Volume 16 Number 22 1988 Nucleic Acids Research

Detection of a nuclear protein that interacts with a metal regulatory element of the mouse metallothionein 1 gene

Carl Séguin[1]* and Jacinthe Prévost[1]

Department of Physiology and Laval University Medical Center, Laval University, Québec, Canada

Received August 15, 1988; Revised and Accepted October 21, 1988

ABSTRACT

Metallothionein (MT) genes contain multiple metal regulatory elements (MREs) that are responsible for metal induction. A protein blotting procedure and a synthetic oligonucleotide have been used to identify nuclear factors interacting with a MRE (MREd) of the mouse MT-1 gene. We report the specific binding of the probe to a protein of apparent Mr 108,000 (p108). The specificity of the interaction was demonstrated by mutation analysis and competition experiments. Furthermore, the probe contains the Sp1 consensus binding sequence 5'CCGCCC3', in addition to the MRE consensus sequence, 5'TGCAC3', and we show that a Simian Virus 40 DNA fragment which contains six Sp1 binding sites did not bind p108 nor did it compete for the protein(s) interacting with MREd in a DNA footprinting assay. These results show that a metal regulatory element of the mouse MT-1 gene interacts specifically with a nuclear protein of Mr 108,000 and that this protein is distinct from the transcription factor Sp1.

INTRODUCTION

Transcription regulation of eukaryotic genes transcribed by RNA polymerase II depends on multiple transcription factors interacting with the sequence elements that form the promoter (1,2). Different combinations of transcriptional factors are thought to mediate differential gene expression in various cell types, physiological conditions, during development or in response to extracellular inducers. Protein-protein interactions and modifications of regulatory proteins such as phosphorylation have been suggested to play an important role in the mechanism by which these proteins act (1). In several systems, such proteins have been identified, extensively purified and, for some of them, the corresponding cDNAs cloned. (2-5). This is the case for the transcription factor Sp1 which binds a GC rich element found in many cellular and viral promoters (6-9) and the major late transcription factor (MLTF) which has been found to be active at the promoter of the adenovirus 2 major late gene (10). MLTF also binds to the promoter of the rat X fibrinogen (11) and the mouse metallothionein 1 genes (12).

The metallothionein (MT) genes provide an interesting system for studying the mechanism of action of transcription factors on gene expression because they are transcriptionally inducible by heavy metals such as cadmium and zinc as well as by a number of other agents including glucocorticoid hormones and interferon (13,14). Mutant deletion analyses and sequence similarities have revealed at least five metal regulatory cis-acting elements (MRE a,b,c,d,and e) which allow transcription of the mouse MT-1 gene to be incuced by heavy metals (15-18). Experiments with synthetic MRE sequences showed that different MREs have different transcriptional efficiencies, MREd being the strongest element for metal-induced transcription (17). It has been suggested that stimulation of MT gene transcription by heavy metals is mediated by the binding of a specific heavy metals- activated regulatory protein (s) to the multiple MREs (13-20). Footprinting experiments indicated that mouse nuclear factors can bind to MREs in a metal-dependant manner (20-21). In vitro, proteins present in L cell crude nuclear extracts bind to MREd and the chelating agent EDTA selectively inhibits the binding of these proteins to the metal element (20). Moreover, in vivo, dimethylsulfate (DMS) protection is enhanced at MREd upon zinc induction (21). While in vitro, EDTA-sensitive footprints have been observed only to the MREd region, in vivo, all MREs show increased DMS protection upon stimulation with zinc. The reasons of the differences between in vivo and in vitro results are still ambiguous. In addition to MRE binding proteins some general transcription factors also interact with the mouse MT-1 gene. Recent in vitro experiments showed that the transcription factors Sp1 (21) and MLTF (12) bind to the mouse MT-1 promoter and that addition of MLTF to in vitro transcription reactions stimulates transcription from mouse MT-1 promoter (12). However, the mechanism by which Sp1, MLTF and the putative MRE-binding protein(s) act to modulate MT gene transcription initiation remains unclear. In an attempt to characterize the molecular nature of a specific MRE-binding protein present in L cell nuclear extracts, we use a protein blotting procedure and a synthetic oligonucleotide corresponding to the MREd region. We show that a mouse nuclear protein of apparent Mr 108,000 binds with high affinity to the MREd region.

MATERIALS AND METHODS

Preparation of nuclear extracts

Heavy metal-resistant mouse L cells (obtained from D.H. Hamer) were grown in suspension in presence of 50 μM $CdCl_2$ and 80 μM $ZnCl_2$ and crude

nuclear extracts were prepared as described (20) with 5 μM $CdCl_2$ in all buffers.

