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Metal-Responsive Elements Act as Positive Modulators of Human Metallothionein-II_A Enhancer Activity

MICHAEL KARIN,†* ALOIS HASLINGER,‡ ADRIANA HEGUY,† THERESE DIETLIN, AND TRACY COOKE†

Department of Microbiology, School of Medicine, University of Southern California, Los Angeles, California 90033

Received 21 July 1986/Accepted 20 October 1986

The human metallothionein II_A ($hMT\text{-}II_A$) gene contains two enhancer elements whose activity is induced by heavy-metal ions such as Cd^{2+}. To determine the nature of the relationship between the metal-responsive elements and the element(s) responsible for the basal activity of the enhancers, the basal-level enhancer element(s), the $hMT\text{-}II_A$ enhancers were subjected to mutational analysis. We show that deletion of the metal-responsive elements had no effect on the basal activity of the enhancer but prevented further induction by Cd^{2+}. On the other hand, replacement of the basal-level enhancer element with linker DNA led to inactivation of the enhancer both before and after treatment with Cd^{2+}. Therefore, the metal-responsive elements seems to act as a positive modulator of enhancer function in the presence of heavy-metal ions. In addition to the two enhancers, the $hMT\text{-}II_A$ promoter contained one other element, the GC box, required for its basal expression. Unlike deletion of the basal-level enhancer element, replacement of the GC box with linker DNA had no effect on the ability of the promoter to be induced by Cd^{2+}.

Metallothionein (MT) genes offer an attractive system for studying the control of gene expression in animal cells. These genes, which form a multigene family, are expressed in essentially every cell type, and their transcription can be further induced in response to a variety of exogenous stimuli, including heavy-metal ions (e.g., Cd, Zn, and Hg), glucocorticoid hormones, interferon, and interleukin 1. MT gene expression is also stimulated by exposure of cells to tumor promoters, inhibitors of protein and DNA synthesis, and ionizing radiation (see reference 14 for a review). To understand the mechanisms that regulate the basal and induced expression of these genes, it is essential to characterize both the *cis*- and *trans*-acting genetic elements involved in MT expression.

Previously we have shown that *cis* elements important for basal and induced expression of the human MT I_A ($hMT\text{-}I_A$) and $hMT\text{-}II_A$ genes are present in their 5′-flanking regions (15, 22). Deletion analysis of the $hMT\text{-}II_A$ control region defined two distinct regulatory elements that can independently mediate induction of the gene by heavy-metal ions and glucocorticoids. Two copies of the metal-responsive element (MRE) were originally detected, yet a single copy was found to be sufficient for induction. The glucocorticoid-responsive element (GRE) was shown to coincide with the DNA-binding site for the glucocorticoid hormone receptor, a *trans* regulator of gene expression (16). We also determined the approximate location of an upstream promoter element, centered around nucleotides −80 to −100, which is important for basal-level expression (16, 17). Similar elements were found to control both the basal and metal-induced transcription of the mouse MT-I (mMT-I) gene (3, 28). Comparison of the human and murine MT promoters reveals considerable nucleotide sequence homology (3, 16, 17, 25), suggesting that they are recognized by cellular proteins common to both species.

The $hM\text{-}II_A$ gene is unique in its much higher levels of both basal and induced expression compared with each of the multiple "MT-I-like" genes (11, 22). Recently it was found that the high basal level of $hMT\text{-}II_A$ expression is due in part to the presence of a basal-level enhancer element (BLE) within its control region (10). In addition, we found that the $hMT\text{-}II_A$ enhancer can compete with the simian virus 40 (SV40) enhancer for one or more cellular factors in vivo (24) and in vitro (W. Lee, A. Haslinger, M. Karin, and R. Tjian, Nature, in press). The in vivo competition is modulated by Cd^{2+}, an inducer of MT gene transcription. This finding suggested that heavy-metal ions control the ability of the $hMT\text{-}II_A$ enhancer to bind a positive factor present in limiting amounts, which is also required for maximal activity of the SV40 enhancer. Two models were proposed to explain these results. According to the first one, a metal-regulatory factor (MRF) stabilized the binding of a limiting factor to the BLE in the presence of heavy-metal ions. In the second model, in the absence of Cd^{2+} the MRF acts as a repressor that binds to the MRE and interferes with the binding of a positive factor to the BLE. In the presence of Cd^{2+}, the MRF no longer binds to DNA and repression is relieved. To discriminate between the two models, we have performed deletion analysis of the $hMT\text{-}II_A$ enhancers and found that in the absence of functional MREs the basal activity of either enhancer remains low and cannot be further increased by Cd^{2+}. While these results are consistent with the first model, they seem to contradict the second model and suggest that the MREs act as positive modulators of enhancer function.

