^{2+} chelator TPEN gave similar results (results not shown).

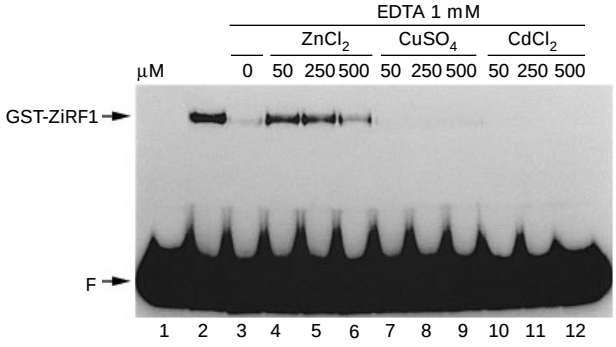

Figure 1 Zn^{2+} restores the DNA-binding activity of EDTA-treated GST–ZiRF1

Binding of EDTA-treated GST–ZiRF1 (lanes 3–12) with labelled $MRE_{d/c}$ was analysed in mobility-shift assays after incubation with increasing concentrations (50, 250, 500 μM) of the metal salts, $ZnCl_2$ (lanes 4–6), $CuSO_4$ (lanes 7–9) or $CdCl_2$ (lanes 10–12). Lane 2, untreated protein. Arrows indicate GST–ZiRF1 activity and F is free probe.

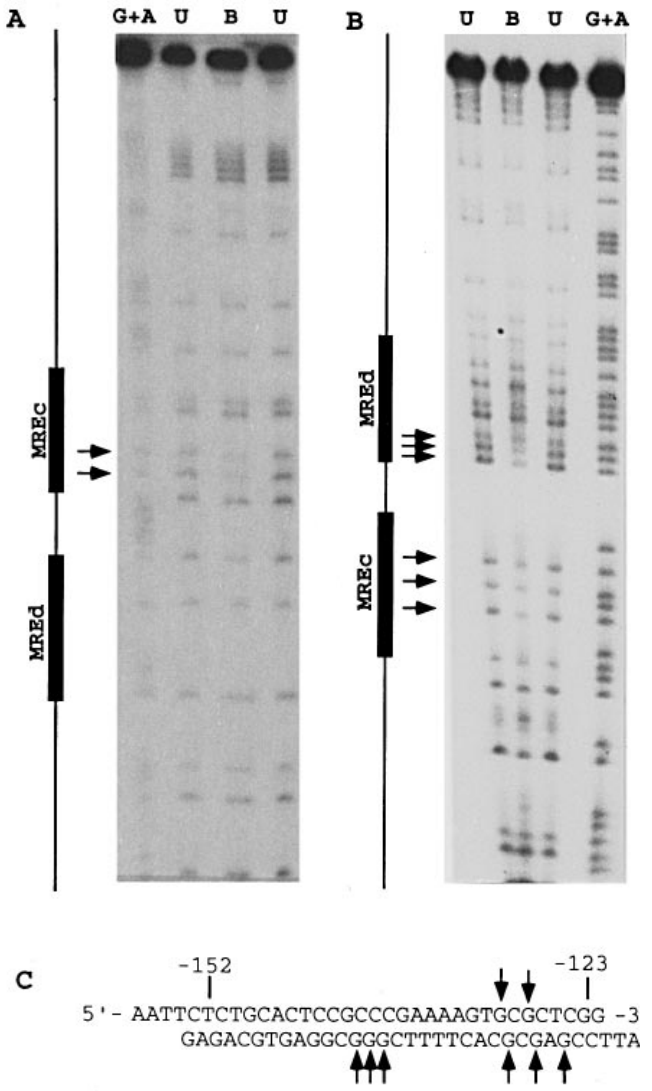

Figure 2 Methylation interference analysis

Methylation interference footprinting was performed by using partially purified GST–ZiRF1 and pBS-$MRE_{d/c}$ fragments labelled on either strand. U represents unbound probe, showing cleavage at all guanines; B represents bound DNA–protein complexes; the G + A ladder shows cleavage at guanine and adenine residues. Arrows indicate guanines interacting with GST–ZiRF1. (**A**) Analysis of the pBS-$MRE_{d/c}$ coding strand fragment; (**B**) analysis of the non-coding strand; (**C**) nucleotide sequence of the $MRE_{d/c}$ region. Arrows indicate guanine residues interacting with ZiRF1.