Protein blotting procedure

$NaDodSo_4$ /PAGE using 8% acrylamide-bisacrylamide (50:1) and 9% acrylamide-bisacrylamide (30:0.8) separating gels was carried out in a Protean II and a mini-Protean II BIORAD apparatus, respectively, as described (22) except that Coomassie blue instead of Pyronin Y was used as the tracking dye. Standard (Figs. 2a,4) and mini (Fig. 2b,c)-PAGE and electro-blotting apparatus were utilized. Identical results were obtained with both systems although standard size apparatus gave sharper bands (compare Fig. 2a, lane 1 with Fig. 2b, lane 1 and Fig. 2c, lane 1). In addition, the gel system used with the standard apparatus gave a better resolution of the proteins in the 100 kD range. Typically, 15 μl aliquots of crude nuclear extracts (6 to 10 mg/ml) were electrophoresed on mini-apparatus while 30 μl aliquots of an extract concentrated to 20 mg/ml with Amicon Centricon-10 microconcentrators prior to electrophoresis were used with standard apparatus. After electrophoresis, gels were incubated for a 1h wash in 200-500 ml of transfer buffer (100 mM Tris/190 mM glycine) with gentle agitation. Blotting procedures and detection of DNA-binding proteins were performed as described (22) with the following modifications. Nitrocellulose strips were incubated for 4h in binding buffer (20 mM Hepes, pH 7.9, 5 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl and 5 μM $CdCl_2$) containing 10^5 cpm of ^{32}P-labeled DNA per ml. The filters were washed in three changes of binding buffer over a period of 1h and then exposed to Kodak XAR-2 films with intensifying screens at -70°C. All procedures were done at 4°C and the binding reaction was always performed within the 24 hours following the transfer. When using mini-gel apparatus, the inner chamber buffer was changed every 15 minutes in order to keep the temperature below 10°C. Molecular weights were determined by running, in an adjacent lane, a set of C-14 labelled protein markers (Amersham). Oligonucleotides were labeled with polynucleotide kinase and [γ-^{32}P]ATP to 2500 cpm/fmol ($\sim$2 x 10^6 cpm/μg). Equimolar quantities of the two strands of oligonucleotides were mixed, boiled and slowly cooled down to room temperature. Aliquots of double- stranded oligonucleotides were electrophoresed through a non-denaturating 15 percent polyacrylamide gel. Typically 80 to 90% of the DNA migrated as double-strand species. Metallothionein, SV40 and pBr322 DNA fragments were labelled the same way to 2000, 6000 and 40,000 cpm/fmol, respectively. Competitor nucleic acids were added to the binding buffer