MATERIALS AND METHODS

Cells and transfection. HeLa cells were grown in Dulbecco modified Eagle medium supplemented with 2.5% fetal calf serum, 2.5% calf serum, penicillin, and streptomycin. To ensure reproducible (less than 30% variation in chloramphenicol acetyltransferase [CAT] expression levels) and efficient transfections, new cultures were started from frozen stocks every 2 to 3 weeks. Cells were transfected with 15 μg of plasmid DNA-calcium phosphate coprecipitate per 100-mm plate (9). All plasmids used for transfections were purified by double CsCl banding. Four hours after transfec-

* Corresponding author.
† Present address: Division of Pharmacology, School of Medicine, University of California, San Diego, La Jolla, CA 92093.
‡ Present address: Institute of Biochemistry, University of Vienna, Vienna, Austria.

tion, the cells were incubated for 2 to 3 min in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline containing 15% glycerol (glycerol shock) and incubated either in normal growth medium or in the presence of 5 μM $CdCl_2$ for 12 to 14 h before being harvested for determination of CAT activity or for RNA analysis.

Analysis of gene expression. Cells were lysed by sonication in 150 μl of 0.25 M Tris hydrochloride, pH 7.8, and 50 to 100 μl of extract was used for determination of CAT activity as described previously (8). CAT activity was quantitated by slicing the thin-layer chromatography plates and determining the amount of radioactivity in the acetylated and nonacetylated forms of chloramphenicol by scintillation counting. One unit of CAT activity is the amount of enzyme required to acetylate 1 pmol of chloramphenicol during 1 h at 37°C. Experiments were repeated at least three times in duplicate, and the activity expressed from pSV2CAT was used as a criterion to determine the efficiency and reproducibility of the transfection. To determine average values of CAT activity, we only used data from highly efficient transfections (expression from pSV2CAT was 3,000 to 5,000 U/mg of protein). For RNA analysis cells were lysed in 5 M guanidinium-thiocyanate buffer, and RNA was pelleted through a CsCl block gradient (5). Total cellular RNA (25 μg) or 5 μg of polyadenylated [poly(A)$^+$] RNA was analyzed by primer extension with 5'-end-labeled synthetic oligonucleotide primers as described by Walker et al. (29). The sequence of the *cat* primer is complementary to nucleotides +15 to +38 of the *cat* gene (1) (5'-ATATCAACGGTGGTATAT CCAGTG-3') and that of the thymidine kinase (TK) primer is complementary to nucleotides +58 to +81 of the *tk* gene 5'-AGGTGCGGGAGTTTCACGCCACCA-3' (20). The samples were analyzed on 8% polyacrylamide–urea gels followed by autoradiography with intensifying screens for 16 to 48 h.

Plasmid constructions. pUCAT2 is a subclone in pUC13 of the enhancerless SV40 early promoter fused to the *cat* gene derived from $pA_{10}CAT$ (18). It has a unique *Bgl*II site at position 139 of the SV40 promoter and a unique *Bam*HI site at the 3' end (past the polyadenylation site) of the transcription unit. Both of these sites were used for insertion of fragments to be tested for enhancer activity (see Fig. 1). The X fragment, containing the proximal hMT-II_A enhancer, is generated by digestion with *Xma*III (−67 to −132), and the Y fragment, containing the distal enhancer, by digestion with *Xma*III and *Ava*I (−129 to −215). The 5' deletion mutants of the proximal repeat were derived from a series of *Bal* 31 5' deletion mutants of an MT-TK fusion gene (16) by digestion with *Hin*dIII (site is 15 base pairs [bp] upstream to the deletion endpoint in the pUC8 polylinker) and *Xma*III (site at −67 in hMT-II_A). The 3' deletion mutants were generated by *Bal* 31 digestion initiated from the *Bam*HI site of hMT-II_A (at +75), a secondary digestion with *Bss*HII (site at −138), and addition of *Bgl*II linkers. The 5' and 3' deletions were cloned into the *Bgl*II site of pUCAT2, and their exact endpoints and orientations were determined by nucleotide sequence analysis (19). The Xh(−130−69) mutants were derived by *Hae*III digestion of the X fragment from XCAT3$^-$, addition of *Bgl*II linkers, and insertion into the *Bgl*II site of pUCAT2. The Yh(−206−150) mutant was derived by *Hha*I digestion of the Y fragment from pYCAT2$^+$, addition of *Bam*HI linkers, and insertion into the *Bgl*II site of pUCAT2. X-LS110/80 was derived from p25.8/6'11 by digestion with *Xma*III, which released the proximal repeat containing the linker-scanning internal deletion mutation. The *Xma*III fragment was cloned by using *Bam*HI linkers into the *Bgl*II site of pUCAT2. p25.8/6'11 itself was generated by ligating an *Hin*dIII (−770 of hMT-II_A)-*Eco*RI (at the 3' end of the *tk* gene) fragment from pMTKΔ5'-80 (16) with the large *Bam*HI (−110)-*Eco*RI fragment of pMTKΔ3'-110 (16). The LS'67/51 mutant was generated by digestion of pMTCATΔ5'-50 (24) with *Hin*dIII, ligation to an *Hin*dIII (−770)-*Bam*HI (−67) fragment from pΔ3'-67 (16), filling in the unligated *Bam*HI and *Hin*dIII sites, and religating. pLS-ES-CAT was generated in a similar way except that the unligated *Bam*HI and *Hin*dIII sites were trimmed by S1 digestion prior to the last ligation step. The Δ65/51 mutant was generated by digestion of pMTKΔ5'-50 with *Hin*dIII, which cuts at the deletion endpoint, and ligation to a filled-in *Hin*dIII (−770)-*Xma*III (−66) fragment from phMT-II_A. The structures of all of these mutants were confirmed by nucleotide sequencing (19) and restriction analysis. The *tk* fusions were generated by replacing the *cat* structural sequences from the vectors described above with *tk* structural sequences present on a filled-in *Bgl*II-*Eco*RI fragment derived from pTK (20). To generate a similar fusion of the wild-type promoter to *tk*, the *Bgl*II-*Eco*RI fragment carrying *tk* sequences was inserted into pHSI (a subclone of the hMT-II_A promoter from −770 to +70 in pUC8; A. Haslinger, unpublished data). This resulted in a fusion whose 5' leader was 10 bp longer than the three GC box mutants, as confirmed by sequence analysis.