The EDTA-dialysed GST–ZiRF1 was then preincubated with various metal ions before the addition of the ^{32}P-labelled $MRE_{d/c}$ (Figure 1, lanes 3–12). In the presence of Zn^{2+} salts the DNA-binding activity was restored to levels comparable with those observed with the untreated protein (Figure 1, lanes 4–6). Inhibition occurred only when Zn^{2+} salts were used at concentrations higher than 500 μM (Figure 1, lane 6). In contrast, the

Table 1 Functional analysis of nucleotide interactions with ZiRF1 by transfection assays

NIH3T3 cells were transfected with a DNA mixture containing 5 μg of the CAT reporter genes, 0.5 μg of CMV–*LacZ* and 4 μg of pBS-SK$^+$. Cells were cultured for 18 h in medium containing 0.1% FBS, and 200 μM $ZnCl_2$ was added for 6 h. CAT activity was measured from β-galactosidase-equivalent portions of cell extracts. The values reported represent means $\pm$S.E.M. for three independent transfections.

	CAT activity (c.p.m.)	
Plasmid	$-Zn^{2+}$	$+Zn^{2+}$
pbl-CAT2	13750 $\pm$ 480	12000 $\pm$ 860
pbl-mutG2	18666 $\pm$ 380	15200 $\pm$ 790
pbl-$MRE_{d/c}$	19000 $\pm$ 180	72000 $\pm$ 1040

same concentrations of either Cu^{2+} or Cd^{2+} salts did not reconstitute binding activity of the EDTA-treated protein (Figure 1, lanes 7–9 and 10–12). Neither did lower concentrations of the toxic Cu^{2+} and Cd^{2+} salts (between 2 and 50 μM) induce any positive effect, and no appreciable effect was revealed when non-EDTA-treated GST–ZiRF1 was preincubated with the same metals (results not shown).

Mapping of ZiRF1 nucleotide contacts with the $MRE_{d/c}$ region by methylation interference footprinting analysis

The methylation interference technique was used to fine-map the contacts between ZiRF1 and the $MRE_{d/c}$ region.

The use of the coding-strand-labelled pBS-$MRE_{d/c}$ fragment as probe showed contacts between GST–ZiRF1 and two guanines located at positions -128 and -130 of the MRE_c element [Figure 2A; compare lanes B (bound) with U (unbound)].

Contacts on the non-coding strand were also revealed with three guanine residues located at positions -140, -139, -138 on the 3′-end of the MRE_d element and at positions -129, -127, -125 of the MRE_c element (Figure 2B; compare lanes U and B). No interactions were detected with the guanine residues present in the flanking regions of the pBS vector, thus demonstrating the ZiRF1-binding activity is restricted to MRE sequences in this context.

The sequence of the $MRE_{d/c}$ region with the sites of interaction with ZiRF1 is shown in Figure 2(C).

Point mutations at the sites of interaction with ZiRF1 render the $MRE_{d/c}$ non-metal-inducible

We further investigated the specificity of the interaction of ZiRF1 with the MRE sequences using transient transfection assays. We synthesized the mutG2 oligonucleotide in which the guanines at positions -140 and -138 were replaced by adenines. Reporter plasmids pBL-CAT2 carrying wild-type $MRE_{d/c}$ or mutG2 were fused to a TK minimal promoter driving the expression of a CAT reporter gene and transfected in the mouse NIH3T3 cells. CAT activity was determined as described in the Experimental section and c.p.m. values representing the mean $\pm$S.E.M. for three different experiments are reported in Table 1. The results show that, whereas the $MRE_{d/c}$ region is able to confer metal responsiveness on a TK minimal promoter, the activity of the reporter gene did not increase in the presence of Zn^{2+} when a mutG2 was used to regulate the pBL-CAT2 reporter.

