A Nuclear Factor That Recognizes the Metal-responsive Elements of Human Metallothionein \( \Pi_A \) Gene*

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Expression of metallothionein (MT) genes is regulated by heavy metals mainly at the transcriptional level, via cis-acting elements called the metal-responsive elements (MREs). A HeLa cell nuclear factor that recognizes MREs of the human MTIIa (hMTIIa) gene, MREBP, was characterized. Mobility shift assay and DNase I footprinting experiments showed that MREBP binds specifically to several MREs present upstream of the hMTIIa gene. Cadmium and zinc ions inhibited binding of MREBP to a MRE at high concentrations, suggesting a role of MREBP in the negative regulation of the hMTIIa gene. MREBP was partially purified by passing the HeLa nuclear extract over heparin-agarose, Sephacryl S-300, and MRE-Sepharose affinity columns. Blotting experiments showed that a polypeptide with an \( M_r \) of 112,000 is responsible for the MREBP activity.

Metallothioneins (MTs) are a family of cysteine-rich metal-binding proteins, which have been postulated to be involved in detoxification of heavy metals such as cadmium and mercury and the homeostatic regulation of essential metals such as zinc and copper (1-5). Synthesis of mammalian MTs has been shown to be induced by heavy metals which they bind (4-7), and the induction occurs mainly at the transcriptional level (8-10). The upstream region of MT genes has ability to confer metal-responsiveness to heterologous promoters (11-13), and the nucleotide sequences responsible for this activation have been identified by deletion mapping experiments; at least two different sites are important for the metal regulation, and these sites involve a DNA sequence called the metal-responsive (or regulatory) element (MRE) (12, 14, 15). Multiple copies of MRE-like sequences have been found in the 5'-flanking regions of various mammalian MT genes (12, 16). The MRE motif by itself confers metal-responsiveness to heterologous promoters (12, 13, 16, 17) and functions as a heavy metal-inducible enhancer (13). Many of the enhancer elements have been shown to be the recognition sites of transcriptional regulatory proteins (18), and MRE is also expected to be the target of a protein(s) that regulates MT gene expression in response to heavy metals. Recently, several papers (13, 19-23) have reported proteins that specifically interact with MREs. In some cases, MRE-binding activity is enhanced by heavy metals such as cadmium (19) and zinc (13, 22) in vitro. Also by in vivo footprinting experiments, metal-dependent protection of MREs has been shown (24, 25). Nevertheless, none of those proteins has yet been identified as a transcriptional regulator of MT genes.

We have recently identified a HeLa cell nuclear factor which binds to the MRE sequences of the human MTIIa (hMTIIa) gene (26). In the present study, we characterized and purified this nuclear factor, MREBP, which possibly acts as a negative regulator of the hMTIIa gene.

MATERIALS AND METHODS

DNA Probes—An oligonucleotide probe containing the MREa sequence of hMTIIa gene (MREa probe) was prepared by end labeling of annealed synthetic oligonucleotides (Fig. 1A and B; generous gifts from Dr. K. Nagata) with [\( \gamma \)-\( ^{32} \)P]ATP (4,500 Ci/mmol) and T4 polynucleotide kinase. AuaI-MuaI probe (AM probe; 95 bp) containing a part of the upstream sequence of hMTIIa gene (Fig. 1A) was prepared by restriction enzyme digestion of a plasmid pHMTIIa (pHMTIIa was constructed by subcloning the 3-kilobase pair HindIII fragment containing the hMTIIa gene) (27) into the HindIII site of pBR322 and labeling at the AuaI site (27). The coding strand was end-labeled as above, and the noncoding strand was labeled by end-filling with Klenow fragment and four deoxyribonucleotides containing [\( \alpha \)-\( ^{32} \)P]dTTP (3,000 Ci/mmol). The DNA fragments were subjected to MspI digestion after labeling at the AuaI site so as to leave the MspI site (\(-116\)) unlabelled.

