

Metal-dependent binding of a nuclear factor to the rat metallothionein-I promoter

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ABSTRACT

Genomic footprinting studies *in vivo* and experiments using synthetic metal regulatory elements (MREs) *in vitro* suggest protein binding to the MREs of the mouse and rat metallothionein I (MT-I) genes. Using gel-retardation assays of promoter fragments, we observe a cadmium-dependent binding factor for the rat MT-I promoter in rat hepatoma cells. This factor is present in extracts from both uninduced and cadmium-induced cells, but requires the presence of cadmium to bind to the promoter. The formation of a cadmium-dependent complex is competed by an oligonucleotide containing two MREs. This competition is lost when one of the MREs is mutated, indicating a requirement for at least two MREs for binding of this factor. The cadmium-dependent factor dissociates more rapidly from the MT-I promoter than does a factor that binds to a consensus Sp1 site present on the same DNA fragment. UV crosslinking analysis using nuclear extracts from cadmium induced cells, in the presence of an oligonucleotide probe containing both 5-bromodeoxyuridine and ³²P-deoxycytidine, identifies a 39 kDalton protein associated with the metal inducible complex.

INTRODUCTION

The rat metallothionein I gene is inducible by a variety of factors, including heavy metals (Cd⁺⁺, Zn⁺⁺), glucocorticoid hormones (see review, ref. 1) and phorbol esters (2) such as tetradecanoyl phorbol acetate (TPA). Transcription of metallothionein (MT) genes is regulated by a complex array of cis-acting promoter and enhancer elements. Deletion analysis of MT genes has been used to characterize sequence elements responsible for basal-level and inducible expression (3,4,5,6). Regulatory proteins that bind to some of the basal-level elements have been identified; one of these proteins is Sp1, which binds

in vitro to a single 'GC box' in the human MT-II_A promoter (7). Genomic footprinting studies suggest that Sp1 protein is bound to the 'GC box' of the rat MT-I promoter under conditions of both basal and metal-stimulated expression (6). Another protein, c-jun/AP-1, binds to the human MT-II_A basal-level enhancer (8). The AP-1 binding site also acts as a TPA inducible enhancer element, and is found in other TPA inducible promoters such as collagenase and SV40 (2). A point mutation in this TPA-responsive element eliminates basal level and TPA induced transcription and disrupts the binding of purified AP-1 (9).

The metal responsive elements (MREs) of the MT genes act as metal inducible enhancers (3), suggesting that a protein involved in metal induction binds to these sites and influences the transcription of the gene. All MT genes contain multiple MREs in their 5' flanking regions. In transfection assays, the activity of the MT gene is dependent upon the number of MREs present (6,10). An additional DNase I hypersensitive site appears in the MT-I gene upon treatment of cells with cadmium (11,12). In several other cases the appearance of a hypersensitive site correlates with the binding of a putative activator (13,14). Cadmium-inducible protection of guanine residues *in vivo* occurs in five MREs in the rat MT-I promoter (1), as well as in the mouse MT-I promoter (15), suggesting that metal induction involves the metal-dependent binding to the MREs of a sequence-specific regulatory factor. Seguin and Hamer (16) and Westin and Schaffner (17) have demonstrated the metal-dependent binding of factors to synthetic oligonucleotides containing an MRE sequence. However, the nature of the interactions between the multiple MREs and the factor(s) that bind to them are not clear.

We have examined the interactions between factors present in nuclear extracts and the multiple metal responsive elements and a basal level element (Sp1 binding element) present on fragments of the rat MT-I promoter. We have identified a factor(s) which forms a metal-dependent DNA complex with the rat MT-I promoter *in vitro*. We identify a component of this complex as a 39 kDalton protein.

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MATERIALS AND METHODS**Preparation of nuclear extracts from rat hepatoma cell**

Nuclear extracts, prepared by modifications of the procedure of Wu (18) were from approximately 10^9 Fao rat hepatoma cells (19). Cadmium-induced extracts were from cells treated for three hours with $3 \mu\text{M}$ CdCl_2 . Unless noted, all procedures were carried out at 0 or 4°C . Briefly, cells were rinsed on the plates with cold PBS, scraped and washed again with PBS. All solutions used subsequently contained 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 100 U/ml aprotinin, $5.0 \mu\text{g/ml}$ leupeptin, and $5.0 \mu\text{g/ml}$ pepstatin A, except for the dialysis buffer, which contained 0.2 mM, 10 U/ml, $0.5 \mu\text{g/ml}$ and $0.5 \mu\text{g/ml}$ of the proteinase inhibitors respectively. All solutions (including the dialysis buffer) used to prepare extracts from cadmium-induced cells contained $3 \mu\text{M}$ CdCl_2 . The cells were centrifuged and lysed by resuspension in 40 ml of RSB (10 mM Tris, pH 7.5, 10 mM NaCl, 5 mM MgCl_2) containing 30% sucrose and 0.5% NP40. The nuclei were washed twice with RSB, pelleted at low speed (2500 rpm in a Dynac clinical centrifuge), and resuspended in 1 nuclear pellet volume (npv) of extraction buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl_2 , 200 mM NaCl, 0.5 mM DTT, and 5% glycerol). One npv of extraction buffer B (10 mM HEPES pH 7.9, 1.5 mM MgCl_2 , 600 mM NaCl, 0.5 mM DTT, and 5% glycerol) was added, and the final NaCl concentration was adjusted to 400 mM with 5 M NaCl. The nuclei were stirred on ice for 30 minutes and the extracted nuclei were pelleted for 1 hour at $100,000 \times g$ (33,000 rpm in a Ti75 rotor). The extract was then dialyzed against 20 mM HEPES pH 7.9, 100 mM NaCl, 20% glycerol, and 0.5 mM DTT, was spun in a microfuge to clarify, frozen in liquid nitrogen, and stored at -70°C .

