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Transcription Factor MBF-I Interacts with Metal Regulatory Elements of Higher Eucaryotic Metallothionein Genes

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Metallothionein (MT) gene promoters in higher eucaryotes contain multiple metal regulatory elements (MREs) that are responsible for the metal induction of MT gene transcription. We identified and purified to near homogeneity a 74-kilodalton mouse nuclear protein that specifically binds to certain MRE sequences. This protein, MBF-I, was purified employing as an affinity reagent a trout MRE that is shown to be functional in mouse cells but which lacks the G+C-rich and SP1-like sequences found in many mammalian MT gene promoters. Using point-mutated MREs, we showed that there is a strong correlation between DNA binding in vitro and MT gene regulation in vivo, suggesting a direct role of MBF-I in MT gene transcription. We also showed that MBF-I can induce MT gene transcription in vitro in a mouse extract and that this stimulation requires zinc.

The metallothioneins (MTs) are a small family of cysteine-rich proteins that bind heavy metals such as cadmium, zinc, mercury, and copper. Although the biological role(s) of MT is still a matter of debate, it is clear that one important function is to protect cells against metal toxicity. An important component of this protective mechanism is the ability of metals to induce MT gene transcription. This homeostatic regulatory mechanism has been conserved in a wide variety of eucaryotes from years to humans (reviewed in references 10, 14, and 25).

In higher eucaryotic organisms, the *cis*-acting DNA sequences that confer metal responsiveness to the MT gene consist of multiple but imperfect repeats of a 12- to 17-base-pair (bp) metal regulatory element (MRE) (4, 15, 17, 34). Studies of the mouse MT-I gene promoter show that different naturally occurring MRE sequences have different strengths as transcription elements. Strong elements, such as MREd, can function as a single copy, whereas weaker elements, such as MREa, must be present in two or more copies to direct metal-inducible transcription (6, 35). Detailed point mutation analysis of mouse MT-I MREa and MREd (mMREa and mMREd) revealed that a highly conserved heptanucleotide core sequence, TGCPuCNC, is crucial for induction by metals. Less highly conserved flanking sequences have some effect on the efficiency of transcription but are not strictly required for metal induction (6, 30).

In the yeast *Saccharomyces cerevisiae*, it is known that the upstream metal regulatory sequences act as binding sites for a metal-activated transcription factor (9), but in higher eucaryotes such factors have not been demonstrated. However, two types of in vivo experiments suggest that MREs also represent binding sites for positively acting, metal-sensitive transcription factors. First, in a cotransfection experiment, MRE constructs repressed metal-induced transcription from the mouse MT-I (mMT-I) promoter (31). This suggests that the MRE is competing for a rate-limiting transcription factor. Second, in situ chromatin-mapping experiments revealed a complex pattern of nuclease-sensitive and -resistant sites around the MRE sequences, and come of these putative DNA-protein contacts are perturbed by metals (1, 24).

Although factors that bind to the MRE in mammalian total nuclear extracts have been reported (32, 33, 37), attempts to purify them and demonstrate their relevance to MT gene transcription have been hampered by two considerations. First, the MRE sequences are interdigitated with a complex series of sites for other *trans*-acting factors (2, 5, 8, 11, 16, 17, 19, 20, 22, 23, 27, 28; J. Imbert, V. Culotta, P. Fürst, L. Gedamu, and D. Hamer, Adv. Inorg. Biochem., in press). Consequently, even factors that bind to relatively short MRE oligonucleotides may in fact be specific for overlapping sequence motifs. Second, metal addition and chelation experiments in crude extracts are complicated by the fact that some general factors, such as the zinc-finger proteins, are metal dependent, whereas others are nonspecifically inhibited. This can lead to misinterpretations due to binding or release of the probe by proteins other than the true metal regulatory factor.

Here, we report the use of a trout MT-B (tMT-B) gene MRE sequence as an affinity reagent to isolate a mouse nuclear factor that specifically binds to certain functional MRE sequences. There were two reasons for selecting this cross-species approach. First, transfection experiments show that the tMT-B gene promoter is functional and metal inducible in human and mouse cells (39; this report). Second, although the trout promoter contains two MRE sequences and one TATA box, it lacks any of the other known upstream promoter elements associated with the mammalian genes. In addition, this promoter is 62% A+T-rich (39) and lacks the multiple G+C-rich sequences which are known to bind to several mammalian transcription factors (12). Using this approach, we purified to near homogeneity a 74-kilodalton (kDa) protein that specifically recognizes trout MREa (tMREa) and certain other functional MRE sequences. We also showed that this factor stimulates in vitro transcription of the tMT-B promoter in a zinc-dependent fashion. We call

