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A nuclear factor binds to the metal regulatory elements of the mouse gene encoding metallothionein-I

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ABSTRACT

The ability of vertebrate metallothionein (MT) genes to be induced by heavy metals is controlled by metal regulatory elements (MREs) present in the promoter in multiple, non-identical copies. The binding specificity of the mouse L-cell nuclear factor(s) that interact with the element MREd of the mouse MT-I gene was analyzed by in vitro footprinting, protein blotting, and UV cross-linking assays. In vitro footprinting analyses revealed that synthetic oligodeoxynucleotides (oligomers) corresponding to the metal regulatory elements MREa, MREb, MREc, MREd and MREe of the mouse MT-I gene, as well as the MRE4 of the human MT-IIA gene and the MREa of the trout MT-B gene, all competed for the nuclear protein species binding to the MREd region of the mouse MT-I gene, the MREe oligomer being the weakest competitor. In addition, protein blotting experiments revealed that a nuclear protein of 108 kDa, termed metal element protein-1 (MEP-1), which specifically binds with high affinity to mouse MREd, binds with different affinities to the other mouse MRE elements, mimicking their relative transcriptional strength in vivo: MREd ≥ MREa = MREc > MREb > MREe > MREf. Similarly, human MRE4 and trout MREa bind to MEP-1. A protein similar in size to MEP-1 was also detected in HeLa-cell nuclear extracts. In UV cross-linking experiments the major protein species, complexed with mouse MREd oligomers, migrated on a denaturating gel with an apparent Mr of 115 000 and was detected using each of the mouse MRE oligomers tested. These results show that a mouse nuclear factor can bind to multiple MREs in mouse, trout, and human MT genes.

INTRODUCTION

Metallothioneins (MTs) are small cysteine-rich proteins that bind heavy metal ions such as Cd^{2+}, Zn^{2+} and Cu^{2+} (1,2). The genes encoding MTs are inducible at the transcriptional level by the same metal ions that the MTs bind. Heavy metal activation of MT gene transcription is dependent on the presence of short cis-acting elements, termed metal regulatory elements (MREs), which are present in six non-identical copies (MREa through MREf) in the 5′ flanking region of the mouse MT-I gene (3–7). Synthetic copies of four of the mouse MT-I MRE sequences are able to confer heavy-metal regulation on a heterologous promoter. MREe and MREf elements are apparently inactive while MREd is the strongest element for metal induction (6,8). In addition, the MREd element has the capacity to respond to the same spectrum of metal ions (Cd^{2+}, Zn^{2+} and Cu^{2+}) as does the complete MT gene promoter, suggesting that all MRE elements are responsive to the different metals and together act to facilitate a strong induction response (9).

Competition experiments suggested that one or more positively acting transcription factors interact with these elements (10,11). The mechanism by which these factors act as positive regulatory proteins in the presence of metals is still unknown. It has been postulated that heavy metals increase transcription by inducing the binding of a regulatory factor to the MREs (3–7, 12–14). However, it is not yet known whether metal induction of MT gene transcription involves different factors binding to different MRE sequences or a single factor binding to different MREs with different affinities. In vivo, dimethylsulphate protection has been observed on all MREs upon stimulation both in the mouse (7) and the rat (14). In vitro, metal-dependent binding of factors has been observed both to the mouse MREd (13,15) and MREa (8) elements as well as to the rat MT gene promoter (16). Imbert et al. (17) have shown that MBF-1, a mouse nuclear factor of 74 kDa purified to near homogeneity, can bind to the MREa element of the trout MT-B gene as well as to MREd and MREe of the mouse MT-I gene. However, this factor was reported not to bind to the other MREs of the mouse gene.

We have previously shown that a mouse nuclear factor of 108 kDa, here designated MEP-1, binds with high affinity to the MREd of the MT-I gene (18). To determine if different MRE elements can bind common nuclear factors, we performed competition experiments in an exonuclease III (ExoIII) footprinting assay. In addition, we performed protein blotting and UV cross-linking experiments to assess the binding properties

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of MEP-1 to different MRE elements. Our results show that the protein(s) binding to the MREd region of the mouse MT-I gene can bind with different affinities to other MRE elements. Moreover, MEP-1 can bind to the different MREs present in the promoter of the mouse MT-I gene as well as to the MREa of the trout MT-B gene and the MRE4 of the human MT-IIA gene.

MATERIALS AND METHODS

Cell culture and footprinting analyses

HeLa (Strain S3, American Type Culture Collection) and heavy metal-resistant mouse L cells (L50) (generously provided by D.H. Hamer) were grown in suspension in SMEM supplemented with glutamine, antibiotics and 10% fetal calf serum. L50 cells were grown in the continuous presence of 15 μM $CdCl_2$ (15) while HeLa cells were treated with 5 μM $CdCl_2$ for 10 hours before harvesting by centrifugation. All buffers used to prepare crude nuclear extracts contained 5 μM $CdCl_2$ (13).