Preparation of end-labelled fragments

DNA restriction fragments of the MT-I promoter were isolated from polyacrylamide gels. Gel fragments were crushed and soaked overnight at 37°C in elutip low salt buffer (20 mM Tris pH 7.5, 0.2 M NaCl, 1.0 mM EDTA) and the DNA was purified over elutip columns (S&S). Fragments were 5' end-labelled by the method of Maxam and Gilbert (20), isolated from acrylamide gels and purified on elutip columns.

Gel retardation assays

Gel retardation assays were carried out as described by Peterson et al. (21). Reactions contained 2000–10,000 cpm of end-labelled probe, 70 mM NaCl, 2–6 μg poly (dI-dC)-poly (dI-dC) (Pharmacia), and 7 μg (unless otherwise noted) of crude nuclear extract in a final volume of 12–16 μl . Binding reactions were for 15 minutes at room temperature. Two μl of dye (30% glycerol, 0.025% bromophenol blue, 0.025% xylene cylenol) were added and the samples were loaded onto gels. The gels used were a Tris-glycine (50 mM Tris, 380 mM glycine, pH 8.6)-acrylamide gel (22). Gels were either 5% or 6% acrylamide, as indicated in the figure legends. Gels were run for 2–3 hours at 20 mAmp (constant current) in $0.1 \times$ Tris-glycine. For the experiments in Figure 2, electrophoresis was done at room temperature. The remaining gel retardation assays were done at 6°C . This lower temperature was necessary in the competition assays where competitor was added after complex formation. For competition experiments, either pBR322 or a specific competitor DNA was preincubated for 5 minutes at room temperature with the nuclear extract and poly(dI-dC)-poly(dI-dC) before the addition of the probe. The 54 bp competitor had the sequence:

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          ---MREd--
CCGAGCCCGAAGAGTGGCTCGGCTCTGCCAAGGACGCTGGCTCGTGAC
          ---MREc--
          CGGGCTTCTCACGCGAGCCGAGACGGTTCTCGCAGCGGAGCACTGGGCT

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The 49-mer competitor with a mutant MREc had the sequence:

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          ---MREd--
GCCCGAAGAGTGGCTCGGCTCTGCCAAGGAATAGTATAGATGTCATGA
          -----
          CGGGCTTCTCACGCGAGCCGAGACGGTTCTTATCATATCTACAGTACT

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Determination of stability of gel retardation complexes

A competition experiment was designed to measure the rate of dissociation of DNA-extract complexes after a 15 minute preincubation to form these complexes. The preincubation was as described above, using either the 109 bp probe or a 135 bp probe (see Figure 1). All preincubations were done at room temperature, followed by cooling to 6°C . Induced extracts were then treated for varying lengths of time with a 536-fold excess of 54-mer oligonucleotide containing MREc and MREd (from –142 bp to –97 bp 5' of the MT-I transcription start site, plus flanking Ava I sites). Total probe was 14 fmol; total competitor was 7.5 pMol. In the experiment shown in Figure 5 the concentration of the 41 bp competitor was 2.4 fmol; total competitor concentration was 7 pMol, giving a 2900-fold excess of the 41-mer competitor. The 41-mer competitor had the sequence:

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          -GC Box-
CCGAGAAAGGGGGCGTCCCGTTGTGCACACCGGGCGC
          ---MREf--
          CTTTCCCCGCCAGGGCAACACGTGTGGCCCGGGCT

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UV cross-linking of nuclear extract-DNA complexes.