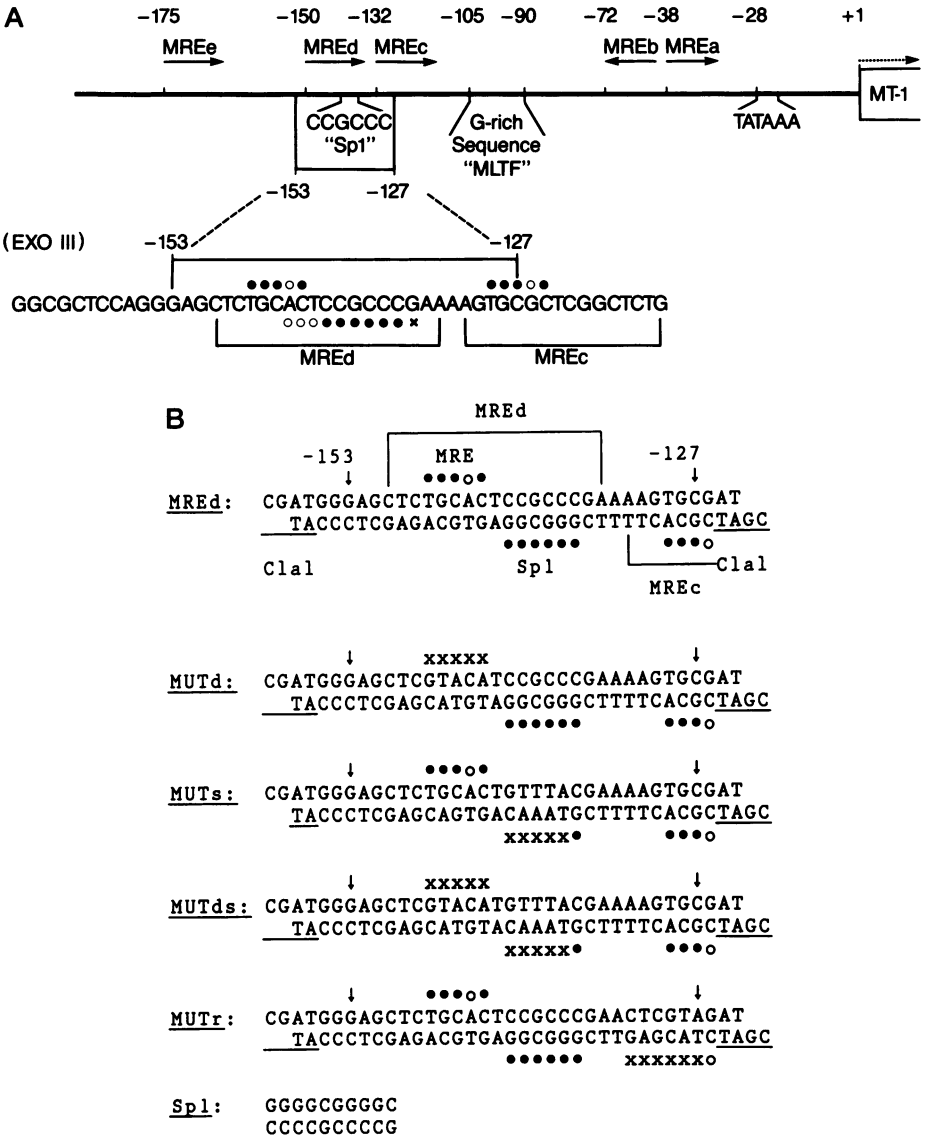

Fig. 1: (A) The top line shows the arrangement of the five metal regulatory elements (arrows) of the mouse MT-1 gene, the G-rich sequence which binds the transcription factor MLTF (10) and the TATA box. The sequence around MREd is shown with brackets above the sequence indicating the area protected from exonuclease III (20) and brackets below the sequence indicating the positions of MREc and MREd. Dots above the sequence indicate the conserved MRE core sequence TGCPuC (Pu, purine). Marks below the sequence indicate homology to the consensus Sp1 binding site; black dots indicate agreement with unique nucleotides, white dots indicate agreements with ambiguous nucleotides and

the cross (X) indicates disagreement at an ambiguous site. B) Sequences of the synthetic oligonucleotides with the top strand listed 5' to 3'. Vertical arrows indicate the boundaries (-153 and -127) of the protected region in an exonuclease III footprinting analysis (20). Brackets indicate the positions of MREd and MREc. Dots indicate the MRE and Sp1 core sequences. Crosses (X) indicate the positions of base substitutions in the mutated sequences. The Sp1 oligonucleotide was the same that had been used to purify the Sp1 transcription factor (8). Oligonucleotides were synthetized as two complementary strands by the phosphoramidite method on a Biosearch DNA synthetizer and purified on 15 percent polyacrylamide 8M urea gels. MRE elements were synthetized with a ClaI site on both ends (underlined).

together with the probe. The non specific nucleic acids competition cocktail was made of E.coli DNA- micrococcal nuclease digest (average length ∿ 1 kbp), yeast tRNA, poly (dI- dC)$_2$ and lambda DNA-MspI digest (20, 20, 2, 1) at the final concentration of 1.7 mg/ml.

Footprinting experiments

Exonuclease III footprinting experiments were performed as previously described (20). The MT probe was 5' end-labeled at position +64 and extends to position -200 (Fig. 1A). Competitors were added together with the probe and binding allowed to proceed for 20 min at 24°C.

RESULTS

Binding of the oligonucleotide probes to nuclear proteins

Figure 1a shows the arrangement of the mouse MT-1 gene metal regulatory elements (MREs) (15-18), the G-rich sequence that interacts with the major late transcription factor (MLTF, 10-12), and the sequence of the protected region around MREd in an exonuclease III footprinting assay (20). The protected region extends from nucleotide -127 to -153 relatively to the start site of transcription and contains, in addition to the conserved MRE core sequence TGCPuC (Pu, purine), a Sp1 consensus binding site CCGCCC (7,8). In order to test the binding capacity of this region with a nuclear factor(s) and possibly identify specific protein species, we used synthetic DNA fragments as probes in a protein blotting procedure that allow the detection of DNA-binding proteins in crude nuclear extracts (22). We synthesized a pair of complementary 36-mer oligonucleotides (MREd) which, when annealed, give a short double stranded DNA fragment containing the sequence of MREd plus the 5'end portion of MREc (Fig. 1b). Also, oligonucleotides containing mutated versions of the MREd probe were synthetized. In oligonucleotide MUTd, all five of the most strongly conserved nucleotides of the MRE consensus sequence have been altered. MUTs has the six conserved nucleotides of the Sp1 site changed, MUTds has both MRE and Sp1 consensus altered and MUTr has the six

most downstream nucleotides of the protected region altered (Fig. 1b). A 309bp-fragment of pBr322 and the BglII-XbaI-mouse MT fragment (+64 to +220; MT-INTRON) covering the 3' end of the first exon and 120 bp of the first intron were used as heterologous controls. Crude nuclear extracts from cadmium resistant mouse L cells, grown in presence of $CdCl_2$ and $ZnCl_2$, were prepared, subjected to $NaDodSO_4$/PAGE, electroblotted onto a nitrocellulose filter and probed with the ^{32}P-labeled oligonucleotides.