Mobility Shift Assay—Each reaction mixture (12.5 \( \mu l \)) contained 10 mM Hepes, pH 7.9, 1 mM dithiothreitol (DTT), 10% glycerol, 50 mM KCl, and 4 fmol of \( ^{32} \)P-probe, unless otherwise stated. Proteins were incubated in the mixture for 10 min at 25\( ^\circ \)C. After addition of 1 \( \mu l \) of polydI-dC (Pharmacia), incubation was continued for a further 10 min. Protein-DNA complexes formed were analyzed by gel electrophoresis as described previously (26). Band intensity of the MREBP-DNA complex was quantified using a Shimadzu CS-9000 densitometer. Activity of MREBP that binds a half of the \( ^{32} \)P-probe is defined as 0.5 unit.

DNase I Footprinting—Binding reaction was carried out in a 25-\( \mu l \) reaction mixture as in the mobility shift assay, except that 10 \( \mu l \) of bovine serum albumin and 10 fmol of \( ^{32} \)P-AM probe were added per reaction. The mixture was then treated with DNase I as described by Dyman and Tjian (28) and analyzed by electrophoresis in 8% polyacrylamide gel with 7 M urea. The gel was dried and autoradiographed by standard procedures.

Cell Culture—HeLa S3 cells (CCL 2.2; obtained from Japanese Cancer Research Resource Bank) were propagated in glass roller bottles as described previously (29). Confluent cells were collected by scraping, washed with PBS(\(-\)) (Dulbecco’s phosphate-buffered saline without Mg\(^{2+}\) and Ca\(^{2+}\)), and stored at -80\( ^\circ \)C until used.

Purification of MREBP—MREBP activity was estimated by mobility shift assay with \( ^{32} \)P-MREa probe as described above. Nuclear extracts of HeLa cells were prepared as described previously (26), except adjusting the buffers to pH 7.6 and omitting the first washing step with the hypotonic buffer (Buffer 1). An ammonium sulfate
fraction (40–80% saturated) of the extract was dissolved in 0.20 the original volume of Buffer A (20 mM Hepes, pH 7.6, 0.5 mM DTT, 20% glycerol) containing 0.1 M KCl and dialyzed against the same buffer. After removing insoluble materials by a brief centrifugation, the supernatant was fractionated in Sephacryl S-300 (Pharmacia). Active fractions were pooled and then passed over a heparin-agarose (Bethesda Research Laboratories) column. After washing with the same buffer, proteins were eluted stepwise by 0.3 and 0.6 M KCl in Buffer A. Active fractions (0.3 M KCl eluate) were adjusted to 0.05 M KCl, and 50 ng of calf thymus DNA/ml in Buffer A and applied to a MREa-Sepharose affinity column, prepared according to Kadonaga and Tjian (30) by coupling ligated MREa oligonucleotides (Fig. 1B) to Sepharose 4B (Pharmacia). After washing with Buffer A containing 0.05 M KCl, proteins were eluted stepwise by 0.2, 0.5, and 0.7 M KCl in Buffer A. The 0.5 M eluate was concentrated with ATTO concen-

Sodium Dodecyl Sulfate (SDS)-Gel Electrophoresis—Protein solutions were mixed with an equal volume of SDS-gel buffer (5 mM Tris-Cl, pH 6.8, 0.2 M DTT, 20% glycerol, 0.05% bromphenol blue), and electrophoresed in SDS, 7.5% polyacrylamide gel as described (32). Proteins were detected by silver staining according to Merrill et al. (33).