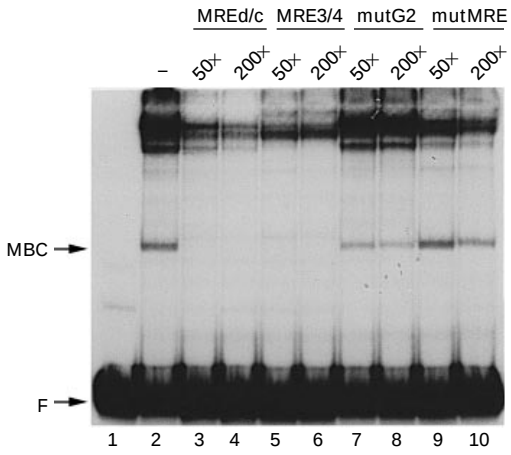

Figure 3 Detection of MBC by mobility-shift assay in mouse L-cells

Nuclear extracts from mouse L-cells were incubated with ^{32}P-labelled $MRE_{d/c}$ and analysed in mobility-shift assays. Lane 1, no added protein; lanes 2–10, 4 μg of nuclear extract. The specificity of interactions with the $MRE_{d/c}$ region was determined by competition with a 50- and 200-fold molar excess of unlabelled wild-type $MRE_{d/c}$ (lanes 3 and 4) and $MRE_{3/4}$ (lanes 5 and 6) or mutant mutG2 (lanes 7 and 8) and $mutMRE_{3/4}$ (lanes 9 and 10). F is free probe.

Identification of an MBC with DNA-binding properties similar to those of ZiRF1 in mouse L-cells

MBCs were detected in nuclear extracts derived from mouse L-cells by the use of the mobility-shift assay (Figure 3, lane 2). Competition experiments showed that binding of a single MBC to the $MRE_{d/c}$ can be displaced by the wild-type $MRE_{d/c}$ (Figure 3, lanes 3 and 4) and $MRE_{3/4}$ (Figure 3, lanes 5 and 6). Incubation of nuclear extracts with a 50- or 200-fold molar excess of the unlabelled non-metal-regulating mutG2 or the $mutMRE_{3/4}$ oligonucleotide in which the MRE core sequence had been extensively mutated (Figure 3, lanes 9 and 10) did not abolish the MBC. Variability of MBC intensity is consistent with the different extent of nucleotide substitutions of the mutG2 and mutMRE oligonucleotides.

Higher-molecular-mass MBCs (hmMBCs) were also revealed, and their binding activity was further analysed as described in the next section.

We found that the identified MBC exhibited properties similar to those of ZiRF1, after treatment of the nuclear extracts with EDTA (Figure 4, compare lanes 2 and 3). Similar results were also obtained with the two other metal chelators 1,10 PHE and the high-affinity zinc chelator TPEN (results not shown). We therefore examined the possibility that the MRE-binding activity of the MBC, like that of ZiRF1, was also modulated by Zn^{2+}. EDTA-treated L-cell nuclear extracts (Figure 4, lane 3) were preincubated in the presence of increasing amounts of Zn^{2+}, Cu^{2+} or Cd^{2+} ions (Figure 4, lanes 4–12). The MBC activity reappeared at Zn^{2+} doses ranging between 250 and 500 μM (Figure 4, lanes 4 and 5). Higher Zn^{2+} concentrations resulted in an inhibitory effect (Figure 4, lane 6). In contrast, preincubation of EDTA-treated extracts with Cu^{2+} salts did not reconstitute MBC binding (Figure 4, lanes 7–9), and Cd^{2+} negatively affected the activity of both hmMBCs and the slowly migrating MBC (Figure 4, lanes 10–12).