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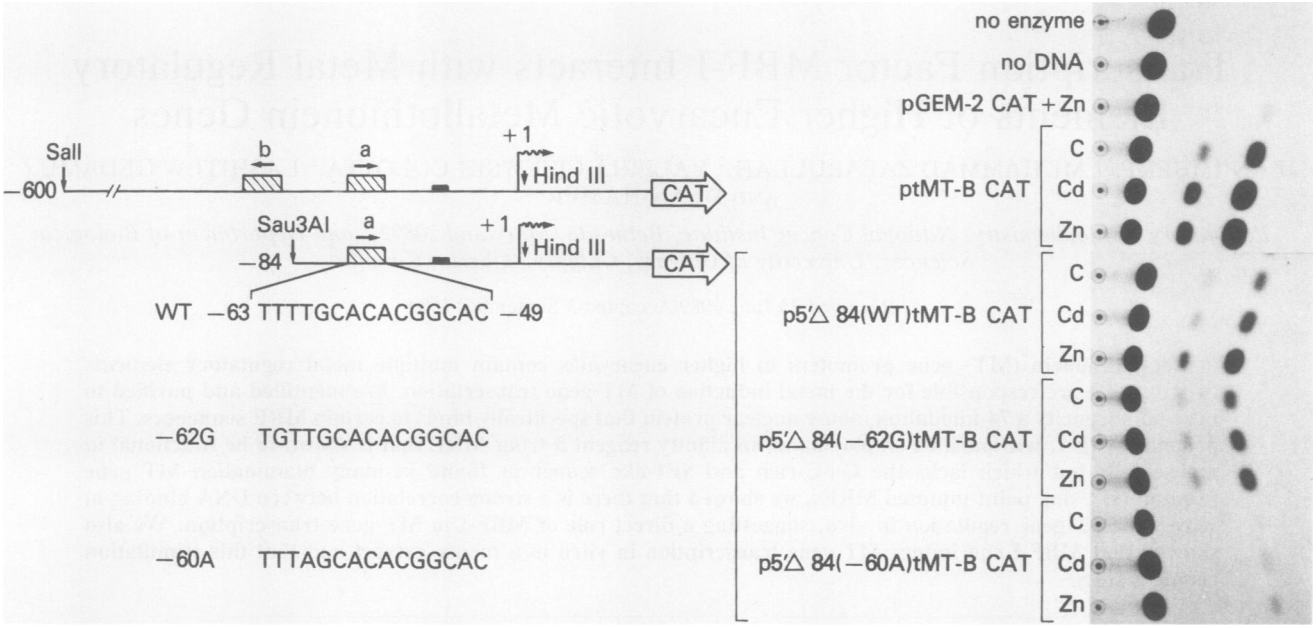

FIG. 1. Metal-dependent activity of the tMT-B promoter in mouse cells. Left, Maps of tMT-B 5′ deletion and point mutant constructs transfected by the calcium phosphate precipitation procedure into L cells. The arrows and boxes above the line show the positions and orientations of MREa and MREb, while the TATA box is indicated by ■. The number +1 refers to the transcription initial point in trout cells (39). Right, CAT activity of tMT-B promoter-*cat* fusion constructs in L cells. DNA (10 μg/100-mm petri dish) of each construct was transfected into L cells (6). After transfection, cells were exposed to 5 μM $CdCl_2$ or 100 μM $ZnCl_2$ for 18 h and harvested. CAT assays were performed with 100 μg of protein extract. The CAT assay reaction products were analyzed by thin-layer chromatography on a silica gel, and the film was exposed for 24 h. The assay for which the results are shown is representative of three independent experiments, all of which gave similar results. WT, Wild type.

this factor metal response element binding factor I (MBF-I) and suggest that it is directly involved in the metal-stimulated transcription of MT genes.

MATERIALS AND METHODS

Cell culture, transfection, and CAT assay. Mouse L cells were grown in monolayer or in suspension in Dulbecco minimal essential medium (D-MEM) or Spinner minimal essential medium (S-MEM) medium (Biofluids) containing 10% fetal calf serum. The conditions for DNA transfection, metal induction, and chloramphenicol acetyltransferase (CAT) assays have been described previously (6, 39).

Site-directed mutagenesis of tMT-B gene promoter. The construction of the tMT-CAT hybrid plasmids was described previously (39). Site-directed mutagenesis was performed by the protocol of Vandeyar et al. (36) on the *Eco*RI-*Hin*dIII fragment of p5′Δ84tMT-B CAT (39) subcloned into M13mp18 cut with the same enzymes. Two synthetic oligonucleotides with a single substitution in tMREa (Fig. 1) were employed for this purpose. Mutants were screened by sequence analysis of all constructs (M13mp18 and pGEM-2-CAT derivatives).

Nuclear extract preparation and DNA-binding factor purification. Nuclear extracts were prepared from exponentially growing mouse L cells by a slight modification of the method of Dignam et al. (7). KCl was substituted for NaCl and 1 μM $ZnSO_4$ was added in all buffers. Buffer III is 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 0.1 M KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride 1 μM $ZnSO_4$, and 20% glycerol. In a typical preparation, 1 to 2 g of nuclear extract (in buffer III) was passed over a heparin-Sepharose Cl6B matrix (Pharmacia, Uppsala, Sweden) column (HR16/10; Pharmacia). Proteins bound to the matrix were eluted at 0.6 M salt. After dialysis against buffer III containing 50 mM KCl, the heparin 0.6 M fraction was passed over a cation-exchange DEAE-cellulose matrix (DE52; Whatman, Inc., Clifton, N.J.) column (HR16/10; Pharmacia) and eluted by a two-step gradient (0.25 and 0.4 M KCl). The DEAE 0.25 M KCl fraction was precipitated with $(NH_4)_2SO_4$ (50%, wt/vol). The pellet was dissolved in buffer III, and insoluble particles were removed by centrifugation (100,000 × *g*, 15 min, 4°C). After incubation in the presence of 100 μg of double-stranded poly (dI-dC) (Pharmacia) per ml at 0°C for 15 min, the fraction was passed over an MRE oligonucleotide affinity column matrix and the bound proteins were eluted by a two-step gradient (0.3 and 1 M KCl). The MRE oligonucleotide affinity matrix was constructed by coupling a synthetic oligonucleotide derived from the tMT-B gene promoter to cyanogen bromide-activated Sepharose Cl4B (Pharmacia) as described previously (38). The oligonucleotide sequence was

5′-GATCTGTTTTGCACACGGCACCC-3′
3′-ACAAAACGTGTGCCGTGGGCTAG-5′

Affinity resin was packed in an HR10/10 (Pharmacia) column. MRE-specific DNA-binding activity purification was observed by DNA affinity labeling and DNase I footprinting assays. The activity of crude nuclear extracts and purified fractions is stable for several months when they are kept at −80°C in buffer III.

Footprint analysis. DNase I footprint analysis was done as described previously (3) with the exception that binding reactions were performed at room temperature in a modified binding buffer ($Z^{e'}$ buffer is 25 mM HEPES [pH 7.5], 0.1 M KCl, 20% glycerol, 0.01% Nonidet P-40, 1 μM $ZnSO_4$,

and 1 mM dithiothreitol). As indicated in the figure legends, 5'-end-labeled *Eco*RI-*Hin*dIII fragments derived from p5'Δ84tMT-B CAT and its respective point mutants were used as probes after gel purification.