Two oligomers were synthesized corresponding to the region surrounding MREc and MREd, an upper strand 32-mer and a lower strand 31-mer:

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          ---MREd--
CGGATCCCCGAAGAGTGGCTCGGCTCTGCC
          AGACGGTTCTCGCAGCGGAGCACTCTAGAC
          ---MREc--

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The oligomers were used in a synthesis reaction with the Klenow fragment of DNA polymerase to incorporate 5-bromo-deoxyuridine triphosphate and ^{32}P -deoxycytidine triphosphate, in addition to dATP and dGTP (23). This DNA probe, after purification, was used in a gel retardation experiment using control and cadmium-induced Fao cell extracts. After a 15 minute incubation with probe, the mix was placed on ice and exposed to UV light ($2500 \mu\text{watt/cm}^2$ at 10 cm) for 20 minutes, in a sterile freezer vial covered with Saran wrap. The sample was electrophoresed under conditions described above for gel retardation. The inducible band complex (present using induced extracts, absent using control extracts) was excised and the labeled complex eluted by crush-and-soak in 2 ml of 10 mM triethylammonium bicarbonate pH 8.0 (TEAB), re-extracted with 1 ml of TEAB and the pooled fractions lyophilized. Samples were

redissolved in TEAB containing 10 mM CaCl₂, 1.5 U of micrococcal nuclease (Pharmacia) and 0.2 μg of DNase (Pharmacia, HPLC-purified) and digested 30 minutes at 37°C. The reaction was stopped by adding EGTA to 15 mM, then dialyzed 6h against 50 mM TEAB. Half of this sample was subjected to treatment with iodoacetic acid to carboxymethylate cysteines, as previously described (24). This was done to prevent the possible oligomerization of factor(s) due to interchain disulfide bonds. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% acrylamide gel.

RESULTS

Gel retardation identifies a metal-dependent complex

The known cis-acting elements of the rat MT-I promoter are shown in Figure 1. Using gel retardation assays, we examined the interaction of nuclear extracts with the rat MT-I promoter. In the first experiment, the 109 bp MT-I promoter fragment containing the MREs b, c, d, e, and a portion of MREa (nucleotides -47 to -155) was used as the labelled probe (see Figure 1). The probe was incubated with extracts prepared from cadmium-induced and uninduced Fao cells. When incubated with the 109 bp DNA probe in the presence of an excess of non-specific competitor DNA, these extracts showed a common retarded complex on a Tris-glycine gel (Figure 2A). The extract prepared from cadmium-induced Fao cells gave rise to an additional complex, seen only faintly in the uninduced extract. When an excess of unlabelled homologous (109 bp) fragment was included as competitor in the reaction, all the retarded complexes with extracts from the cadmium-induced cells disappeared (Figure 2B), suggesting that the complexes were due to sequence-specific binding of a factor(s) to the labelled probe.

To examine the role of cadmium in the formation of the metal-dependent gel retardation complex, we incubated the 109 bp DNA probe, in the presence or absence of cadmium, with extract prepared from uninduced cells (Figure 2C). Formation of the metal-dependent complex could occur if uninduced extract was incubated with probe in the presence of added cadmium. These data suggest that a factor, only capable of binding the MT promoter in the presence of metal, is present in both induced and uninduced Fao cells. We have performed gel retardation experiments using nuclear extracts from control and cadmium-treated Fao cells in which the electrophoresis was done in a Tris-Borate-EDTA buffer system. Although retarded bands were present, no cadmium-inducible complex was ever observed (data not shown). Thus, the inducible complex is disrupted in a gel buffer system which includes a metal ion chelator (see Discussion).

Two or more MREs are required for formation of the metal inducible complex

In order to establish which part of the MT-I promoter sequence in the 109 bp fragment is associating with a nuclear factor in a metal-dependent fashion we carried out competition experiments. Nuclear extract from induced cells was incubated with a synthetic oligonucleotide containing MREc and MREd (nucleotides -94 to -142) followed by addition of labeled 109 bp fragment as probe. Electrophoresis was carried out at 6°C. Under these conditions, the metal inducible complex was resolved as a doublet, while the common upper complex appeared as multiple bands (Figure 3, lane 1). Incubation with the

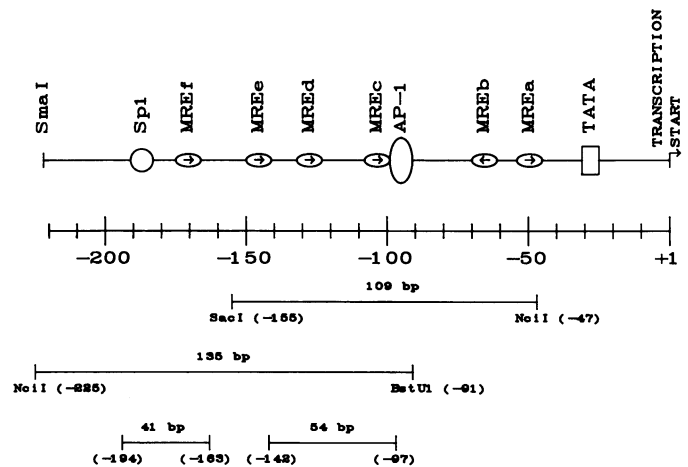


Figure 1. The rat MT-I promoter region. Regulatory element consensus sequences are marked. Probes and competitors used in these experiments are shown below the diagram of the MT-I promoter.