RESULTS

hMT-II_A gene contains metal-inducible enhancer elements. The 5' control region of the hMT-II_A gene contains two direct but imperfect repeats, located from positions −67 to −140 and −140 to −214, capable of acting like enhancer elements (10) (Fig. 1). Because the hMT-II_A enhancers can compete in vivo with the SV40 enhancer for binding of a positive factor in a Cd^{2+}-dependent manner (24), we examined the effect of this ion on the activity of these enhancers. Various fragments of hMT-II_A 5'-flanking DNA were inserted either upstream or downstream of the *cat* gene in the vector pUCAT2 (Fig. 1), transfected into HeLa cells, and examined for CAT activity before and after induction with Cd^{2+}. The presence of the SV40 enhancer within the vector led to an approximately 200-fold increase in CAT expression (Fig. 2). As expected, the level of CAT expression from the SV40 early promoter in either the absence or presence of the viral enhancer was not significantly affected by treatment of transfected cells with Cd^{2+}. By contrast, insertion of either the proximal (x) or the distal (y) repeats of the hMT-II_A gene (10) into pUCAT2 upstream or downstream of the *cat* gene resulted in stimulation of CAT activity and a significant response to Cd^{2+} (Fig. 2). As observed before (10, 27, 30), polymerization of these elements increased their activity. Primer extension analysis (Fig. 3) also revealed an increase in the level of *cat* transcripts that were correctly initiated from the same major early start sites utilized by the SV40 early promoter under control of the viral enhancer (2). The two transcripts initiated from the early promoter were not well resolved and migrated as a doublet (Fig. 3A), which was clearly separated after prolonged electrophoresis (Fig. 3B). No effect of Cd^{2+} was observed on *cat* or *tk* mRNA expressed under control of the SV40 enhancer. Since induction by Cd^{2+} was observed regardless of the orientation or position of these repeats and without affecting the locations of the transcriptional start sites, the hMT-II_A repeats can be regarded as metal-inducible enhancer elements. Similar results were obtained by Serfling et al. (27), who used the