DNA affinity labeling. A modified protocol of DNA affinity labeling by UV cross-linking of protein-DNA complexes was developed based on those described previously by Safer et al. (29). The probe was prepared by hybridizing a 21-base oligonucleotide of the coding strand of MREd from the mMT-I gene (positions −154 to −134) to an 8-bp complementary primer (positions −141 to −134). This oligonucleotide was made completely doubled stranded by incubation with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dCTP and the three other cold deoxynucleotides. Each 20-μl reaction mixture contained 10 fmol of oligonucleotide probe (5×10^4 cpm), 10 μg of bovine serum albumin, 10 μg of tRNA, and the indicated amounts of DNA competitor and of MBF-I protein fraction in Z$^{e'}$ buffer. After DNA-protein binding and UV irradiation (254-nm filter, 10 min at room temperature), reaction mixtures were incubated for 10 min at 37°C in the presence of 10 mM $MgCl_2$ and 1,000 U of DNase I (Worthington Diagnostics, Freehold, N.J.). Fractions were resolved by 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and analyzed by autoradiography.

Competition assays. A set of 21-bp synthetic oligonucleotides corresponding to mMREd and single point mutations (6) was used to perform competition experiments in DNase footprinting and DNA affinity labeling assays. Oligonucleotide competitors were annealed to form double-stranded DNA in 0.1 M NaCl–5 mM HEPES (pH 7.9) by slow cooling from 100°C to room temperature. After preincubation in the presence of variable amounts of DNA competitors for 10 min at room temperature, the labeled probes were added to binding reactions and DNase footprinting and DNA affinity labeling were performed as described above.

Renaturation assay. Approximately 1 μg of MBF-I was precipitated with trichloroacetic acid and fractionated in a preparative SDS-8% polyacrylamide gel. The regions of the gel containing the p74 putative MBF-I polypeptide and the other polypeptides revealed by Coomassie blue staining were excised and renatured as described previously (3), with the exception that the renaturation buffer contained 50 μM $ZnSO_4$. DNA-binding activity was assayed by UV cross-linking.

In vitro transcription. Nuclear extracts were prepared from exponentially growing mouse L cells by a slight modification of the method of Parker and Topol (26). The extract buffers contained 0.2 mM phenylmethylsulfonyl fluoride and 1 mM sodium metabisulfite protease inhibitors, while the dialysis buffer contained 57 mM $(NH_4)_2SO_4$ and 1 mM $MgCl_2$ in place of KCl. Depletion of MRE-binding proteins from nuclear extracts was performed as described previously (18), using a tMREa oligonucleotide affinity matrix. In vitro transcription assays (20 μl) contained 4 μl of the mouse nuclear extract, 100 ng of supercoiled template DNA, 0.5 to 2.0 μg of supercoiled pUC DNA carrier (amount required varied with extract preparation), 500 μM each rATP, rUTP, rGTP, and rCTP, 16 units of RNasin (Promega Biotec, Madison, Wis.), and a final buffer of 12.5 mM HEPES (pH 7.9), 7.5 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 30 mM KCl, 10% glycerol, and 2 mM dithiothreitol. Reaction mixtures were also supplemented with 50 μM $ZnSO_4$ as specified, and the indicated amounts of MBF-I which had been desalted to 50 mM KCl by being passed through a fast protein liquid chromatography desalting column (Pharmacia). After a 60-min incubation at 30°C, reactions were terminated by adding 300 μl of a solution containing 200 mM NaCl, 20 mM EDTA, 1.0% SDS, and 100 μg of yeast tRNA per ml, and nucleic acids were successively extracted with phenol and chloroform and precipitated at room temperature with isopropanol. The amount and initiation site of the transcripts were determined by hybridization of the RNA to a single-stranded ^{32}P-end-labeled *cat* probe (pSV2CAT residues 4994 to 4927) and extension with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) as described previously (6). The transcription products were quantified by densitometric tracings of the relevant autoradiograms.

RESULTS

tMREa is functional in mouse cells. The tMT-B gene promoter contains two MRE sequences, identified both by homology to mammalian MREs and by deletion mapping (39), but lacks the G+C-rich sequences that are closely associated with the MRE sequences of mammalian MT genes (Imbert et al., in press). Because we wished to use the tMT-B gene MREa as an affinity reagent for purification of mouse metal regulatory factors, it was first necessary to determine whether this sequence is functional in mouse cells. For this purpose, we transfected mouse L cells with a *cat* fusion gene containing 600 bp of MT-B gene 5'-flanking sequences, incubated the cells in the absence or presence of metals, and then performed CAT assays. This construct gave a substantial basal level of expression in the absence of added metals, and this was induced by Cd (2.5-fold) and Zn (3-fold) (Fig. 1). To determine whether tMREa alone is sufficient for metal induction, we repeated the assay using a −84 deletion mutant that lacks MREb (39). This construct was expressed somewhat less efficiently than the complete promoter but was still inducible by metals. To test more specifically the role of tMREa in metal induction, we used site-directed mutagenesis to introduce individual point mutations into either the core or flanking sequences of tMREa. The core mutation −60A, which is analogous to the nonfunctional −147A mutation of mMREd (6), substantially affected both the basal and metal-induced transcription of the fusion gene. In contrast, the flanking sequence mutation −62G, which is analogous to the functional −149G mutation of mMREd, gave approximately the same expression levels as the wild-type tMREa. These results showed that tMREa acts as a metal-responsive transcription element in mouse cells and further that the mouse transcription factor has the same sequence specificity for the homologous and heterologous MRE sequences. The effect of the –60A core mutation on basal as well as induced transcription suggests that tMREa is the dominant transcription element in the trout promoter; the analogous −147A mutation in mMREd did not affect basal transcription, presumably owing to the presence of additional basal transcription elements in the mouse promoter (6).