Protein Blotting—The compositions of the buffers used were: sample buffer, 25 mM Tris-Cl, pH 6.8, 0.1 M DTT, 10% glycerol, 2.5% sodium lauroyl sarcosinate, and 0.25% bromphenol blue; transfer buffer, 25 mM Tris and 190 mM glycine; blocking buffer, 25 mM Hepes-Na, pH 7.9, 50 mM NaCl, 1 mM DTT, and 0.4 mg/ml bovine serum albumin (Sigma). Each treatment was done at room temperature, unless otherwise stated. The samples or pre-stained molecular weight standards (Amersham) were mixed with an equal volume of 2-fold concentrated sample buffer. The samples were allowed to stand at room temperature for 10 min, whereas the standards were boiled for 5 min. The protein solutions (20 µl) were electrophoresed in SDS, 7.5% polyacrylamide gel (32) at 30 mA for 2 h at 6 °C.

RESULTS

Specific Binding of MREBP to MRE—We have previously observed that a HeLa nuclear protein, MREBP, binds to oligonucleotides containing the MRE sequences of the hMTTI1 gene (26). For heavy metal-induced transcription, TGGCRTCNC (R, purine) within the MRE sequence (termed “MRE core” hereafter) has been shown to be particularly important using point mutants of MREd of mouse MT-1 gene (34). To define the sequence essential for MREBP binding, we examined the effects of mutations in the target MREa sequence of the hMTTI1 gene by mobility shift assay. A short oligonucleotide containing the MREa sequence was used as a probe (Figs. 1, A and B), and a crude extract of HeLa cells was used as the source of MREBP. Results are shown in Fig. 1C. A retarded band by MREBP was observed after incubation of the probe and the proteins (lane 2), reported (26). This complex was competed out by the cold MREa oligonucleotide (lane 3). An oligonucleotide with mutations in GC in the MRE core (M-1 in Fig. 1B) was unable to compete with the probe efficiently (lane 4), whereas those with mutations in the other sites in the probe (M-2 and M-3 in Fig. 1B) were not (lanes 5 and 6). These results indicate the sequence-specific binding of MREBP to MREa, and the bases within the MRE core are important for MREBP binding. When competitor oligonucleotides were added to reactions, a part of the 32P-probe did not enter the gel (lanes 3–6), suggesting the formation of large protein-DNA complexes at high concentrations of oligonucleotides.

To confirm the specific binding of MREBP, we also tested another 32P-labeled DNA probe (AM probe) which covers a part of the hMTTI1, upstream sequence containing multiple copies of MRE (95-bp Aval/Msal fragment; Fig. 1A). We chose this fragment since it contains three copies of MREs (MREc, d, and e) but no other known regulatory elements except an AP-2 site (Fig. 1A). In this experiment, a partially purified MREBP preparation (0.3 M KCl eluate from a heparin-agarose column, described later) was used. As shown in Fig. 1D, three protein-DNA complexes were observed. As the
concentration of the nuclear proteins was increased, distribution of the complexes shifted to the slower migrating species (Fig. 1D, lane 2). Since these bands were competed out by the mutant MREa oligonucleotides in the same way as shown in Fig. 1C (data not shown), it is likely that these retarded bands result from binding of MREBP, possibly to multiple sites in the AM probe.

Footprints of MREBP—The binding sites of MREBP were then analyzed by DNase I footprinting, using the AM probe. On both the coding and noncoding strands, the region around MREc, as well as another region which contains an MRE-like sequence (named MREg), was strongly protected (Fig. 2A, lane 1, data not shown for the noncoding strand). These data indicate the specific binding of MREBP to the MREs in the AM probe, but with different affinity to each MRE. The AP-2 site was not protected in this assay. Results obtained from these and repeated footprinting experiments are summarized in Fig. 2C.