The molecular components of the MBC were further analysed by Western blot (Figure 5) as described in the Experimental section. Polyclonal rabbit antibody DE034, raised against the

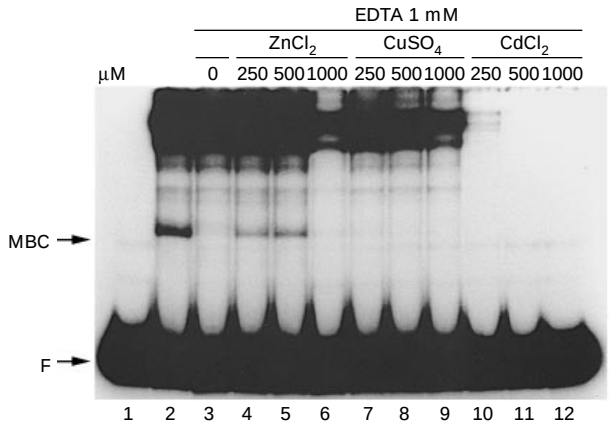

Figure 4 MBC-binding activity is dependent on Zn^{2+}

Mobility-shift assays were performed on EDTA-treated L-cell nuclear extracts preincubated with increasing concentrations of $ZnCl_2$ (lanes 4–6), $CuSO_4$ (lanes 7–9) or $CdCl_2$ (lanes 10–12). Lane 1, no added protein; lane 2, untreated L-cell nuclear proteins; lanes 3–12, EDTA-dialysed L-cell nuclear extracts. F is free probe.

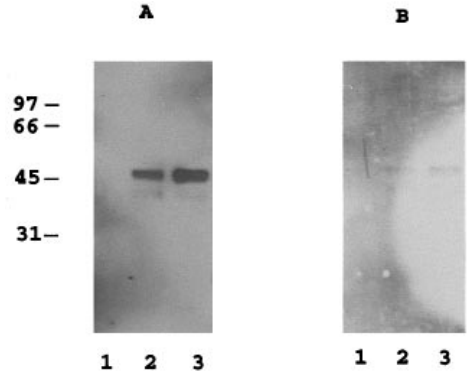

Figure 5 ZiRF1 is a component of the MBC

Equal amounts of the bacterially expressed GST (lanes 1), ZiRF1 with the GST moiety removed by thrombin digestion (lanes 2) and the gel-purified molecular components of the MBC (lanes 3) were separated by SDS/PAGE and analysed by the Western-blot procedure. (**A**) Analysis with polyclonal antibody DE034 raised against purified GST–ZiRF1 or with (**B**) preimmune serum. Relative positions of molecular-mass (kDa) standards are indicated.

partially purified GST–ZiRF1, was able to recognize the MBC component as well as the bacterially expressed form of ZiRF1 (Figure 5A, compare lanes 2 and 3). MBC showed the same predicted electrophoretic mobility as ZiRF1 after removal of the GST portion by thrombin treatment before the gel separation. Antibody specificity was shown by their inability to bind the GST moiety (Figure 5A, lane 1). No signal was detected after treatment of the filter with preimmune serum (Figure 5B).

ZiRF1 and SP1 transcription factors mutually interact with the mouse $MRE_{d/c}$ region

We extended our analyses of the detected MBC complexes further, by using nuclear extracts obtained from the mouse fibroblast NIH3T3. The use of these extracts revealed an improved resolution of the hmMBCs. Oligonucleotides corresponding to the high-affinity binding sites for the SP1 [29] and mMTF1 [25] factors (referred to as SP-HSV and MRE-s respectively) were used as competitors in addition to the formerly described wild-type $MRE_{d/c}$ and the mutg2 oligomers.