ExoIII footprinting analyses were performed as described by Séguin and Hamer (13). The MT fragment used as the probe was 5′ end-labelled at position +64 and extends to position −200 relative to the start point of transcription. Gel-purified oligodeoxynucleotide (oligomer) competitors (Fig. 1B) were annealed to form double-stranded DNA in medium salt buffer (19) by slow cooling from 95°C to room temperature. The concentration of each single stranded oligomer DNA was measured by spectrophotometry. The percentage of double stranded molecules was evaluated by gel electrophoresis and was always greater than 95%. Competitors were added together with the probe and binding was allowed to proceed for 20 min at 24°C. The samples were loaded and electrophoresed at a constant current of 30 mAmp on 5% sequencing gels (38:2 acrylamide:bisacrylamide). The gels were dried and autoradiographed by standard procedures. Densitometry was performed on multiple exposures of each gel using a Joyce-Loebl Chromoscan 3 densitometer.

Protein transfer analyses

The protein blotting procedure (South Western) was performed with double stranded oligomers (Fig. 1B) freshly labelled with polynucleotide kinase and [γ^{32}P]-ATP at a specific activity of 2000 to 2500 cpm/fmol (18). Typically, 30 μl of crude nuclear

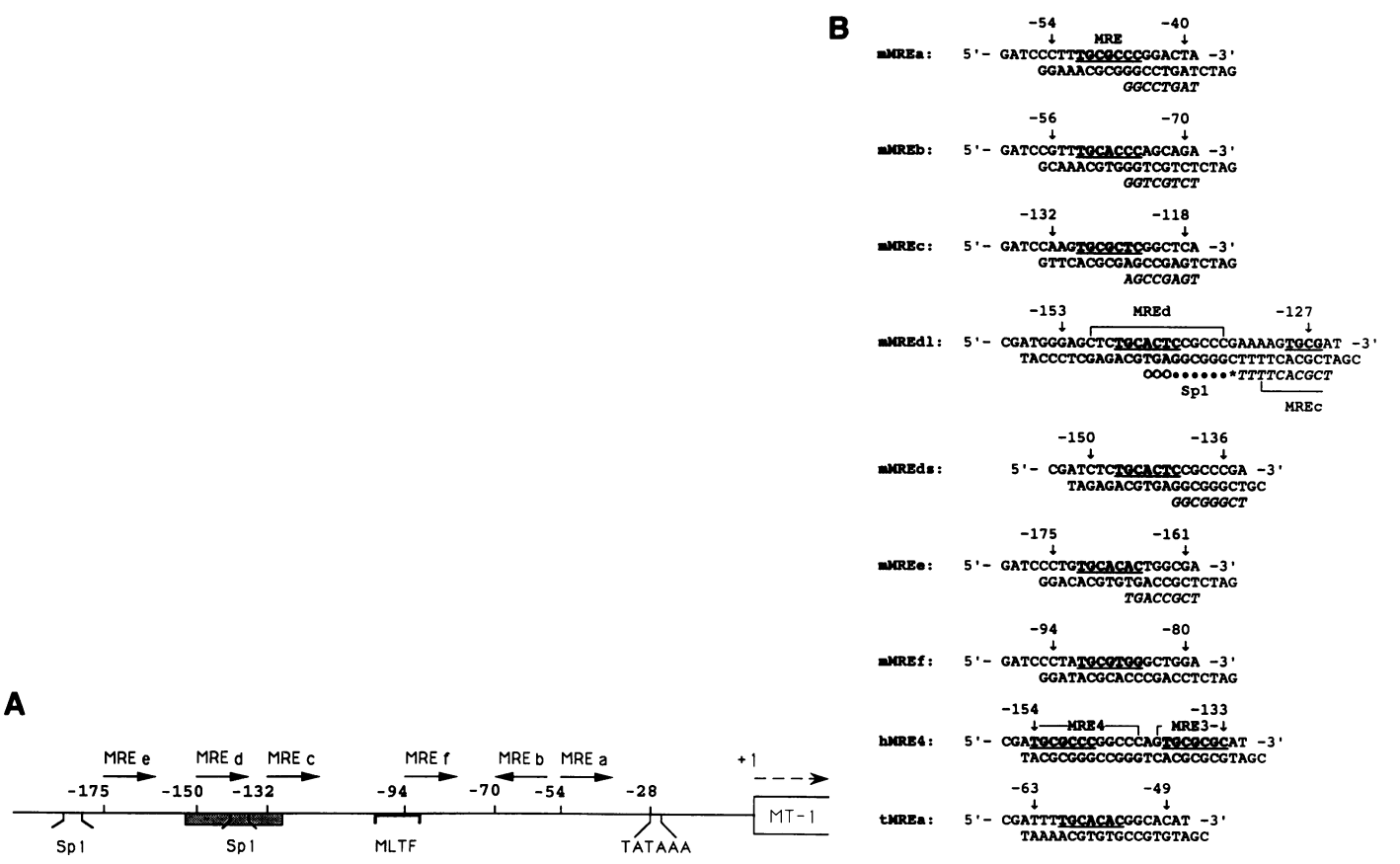

Figure 1. A) Arrangement of the six metal regulatory elements (arrows) of the mouse MT-I gene, the binding sites for the transcription factor Sp1 (7,23) the G-rich sequence interacting with the transcription factor MLTF (22) and the TATA box. The hatched box indicates the protected region around MREd in ExoIII footprinting experiments. B) Sequences of the synthetic oligomers. The underlined nucleotides correspond to the conserved MRE core sequence TGCRCNC (R, purine; N, any nucleotide). On the mMREdl oligomer, dots below the sequence indicate similarity with the consensus Sp1 binding site; black dots indicate agreements with unique nucleotides, white dots indicate agreements with ambiguous nucleotides and the asterix indicates disagreement at an ambiguous nucleotide. Vertical arrows indicate the boundaries (−153 and −127) of the protected region in an ExoIII footprinting analysis (13). The arrows on the other MRE elements indicate the region covering the MRE consensus sequences (6). Brackets indicate the positions of MREd and MREc consensus sequence on the mMREdl oligomer and the positions of MRE4 and MRE3 core sequence on the hMRE4 synthetic DNA. Shorter italicized DNA sequences beneath each paired MRE