Purification of MBF-I. We used conventional and affinity chromatography methods to partially purify a mouse nuclear factor that binds to tMREa. The purification protocol included chromatography on heparin-Sepharose, DEAE-cellulose, and Sepharose-tMREa oligonucleotide columns (Fig. 2A). The factor, which we term MBF-I, was assayed by DNase footprinting (Fig. 2C) or by UV cross-linking with tMREa or mMREd oligonucleotide probes (see Fig. 4A). The extent of purification from the nuclear extract was 300-fold with a yield of 4% (Table 1). A sample containing 25

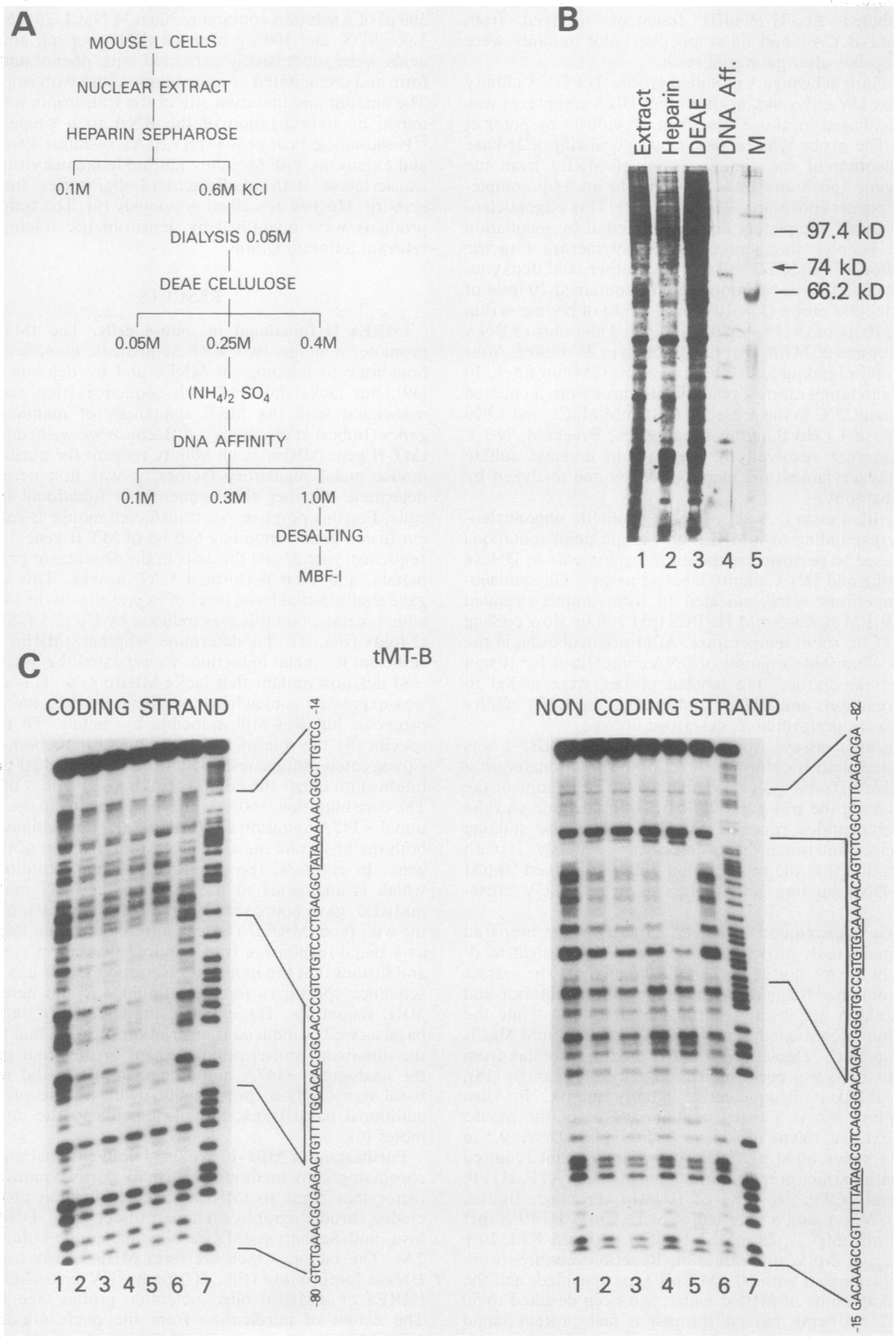
A
MOUSE L CELLS
NUCLEAR EXTRACT
HEPARIN SEPHAROSE
0.1M
0.6M KCl
DIALYSIS 0.05M
DEAE CELLULOSE
0.05M
0.25M
0.4M
(NH4)2 SO4
DNA AFFINITY
0.1M
0.3M
1.0M
DESALTING
MBF-I
B
Extract
Heparin
DEAE
DNA Aff.
M
97.4 kD
74 kD
66.2 kD
1 2 3 4 5
C
tMT-B
CODING STRAND
-14
-80 GTCTGAACGCGAGACTGTTTTGCACACGGCACCCGTCTGTCCCTGACGCTATAAAAACGGCTTGTCG
1 2 3 4 5 6 7
NON CODING STRAND
-82
-15 GACAAGCCGTTTTTATAGCGTCAGGGACAGACGGGTGCCGTGTGCAAAACAGTCTCGCGTTCAGACGA
1 2 3 4 5 6 7

TABLE 1. Purification of MBF-I

Step	Total protein (mg)[a]	Activity		Purification factor	Cumulative yield (%)
		Total activity (U)[b]	Sp act (U/mg)		
Mouse L cells (25 g)	1,250				
Nuclear extract	157				
Heparin-Sepharose	48	22,000	456	3	100[c]
DEAE-cellulose	24	14,000	588	1.3	64
DNA affinity	0.02	1,000	41,600	71	4

[a] Protein concentrations were estimated by the method of Bradford (Bio-Rad protein assay) for intermediate steps and by silver staining of an SDS gel for affinity-purified MBF-I.