Results obtained with the different oligonucleotides are shown in Figure 2a. The oligonucleotide MREd (lane 1, see also Fig. 2b, lane 1 and Fig. 2c, lane 1) interacted principally with a group of proteins that migrate with apparent Mr 105,000-110,000 with a major signal at 108,000 (p108, arrow) and a protein that migrates with Mr 45,000 (p45, arrow head). Other bands were also observed with lower intensity (Fig. 2b, lane 1 and Fig. 2c, lane 1). The binding of the MREd probe to these other proteins could be abolished by increasing the NaCl concentration in the binding buffer to 300 mM. The binding to p45 was similarly abolished and could be observed with heterologous probes such as pBr322 (Fig. 2c, lanes 4-6) and MT-INTRON (data not shown). Moreover, while single stranded MREd oligonucleotides bound to p45 and to the other protein species, it did not interact with p108 (data not shown). These data suggest that the binding of MREd to p45 and to the minor proteins was the result of non-specific interactions with general nucleic-acid binding proteins.

In order to assess the specificity of the binding to p108, we used mutated oligonucleotides. Mutations in the MRE consensus sequence TGCAC, in the Sp1 site or in both core sequences led to a complete inhibition of the binding to p108 (Fig. 2a, lanes 2-4). Effects of the different mutations on the binding to p45 was not reproducible. For instance, MUTd did not bind to p45 in some experiments (Fig. 2a, lane 2, arrowhead) while in others the signal was not affected. Mutation in six nucleotides located in the MREc consensus sequence (MUTr) led to a 40 to 50% inhibition of the binding to p108, to a strong inhibition of the interaction to p45 and to the detection of a new protein species of apparent Mr 60,000 (Fig. 2a, lane 5). The detection of a new protein species by MUTr can be attributable to the fortuitous generation of a binding site for a DNA binding protein interacting with a related DNA sequence in the genome. These results show that this protein blotting procedure allows the MREd probe to bind specifically to a population of nuclear protein of Mr 108,000. In addition, a protein of Mr 45,000 interacts non selectively with the different probes. The specificity of the interaction