element correspond to the primer used to generate the probe for the UV cross-linking analyses. MT sequences extend to positions −155 and −126 on mMREdl and to position −134 on mMREds. The mMREb oligomer is in the inverse orientation. MRE oligomers were synthesized with a *BamHI* (mMREa, mMREb, mMREc, mMREe and mMREf) or a *ClaI* (mMREdl, mMREds, hMRE4 and tMREa) site on both ends. The trout element is from the MT-B gene and the human element from the MT-IIA gene.

extracts (10 mg protein/ml, as determined by the BioRad protein assay) were electrophoresed on $NaDodSO_4$/PAGE using 8% acrylamide-bisacrylamide (50:1) separating gels and transferred to Immobilon-PVDF membranes (Millipore). Filter strips were incubated for 4h in binding buffer containing 10^5 cpm of ^{32}P-labelled DNA probe per ml. The relative intensity of the signal obtained with the different MRE probes was evaluated by laser densitometry and a value of one was arbitrarily assigned to the signal obtained with the mMREdl oligomer.

UV cross-linking

A UV cross-linking protocol to determine the molecular weight of the MRE-binding protein(s) in crude nuclear extracts was developed, based on those described by others (17,20,21). The probe was prepared by hybridizing a 21-base oligomer of the coding strand of the different MRE elements from the mouse MT-I gene to an 8- or 10-base complementary primer (Fig. 1B). These oligomers were made completely doubled stranded by incubation with the Klenow fragment of DNA polymerase I in the presence of the appropriate [$\alpha^{32}P$]-dNTP and the three other unlabelled nucleotides. The [$\alpha^{32}P$]-labelled nucleotide was dCTP for MREa and MREds, dTTP for MREb, MREdl and MREe, and dATP for MREc. Each reaction mixture contained 20 fmol of oligomer probe (5×10^4 to 5×10^5 cpm), 10 μg of yeast tRNA (Gibco-BRL), 1 μg of $p(dN)_5$ (Pharmacia), 0.5 μg of *Msp*I digest of lambda DNA and 20 μl of crude L50-cell nuclear extracts (10 mg/ml) in buffer III (13). The final reaction volume was brought to 50 μl with binding buffer (20 mM HEPES, pH 7.9; 50 mM NaCl; 1 mM DTT; 5 mM $MgCl_2$; 5% glycerol; 0.1 mM EGTA). After DNA protein binding (20 min, 24°C), reaction mixtures were irradiated for 20 min at 4°C with a 254 nM UV lamp (600 μW/cm). When specified, DNase I (Worthington Diagnostics, 2064 units/mg) was added as indicated in the figure legends and the mixture incubated at 37°C for 15 min. Fractions were resolved by 8% $NaDodSO_4$-polyacrylamide (acrylamide/bisacrylamide: 30−0.8) gel electrophoresis and analyzed by autoradiography.