Effects of Heavy Metals on MREBP Binding—In our earlier experiments, low concentrations of cadmium did not show any marked effects on binding of MREBP to MRE sequences (26). However, in the course of more detailed study of heavy metal effects, we found that ZnSO4 (100 μM) inhibits binding of MREBP to MREa oligonucleotide (Fig. 3A, lane 4). This effect was abolished by the addition of EDTA (lane 3), although EDTA itself showed a weak inhibitory effect on MREBP/MRE binding (lane 2). Neither Na2SO4 (lane 5), K2SO4 (lane 6), nor MgSO4 (lane 7) showed any inhibitory effect, indicating that the inhibition is not due to the sulfate ion and is specific to the zinc ion. We then examined the effects of various concentrations of ZnSO4 and CdSO4 on MREBP activity (Fig. 3B). Low concentrations of zinc

![Fig. 2. DNase I footprints of MREBP. A, footprinting with the 32P-AM probe labeled at the 5' end of the coding strand. All reactions except lane 1 contain 3 μg of the heparin-agarose fraction. The competitors (500 fmol) were added as follows. Lanes: 2, no competitor; 3, wild type MREa; 4, M-1,5, M-3. The arrows indicate the locations of the MREs. B, footprinting with the 32P-AM probe labeled at the 3' end of the noncoding strand. Lanes: 1, no protein; 2, 1.5 μg of the heparin-agarose fraction. C, summary of the footprinting. The DNA sequence of the AM probe is indicated with uppercase letters. The protected regions in the coding and noncoding strands are indicated with the overlines and underlines, respectively. The dotted lines indicate the weakly protected regions. The MRE core and the flanking GC-rich sequence (34) are indicated as a MRE.](image)

![Fig. 3. Effects of metals on DNA binding of MREBP. A, effects of metal ions on DNA binding of MREBP. The heparinagarose fraction (0.15 μg of protein) was analyzed by mobility shift assay with the 32P-MREa probe. The additions were as follows. Lanes: 1, none; 2, 20 mM EDTA; 3, 20 mM EDTA and 100 μM ZnSO4; 4, 100 μM ZnSO4; 5, 100 μM Na2SO4; 6, 100 μM K2SO4; 7, 100 μM MgSO4. The arrowhead indicates the retarded band by MREBP. F indicates the free probe. B, dose effects of ZnSO4 and CdSO4 on DNA binding of MREBP. The heparin-agarose fraction (0.15 μg of protein) was analyzed by mobility shift assay with the 32P-MREa probe at the metal concentrations indicated in the abscissa. MREBP activity was quantified densitometrically and normalized to the controls without the metals (indicated as 100 in the ordinate). Three independent experiments are shown with different symbols (●, ○, and ×) for ZnSO4 (left panel) and CdSO4 (right panel), respectively.](image)
slightly (about 1.5-fold around 50 μM) enhanced MREBP activity (Fig. 3B, left panel). However, the inhibitory effect of zinc at higher concentrations was more prominent; MREBP activity sharply decreased at zinc concentrations between 50 and 100 μM. Cadmium also enhanced MREBP activity up to 2.2-fold in the low concentration range (~20 μM), but inhibited it at higher concentrations (Fig. 3B, right panel). Cadmium inhibited MREBP activity at lower concentrations (>25 μM) than zinc. From these results, it is clear that binding of MREBP to the MRE sequence is specifically modulated by the heavy metals.

The responsiveness of MREBP to heavy metals is relatively unstable. Preparations of MREBP occasionally lost the metal responsiveness without losing the ability to bind DNA in the absence of the metals. DTT treatment failed to recover the metal dependence of these proteins (data not shown).

**Purification of MREBP**—MREBP activity detected by mobility shift assay with the 32P-MREa oligonucleotide probe was purified from nuclear extracts of HeLa cells. Results of a typical purification are shown in Fig. 4. Preliminary experiments showed that the majority of MREBP activity is recovered in a 40–80% saturated ammonium sulfate fraction (data not shown). This fraction of the crude extract was fractionated by Sephacryl S-300 gel filtration (Fig. 4A). Although MREBP activity was widely distributed, this step was effective in removing high molecular weight proteins. The pool of the active fractions was then passed over a heparin-agarose column (Fig. 4B). The activity was concentrated in the 0.3 M KCl eluate. This fraction was further purified by affinity chromatography using an affinity matrix prepared by coupling multimeric MREa oligonucleotides to Sepharose 4B (Fig. 4C). MREBP activity was recovered mainly in the 0.5 M KCl eluate, although appreciable amounts of activity were detected also in the flow-through fraction and the 0.3 M KCl eluate. The activity in the 0.5 M KCl eluate was recovered only in the 0.5 M KCl fraction when re-chromatographed through another MREa-Sepharose column (Fig. 4D). Rechromatography of the activity eluted at lower than 0.5 M KCl confirmed the existence of activities with lower affinities to the column, rather than overloading of the first column (data not shown). These activities were not studied further.