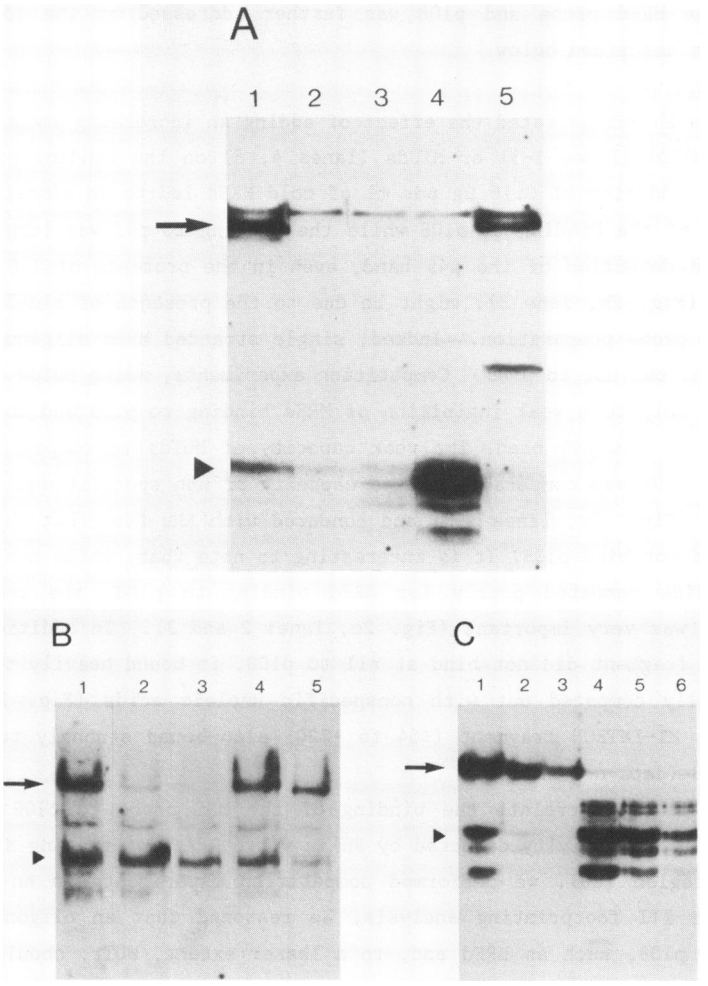

Fig. 2: A) Binding of the oligonucleotide probes to nuclear proteins. Lanes: 1, MREd; 2, MUTd; 3, MUTs; 4, MUTds; 5, MUTr. The arrow corresponds to Mr 108,000 and the arrowhead indicates the position of Mr 45,000. B) Analysis of the binding specificity of the MREd oligonucleotide to nuclear proteins using MREd (1-2 ng/ml) as probe and cold MREd (lanes 1 to 3) or MUTds (lanes 4 and 5) as competitor. Competitor concentrations, lanes 1, 0 µg; 2, 0.16 µg/ml; 3, 1.6 µg/ml; 4, 0.26 µg/ml; 5, 2.6 µg/ml. C) Competition experiments using heterologous probe and non specific nucleic acids as competitors. Probes: Lanes, 1-3, MREd (1 ng/ml); 4-6, a 309 bp-pBr322 MspI fragment (0.2 ng/ml). Non-specific nucleic acid competition cocktail. Lanes; 1 and 4, 0 µg; 2 and 5, 0.8 µg/ml, 3 and 6, 4 µg/ml. Arrows and arrowheads as in fig. 2a. Binding of the MREd probe to p108 was highly reproducible and was observed in more than 20 independent experiments. The binding capacity of the mutant probes was assessed in four different experiments.

between the MREd probe and p108 was further addressed by the competition experiments described below.

Competition

Figure 2b demonstrated the effect of adding an increasing amount of cold competing MREd (lanes 1-3) or MTUds (lanes 4, 5) on the binding of MREd to p108. The addition of 0.16 µg per ml of cold MREd led to an almost complete inhibition of the binding to p108 while the binding to p45 was largely unaffected. The detection of the p45 band, even in the presence of 1.6 µg/ml of cold MREd (Fig. 2b, lane 3), might be due to the presence of single-strand-DNA in the probe preparation. Indeed, single stranded MREd oligonucleotides bound to p45 but not to p108. Competition experiments, using MUTds as competitor, led only to a weak inhibition of MREd binding to p108 and to a strong diminution of the p45 band. The weak capacity of MUTds to compete for MREd binding to p108 was comparable to the capacity of non specific nucleic acids to compete (Fig. 2c, lanes 1-3) and concured with the fact that this mutant (MUTds) did not bind p108. It is interesting to note that, while non specific nucleic acids competed poorly for MREd binding to p108, the competition toward p45 was very important (Fig. 2c, lanes 2 and 3). In addition, while the pBr322 fragment did not bind at all to p108, it bound heavily to p45 and was partially competed out with nonspecific nucleic acids (Fig. 2c, lanes 4-6). The MT-INTRON fragment (+64 to +220) also bound strongly to p45 but not to p108 (data not shown).

In order to correlate the binding of the MRE probe to p108 with the observed binding activity detected by an in vitro DNA footprinting assay onto the MREd region (20), we performed competition experiments in an in vitro exonuclease III footprinting analysis. We reasoned that an oligonucleotide that binds p108, such as MREd and, to a lesser extent, MUTr, should compete for the reported binding activity to MREd detected in a crude extract (20) while mutants such as MUTd, MUTs, and MUTds should not compete. Figure 3a shows that, while MREd and MUTr competed very efficiently, MUTd, MUTs and MUTds showed very little competition activity. It is interesting to note that in the concentration range of competitor DNAs used for these experiments, the only band that was readily competed out was the MREd band at -153. The remaining of the footprinting remained unmodified.

The protein detected by MREd is different from the transcription factor Sp1

As shown in Figure 1a, the MREd region contains a putative binding site (5'CCGCC3') for the transcription factor Sp1 and Mueller et al. (21) have shown that, in vitro, purified Sp1 weakly binds to this region. The Sp1

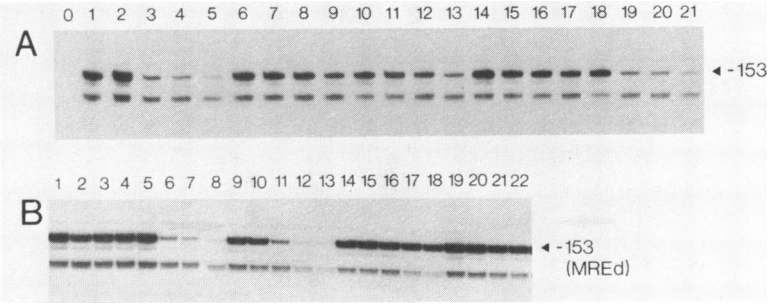

Fig. 3: Competition experiments in an exonuclease III footprinting assay. Arrowheads indicate the exonuclease III stop at the -153 boundary. A) Competition was performed with cold oligonucleotides. Competitors. Lanes: 0, no extract; 1, standard, no competitor; 2-5, MREd; 6-9, MUTd; 10-13, MUTs; 14-17, MUTds; 18-21, MUTr. In each case, 0, 20, 40 and 100 ng of competitors were used in a final reaction volume of 50 µl. B) Competitors used in competition experiments. Lanes 1-4, the oligonucleotide Sp1 (0, 20, 40, 100 ng); 5-8, MREd (0, 20, 40, 100 ng); 9-13, the same MT fragment (-200 to +64) as the one used as probe in the exonuclease III assay (0, 1, 10, 50, 100 ng); 14-18, the PvuII-BglI fragment (270 bp) of Simian Virus 40 DNA (0, 3.5, 35, 175, 350 ng); 19-22, a 309 bp-pBr322- MspI fragment (0, 17, 34, 51 ng). Exonuclease III footprinting experiments were performed with the same nuclear extract preparation used in the experiments described in Fig. 2.