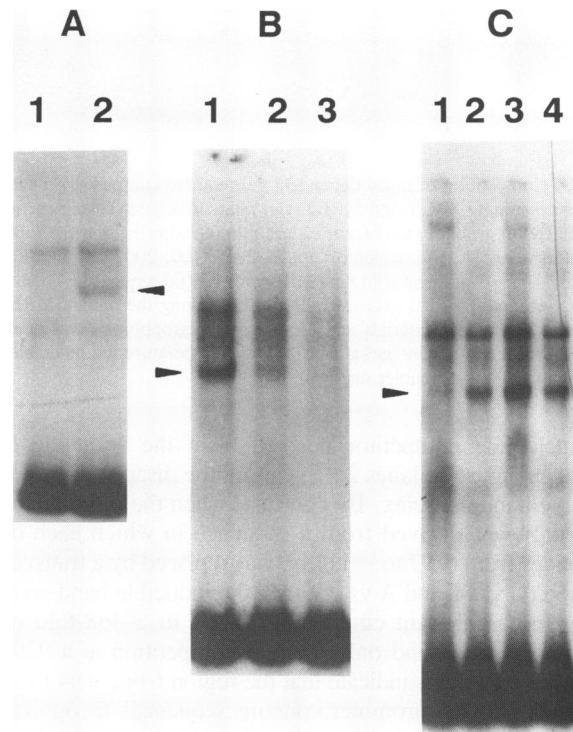


Figure 2. Gel retardation assay with control and cadmium induced extracts analyzed in a Tris-glycine gel system. A: A gel retardation assay was carried out using 17.5 μg of nuclear extract from either control (lane 1) or cadmium induced (lane 2) cells, 18,000 cpm of the end-labelled 109 bp probe, and 10 μg poly (dI-dC). The reactions were loaded on a 6% acrylamide Tris-glycine gel and run for 3 hours at 20 mAmp constant current. B: A gel retardation assay was carried out with extract from cadmium induced cells, following preincubation of the reaction mix with a 275× (lane 2) or 825× (lane 3) molar excess of unlabelled 109 bp fragment. Probe and electrophoresis were performed as in A. Lane 1 had no competitor DNA. C: Gel retardation assays were performed as above. Lanes 1 and 2 show experiments with extracts from uninduced cells; Lanes 3 and 4 show experiments with extracts from cells exposed to cadmium for three hours prior to preparation of nuclear extract. The inducible band complex in each panel is indicated by the arrow. Five μM CdCl₂ was added to the reaction mixture prior to electrophoresis in lanes 2 and 4; no addition was made to the reaction mixtures used in lanes 1 and 3.

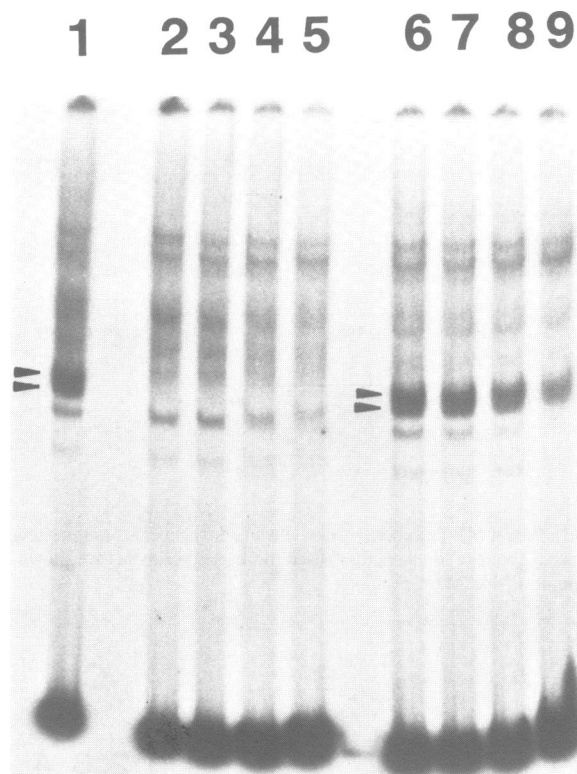


Figure 3. Competition of metal-dependent gel retarded complex by a synthetic oligomer containing MREc and MREd. Gel retardation assays were carried out with the 109 bp probe, extract from cadmium induced cells, and the following competitors: Lane 1: no competitor; Lanes 2–5: 100, 200, 400 and 800-fold excess of 54-mer containing wild type MREc and MREd sequences; Lanes 6–9: 92, 184, 368 and 920-fold excess of 49-mer containing the mutant MREc and wild type MREd (see Materials and Methods). Electrophoresis was done in a 5% acrylamide-Tris-glycine gel at 6°C. At this temperature the inducible band complex appears as a doublet (arrows).

oligonucleotide competitor derived from the authentic MT-I sequence (Figure 3, lanes 2–5) caused the disappearance of the inducible band complex. By contrast, when the oligonucleotide competitor was derived from a sequence in which each of the nucleotides from –97 to –111 bp was replaced by a transversion mutation (G vs. T and A vs. C) then the inducible band complex showed no significant competition at up to a 368-fold molar excess over probe and only a slight competition at a 920-fold excess. These results indicate that the region from –94 to –142 bp in the rat MT-I promoter contains sequences recognized by a factor(s) involved in the formation of the inducible band complex. Furthermore, when MREc, spanning nucleotides –97 to –111, is mutated, then the loss of recognition at this site is sufficient to prevent competition from occurring. The data suggest that the cadmium-inducible binding occurs at MRE sequences and that both MREc and MREd are necessary to compete for binding to the 109 bp probe.