RESULTS

Exonuclease III footprinting competition experiments

Fig. 1A shows the arrangement of the six MRE elements on the mouse MT-I gene (3,5,7,12), the G-rich sequence that interacts with the major late transcription factor MLTF (22) and the two Sp1 sites (7,23). Using an in vitro ExoIII footprinting assay, we have previously shown (13) that one or more nuclear proteins bind to the MREd region between nucleotides −127 and −153 relative to the start point of transcription (Fig. 1A, hatched box). The DNA sequence recognized by this factor is the same as that required for in vivo transcriptional activity of MREd (9), since substitution of residues G or C of mMREds (nucleotides −146 and −143, see Fig. 1B) by A (MUT2) or T respectively, completely abolished binding activity (15). Zinc ions are required for specific in vitro DNA binding of the MREd-binding protein(s) the latter which is different from the transcription factor Sp1 (15,18, Labbé and Séguin, unpublished results). To determine if the MREd-binding protein(s) present in mouse extracts can also bind to the other MREs of the mouse MT-I gene or to MRE elements present in MT genes from other species, we performed competition experiments with synthetic oligomers corresponding to the individual MREs. The sequences of the different oligomers used are shown in Fig. 1B: mMREdl corresponds to the protected region, as assayed by ExoIII footprinting, in the mouse MREd region, namely the entire MREd sequence and the 5′ end portion of MREc (nucleotides −127 to −153); mMREds contains only the MREd consensus sequence (nucleotides −134 to −150); mMREa, mMREb, mMREc, mMREe and mMREf correspond to the different MREs present in the mouse MT-I gene. Oligomers corresponding to the MRE3-MRE4 region of the human MT-IIA gene (hMRE4) and MREa of the trout MT-B gene (tMREa) were also synthesized.

All of the MREs tested could compete with the protein(s) binding to the mouse MREd region (Fig. 2), indicating that the same cellular factor binds to all of these MRE elements; mMREdl, mMREds, mMREa, mMREb and mMREc showed

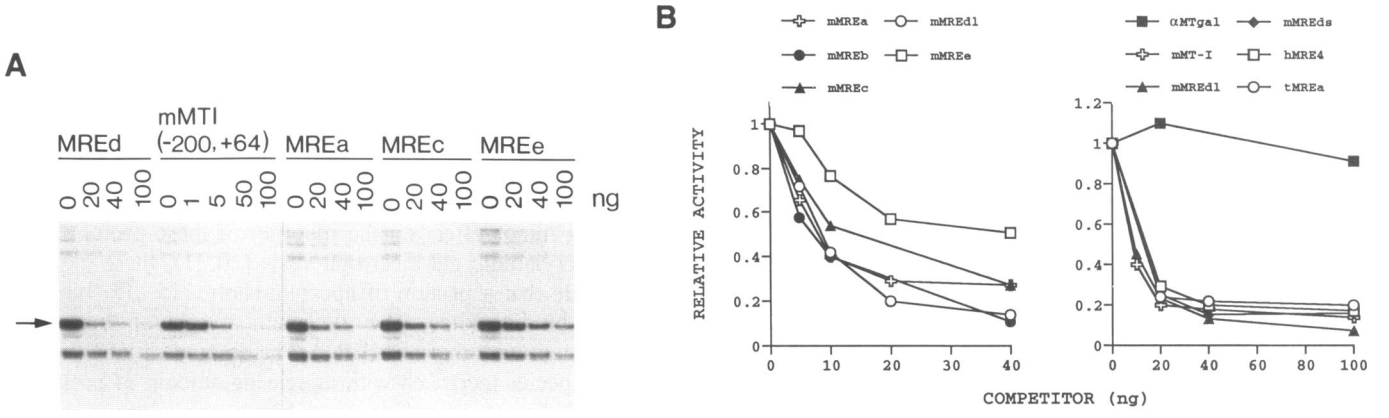

Figure 2. A) ExoIII footprinting assay of a representative competition experiment using crude nuclear extracts prepared with heavy metal-resistant mouse L cells (L50) grown in the continuous presence of 15 μM $CdCl_2$, as described in Material and Methods. Competition was performed with double-stranded unlabelled oligomers corresponding to the various mouse MRE elements, as indicated, or with the same MT DNA fragment (mMTI) (−200 to +64) used as a probe in the ExoIII assay. The amount of competitor oligomer used in each reaction is shown above the lanes. The probe was used at a concentration of approximately 1 ng per assay. The MREd oligomer used in this experiment is mMREdl. The arrow indicates the ExoIII stop at the −153 boundary. B) Graphic representations of ExoIII footprinting competition experiments performed, as in Fig. 2A, with induced L50-cell nuclear extracts and unlabelled oligomers corresponding to the indicated MRE elements. The DNA competitor αMTgal is a plasmid in which most of the MT promoter sequences have been replaced by a fragment of the human α-globin gene (10); no MRE element is present in this DNA. The intensity of the band at −153 (arrow in Fig. 2A) relative to that with no competitor DNA (relative activity) is shown as a function of the concentration of competitor DNA. The intensity of the bands was measured by scanning the films of multiple exposures of each gel with a transmission densitometer.