factor has been purified to more than 95 percent homogeneity and two polypeptides of 105 and 95 kilodaltons have been identified (8,9). The possibility that p108 was in fact Sp1 was tested by using, in the protein blotting procedure, a specific DNA probe known to bind Sp1. Figure 4 shows that, while MREd (lane 1) and a mouse MT-fragment, positions -200 to +64 (lane 3), containing all the regulatory sequences of the promoter, bound p108, the PvuII-BglI enhancer-DNA fragment of Simian Virus 40 (SV40) (map units 0.712 to 0.659) which contains six Sp1 binding sites (23) did not bind p108 (lane 2) nor did it compete for the protein(s) that protected the MREd region against digestion by the enzyme exonuclease III (Fig. 3b, lane 14-17). Similarly, the Sp1 oligonucleotide did not compete for the proteins binding to the MREd region (Fig. 3b, lanes 1 to 4) despite that the sequence GGGGCGGGGC (Fig. 1b) has been determined to be the highest affinity Sp1-binding sequence studied to date (7). The detection of a protein species comigrating with p108, when both MREd and the SV40 DNA were used together as probes (Fig. 4, lane 4), demonstrates that the SV40 DNA fragment did not bind p108. In the protein blotting experiment, the SV40 probe detected some proteins with major bands migrating with apparent Mrs 95,000 and 105,000 (Fig. 4, lane 2, thin

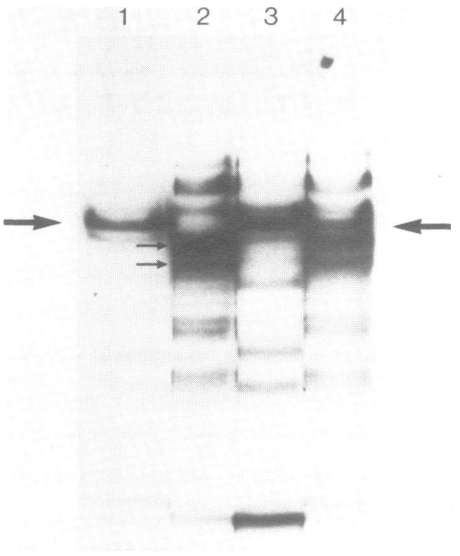

Fig. 4: Binding of various ^{32}P-labeled DNAs to nuclear proteins. Lanes: 1, MREd oligonucleotide; 2, PvuII-Bgl1 SV40 DNA fragment; 3, MT fragment extending from -200 to +64; 4, both MREd oligonucleotide and SV40 fragment. Heavy arrows correspond to Mr 108,000 and thin arrows indicate nuclear protein species that migrate with apparent Mrs 95,000 and 105,000. Blotting procedures and detection of DNA-binding proteins as in Fig. 2a, except that MT and SV40 probe concentrations were 10^4 cpm per ml of binding buffer.

arrows) which is in agreement with the observed molecular weights of purified Sp1 (vide supra). The Sp1 oligonucleotide (Fig. 1b) also bound these protein species (data not shown). The nature of the other proteins detected by the SV40 DNA probe has not been investigated but could represent other DNA binding proteins interacting with this SV40-enhancer DNA fragment. The detection of p108 by the MT fragment (-200 to +64) excluded that a 200-300 bp-DNA fragment could not bind p108 on nitrocellulose filters due to steric hindrance, thus excluding the likelihood that the SV40 probe did not bind p108 for similar steric reasons. These results demonstrate that despite the presence of a seven out of a eight base pair match with the Sp1 binding site consensus sequence ($\overset{T}{C}CCGCCC\overset{A}{C}$), the Sp1 transcription factor did not interact with the MREd probe and therefore is different from the herein detected binding protein p108.

DISCUSSION

We have used a protein blotting procedure and synthetic oligonucleotides to detect nuclear proteins that specifically interact with the MREd region of