The metal inducible complex is less stable than complexes involving transcription factor Sp1

When the metal-inducible complex found with the 109 bp fragment was examined, either by methylation interference (25) or copper-orthophenanthroline footprinting (26), we were unable to demonstrate interference or protection (data not shown) over the metal responsive elements. We therefore examined, in a gel retardation assay, the stability of the metal-inducible complex,

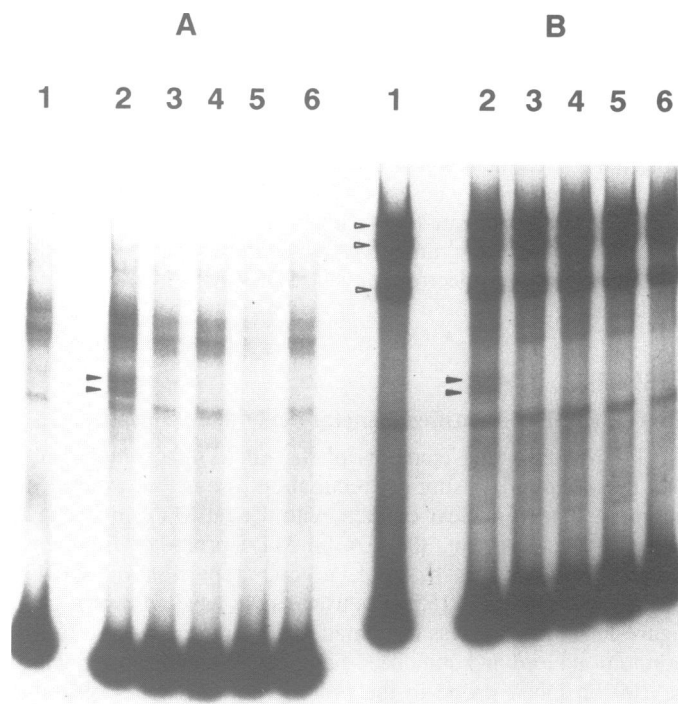


Figure 4. Stability of metal-dependent gel retarded complex. Panel A: 109 bp probe containing MREb, c, d, and e; Panel B: 135 bp probe (–91 to –225 bp) containing MREc, d, e, and f and the Sp1 binding site. Lanes 1; control extract; lanes 2; cadmium-induced extract; lanes 3–6; induced extract with 54 bp competitor incubated at 6°C, after complex formation for 15 minutes at room temperature, for 5, 15, 30 and 60 minutes, respectively. Electrophoresis conditions were the same as in Figure 3. The inducible band complex is indicated by the filled arrows. The Sp1 complexes are indicated by the unfilled arrows.

and compared the stability of this complex with a presumptive Sp1 protein-DNA complex.

Extracts were preincubated, as described in Methods, with labeled 109 bp probe prior to addition of competitor. The metal-inducible doublet bands disappeared within 5 minutes after the addition of the 54 bp competitor DNA fragment containing MREc and MREd (Figure 4A, lanes 3–6), compared with no addition (Figure 4A, lane 2). Since the competitor sequence was added after formation of the DNA-factor complex, disappearance of the metal-inducible retarded bands is the consequence of dissociation of the complex between factor and labeled DNA probe, and subsequent formation of complexes with competitor DNA.

A 135 bp fragment containing MREs c, d, e, and f and the distal 'GC box' (see Figure 1) was used as a labeled probe, in a similar competition experiment. Complexes migrating more slowly during electrophoresis are present in extracts prepared from both uninduced and metal induced cells (Figure 4B, lanes 1 and 2, respectively). The slower migrating retarded bands with the 135 bp probe are likely to be due to complexes between the 'GC box' and Sp1 protein. Footprint analysis of each of these three upper complexes, isolated from a gel retardation run using a methylated 135 bp probe (25), show methylation interference at G residues at –185 and –187 to –189 bp (data not shown), located in the upstream GC box. Footprinting experiments *in vivo* demonstrate that, as seen here with extracts from Cd-induced and uninduced cells, this interaction also occurs in both induced and uninduced cells (6). As with the 109 bp probe, the cadmium-