[b] One unit of activity is defined as the amount of protein required to provide complete protection of ttMREa from digestion by DNase I under standard conditions (see Materials and Methods).

[c] On the basis that there is 100% recovery of MBF-I after heparin-Sepharose; determination of activity cannot be made on the nuclear extract.

footprinting units of the most highly purified fraction gave three major bands on a silver-stained SDS gel (Fig. 2B).

The footprint of the 1 M KCl affinity-purified fraction of MBF-I on the tMT-B gene promoter is shown in Fig. 2C. The protected region extends from −66 to −48 on the coding strand and from −71 to −41 on the noncoding strand and thus includes the ttMREa sequence. Except for a weaker interaction over tMREb, no other protected regions on the trout promoter were observed (data not shown).

MBF-I specifically binds to functional MRE sequences. The DNA-binding specificity of MBF-I was tested by both competition and direct binding assays with point-mutated oligonucleotides and probes. Figure 3A shows an experiment in which the footprint of MBF-I over ttMREa was competed for with a set of mMRE oligonucleotides whose in vivo activities have previously been characterized by transfection experiments (6). The wild-type MREd oligonucleotide, the functional point mutants −149G and −140T of MREd, and tMREa all showed significant inhibition of the footprint. For MREd and −149G, competition was complete at a 100-fold molar excess, whereas for −140T and tMREa, competition was less efficient and was not complete even at a 1,000-fold excess. In contrast, the nonfunctional mMREd core mutants −147A and −143T showed little or no competition, even at a 1,000-fold excess. An oligonucleotide containing the SP1-binding site and calf thymus DNA also failed to compete even at high concentrations.

To further test the specificity of MBF-I, we carried out DNase I footprint analyses on tMT-B gene promoter probes bearing point mutations in ttMREa. The nonfunctional construct bearing the −60A core mutation was devoid of a footprint over ttMREa, whereas the functional mutant −62G footprinted normally (Fig. 3B). These experiments showed that the same nucleotides required for MRE function in vivo are required for MBF-I DNA binding in vitro.

Identification of MBF-I as a 74-kDa polypeptide. We used a modified DNA affinity labeling by UV cross-linking method to identify the polypeptide responsible for the DNA-binding activity of MBF-I. This procedure involved the transfer of ^{32}P from specific phosphodiester bonds at the DNA-binding site to the polypeptide chain (29). In total nuclear extract, the predominant labeled protein migrated at approximately 78 kDa, and this protein copurified with the MBF-I DNA-binding activity (Fig. 4A). The specificity of the UV cross-linking reaction was tested by competition with point mutant oligonucleotides. Figure 4B shows that, as expected, mMREd, the functional mutants −149G and −140T, and mtMREa all competed successfully, whereas the nonfunctional core mutant −147A did not. In agreement with the footprint competition experiments (Fig 3A), the −140T mutant was a poorer competitor than mMREd or −149G. Surprisingly, the −143T flanking sequence mutant of mMREd also competed in this and several repeats of this experiment; at present, we have no explanation for the discrepancy between the footprinting and cross-linking results obtained with this mutant.

The radiolabeled p78 migrated just above the predominant 74-kDa band seen in silver-stained gels (Fig. 2B). To identify unambiguously which polypeptide in the MBF-I preparation is responsible for DNA-binding activity, we excised slices from a Coomassie blue-stained preparative gel from regions containing or not containing a detectable polypeptide. The polypeptides were then eluted and renatured, and DNA-binding activity was tested by affinity labeling. Only the 74-kDa polypeptide was radiolabeled, and its apparent mobility shifted to 78 kDa (Fig. 4C, lane 4). In contrast, the 78-kDa region of the gel did not yield any detectable DNA-binding activity (Fig. 4C, lane 3). The recovery of DNA cross-linking activity after SDS-gel electrophoresis, elution, and renaturation was approximately 2%. These findings suggest that the DNA-binding activity of MBF-I is associated with the 74-kDa protein.

MBF-I activates transcription in vitro. To determine whether MBF-I is a transcriptional activator and to test whether its activity is metal dependent, we added purified MBF-I to in vitro transcription reactions containing the tMT-B 5′Δ84 template and containing or lacking various metal ions. In the absence of metals, MBF-I had no effect on production of the expected transcript represented by the 135-nucleotide reverse transcriptase product (Fig 5A). However, the addition of Zn(II) stimulated transcription 2-fold, and the further addition of MBF-I resulted in a further 3.4-fold stimulation. Although the effect of MBF-I was small, it was reproducible in seven independent experiments with three different extracts (mean ± standard deviation = 2.4 ± 0.7, as determined by densitometric tracing). The metal stimulation effect was apparently specific for Zn(II) since other divalent cations such as Cu(II) or Cd(II) had no effect (data not shown). As a control for this experiment, the

FIG. 2. Purification of MBF-I. (A) Scheme for purification of MBF-I from nuclear extracts of L cells. All column fractions were assayed for MBF-I by DNA affinity labeling and DNase I footprint reaction. (B) Silver-stained SDS-polyacrylamide gel of fractions at various stages of purification. Lanes: 1, total nuclear extract (7 μg); 2, heparin-Sepharose, 0.6 M (7 μg); 3, DEAE-cellulose, 0.25 M (9 μg); 4, ttMREa DNA affinity, 1.0 M (25 activity units as defined in Table 1); 5, molecular size markers (kD, kilodaltons). The arrow designates the 74-kDa MBF-I DNA-binding polypeptide. This SDS-8% polyacrylamide gel was run under reducing conditions. (C) Footprint of MBF-I on the tMT-B promoter. Coding strand and non-coding strand DNA probes (102 bp) were 5′ end labeled with [γ-^{32}P]ATP and polynucleotide kinase at the *Eco*RI (−95) and *Hin*dIII (+7) sites of the p5′Δ84tMT-B CAT construct, respectively. Lanes: 1 and 6, no purified mouse nuclear factor added; 2 to 5, increasing amounts of affinity-purified MBF-I (1, 2, 4, and 8 μl, respectively); 7, A+G Maxam-Gilbert sequence ladder (21). DNase I reactions were performed as described previously (3) in the presence of 20 μg of bovine serum albumin as the carrier. The core sequences of the ttMREa and of the TATA box are underlined on each DNA strand. The extent of the protected area is marked above each DNA strand