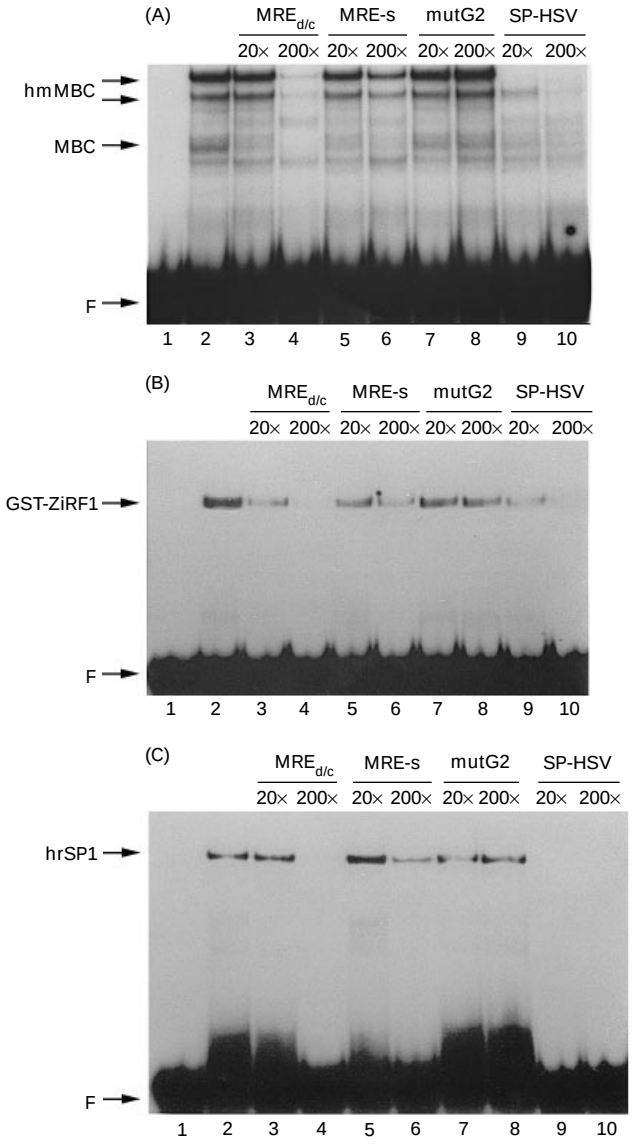

Figure 6 Binding of nuclear and recombinant ZiRF1 and SP1 factors to the $MRE_{d/c}$ MT-I promoter region

(**A**) Nuclear extracts from mouse NIH3T3 cells were incubated with a ^{32}P-labelled $MRE_{d/c}$ and analysed in mobility-shift assays. Lane 1, no added protein; lanes 2–10, 4 μg of nuclear extract. Competition with a 20- and 200-fold molar excess of $MRE_{d/c}$ (lanes 3 and 4), MRE-s (lanes 5 and 6), mutG2 (lanes 7 and 8) and the SP1-binding site, SP-HSV (lanes 9 and 10) is shown. Arrows indicate hmMBCs, MBC and free probe, F. (**B**) Binding of the GST–ZiRF1 to $MRE_{d/c}$: lane 1, no added protein; lanes 2–10, 10 ng of bacterially expressed GST–ZiRF1. Competition with 20- and 200-fold molar excess of the unlabelled $MRE_{d/c}$ (lanes 3 and 4), MRE-s (lanes 5 and 6), mutG2 (lanes 7 and 8) and SP-HSV (lanes 9 and 10) is shown. Arrows indicate GST–ZiRF1–MRE complex and free probe, F. (**C**) Binding of the recombinant human SP1 to the ^{32}P-labelled wild-type $MRE_{d/c}$ MT-I region. Lane 1, no added protein; lanes 2–10, 10 ng of purified human SP1. Lanes 3–10 same competitors as in (**B**). The sequence of the $MRE_{d/c}$ with the relative positions of the MRE-s- and SP1-binding sites (upper and lower lines respectively) is the following:

MRE-s
5′-AATTCTCTGCACTCCGCCCGAAAAGTGCGCTCGG-3′
SP1

NIH3T3 nuclear extracts revealed three distinct complexes with $MRE_{d/c}$ (Figure 6A, lane 2). As Figure 6(A) shows,

incubation with a 20-fold molar excess of the wild-type $MRE_{d/c}$ specifically inhibited the MBC, whereas a 200-fold molar excess was required to prevent the binding of the two hmMBCs (Figure 6A, lane 4). As with the $MRE_{d/c}$, both 20- and 200-fold of the MRE-s specifically competed with the binding of MBC (Figure 6A, lanes 5 and 6). A weak inhibition of the two hmMBCs appeared in the presence of a 200-fold excess of unlabelled competitor. The high-affinity SP1 site was more active with the two hmMBCs than the wild-type $MRE_{d/c}$ and the MRE-s (Figure 6A, lanes 9 and 10).

We analysed the effects of the same set of competitors on the GST–ZiRF1–$MRE_{d/c}$ interaction (Figure 6B). $MRE_{d/c}$ and the SP-HSV exhibited comparable levels of binding inhibition, suggesting common sequence recognition properties between the two factors (Figure 6B, compare lanes 3, 4 and 9, 10). Conversely, the point-mutated $MRE_{d/c}$, mutG2, did not affect the MRE-binding activity (Figure 6B, lanes 7 and 8). The high-affinity binding site of mMTF1, MRE-s, gave rise to slightly increased inhibition of the ZiRF1–$MRE_{d/c}$ interaction compared with the wild-type competitor (Figure 6B, compare lanes 3, 4 and 5, 6). Such differences in the efficiency of competition between the two MREs may reflect different sequence composition between $MRE_{d/c}$ and MRE-s.

The potential of transcription factor SP1 to interact with the $MRE_{d/c}$ was also investigated using human recombinant SP1 protein [29]. Competition experiments showed that the specificity of SP1 toward the $MRE_{d/c}$ was comparable with that displayed by ZiRF1 (compare Figure 6B with Figure 6C). The inability of the mutG2 to inhibit SP1 binding indicates similar DNA recognition by the two factors. Moreover, the SP1–$MRE_{d/c}$ complex displayed similar resistance to the competition with the MRE-s. Similar results were also obtained by comparing the interaction of both SP1 and GST–ZiRF1 with the high-affinity MTF1-binding site, MRE-s, thus suggesting similar MRE-recognition properties in the metal-regulated mMTF1 and ZiRF1. Like ZiRF1, the human recombinant SP1 was also able to bind the MRE-s, which indicates that the basal SP1 transcription factor displays many characteristics of an MRE-binding protein (results not shown).

DISCUSSION

We previously isolated the mouse M96 cDNA clone that encodes a novel MRE-binding factor, the Zn^{2+}-regulated factor, ZiRF1 [28]. Analysis of the M96 cDNA coding sequence revealed the presence of regions particularly rich in cysteine and histidine residues (22 cysteines and 12 histidines). These residues could enable the factor to both bind bivalent cations and form zinc finger structures. In this paper, we therefore examined the interactions of ZiRF1 with MRE sequences and the role of Zn^{2+} ions in modulating its MRE-binding activity.

The observation that ZiRF1 exhibits its functional activity as a metal-bound protein and not as an apoprotein and the finding that the EDTA-treated GST–ZiRF1 fusion protein was able to recover full MRE-binding activity *in vitro* exclusively in the presence of Zn^{2+} ions suggest a key role for this metal in the activation of ZiRF1.