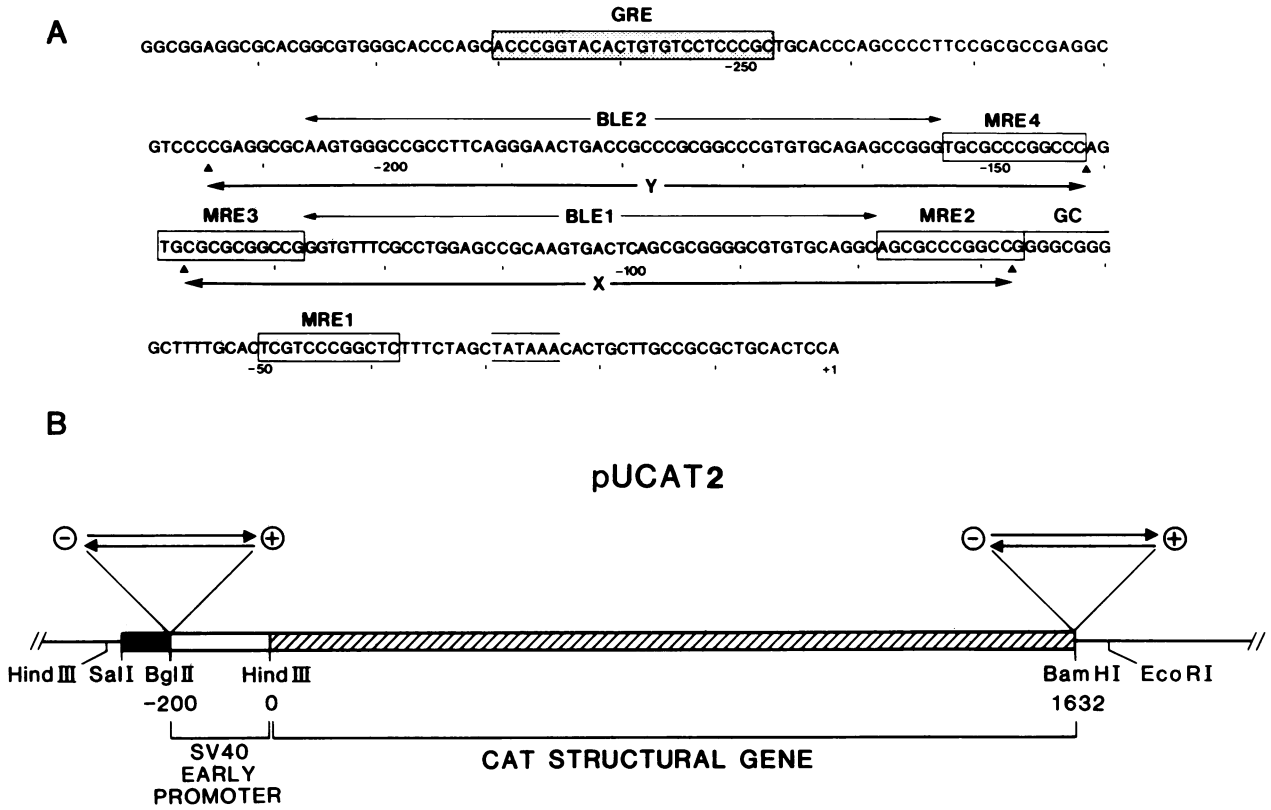

FIG. 1. Structure of the hMT-II$_A$ control region and the pUCAT2 vector. (A) Sequence of the hMT-II$_A$ 5′ control region from position +1 (the major start site of transcription) to position −300. Shown are the locations of the TATA and GC boxes, the MREs, and the BLEs. Also indicated are the regions referred to as the proximal repeat (X fragment) and the distal repeat (Y fragment). (B) Structure of pUCAT2. The hatched box indicates the *cat* structural gene, including splicing and polyadenylation signals from SV40. The open box denotes the SV40 early promoter (the TATA box and the 21-bp repeats), and the solid box a fragment of pBR322 DNA. Fragments tested for enhancer activity were inserted in either orientation into the *Bgl*II (upstream location) or *Bam*HI (downstream location) site.

"enhancer trap" approach (30) to find metal-inducible enhancers in the mMT-I and hMT-II$_A$ genes. Because the CAT assay allows better quantitation of expression than the primer extension assay, we used it in the experiments described below to determine the quantitative effects of mutations in the hMT-II$_A$ control region.

Analysis of 5′ and 3′ deletions of the proximal repeat. Because both repeats act as similar metal-inducible enhanc-