the mouse MT-1 gene. We have shown that a nuclear protein with an apparent Mr 108,000 binds specifically to MREd and that this protein is different from the transcription factor Sp1 for which there is a consensus binding site within the MREd region. It is unlikely that p108 corresponds to the transcription factors AP1, AP2 and AP4 which can bind the related human MTIIA gene (24-26) because the reported Mrs for these proteins are 47 kD, 52 kD and 48 kD respectively. Moreover, there is no binding site for these factors in the MREd region of the mouse MT-1 gene. The blotting procedure used in this study has been used by Miskimins et al. (22) to detect specific, high affinity DNA-protein interaction with the transferrin receptor gene promoter and by Silva et al. (27) to study human glucocorticoid receptor interactions with DNA. Salt and blocking conditions used in the procedure select for specific, high affinity DNA protein interactions. The apparent high affinity of p108 is indicated by its ability to bind MREd in the presence of up to 300 mM NaCl and 50 mM $MgCl_2$ and by the fact that an excess of nonspecific nucleic acids in the binding buffer does not effectively block the interaction of labeled MREd with this protein. The specificity of the interaction of MREd to p108 is shown by the inability of mutated oligonucleotides to bind to p108. Both the MRE and Sp1 consensus sequences TGCAC and CCGCCC are required for efficient binding of MREd to p108. Mutations in the nucleotides, located downstream from the Sp1 site, only led to partial inhibition of the binding. The precise identification of the nucleotides involved in the binding of p108 to MREd will require more systematic mutagenesis of the eleven nucleotides forming the MREd-Sp1 consensus region.

We have previously shown that some component in the mouse extract, as assayed by protection from exonuclease III digestion and gel shift assay, was sensitive to EDTA (20). We attempted to abolish the binding of the oligonucleotide MREd to p108 by adding EDTA to the different buffers. While the binding was weaker in presence of EDTA it was not completely inhibited. The apparent contradiction between the different assays for DNA binding proteins could be explained if the denaturation and the immobilization of the proteins in the blotting procedure have partially modified some of the biochemical properties of p108 toward its ligand. A study of the dependence of p108 on heavy metals to bind MREd will require the purification of the protein and kinetic studies in solution.

MREs binding proteins have been detected by footprinting analyses in mice (20,21) and rats (28) while, in humans, a factor which binds to the MREs has not been detected yet (29,30). However, the capacity of human MTIIA-promoter DNA to compete efficiently for the protein(s) binding to the MREd

region in an exonuclease III footprinting assay (Séguin, C. and Reimnitz, G., unpublished results) reveals the existence of binding DNA sequences for the same protein(s) on the human MTIIA promoter as well. Whether or not these sequences are one of the MREs present in the human gene (29-31) or other control elements remains to be shown.

In humans, the transcription factor Sp1 has been shown to bind to a GC element of the MTIIA gene and to activate RNA synthesis in vitro (24). In the mouse, purified Sp1 has been shown to weakly bind, in vitro, to the Sp1 binding site centered at -139 in the MREd region (21). A high affinity Sp1 binding site has also been mapped at -182 (21). However, the absence of a characteristic Sp1-dimethylsulfate in vivo footprint pattern at the MREd region has led Mueller et al. (21) to suggest that it is unlikely that Sp1 bind to this region in vivo. The protein blotting procedure, used in this study, shows that the MREd region selectively binds to a protein species distinct from Sp1. Moreover, Sp1-binding DNAs do not compete for the protein(s) binding to the MREd region in a DNA footprinting assay (Fig. 3b, lanes 1-4 and 14-18). Altogether, these data do not support the contention that the Sp1 factor binds to the MREd region in vivo. It has been suggested that the ability of MREs to modulate transcription in response to heavy metals depends upon the ability of a specific metal regulatory protein (referred to as the MRP or MRF) to bind to the sequence in presence of heavy metals and induce transcription (13-20). Mueller et al. (21) have suggested that in L cells, for the case of the MREd-Sp1 region, there is a balance between active Sp1 factor and MRP that favors binding by MRP. A major question is whether the protein p108 corresponds to MRP. Three pieces of evidence suggest that p108 is a central component of the transcriptional machinery involved in the modulation of the mouse MT gene transcription. First, the high affinity of p108 for MREd as shown by its capacity to bind to MREd in this protein blotting procedure; second, its strong binding onto a MREd-affinity column (Séguin, C. and Prévost, J., unpublished results) and finally, the fact that the only protein(s) that is sensitive to EDTA and readily competed out in a DNA footprinting assay is the protein(s) binding to the MREd region (20). It cannot be excluded, however, that other proteins, together with p108, contribute to the protection activity detected by DNA footprinting analysis around the MREd region. Indeed, the detection of only one class of specific DNA binding protein by the MREd probe does not exclude that other proteins interact with the MREd region but have not been detected by the protein blotting procedure due to lower affinity for their target DNA.

In addition, the binding of multiple factors to the MREd region might require cooperative interactions which could not take place between nitrocellulose-immobilized proteins. Other proteins, such as MLTF that has been reported to bind *in vitro* (12) and *in vivo* (21) to the mouse MT promoter and to induce transcription of the gene *in vitro* (12) may act synergistically with p108 in regulating MT gene expression. *In vitro* transcription studies using purified or partially purified preparations of p108 will be required to demonstrate the role of p108 in the modulation of MT gene transcription. Together with the DNA footprinting assay (20), this blotting procedure should facilitate the purification of this MRE-binding protein and the analysis of its mechanism of action.