Our data demonstrate the specific interactions of ZiRF1 with the functional $MRE_{d/c}$ region that spans from nucleotides -150 to -123 of the mouse MT-I promoter. Both *in vivo* and *in vitro* footprinting analyses showed that nuclear proteins whose binding is enhanced by Zn^{2+} could interact with this metal-regulatory region [14]. Interestingly, nucleotide changes in the sites of interaction with ZiRF1, which were not investigated in the earlier analysis of the point mutations of MRE regions [11], also abolished the $MRE_{d/c}$ metal-dependent activation of a heterologous promoter.

We demonstrated that antibodies raised against ZiRF1 recognize this protein as a major component of the MBC revealed in L-cell extracts and that sequence specificity and Zn^{2+}-dependence, two features of ZiRF1, characterize the interactions of MBC with this MRE region.

We also detected other MRE-binding complexes of higher molecular mass (hmMBCs), but only the MBC, like ZiRF1, appeared to be modulated by Zn^{2+} ions. Both the mouse MEP1 and the rat ZAP factors show Zn^{2+}-dependent MRE-binding activity [15,22], but their higher molecular mass indicates that ZiRF1 is a distinct factor. Further examination of the nuclear extracts obtained from the mouse fibroblast NIH3T3 cell line by competition experiments with oligonucleotides matching the high-affinity binding sites for both mMTF1 and SP1 indicated that, since mMTF1 does not bind to the SP1 site, SP1 or other proteins with similar binding activities are probably hmMBC components.

Taken together, our results indicate the mutual interactions of the transcription factors ZiRF1 and SP1 with the $MRE_{d/c}$ region of the mouse MT-I promoter and support the hypothesis that metal-dependent activation of MT genes may involve more than one protein with different affinities for MREs and/or multiple mechanisms of activation after metal treatment [39]. Metal-regulated factors and basal SP1 bind to this region, as demonstrated also by Mueller et al. [14]. Analysis of MRE interactions with purified human SP1 confirms these results and indicates that this transcription factor displays the properties of an MRE-binding protein.

We also found that the SP1–MRE interaction was more resistant to treatment with metal chelators when compared with ZiRF1 (results not shown). Therefore it seems likely that, in the case of SP1, Zn^{2+} ions may have a structural role, i.e. they stabilize the zinc finger domains [40]. Conversely, the MRE-binding properties of ZiRF1 suggest that Zn^{2+} ions may act as allosteric effectors during the interaction with a Zn^{2+}-sensitive domain, and this ensures the correct functional structure of the factor.

The features of ZiRF1 suggest that it may be activated in a manner analogous with the yeast MT regulator ACE1/CUP2 [41,42]. Zn^{2+} ions may therefore interact with the metal-binding domain of ZiRF1, which pre-exists as an apoprotein. In this case, the apo-ZiRF1 could act as a molecular sensor of the intracellular metal content.

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REFERENCES

1 Hamer, D. H. (1986) Annu. Rev. Biochem. **55**, 913–951
2 Bremner, I. and Beattie, J. H. (1990) Annu. Rev. Nutr. **10**, 63–83
3 Kagi, J. H. R. (1991) Methods Enzymol. **205**, 613–626
4 Karin, M. (1985) Cell **41**, 9–10
5 Durnam, D. M. and Palmiter, R. D. (1981) J. Biol. Chem. **256**, 5712–5716
6 Stuart, G. W., Searle, P. F., Chen, H. Y, Brinster, R. L. and Palmiter, R. D. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 7318–7322
7 Stuart, G. W., Searle, P. F. and Palmiter, R. D. (1985) Nature (London) **317**, 828–831
8 Searle, P. F., Davidson, B. L., Stuart, G. W., Wilkie, T. M., Norstedt, G. and Palmiter, R. D. (1984) Mol. Cell. Biol. **4**, 1221–1230
9 Zafarullah, M., Bonham, K. and Gedamu, L. (1988) Mol. Cell. Biol. **8**, 4469–4476