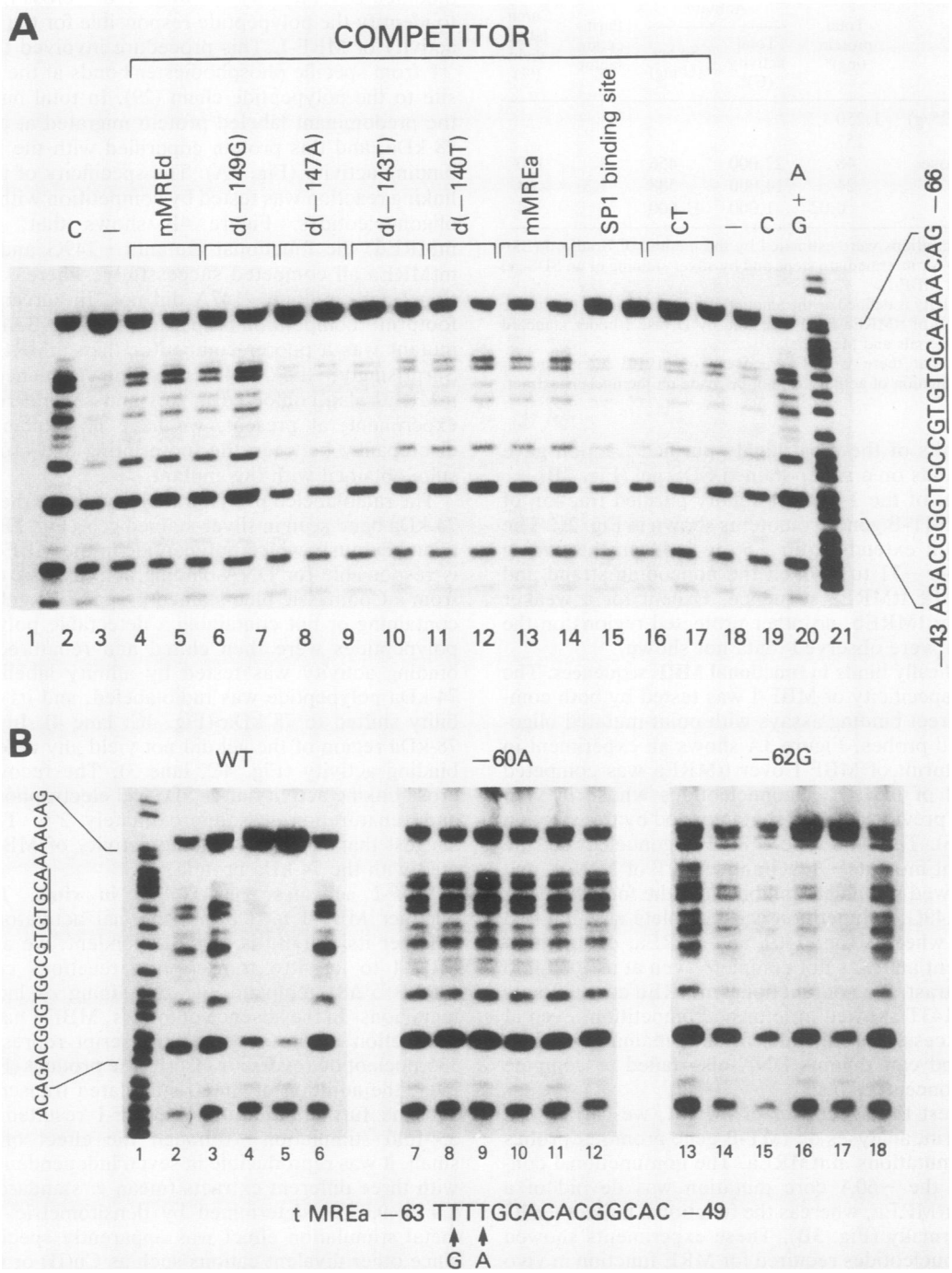

FIG. 3. MBF-I binding to functional MREs. (A) Footprinting in the presence of different competitor DNAs. Each reaction contained 5 fmol of end-labeled DNA probe (p5′Δ84tMT-B CAT *Eco*RI-*Hin*dIII fragment), 5 μl of MBF-I, and 0, 500, or 5,000 fmol of competitor DNA. The metal inducibility of each MRE oligonucleotide (+, metal inducible; –, noninducible) was determined previously (6). Lanes: 1 and 20, no MBF-I; 2 and 19, no competitor; 3, 100-fold excess, and 4, 1,000-fold excess of wild-type mMREd oligonucleotide (+); 5 and 6, (–149G)mMREd (+); 7 and 8, (–147A)mMREd (–); 9 and 10, (–143T)mMREd (–); 11 and 12, (–140T)mMREd (+); 13 and 14, mtMREa (+); 15 and 16, SP1-binding site; 17 and 18, sonicated calf thymus (CT) DNA; 21, A+G Maxam-Gilbert sequence ladder (21). (B) DNase footprinting analysis of point-mutated tMREa tMT-B promoter template. Each reaction contained 10 fM end-labeled DNA probe (p5′Δ84tMT-B CAT *Eco*RI-*Hin*dIII fragment), 100 ng of double-stranded poly(dI-dC), and various amounts of MBF-I. Lanes: 1 to 6, wild-type (WT) ttMREa template (1, A+G Maxam-Gilbert sequence ladder [21]; 2 and 6, no MBF-I; 3 to 5, 1, 2, and 4 μl of MBF-I, respectively); 7 to 12, –60A mutant template (7 and 12, no MBF-I; 8 to 11, 1, 2, 4, and 8 μl of MBF-I, respectively); 13 to 18, –62G mutant template (13 and 18, no MBF-I; 14 to 17, 1, 2, 4, and 8 μl of MBF-I, respectively). The sequence of ttMREa and the positions of the site-directed mutations –60A and –62G are shown below.