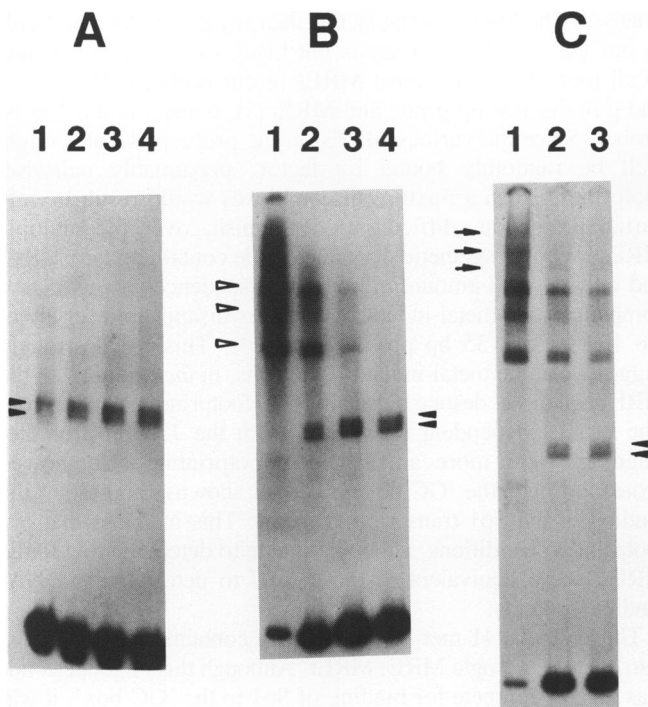


Figure 5. Time course of competition of Sp1 containing complexes. A time course of competition, using the 41 bp oligomer containing a GC box and MREf (see Materials and Methods) was carried out as in Figure 4. Panel A: The 109 bp probe. Panel B: The 135 bp probe. Lane 1 in each panel is cadmium-induced extract; lanes 2–4 in Panels A and B are cadmium-induced extract with 41 bp competitor incubated at 6°C, after complex formation for 15 minutes at room temperature, for 5, 15 and 30 minutes, respectively; Panel C: A repeat experiment of Panel B, lanes 1–3, showing more clearly the formation of upper bands in the absence of competitor (lane 1). The inducible band complex is indicated by the solid arrows without tails. The Sp1 complexes are indicated by the unfilled arrows. The presumed double complex is indicated, in Panel C, by the solid arrows with tails.

dependent complex was rapidly competed away, in less than five minutes, by the 54-mer competitor containing MREc and MREd (Figure 4B, lanes 3–6). As expected, the putative Sp1/DNA complex was not competed when the 54 bp competitor containing only MRE sequences was used.

To analyze the dissociation rate of the presumptive Sp1-DNA complex we used as a competitor a 41 bp oligomer containing both the GC box and MREf. In this experiment the protein-DNA complexes were once again preformed, prior to the addition of unlabelled competitor. Like the mutated competitor used in Figure 3, this 41 bp competitor was unable to compete for binding of the metal-inducible factor to the 109 bp probe, further suggesting that multiple MREs are required for the binding of the metal-inducible factor (Figure 5A). In contrast, the 41 bp oligomer could compete the presumptive Sp1 binding from the 135 bp probe (Figure 5B, 5C). However, while competition for the cadmium-inducible binding protein by an appropriate fragment (the 54 bp oligomer containing two MREs) was complete within five minutes (Figure 4), complete dissociation of the upper bands from the 135 bp probe in the presence of the 41 bp competitor containing the GC box required 30 minutes (Figure 5B, 5C).

In the experiment shown in Figure 5, the probe amounts were reduced six-fold as compared to the experiment shown in Figure 4. Under these conditions the cadmium-dependent complex was not apparent, in the absence of competitor, using the 135 bp probe