similar competition strength, while mMREe was approximately 50% weaker. Competition experiments were also performed with synthetic oligomers corresponding to the MREa of the trout MT-B gene and to a region of the human MT-IIA gene covering the MRE4 element and the 5′ end portion of MRE3 (nucleotides −133 to −154), a region interacting with mouse and human nuclear proteins as assayed by Dnase I footprinting (Remondelli, P., Séguin, C. and Leone, A., unpublished results). All of these oligomers competed equally well and as strongly as mMREdl (Fig. 2B). The heterologous competitor DNA, αMTgal, is a Simian Virus 40-based plasmid, containing human α-globin promoter sequences fused to a minimal MT promoter at position −34 (10); this construct does not contain any MRE elements and did not compete for the MREd-binding protein(s) (Fig. 2B).

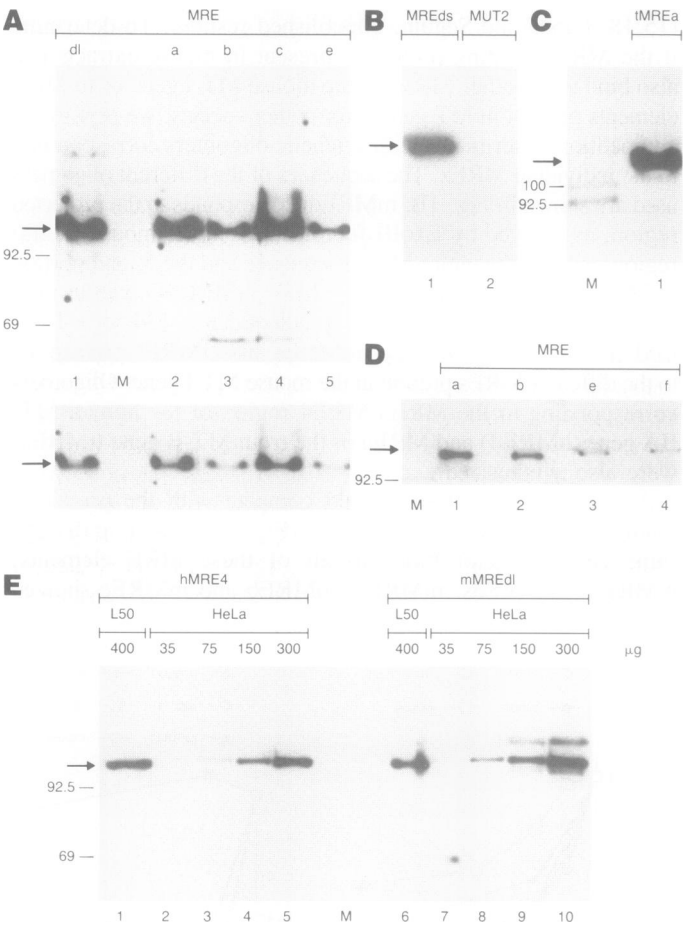

Figure 3. Binding of oligomer MRE variants to L50-cell nuclear proteins as assayed by the protein blotting procedure (South-Western) A) Mouse MRE oligomer probes MREdl to MREe as designated above the lanes. A shorter exposure of the band corresponding to the 108 kDa protein species (MEP-1) is shown at the bottom of the figure. M: ^{14}C labelled protein markers. B) Binding of the mouse MREds (lane 1) and the MUT2 (lane 2) oligomer probes to MEP-1. MUT2 is a nonfunctional mutant in which the G at position −146 has been replaced by an A (15). C) Binding of the tMREa oligomer probe to L50-cell nuclear proteins (lane 1). The gel was loaded with 300 µg of extract. D) Relative binding affinity of the mouse MREf oligomer. The mouse MRE probes are designated above the lanes. E) Analysis of the binding of human and mouse MRE oligomers to nuclear proteins using hMRE4 (lanes 1−5) or mMREdl (lanes 6−10) as the ligand. Nuclear extracts: lanes 1 and 6, L50-cell crude nuclear extracts; lanes 2−5 and 7−10, HeLa-cell crude nuclear extracts. The indicated amount of protein (in µg) was loaded on the gel. Arrows correspond to MEP-1.

Thus, the protein(s) binding to the mouse MREd region can bind the other MREs present in the mouse gene as well as MREs from MT genes of other species.