To estimate the recovery of MREBP activity accurately, the serial dilutions of the pooled active fractions from each purification step were subjected to mobility shift assay, and the yield and specific activity were calculated. The results are summarized in Table I. The overall recovery of the activity was 1.7%. The affinity-purified MREBP showed exactly the same footprints as the heparin-agarose fraction (data not shown).

Proteins in each purification step were electrophoresed in...
Table I

Summary of purification of MREBP
A packed volume of 30 ml of HeLa cells was used as the starting material. MREBP activity was measured by mobility shift assay with 32P-MREa probe.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (Total Specific Activity/Specific activity) %</th>
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<tr>
<td>Nuclear extract</td>
<td>41.0</td>
<td>88.2</td>
<td>205,000</td>
<td>2,330</td>
<td>100</td>
</tr>
<tr>
<td>(NH4)2SO4</td>
<td>11.2</td>
<td>38.1</td>
<td>56,000</td>
<td>1,470</td>
<td>27</td>
</tr>
<tr>
<td>Sephacryl S-300</td>
<td>68.5</td>
<td>14.4</td>
<td>68,500</td>
<td>4,760</td>
<td>33</td>
</tr>
<tr>
<td>Heparin-agarose</td>
<td>5.8</td>
<td>9.4</td>
<td>87,000</td>
<td>9,260</td>
<td>42</td>
</tr>
<tr>
<td>MREa-Sepharose 1*</td>
<td>4.3</td>
<td>0.02</td>
<td>2,150</td>
<td>108,000</td>
<td>2.9</td>
</tr>
<tr>
<td>MREa-Sepharose 2*</td>
<td>4.2</td>
<td>1.220</td>
<td>1</td>
<td>4,620</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* In the steps of MREa-Sepharose chromatography, 36% of the heparin-agarose pool was processed.

** The value was estimated by silver staining.

The values are calculated assuming that all of the heparin-agarose pool was processed.

Fig. 5. SDS-polyacrylamide gel electrophoresis of purified MREBP. Proteins in the pooled active fractions from each purification step were analyzed by SDS, 7.5% polyacrylamide gel electrophoresis and silver staining. Lanes: 2, crude nuclear extract; 3, 40-80% ammonium sulfate precipitate; 4, Sephacryl S-300; 5, heparin-agarose; 6, MREa-Sepharose. The M, standards (Pharmacia) shown in lane 1 are phosphorylase b (94,000), bovine serum albumin (67,000), and ovalbumin (43,000). For lanes 2-5, 5 µg of proteins were applied. For lane 6, 5 µl of the sample was used. The positions of the M, standards are indicated on the left.

Fig. 6. Identification of the polypeptide with MREBP activity. HeLa nuclear extract (6.1 units of MREBP, 13 µg of protein; lane 1) and the affinity-purified MREBP (1.1 units of MREBP, 10 µg; lane 2) were electrophoresed, transferred to nitrocellulose membrane, and probed with the 32P-labeled 28-bp MRE oligonucleotide. The positions of M, standards (Amersham) are indicated on the left: myosin (200,000), phosphorylase b (97,400), bovine serum albumin (69,000), and ovalbumin (46,000). The arrowhead indicates the protein band with an M, of 112,000. The two lanes are next to each other on the same gel, but lane 2 was exposed to x-ray film longer (7.3 h) than lane 1 (2 h).