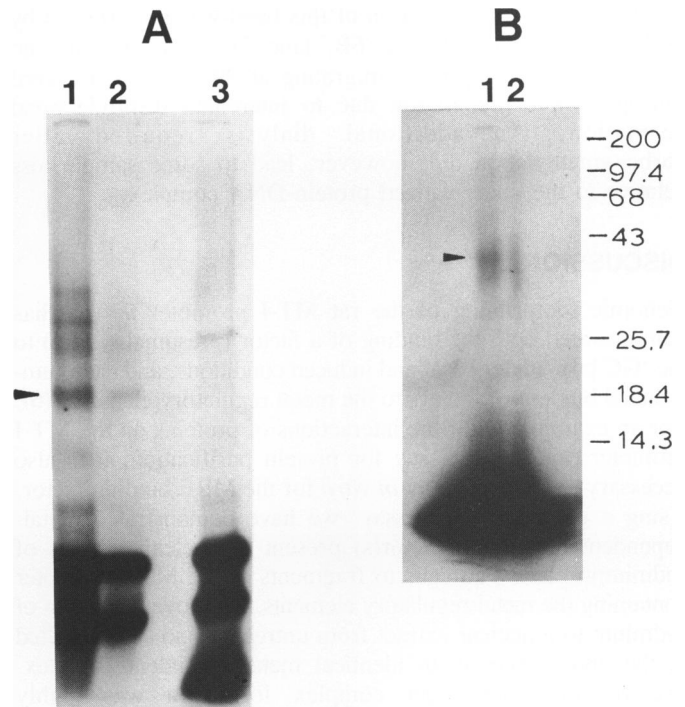


Figure 6. UV cross-linking analysis of metal-dependent complexes. The metal inducible complex prepared from cadmium-treated Fao cell nuclear extract, containing the labelled, BUdR-substituted, 57 bp probe containing MREs c and d, was cross-linked with UV light, extracted from a retardation gel and separated by SDS-PAGE (see Materials and Methods). Panel A: Preparative gel retardation separation of DNA-protein complexes. Lanes 1 and 2: Cadmium-treated nuclear extract (lane 2 was intentionally underloaded). Lane 3: Control nuclear extract. Panel B: SDS-PAGE separation of the metal inducible complex (indicated by the arrow in Panel A, lanes 1–2). Lanes 1 and 2: samples untreated and treated with iodoacetic acid, respectively. The strong band of radioactivity at the bottom is from oligonucleotides released during DNase and micrococcal nuclease digestion. The 39 kDalton affinity-labeled product is indicated by the solid arrow. Protein molecular weight standards are indicated in kDaltons.

(Lane 1 of Figure 5B, 5C). Instead, additional retarded bands appeared above the Sp1-DNA complexes. This was not observed, however, with the 109 bp probe (Lane 1 of Figure 5A). We suggest that, at this probe to extract ratio, the additional bands present with the 135 bp probe, in the absence of competitor, are complexes in which Sp1 and the metal inducible factor are bound to common probe molecules (see Discussion).

UV crosslinking analysis reveals a 39 kDalton protein associated with the metal inducible complex

We established the size of a factor involved in formation of the metal inducible complex by the method of UV crosslinking (23). Using a 57 bp fragment of DNA spanning MREc and MREd of the rat MT-I promoter, we could demonstrate the formation of a metal inducible complex similar to that shown with the 109 bp probe, in gel retardation analyses (Figure 6A, lanes 1 and 2). This complex was also competed by excess 57 bp DNA. Using this 57 bp probe, uniformly labeled and containing bromodeoxyuridine, we could chemically cross-link this complex with UV light prior to gel retardation electrophoresis (see Materials and Methods). Subsequent separation of the inducible band complex, recovered from the gel retardation run, by SDS-PAGE revealed a single band at 39 kDalton (Figure 6B, lane

1). The position of migration of this band was not affected by carboxymethylation (Figure 6B, lane 2), indicating that no oligomerization of protein migrating at 39 kDalton occurred during sample preparation due to interchain disulfide bond formation. An additional dialysis required after carboxymethylation did, however, lead to some sample loss relative to the underivatized protein-DNA complex.

DISCUSSION

Genomic footprinting of the rat MT-I promoter *in vivo* has demonstrated both the binding of a factor (presumably Sp1) to the 'GC box' under basal and induced conditions, and cadmium-induced binding of factors to the metal regulatory elements (6). For an examination of the interactions of proteins on the MT-I promoter, and as an assay for protein purification, it is also necessary to have an assay *in vitro* for the MRE binding factor. Using a gel retardation assay, we have demonstrated metal-dependent binding of factor(s) present in nuclear extracts of cadmium-treated Fao cells to fragments of the MT-I promoter containing the metal regulatory elements. Moreover, addition of cadmium to a nuclear extract from untreated Fao cells resulted in the appearance of an identical metal-dependent complex. Specific metal-dependent complex formation was highly dependent upon the buffer conditions used in the assay (27). In a Tris-glycine buffer system, metal-dependent complex formation occurred. We were unable to detect any metal-dependent binding in a Tris-borate-EDTA buffer system (data not shown), possibly because a direct interaction between cadmium ion and the binding factor, necessary for the factor to bind to the DNA, may be disrupted by EDTA.

In addition to the metal-dependent complex in the Tris-glycine buffer system, we observed common metal-independent complexes formed with proteins from nuclear extracts of both control and cadmium-treated Fao cells, using labelled probes derived from the MT promoter. When the probe contained the distal 'GC box' of the MT promoter, we identified Sp1 complexes by footprint analysis (data not shown). These data are consistent with the protection, observed in genomic footprinting in both control and metal-treated cells (6), over the 'GC' box. Deletion analysis has shown this distal element to increase basal level expression of the MT-I gene (6).