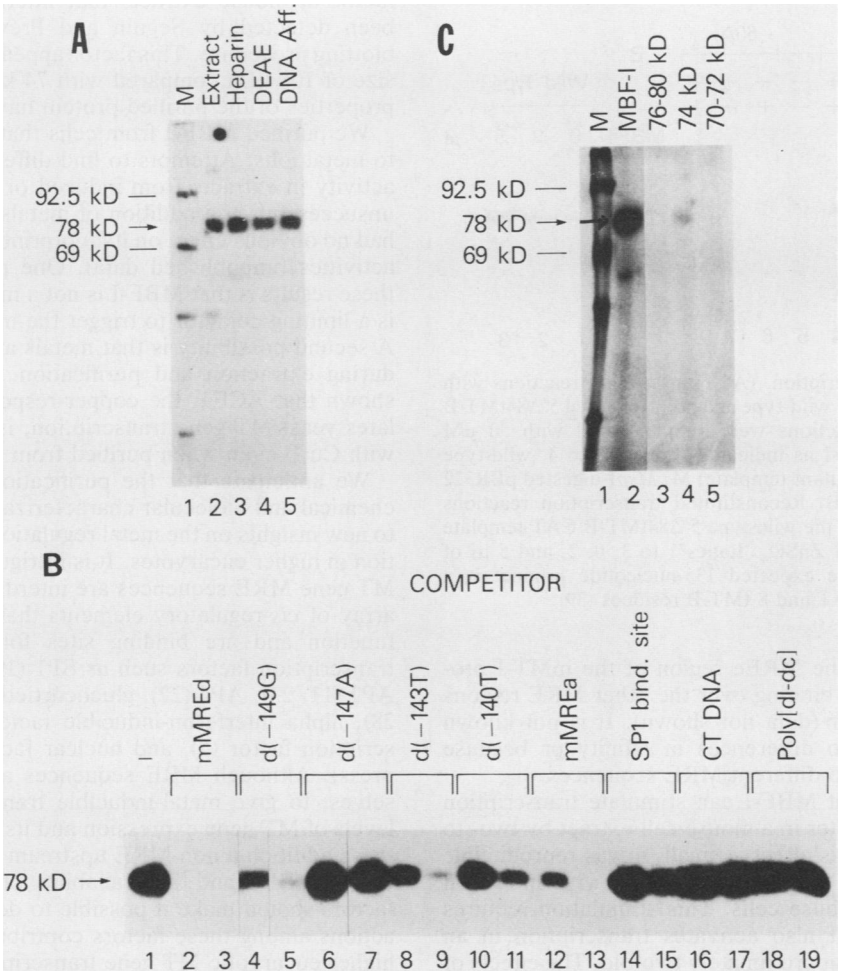

FIG. 4. Identification of MBF-I DNA-binding activity as a 74-kDa polypeptide. MRE affinity labeling was performed by UV cross-linking as described in Materials and Methods. (A) DNA affinity labeling of mMREd-binding factors at different stages of MBF-I purification. Lanes: 1, ^{14}C-labeled molecular size standards (Amersham Corp., Arlington Heights, Ill.); 2, nuclear extract; 3, heparin-Sepharose, 0.6 M; 4, DEAE-cellulose, 0.25 M; 5, ttMREa DNA affinity, 1 M. Each lane contained 1 activity unit of MBF-I. (B) Competition of affinity-labeled MBF-I by functional (+) and nonfunctional (−) MREs. Internally labeled mMREd (50 fmol) was added after 10 min of incubation of the UV cross-linking reaction mixture in the absence or presence of competitor DNAs (250 or 2,500 fmol). Lanes: 1, no competitor; 2, 5-fold excess, and 3, 50-fold excess of wild-type mMREd (+); 4 and 5, (−149G)mMREd (+); 6 and 7, (−147A)mMREd (−); 8 and 9, (−143T)mMREd (−); 10 and 11, (−140T)mMREd (+); 12 and 13, mtMREa (+); 14 and 15, SP1-binding site; 16 and 17, sonicated calf thymus (CT) DNA; 18 and 19, double-stranded poly(dI-dC). (C) Renaturation of MBF-I DNA-binding activity. DNA-binding activity of renatured MBF-I polypeptides (see Materials and Methods) was assayed by UV cross-linking. Lanes: 1, molecular size standards; 2, native MBF-I; 3, 76 to 80-kDa area; 4, 74-kDa MBF-I polypeptide; 5, 68- to 72-kDa area. kD, Kilodaltons.

same reactions were performed with a template with the −60A core mutation of tMREa. With this mutant, neither Zn(II) nor MBF-I had any effect on transcription.

The inability of the mutant template to respond to Zn(II) suggested that stimulation of the wild-type template by zinc alone might be due to endogenous MBF-I activity in the transcription extract. In an attempt to assay more cleanly the transcriptional activation capacity of MBF-I, we passed the transcription extract over a ttMREa oligonucleotide affinity column. With this depleted extract, purified MBF-I stimulated transcription up to sevenfold in the presence of Zn(II) (mean ± standard deviation = 4.9 ± 1.9, n = 3) (Fig. 5B).