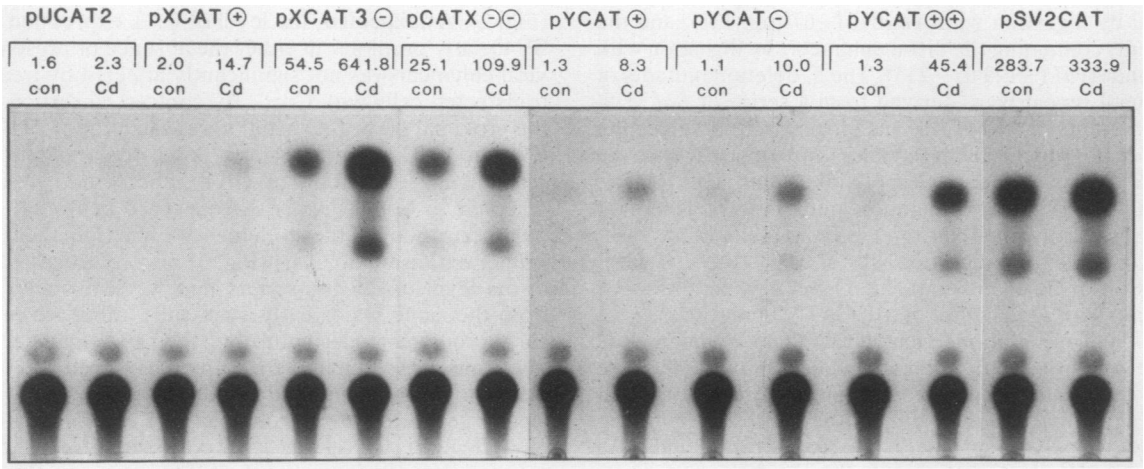

FIG. 2. hMT-II$_A$ control region contains a metal-inducible enhancer. The expression of different constructs containing either the proximal (X) or distal (Y) hMT-II$_A$ repeats inserted in either the sense (+) or antisense (−) orientation upstream (XCAT and YCAT) or downstream (CATX) from the CAT transcription unit was examined after transfection of supercoiled DNA onto HeLa cells. Shown are the results of a typical CAT assay. Unconverted [^{14}C]chloramphenicol is at the bottom and the different acetylated forms are at the top. Symbols: + and −, single inserts; ++ and −−, double inserts; 3, triple inserts. Cells were incubated with (Cd) or without (con) 5 μM $CdCl_2$ after the transfection. The levels of CAT activity (10^1 picomoles of chloramphenicol converted per milligram of protein per hour) are indicated above each lane.

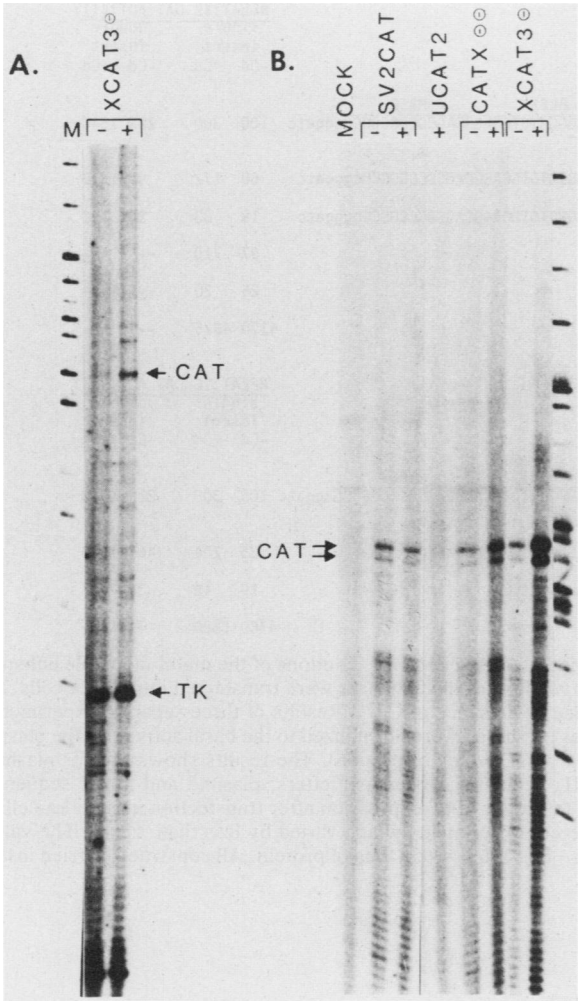

FIG. 3. Primer extension analysis of *cat* mRNA expression. HeLa cells were transfected with (A) a mixture of pXCAT3^{--} and pSVE/TK or (B) with either pSV2CAT, pUCAT2, pCATX, or pXCAT3^{-}. Total cellular RNA was prepared from induced (+) and uninduced (−) cells 12 h after the glycerol shock, and 30-μg samples were analyzed by primer extension with 5′-end-labeled synthetic *cat* and *tk* primers. The bands which correspond to correctly initiated CAT and TK transcripts are indicated. Note that the closely initiated CAT transcripts were not well separated in panel A, but after longer electrophoresis, as in panel B, they were clearly separated. Markers are end-labeled *Hpa*II fragments of pBR322 DNA.

ers, we chose for the sake of simplicity the proximal repeat (−67 to −140) as our major target for mutational analysis to determine the relationship between the MREs and the BLE.

Progressive 5′ and 3′ enhancer deletions were constructed and inserted either as a single copy or as a tandem repeat in both orientations, upstream of the SV40-*cat* transcription unit. While a single copy of the proximal (or distal) enhancer was active, the use of a tandem repeat may be more similar to the natural promoter, which contains two repeats. To avoid any bias in the results due to the effect of the deletions on the extent of competition between the hMT-II$_A$ enhancer, which is recognized by several distinct factors (Lee, in press), and other apparently unrelated enhancer elements (24), we purposely avoided the use of cotransfected plasmids as internal controls in these experiments. However, the experiments were repeated several times with different plasmid DNA preparations, and all transfected cells were split randomly between the control and Cd^{2+}-treated groups. Figure 4A shows the quantitation of CAT activity expressed by a set of 5′ deletion mutants before and after metal induction. For comparison we also determined the basal and induced activities of a construct harboring a single copy of a portion of the 5′ control region (−214 to −78) containing both the proximal and distal repeats. The duplicated proximal repeat led to approximately twice as much CAT activity as the combination of proximal and distal repeats, both before and after induction. Deletion of sequences located between positions −138 and −105 resulted in approximately a twofold decrease in the basal level of CAT expression and a similar decrease in the induced level of constructs with a single insert. However, in constructs with tandem inserts the induced activity was not significantly affected by this mutation. A major decrease (five- to eightfold) in both basal and induced activity was observed when the nine residues between positions −105 and −96 were deleted. Similar effects were observed for constructs with either single or tandem inserts. These results suggest that the 5′ border of a control sequence important for both basal and induced enhancer activity is located between positions −105 and −96. An additional control element that appears to affect mainly basal activity may be located between −138 and −105.