We attempted, by DNA methylation protection (25), DNase I protection (8) and 1,10-phenanthroline-copper nuclease protection (29), to footprint the metal dependent complex, with the intent of determining the binding site of the metal-dependent factor(s). In no case did we find protection over the MREs. Under similar conditions we could clearly footprint the binding to the 'GC' box. These results were initially puzzling, as our footprinting experiments *in vivo* have clearly demonstrated the binding of factors to the MREs (6). Examination of the stability of the cadmium-dependent protein/DNA complex *in vitro* suggested that the metal-dependent factor has a fast off-rate and/or a low affinity for binding the MT promoter when compared to the metal independent complexes observed with the 135 bp probe containing the MT-I 'GC box'. Complete competition for the 'GC' box requires 30 minutes. This is in contrast to the MRE factor, which shows complete competition in less than 5 minutes, when a competitor fragment containing two MREs is used. This difference in dissociation rates may make the metal-dependent complex difficult to footprint *in vitro*. In addition, if there are multiple MRE sequences on a DNA probe, present in large excess

relative to the MRE binding factor, then any given probe molecule in our gel retardation assay is not likely to have factor bound to all four of the functional MREs in our probes (MREb, c, d and e in the 109 bp probe and MREc, d, e and f in the 135 bp probe). Since the various MREs on the probe molecules might well be randomly bound by factor, presumably pairwise, footprinting such a mixture of complexes would result in only partial protection, difficult to distinguish, over the multiple MREs. When a synthetic oligonucleotide containing two MREs and only a small amount of additional sequence was used as a competitor, the metal-inducible complex disappeared for either the 109 bp or 135 bp probe (Figure 4). This result strongly suggests that the metal-inducible factor is, in fact, binding to the MRE sequences, despite our inability to footprint this interaction. The metal-independent complexes from the 135 bp promoter fragment were more amenable to footprinting and showed protection over the 'GC box' (data not shown), suggesting the binding of the Sp1 transcription factor. This indicates that our footprinting conditions, although unable to detect binding to the MREs, were equivalent to those used to detect many DNA-binding proteins.

The synthetic 41-mer oligonucleotide containing the 'GC box' also contains a single MRE, MREf. Although this oligonucleotide was able to compete for binding of Sp1 to the 'GC-box', it was unable to compete for binding of the metal-inducible factor, suggesting that a single MRE is insufficient for efficient binding of the metal-inducible factor. Similarly, mutation of MREc, one of the two MREs present in an effective competitor, eliminated the ability of this oligomer to compete for the binding of the metal dependent factor. The single functional MRE still present in this altered oligomer, MREd, was not sufficient, by itself, to compete for binding. These data are consistent with the experimental results of Searle et al (10), demonstrating that multiple MREs are required for metal-inducibility of the mouse MT-I gene.

With a limiting amount of the 135 bp probe, the cadmium-inducible complex is absent, while larger complexes are present (compare lane 1 of Figure 5B and 5C with lane 2 of Figure 4A and 4B). Under these conditions the cadmium inducible band appears only after the addition of a competitor containing the 'GC' box. This is most easily explained by assuming that the Sp1 factor binds quantitatively to probe molecules (which are now limiting) that are also complexed with the cadmium-inducible binding factor and shifts the inducible band complexes, to produce complexes above the Sp1 bands. When the 41 bp competitor containing the 'GC box' is added, only the Sp1 factor is competed away. Following dissociation of Sp1, the remaining cadmium-inducible complex migrates at its characteristic position. These data also indicate that there is no apparent cooperativity between Sp1 and the MRE binding factor(s) for binding to probe molecules.

Two recent reports provide evidence for the binding of a transcription factor to the MREd of the mouse MT-I gene (17,30). Unlike the cadmium-dependent binding which we observe with the 109 bp rat MT-I promoter region, the complex with MREd is formed in the presence of zinc but not cadmium (17). The authors demonstrate that this factor, found in HeLa cell extracts, resembles Sp1 in its binding sequence specificity. The lack of inducibility by cadmium ion, in contrast to our results, suggests that this factor differs from the one we have identified in Fao cell extracts. HeLa cell extracts also contain nuclear factors that bind to an Sp1 binding site adjacent to MREd in the mouse gene (17). The identical sequence is present adjacent to the equivalent

MREs in the rat MT-I gene (6). However, both with a footprinting assay *in vivo* (1), and an assay *in vitro* with Fao extracts (data not shown), we have been able to identify a binding site for Sp1 only at the upstream GC box, 5' of MREf. Studies using MREs from the trout metallothionein gene have identified a 75 kDalton protein from mouse L-cell extracts which binds to trout MRE sequences (31). This factor binds only weakly to mouse MT-I MRE sequences, however, and its binding is not inducible by metal ions.

Our UV cross-linking experiments identify a factor in Fao nuclear extracts with a size of 39 kDalton, which binds to the MT-I promoter in a metal dependent manner. Competition studies indicate that this factor binds to MRE sequences and that at least two MREs are required for binding. It is likely that this factor is a part of the complex which, *in vivo*, forms with MREs of the rat MT-I gene. The requirement for metal ion suggests that this factor may be the initial signal which leads to metal induction of transcription of the metallothionein gene. The existence of other factors, with different sizes and properties, which are involved in binding to MREs in metallothionein genes suggests metal regulation of MT gene transcription may be a complex process involving interactions among these factors.

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