South Western analyses

We have previously shown, using a protein-blotting procedure (South Western), that a mouse protein of 108 kDa (p108), distinct from Sp1, binds with high affinity to the mMREdl oligomer (18). To assess more directly the binding properties of the different MREs, South Western analyses were performed with the same MRE oligomers used in the ExoIII footprinting competition experiments. The oligomers mMREdl, mMREa, mMREc (Fig. 3A) and mMREds (Fig. 3B, lane 1) bound strongly to the 108 kDa protein species, while the binding was 3 fold lower for mMREb, 6 fold lower for mMREe and more than 20 fold lower for mMREf (Fig. 3A and 3D). The nonfunctional core mutant MUT2, which is inactive in vivo (9), does not compete for the MREd-binding protein(s) in an ExoIII footprinting assay (15), and did not bind to p108 (Fig. 3B, lane 2). These results correlate very well with the relative strength as metal-dependent cis-acting transcriptional elements of these MREs in vivo. Strong binding of p108 to hMRE4 (Fig. 3E, lane 1) and tMREa (Fig. 3C) probes was also detected. As shown in Fig. 3E, the mMREdl and hMRE4 oligomers also bound to a HeLa-cell nuclear protein of 108 kDa. The p108 MRE-binding protein detected by the protein blotting procedure will be henceforth referred as to MEP-1 (metal element protein-1).

Detection of MEP-1 by UV cross-linking analyses

A mouse nuclear protein of 74 kDa, MBF-1, binds to the mMREds oligomer, as assayed by UV cross-linking (17). However, MBF-1 was not detected by the protein-blotting procedure used in this study. To compare the molecular weight of the MRE-binding protein species detected with the two assays, UV cross-linking experiments were performed with crude L50-cell nuclear extracts. The major protein species complexed with the mMREdl (Fig. 4A, lane 1 and 4B, lanes 1−7, arrows) or mMREds (Fig. 4B, lane 8) oligomers (and cross-linked by UV irradiation) migrated on a denaturating gel with an apparent Mr of 115 000. When probe DNA was irradiated in the absence of protein or when UV irradiation was omitted, no labelled species were generated (data not shown). Moreover, using this UV irradiation assay, all of the mouse MRE elements tested were able to form the same complex of 115 kDa (Fig. 5A and 5B). Since the covalent attachment of short oligomers to proteins has only a minor effect on the mobility of these proteins in SDS-polyacrylamide gel electrophoresis (20,21), these experiments indicate that a protein of approximately 115 kDa binds to the MRE-binding sites. The molecular weight of this protein is consistent with that of MEP-1. The amount of label in this 115 kDa-species increased with increasing amount of probe and, at high probe concentrations, some lower molecular weight proteins became radiolabelled (Fig. 5); these protein species were not further analyzed. Furthermore, while the chelating agent EDTA led to a 45 percent inhibition of complex formation with the mMREdl oligomer, Zn^{2+} could restore the binding activity of chelated extracts to 135 percent of the control (data not shown). Finally, the sensitivity of the complex to DNase digestion was analyzed. In contrast to the complexes formed with MBF-1, the UV-irradiated protein-DNA complex of 115 kDa detected in these experiments was very sensitive to DNase I digestion and 10 units

of DNase I was sufficient to destroy most of the complex (Fig. 4A, lane 2 and Fig. 4B, lanes 5–7).

DISCUSSION

We have used an ExoIII footprinting assay to ask whether the mouse nuclear protein binding to the MREd region of the mouse MT-I gene promoter can bind to other MRE elements. We have demonstrated that all of the mouse MRE elements tested, as well as MREs present in human and trout MT genes, are able to compete for the protein species binding to the mouse MREd region. In addition, protein blotting experiments demonstrated that MEP-1 binds with different affinities to the six MRE elements of the mouse MT-I gene as well as to a human and a trout element. MEP-1 was also detected in HeLa cells using either a mouse or a human oligomer as probe. By UV cross-linking analyses, we have detected a DNA-protein complex migrating on SDS-polyacrylamide gels with a size of approximately 115 kDa, which is consistent with the size of MEP-1.

Using synthetic mouse MRE sequences, it has been shown that, in vivo, MREd is the strongest element , MREa and MREc are 50 to 80 percent weaker, MREb is 90 percent weaker and MREe is apparently non-functional (6). Here, we have shown that, in footprinting assays, the mMREdl, mMREds, mMREa, mMREb, mMREc, hMRE4, and tMREa oligomers all competed with similar strength for the protein species binding to the mouse MREd region (Fig. 2). Only mMREe, a non functional element, clearly showed a weaker competitive activity. Moreover, the binding affinity of MEP-1, as assayed by South Western analysis, is equivalent for the mMREdl, mMREds, mMREa, mMREc, hMRE4 and tMREa oligomers, while it is 3 fold lower for mMREb and 6 to 20 fold lower for mMREe and mMREf (Fig. 3). The low binding affinity of mMREf is in agreement with the inability of this element to confer regulatory activity in vivo (8). Overall, there is a good correlation between the binding affinity of MEP-1 toward the different mouse MREs, as assayed in vitro by South Western analyses, and their relative strength for transcriptional metal induction in vivo (6). This suggests a central role of MEP-1 in the regulation of MT genes transcription.