A 3′ deletion of a 16-bp sequence (between positions −67 and −83) that exhibits homology to the MRE consensus (Table 1) led to a twofold increase in the basal level of expression and a 25% decrease in the induced level in constructs containing a tandem insert (Fig. 4B). On the other hand, constructs with a single insert did not reveal any significant increase in basal level, but their induced expression was decreased by approximately 30%. Further deletion of 10 bp between positions −83 and −93 led to approximately a 5-fold decrease in both basal and induced expression for constructs with a single insert and an approximate 10-fold decrease in basal and induced expression of constructs with tandem inserts. These results place the 3′ border of a major control signal, involved in both basal and induced expression, between positions −83 and −93.

Role of the MRE in enhancer function. The 5′ and 3′ deletion analysis described above suggested a role for the element present between positions −105 and −83 in controlling the basal level of enhancer activity. However, these mutants did not yield much information on the relationship between the MREs and the BLE. Therefore, we constructed several additional mutants (Fig. 5) and analyzed their expression phenotypes. In mutant Xh(−130−69), the two MREs which flank the proximal repeat were mutated by short deletions which led to substitution of a single base pair in MRE2 and 8 bp in MRE3. These substitutions were sufficient to prevent metal induction of constructs containing a single insert of the mutant enhancer without affecting basal activity. Increasing the copy number of the mutant repeat restored metal responsiveness, but to a lower extent (approximately 50%) than that observed for the wild-type repeat. These results indicate that the mutated MRE2 was probably still functional. Again, no significant differences were observed in the basal level of CAT expression by these constructs compared with that in constructs containing the wild-type repeat.

Similar mutations were introduced in the distal repeat. While the wild-type repeat (−215 to −128), including MREs 3 and 4, led to four- to eightfold induction of CAT activity in response to Cd^{2+}, the mutated repeat Yh(−206−150), which contained only a small part of MRE4, was not metal respon-

A. 5' Deletion Mutants:

		RELATIVE CAT ACTIVITY single insert -Cd	+Cd	double insert -Cd	+Cd
X(-138-67)=WT	gatcccGCGCGCGGCCCGGGTGTTTCGCCTGGAGCCGCAAGTGACTCAGCGCGGGGCGTGTGCAGGCAGCGCCCGGCCCgaggatc	100	300	228	1540
X(-105-68)	gatcccgagcttggctgcaggtGTGACTCAGCGCGGGGCGTGTGCAGGCAGCGCCCGGCCCcgggatc	60	172	93	1570
X(-96-68)	gatcccgagcttggctgcaggtCGCGGGGCGTGTGCAGGCAGCGCCCGGCCCcgggatc	18	33	20	191
YX(-214-78)		97	710	---	---
pUCAT2		26	30	---	---
pSV2CAT		4170	4375	---	---

B. 3' Deletion Mutants:

		RELATIVE CAT ACTIVITY single insert -Cd	+Cd	double insert -Cd	+Cd
X(-138-67)=WT	gatcccGCGCGCGGCCCGGGTGTTTCGCCTGGAGCCGCAAGTGACTCAGCGCGGGGCGTGTGCAGGCAGCGCCCGGCCCgaggatc	100	308	212	1920
X(-138-83)	gatctGCGCGCGGCCGGGGTGTTTCGCCTGGACGCCGCAAGTGACTCAGCGCGGGGCGTGTGCcggatcc	125	204	465	1480
X(138-93)	gatctGCGCGCGCCGGGTGTTTCGCCTGGAGCCGCAAGTGACTCAGCGCGacggatcc	19	48	39	127
pSV2CAT		4760	5680	---	---