DISCUSSION

In the present work, we characterized a HeLa cell nuclear factor, MREBP, which specifically binds to MREs of hMTIIa gene. The recognition sequence of MREBP was examined by competition experiments with mutated oligonucleotides, and the results showed that the most conserved part of the MRE sequence (MRE core) is important for binding of MREBP. Other MRE-binding factors reported so far (MTF-I, Ref. 13; p108, Ref. 20; MBF-I, Ref. 21; ZAP, Ref. 22) also require the MRE core for their efficient binding to MREs. However, there are some differences in the sequence specificity between MREBP and those factors. Some of other factors also require the adjacent GC-rich region (13, 20), whereas MREBP does not. The mutation at the 3' end of the MRE core does not affect MREBP binding, although this base appears to be important for heavy metal-dependent transcripational activation (34) and binding of ZAP to a MRE (22).

The most striking feature of MREBP is its unique response to heavy metals. The binding affinity of MREBP to a MRE sharply decreased at high concentrations of cadmium and zinc, although 1.5-2-fold stimulation was observed at lower concentrations of the metals. Copper (divalent) also inhibited the DNA binding of MREBP with a kinetics similar to that for zinc. None of other known MRE-binding factors are reported to be inhibited by heavy metals; in contrast, some of

2 S. Koizumi, unpublished results.
these factors are activated by cadmium (19) or zinc (13, 22) in binding to MREs in vitro, and the regulation appears to be specific to those particular heavy metals. Recently, we also identified another HeLa cell nuclear factor whose binding to a MRE is enhanced only by zinc.3 The apparent difference in the metal response suggests that MREBP is distinct from the other MRE-binding factors that are activated by heavy metals.

The fact that MREBP binds to a MRE in the absence of heavy metals, whereas it does not in the presence of high concentrations of heavy metals, suggests a possibility that it functions as a negative regulator of MT genes. On the other hand, it is likely that the transcriptional regulation of MT genes involves a positive regulator(s), as suggested by in vivo competition experiments (35, 36) and the presence of MRE-binding proteins that are activated by heavy metals (13, 19, 22). These considerations lead to a hypothesis that MT genes are controlled by both positive and negative regulators, in a competitive fashion for the overlapping target sequences around the MRE core. This system would provide a more precise regulation than achieved by only one regulator; affinity of each factor to a MRE varies independently in response to heavy metal levels, and the equilibrium of both factors at a given metal concentration would determine the transcriptional activity. At higher concentrations of the metals where larger amounts of MTs need to be produced, MREBP loses an affinity to MREs, which would be fully accessible to positive regulators. When the metal concentration returns to a low level, affinity of positive factors to MREs decreases, and MREBP could act as a “safety valve” so as not to allow unnecessary expression of MT genes any more.

It is noteworthy that in the dose-response curves of MREBP activity versus heavy metal concentration, the minimal concentration of cadmium required for the inhibition of MREBP binding is much lower than that of zinc. This is consistent with the fact that the concentration of cadmium required for induction of MTs is much lower than that of zinc (7, 37), suggesting the involvement of MREBP in the regulation of MT genes in vivo.

Although the results of the binding experiments presented here thus predict that MREBP acts negatively in the transcriptional regulation of MT genes, we have no direct evidence indicating that MREBP actually functions in the transcription machinery. To obtain a definite conclusion for the nature of MREBP, further functional studies using experimental systems such as transfection of cloned genes or in vitro transcription assay will be necessary.

In DNase I footprinting experiments, affinity of MREBP to different MREs was apparently varied; MREc and MREG were strongly protected by MREBP, whereas MREF was protected only partially, and MREe was not. This fact implies that, if MREBP acts as a negative regulator as discussed above, it will have a role in selection of MREs available for positive regulators. MREs with low affinities to MREBP would be accessible to positive factors even at a low concentration of heavy metals, whereas other MREs with high affinities to MREBP would not. The differential MREBP binding thus could contribute to the fine regulation of MT genes.

Blotting experiments suggest that the polypeptide respon-

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