Two kinds of evidence suggest that MEP-1 is one of the proteins which is competed out of the MREd region by the different MRE oligomers, as assessed by footprinting (Fig. 2). First, MEP-1 binds to MREd probes with high affinity. Second, the relative binding affinity of any MRE oligomer for MEP-1 corresponds reasonably well to the relative ability of that oligomer to compete with the MREd-binding proteins. In addition, mutants such as MUTd, which has all five of the most strongly conserved nucleotides of the MRE consensus sequence changed (18), or MUT2 which contains a transition at the nucleotide −146 in the core MRE consensus region (15) do not bind MEP-1 (18 and Fig. 3B) and do not compete for the MREd-binding protein(s) (15,18). Thus, nucleotides important for competing out the factors binding to the MREd region are also required for binding to MEP-1. Consequently, it is likely that MEP-1 contributes to the footprint observed in the MREd region. However, further experiments will be required to provide definitive proof of this.

Three other nuclear proteins have been reported to bind the mouse MREd element, namely MBF-1 (17), ZAP (8) and MTF-1 (24). MBF-1 is a mouse nuclear factor of 74 kDa which has been detected by UV cross-linking analyses, using a mouse MREd oligomer as probe, and was purified to near homogeneity employing, as an affinity reagent, the trout MREa element. However, it is not yet known whether MBF-1 can bind directly to MRE sequences other than the trout MREa. Imbert et al. (17) have observed only a weak footprint over the trout MREb element and a strong footprint over the mouse MREe region but no apparent binding over the other MRE regions of the mouse promoter. It is not clear why MBF-1 was not detected by any of the binding assays used in this study since the mouse MREd oligomer used to detect MBF-1 by UV cross-linking analyses was identical to mMREds which bound MEP-1 as shown both by South Western (Fig. 3B) and UV cross-linking (Fig. 4B) analyses. A lower affinity of MBF-1 for the MREd sequences could explain why it was not detected in the South Western assay

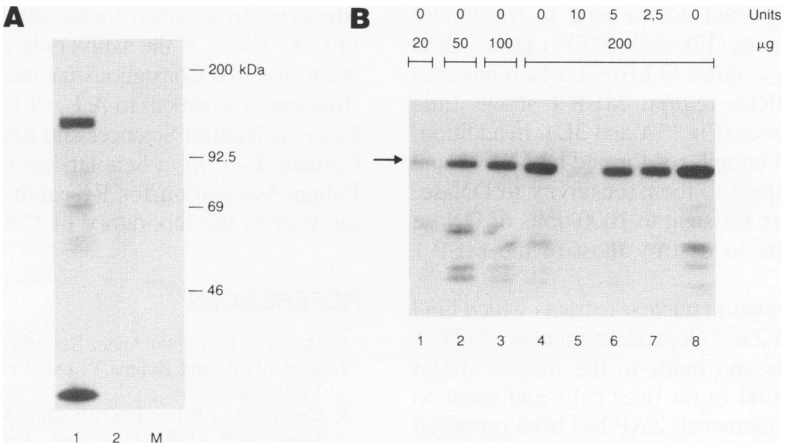

Figure 4. MRE affinity labelling performed by UV cross-linking as described in Materials and Methods A) DNA affinity labelling of mMREdl-binding factors in a L50-cell crude nuclear extract incubated for 20 min at 24°C followed by an incubation in the presence of 10 mM $MgCl_2$ and 0 (lane 1) or 20 (lane 2) units of DNase I. The band at the bottom of lane 1 corresponds to the free oligomer probe. B) The effect of increasing the amount of nuclear proteins or incubating with different amounts of DNase I. The amount of protein extract in the binding reaction (20–200 µg) and the amount (0–10 units) of DNase I are shown above the lanes. Incubation with DNase I was for 15 min at 37°C. The oligomer probe was mMREdl (lanes 1–7) or mMREds (lane 8). Arrows indicate the approximately 115 kDa DNA-protein complex.