FIG. 4. Analysis of 5′ and 3′ deletion mutants of the metal-inducible enhancer. (A) A series of 5′ deletions of the metal-inducible enhancer was prepared by *Bal* 31 digestion. Plasmids carrying wild-type and truncated versions of the enhancer were transfected into HeLa cells, and levels of CAT activity were determined after incubation of cells in the absence or presence of Cd^{2+}. Results of three separate experiments, each done in duplicate, differing by less than ±20%, were averaged. The values presented were normalized to the basal activity of the plasmid carrying a single insert of the wild-type (WT) enhancer (−138 to −67); 100% = 113 U/mg of protein). The results shown are for plasmids carrying the inserts in the antisense (−) orientation. Capital letters, hMT-II_A sequence; lowercase letters, plasmid and linker sequences included in the inserts. (B) 3′ deletions of the metal-inducible enhancer were tested for CAT expression after transfection into HeLa cells in the absence or presence of Cd. The results shown are averages of three different experiments which varied by less than ±20%. The values were normalized to the basal activity of the wild-type (WT) enhancer (−138 to −67); 100% = 95 U/mg of protein. All constructs carried inserts in the antisense orientation.

TABLE 1. Nucleotide sequences of MREs of MT genes[a]

Gene	MRE no.	Sequence												
hMT-II_A	1	A	C	T	C	G	T	C	C	C	G	G	C	T	C
	2	G	C	A	G	C	G	C	C	C	G	G	C	C	G
	3	A	G	T	G	C	G	C	G	C	G	G	C	C	G
	4	G	G	T	G	C	G	C	C	C	G	G	C	C	C
hMT-I_A	1	T	T	T	G	C	G	T	C	C	G	G	C	C	C
	2	T	G	T	G	C	G	C	C	T	T	G	C	C	T
hMT-I_B	1	T	C	T	G	C	A	C	C	C	C	A	C	C	A
	2	A	C	T	G	C	T	C	A	T	G	G	C	C	C
hMT-I_E	1	T	C	T	G	C	G	C	C	C	G	G	C	C	T
	2	G	G	T	G	C	G	C	C	C	G	G	C	C	C
hMT-I_F	1	T	C	T	G	C	G	C	C	C	G	G	C	C	C
	2	T	C	T	G	C	G	C	C	C	G	G	C	C	C
mMT-I	1	T	T	T	G	C	G	C	C	C	G	G	A	C	T
	2	A	G	T	G	C	G	C	T	C	G	G	C	T	C
mMT-II		T	T	T	G	C	G	C	T	C	G	A	C	C	C
Consensus		(T)	Y	T	G	C	G	C	C	C	G	G	C	C	(C)
		8	10	14	14	14	12	14	11	13	13	13	14	13	9

[a] The nucleotide sequences of several MREs from different metallothionein genes are aligned to show homology. The hMT-II_A sequence is shown in reference 10; MRE1 is located between nucleotides −51 and −38, MRE2 between −80 and −67, MRE3 between −141 and −128, and MRE4 between −155 and −142. The remaining genes have either one or two MREs, around the −50 and the −150 regions. The hMT-I_A MREs are situated between nucleotides −57 to −44 and −167 to −154 (22). The hMT-II_B MREs are located at nucleotides −54 to −41 and −128 to −115 (11). The hMT-I_E and hMT-I_F sequences are shown in reference 23, and their respective MREs are located between −56 to −45 and −145 to −132 and between −54 to −41 and −145 to −132. The mMT-I gene (25) has two MREs located between nucleotides −155 and −42 and −132 and −119. The mMT-II gene (25) contains only one MRE at positions −57 to −44. The numbers below the nucleotides in the consensus sequence indicate the number of times each nucleotide is present in the 15 sequences shown above.

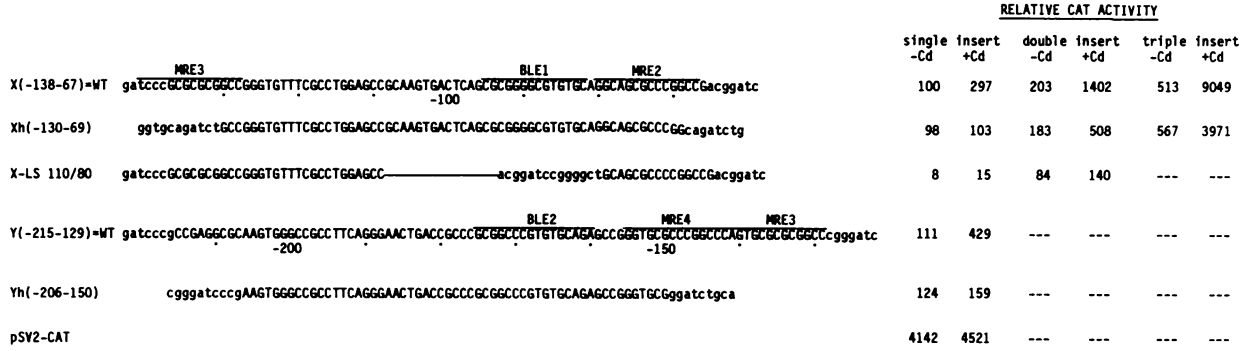

FIG. 5. Analysis of MRE and BLE mutants. Short deletions in the proximal [Xh(−130−69)] and distal [Yh(−206−150)] repeats were generated to test the role of MREs in enhancer function. In mutant LS(110/80), the BLE region was replaced by a segment of the polylinker of pUC8. The sequences of all of the mutants are shown; capital letters indicate hMT-II$_A$ sequences, and lowercase letters indicate plasmid and linker sequences. The locations of the MREs and the BLEs are indicated. All of the constructs were transfected and tested for CAT activity as described in the text. The results shown are averages of three different experiments. The values were normalized to the basal activity of the wild-type (WT) proximal repeat (−138 to −67); 100% = 63 U/mg of protein.