DISCUSSION

We reported the identification and purification to near homogeneity of MBF-I, a 74-kDa mouse nuclear factor that specifically binds to certain functional MRE sequences. MBF-I binds to the metal-inducible tMT-B gene promoter over a region centered around the TGCACAC core sequence of ttMREa. The specificity of MBF-I was demonstrated by its inability to footprint over a core point mutant of ttMREa which is nonfunctional in vivo. Further evidence for specificity was obtained from competition experiments with oligonucleotides containing point mutants of mMREd. These experiments showed a good correlation between the ability of an MRE sequence to function in vivo and its ability to bind to MBF-I. It seems unlikely that the correlation between in vivo and in vitro activity is specious since several point mutations that are functional in vivo also bound to MBF-I. However, the ability of MBF-I to bind directly to MRE sequences other than ttMREa is not yet clear. To date, we have observed only a weak footprint over tMREb and a

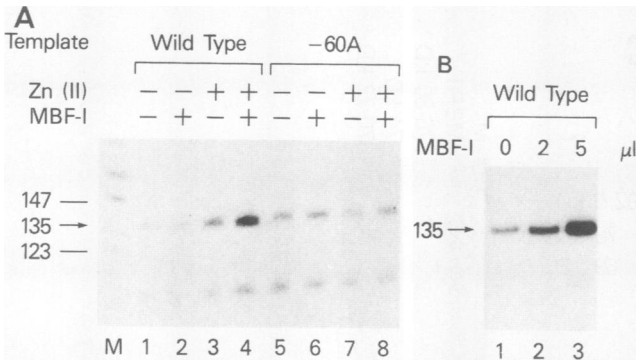

FIG. 5. In vitro transcription. (A) Transcription reactions with total nuclear extract of the wild-type and point-mutated 5′Δ84tMT-B CAT templates. The reactions were supplemented with 50 μM $ZnSO_4$ and 5 μl of MBF-I as indicated. Lanes: 1 to 4, wild-type template; 5 to 8, −60A mutant template; M, *Msp*I-digested pBR322 end-labeled fragments. (B) Reconstituted transcription reactions with depleted extract and the wild-type 5′Δ84tMT-B CAT template in the presence of 50 μM $ZnSO_4$. Lanes 1 to 3, 0, 2, and 5 μl of MBF-I, respectively. The expected 135-nucleotide product is a fusion of 124 pGEM-2-CAT and 8 tMT-B residues (39).

strong footprint over the MREe region of the mMT-I promoter but no apparent binding over the other MRE regions of the mouse promoter (data not shown). It is not known whether this is due to differences in affinity or because different factors bind to different MRE sequences.

We also showed that MBF-I can stimulate transcription from the tMT-B promoter in a mouse cell extract by two- to sevenfold. Although this effect is small, it was reproducible in several experiments and is close to the in vivo induction ratio for ttMREa in mouse cells. This stimulation requires Zn(II), which by itself also activates transcription in an unfractionated extract approximately twofold. The effects of both MBF-I and Zn(II) were shown to be specific by the lack of response of a core point mutant of tMREa. However, the precise role of Zn(II) in transcriptional activation by MBF-I is not known. While it is possible that Zn(II) stimulates DNA binding, we have so far not seen any effect of Zn(II) or other metals on the DNA affinity of MBF-I either by footprinting or cross-linking assay. A second possibility is that Zn(II) interacts with MBF-I and alters its ability to activate transcription. A third possibility is that Zn(II) affects some other transcription factor that interacts with MBF-I.

Our strategy for the purification of MBF-I was to use as an affinity reagent ttMREa, which lacks the G+C-rich, SP1-like sequence associated with many mammalian MREs (13; Imbert et al., in press). Recently, Westin and Schaffner (37) have reported the detection in human nuclear extracts of an mMREd-binding activity, termed MTF1, that appears to be stimulated by Zn(II). This factor binds to a sequence that overlaps the SP1-binding site but can be distinguished from SP1 by subtle differences in methylation interference and band-shift experiments. Although we do not know whether MBF-I and MTF-1 are related, and the molecular weight of MTF-1 has not been reported, we have preliminary evidence that MBF-1 binds to the simian virus 40 21-bp repeats with a footprint identical to that of SP1. However, this MBF-I footprint is blocked only by MRE oligonucleotides and not by SP1 oligonucleotides (unpublished data). Interestingly, Westin and Schaffner (37) have observed that the simian virus 40 early promoter is somewhat metal inducible, a property that could be mediated by an MBF-I-like factor. A factor in mouse extracts that interacts with mMREd has been detected by Seguin and Prévost (33) by a protein blotting procedure. This factor appeared to have a molecular size of 108 kDa compared with 74 kDa for MBF-I, and the properties of the purified protein have not been described.

We purified MBF-I from cells that had not been exposed to metal ions. Attempts to find differences in DNA-binding activity in extracts from induced or noninduced cells were unsuccessful, and addition of metals to the purified protein had no obvious effect on its footprinting or UV cross-linking activities (unpublished data). One possible explanation of these results is that MBF-I is not a metalloprotein but rather is a limiting cofactor to trigger the metal induction process. A second possibility is that metals are bound to the protein during extraction and purification. Recently, it has been shown that ACE1, the copper-responsive factor that regulates yeast MT gene transcription, is almost fully saturated with Cu(I) even when purified from noninduced cells (9).

We anticipate that the purification and subsequent biochemical and molecular characterization of MBF-I will lead to new insights on the metal regulation of MT gene transcription in higher eucaryotes. It is intriguing that all mammalian MT gene MRE sequences are interdigitated with a complex array of *cis*-regulatory elements that can provide enhancer function and are binding sites for already-characterized transcription factors such as SP1 (19, 24), AP1 (2, 19, 22), AP2 (11, 23), AP4 (22), glucocorticoid receptor (16, 17, 26, 28), alpha interferon-inducible factor (8), major late transcription factor (5), and nuclear factor I (Imbert et al., in press). Although MRE sequences are sufficient, by themselves, to give metal-inducible transcription, the absolute levels of MT gene expression and its fine balance depend on these additional non-MRE upstream elements. The purification of MBF-1 and the availability of the other transcription factors should make it possible to determine whether interactions among these factors contribute to the regulation of higher eucaryotic MT gene transcription.

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