10 Searle, P. F., Stuart, G. W. and Palmiter, R. D. (1985) Mol. Cell. Biol. **5**, 1480–1489
11 Culotta, V. C. and Hamer, D. H. (1989) Mol. Cell. Biol. **9**, 1376–1380
12 Carter, A., Felber, B., Walling, M., Jubier, M., Schmidt, C. and Hamer, D. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 7391–7396
13 Karin, M., Haslinger, A., Heguy, A., Dietlin, T. and Cooke, T. (1987) Mol. Cell. Biol. **7**, 606–613
14 Mueller, P. R., Salser, S. J. and Wold, B. (1988) Genes Dev. **2**, 412–427
15 Andersen, R. D., Taplitz, S. J., Wong, S., Bristol, G., Larkin, B. and Herschman, H. R. (1987) Mol. Cell. Biol. **7**, 3574–3581
16 Westin, G. and Schaffner, W. (1988) EMBO J. **7**, 3763–3770
17 Séguin, C. and Prèvost, J. (1988) Nucleic Acids Res. **16**, 10547–10560
18 Imbert, J., Zafarullah, M., Culotta, V. C., Gedamu, L. and Hamer, D. (1989) Mol. Cell. Biol. **9**, 5315–5323
19 Searle, P. (1990) Nucleic Acids Res. **18**, 4683–4690
20 Czupryn, M., Brown, W. E. and Vallee, B. L. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 10395–10399
21 Koizumi, S., Suzuki, K. and Otsuka, K. (1992) J. Biol. Chem. **267**, 18659–18664
22 Séguin, C. (1991) Gene **97**, 295–300
23 Labbè, S., Prévost, J., Remondelli, P., Leone, A. and Séguin, C. (1991) Nucleic Acids Res. **19**, 4225–4231
24 Minichiello, L., Remondelli, P., Cigliano, S., Bonatti, S. and Leone, A. (1994) Gene **143**, 289–294
25 Radtke, F., Heuchel, R., Georgiev, O., Hergersberg, M., Gariglio, M., Dembic, Z. and Schaffer, W. (1993) EMBO J. **12**, 1355–1362
26 Palmiter, R. D. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 1219–1223
27 Xu, C. (1993) DNA Cell Biol. **12**, 517–525
28 Inouye, C., Remondelli, P., Karin, M. and Elledge, S. (1994) DNA Cell Biol. **13**, 731–742
29 Jones, K. A. and Tjian, R. (1985) Nature (London) **317**, 179–182
30 Luckow, B. and Schults, G. (1987) Nucleic Acids Res. **15**, 5490
31 Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. A. and Struhl, K. (1988) in Current Protocols in Molecular Biology, pp. 29–53, Greene/Wiley Interscience, New York
32 Smith, D. B. and Johnson, K. S. (1988) Gene **67**, 31–40
33 Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G. and Chasin, L. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 1373–1376
34 An, G., Hidaka, K. and Siminovitch, L. (1982) Mol. Cell. Biol. **2**, 1628–1632
35 Fried, M. and Crothers, A. D. (1981) Nucleic Acids Res. **9**, 6505–6525
36 Pabo, C. O. and Sauer, R. T. (1984) Annu. Rev. Biochem. **53**, 293–321
37 Dignam, J. P., Lebovitz, R. M. and Roeder, R. G. (1983) Nucleic Acids Res. **11**, 1475–1489
38 Gilman, M. Z., Wilson, R. N. and Wienberg, R. A. (1986) Mol. Cell. Biol. **6**, 4305–4316
39 Thiele, D. J. (1992) Nucleic Acids Res. **20**, 1183–1191
40 Gibson, T. J., Postma, J. P. M., Brown, R. S. and Argos, P. (1988) Protein Eng. **2**, 209–218
41 Fürst, P., Hu, S., Hackett, R. and Hamer, D. (1988) Cell **55**, 705–717
42 Buchman, C., Skroch, P., Welch, J., Fogel, S. and Karin, M. (1989) Mol. Cell. Biol. **9**, 4091–4095

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