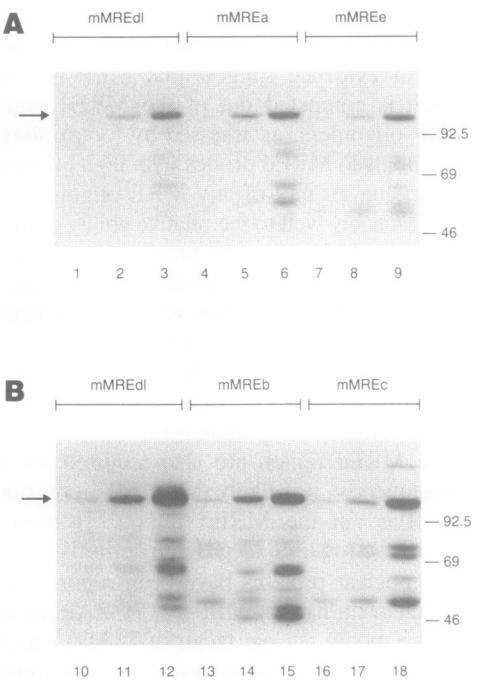

Figure 5. UV cross-linking experiments using the different mouse MRE elements as probes. Oligomer probes. A) Lanes: 1–3, mMREdl; 4–6, mMREa; 7–9, mMREe; B) Lanes: 10–12, mMREdl; 13–15, mMREb; 16–18, mMREc. Increasing amounts (1.2, 2.5 and 5.0×10^5 cpm) of labelled oligomers where incubated with 150 μg of a L50-cell nuclear extract. The film in B) was exposed twice as long as in A)

while differences in the binding buffer, the assay conditions and the cell types used to prepare the nuclear extracts could explain the difference in binding activities detected by us and by Imbert et al., (17) in UV cross-linking experiments. Nevertheless, these results show that MRE elements like mMREd and tMREa can bind at least two protein species, namely MBF-1 and MEP-1.

There is strong evidence that MBF-1 is distinct from MEP-1 and does not represent a degradation product of MEP-1 and that MEP-1 does not represent a dimerization complex of MBF-1. Indeed, MBF-1 has been reported not to bind to the mouse MREa, MREb or MREc elements (17) while MEP-1 clearly does (Figs. 3 and 5). Moreover, in contrast to MBF-1 which interacts strongly with the mouse MREe region, MEP-1 shows little affinity for the mMREe oligomer (Fig. 3A and 3D). In addition, the mMREd oligomer-protein complexes formed by MBF-1 and MEP-1 are different with respect to their sensitivity to DNase. While MBF-1 complexes were resistant to 1000 units of DNase I (17), 10 units was sufficient to destroy most of the MEP-1 complexes (Fig. 4B).

Two factors have been detected in nuclear extracts which bind to mouse MRE elements in a Zn^{2+}-dependent manner. MTF-1 (24) is present in HeLa cells and binds to the mouse MREd element while ZAP (8) is found in rat liver cells and binds to the mouse MREa and MREd elements. ZAP has been proposed to be the rat equivalent of MTF-1 (8). While the molecular weight of MTF-1 has not been reported, preliminary protein blots suggest a size for ZAP similar to that of MEP-1 (8). However, more studies will be required to established the precise relation between MEP-1, ZAP and MTF-1.

Recently, Andersen et al. (16), using gel retardation and UV cross-linking procedures have demonstrated Cd^{2+}-dependent binding of a 39 kDa factor (p39), present in nuclear extracts of rat Fao hepatoma cells, to an oligomer containing the MREc and MREd elements of the rat MT-I gene. Contrary to what we observed for MEP-1, a single MRE is insufficient for efficient binding of this 39 kDa protein and at least two MREs are apparently required for binding. In *Saccharomyces cerevisiae*, Cu^{2+}-inducible transcription of the yeast MT gene *CUP1* requires Cu^{2+}-dependent binding of the *ACE1* protein to specific sites in the promoter region (25–31); in addition, a gene designated *ACE2*, plays a role in regulating basal-level expression of *CUP1* in Cu^{2+}-independent fashion (32).

The phenomenon of multiple factors that can bind to the same DNA site appears to be common in higher eukaryotes (33) and a number of proteins that bind to ATF sites (34), CCAAT/enhancer binding sites (35), octamer sites (36), Sp1 sites (37,38) and homeotic protein-binding sites (39) have been identified. The apparent overlapping DNA binding specificities of MBF-1 and MEP-1 at the level of MREd is intriguing and raises the problem of assessing the role of the different proteins in the regulation of MT genes expression by heavy metals. Moreover, the role of the different MREs and the nature of the interaction between the factors that bind to them are not clear. Two possible mechanisms can be envisioned. (i) Both MEP-1 and MBF-1 play a role in regulating induced-level expression of MT transcription or alternatively, (ii) MEP-1 and MBF-1 may modulate induced- and basal transcription in a way similar to *ACE1* and *ACE2* in yeast. These hypotheses do not exclude the involment of other putative MRE-binding proteins, such as p39 (16), or non DNA-binding factor(s) acting as 'co-activator' proteins (40). Analysis of the action of MEP-1 on MT transcription and its potential interactions with MBF-1 will require precise definition of the recognition sequence of both proteins and in vitro transcription reconstitution experiments using purified MBF-1 and MEP-1 and specific antibodies.

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