ACKNOWLEDGEMENTS

We thank Guy Reimnitz for his expert technical assistance, Dean H. Hamer for providing the mouse MT gene and the heavy metal-resistant-L cell line, Antonella Maresca for fruitful discussions, Serge Côté and Adolfo Ruiz-Carillo for comments on the manuscript and Elaine Leclerc and Aline Douville for excellent typing. This work was supported by grants from the Medical Research Council of Canada, the Natural Sciences and Engineering Research Council of Canada and Le Fonds de la Recherche en Santé du Québec. C.S. is a Scholar from the Medical Research Council of Canada.

[1]Present address: Centre de Recherches en Cancérologie de l'Université Laval, L'Hôtel-Dieu de Québec, 11 Côte du Palais, Québec, Qc, G1R 2J6, Canada.

* To whom correspondence should be addressed.

REFERENCES

1. Maniatis, T., Goodbourn, S. and Fisher, J.A. (1987) Science 236, 1237-1245
2. Jones, NC., Rigby, P.W. J. and Ziff, E.B. (1988) Genes Dev. 2, 267-281
3. Singh, H., LeBowitz, J.H., Baldwin, A.S. Jr., and Sharp, P.A. (1988) Cell 52, 415-423
4. Landschulz, W.H., Johnson, P,F., Adashi, E.Y., Graves, B.J. and McKnight S.L. (1988) Genes Dev. 2, 786-800
5. Santaro, C., Mermod, N., Andrews, P.C. and Tjian, R. (1988) Nature 334, 218-224.
6. Dynan, W. S. and Tjian, R. (1985) Nature 316, 714-778
7. Kadonaga, J.T., Jones, K.A. and Tjian, R. (1986) Trends Biochem. Sci. 11, 20-23
8. Briggs, M.R., Kadonaga, J.T., Bell, S.P. and Tjian, R. (1986) Science 234, 47-51
9. Kadonaga, J.T., Carner K.R., Masiarz, S.R. and Tjian, R, (1987) Cell 51, 1079-1090
10. Chodosh, L.A. Carthew, R. W. and Sharp, P.A. (1986) Mol. Cell. Biol. 6, 4723-4733
11. Chodosh, L.A., Carthew, R.W., Morgan, J.G. Crabtree, G.R. and Sharp,P.A. (1987) Science 238, 684-688

12. Carthew, R.W., Chodosh, L.A. and Sharp, P.A. (1987) Genes Dev. 1, 973-980
13. Hamer, D. (1986) Annu. Rev. Biochem. 55, 913-951
14. Kagi, J.H.R. and Kojima, Y. (eds.) (1987) Metallothionein II, Birkhäuser Verlag, Basel
15. Stuart, G.W., Searle, P.F., Chen, H.Y., Brinster, R.L. and Palmiter, R.D. (1984) Proc. Natl. Acad. Sci. USA 81, 7318-7322
16. Carter, A.D., Felber, B.K., Walling, M.J., Jubier, M.F., Schmidt, C.J. and Hamer, D.H. (1984) Proc. Natl. Acad. Sci. USA 81, 7392-7396
17. Stuart, G.W., Searle, P.F. and Palmiter, R.D. (1985) Nature 317, 828-831
18. Searle, P.F., Stuart, G.W. and Palmiter, R.D. (1985) Mol. Cell. Biol. 5, 1480-1489
19. Séguin, C., Felber, B.K., Carter, A.D. and Hamer, D.H. (1984) 312, 781-785
20. Séguin, C. and Hamer, D.H. (1987) Science 235, 1383-1387.
21. Mueller, P.R., Salser, S.J. and Wold, B. (1988) Genes Dev. 2, 412-427
22. Miskimins, W.K., Roberts, M.P., McClelland, A. and Ruddle, F.H. (1985) Proc. Natl. Acad. Sci. USA 82, 6741-6744
23. Dynan, W.S. and Tjian, R. (1983) Cell 35, 79-87
24. Lee, W., Haslinger, A., Karin, M. and Tjian, R. (1987) Nature 325, 368-372
25. Mitchell, P.J., Wang C. and Tjian, R. (1987) Cell 50, 847-861.
26. Mermod, M., Williams, T.J. and Tjian, R. (1988) Nature 332, 557-561.
27. Silva, C.M., Tully, D.B., Petch, J., Jewell, C.M. and Cidlowski, J.A (1987) Proc. Natl . Acad. Sci. USA 84, 1744-1748
28. Andersen, R.D., Tapliz, S.J., Wong, S., Bristol, G., Larkin, B. and Herschman, H.R. (1987) Mol. Cell. Biol. 7, 3574-3581
29. Scholer, H., Haslinger, A., Heguy, A., Holtgreve, H. and Karin, M. (1986) Science 232, 76-80
30. Karin, M., Haslinger, A.,, Heguy, A., Dietlin, T. and Cooke, T. (1987) Mol. Cell. Biol. 7, 606-613
31. Haslinger, A. and Karin, M. (1985) Proc. Natl. Acad. Sci. USA 82, 8572-8576.