sive. As observed for the proximal repeat, these deletions also did not affect the basal level of expression. In agreement with previous analyses (3, 16, 28), these results indicate that the sequences with homology to the MRE consensus (Table 1) have a role in metal induction.

Most important, these results indicate that the control elements responsible for the basal activity of the metal-inducible enhancer can function independently of the MREs. The basal activity of the MRE-containing enhancer in the absence of Cd^{2+} was essentially identical to that of the MRE-devoid enhancers. Therefore, the increased basal activity of mutant Δ3′(−138−83) observed earlier was probably caused by the novel junction sequence unique to this mutant and not a common property of other mutants which suffered deletion of the MREs.

To further examine the relationships between the MREs and the BLE, we generated mutant X-LS(110/80) in which the 30 bp between positions −110 and −80 were replaced with 14 bp derived from the polylinker region of pUC8. This mutation led to almost complete inactivation of enhancer activity (some residual activity was still observed after duplication of the mutant element). Even though the two MREs were not affected by this mutation, only a marginal response to Cd^{2+} was observed (twofold or less, compared with eightfold induction of a construct containing a tandem insert of the wild-type repeat). These results suggest that the region between −110 and −80 is essential for enhancer activity both before and after induction. In the absence of the BLE, the MREs exerted only a very small effect on their own on the SV40 early promoter from a distance even when they were duplicated. Together, the results (Fig. 5) suggest that the MREs act as positive modulators of the activity of the BLE in response to heavy-metal ions, but cannot function as independent enhancer elements in its absence.

hMT-II$_A$ promoter contains a functional GC box. The metal-inducible enhancer is separated from the first MRE (31) by 16 bp of DNA (Fig. 1). Interestingly, this short region contains a sequence (GC box) that shows perfect agreement with the consensus sequence of SP1 binding sites (13). Since the binding of SP1 to the GC box was shown to be important for optimal transcription of several viral and cellular promoters (see reference 13 for a review), it was logical to investigate whether this sequence is also an important component of the hMT-II$_A$ promoter. To this end, we constructed two mutants of the natural hMT-II$_A$ promoter (Fig. 6A). In mutant LS-ES, the GC box sequence was replaced with 17 bp of pUC8 DNA, and in mutant Δ66/51, the GC box sequence was deleted (see Materials and Methods for details). The activity of these mutant promoters fused to either the *cat* or herpes simplex virus *tk* gene was compared with that of the wild-type hMT-II$_A$ promoter (HSI-TK or HSI-CAT) after transfection into HeLa cells. Both of these mutations led to a severe decrease (6- to 15-fold) in basal and induced CAT activity (Fig. 6A). Primer extension analysis of *tk* mRNA revealed that they also led to a decrease in the amount of correctly initiated transcripts derived from these promoters (Fig. 6B). The difference in length between transcripts transcribed from the wild-type construct (HSI-TK) and the mutants (pLS-ES-TK and pΔ66/51-TK) was due to insertion of 10 bp of DNA into the 5′ leader of the wild-type fusion (see Materials and Methods for details). Unlike the previously described mutation in the BLE [LS-(110/80)], lesions in the GC box resulted in a severe decrease in expression under both basal and induced conditions without affecting the extent of induction by Cd^{2+}. Similar results were obtained with a third mutant, LS-(67/51), in which a 21-bp sequence from pUC8 replaced the hMT-II$_A$ sequence between −51 and −67, leading to a 10-fold decrease in CAT expression (data not shown).

DISCUSSION

The 5′-flanking region of the hMT-II$_A$ gene contains two somewhat divergent direct repeats which function as enhancer elements (10). As shown in this paper, the activity of these enhancers was increased further in the presence of Cd^{2+}. Deletion analysis of the proximal repeat indicated that it was composed of at least two transcriptional control elements: a BLE, responsible for its basal activity, and an MRE, responsible for its metal inducibility. While the distal repeat was not subjected to as extensive an analysis as the proximal repeat, it also contained a region, located between nucleotides −150 and −206, which functioned as a BLE (referred to as BLE2 in Fig. 1) and an MRE. The present results, together with the results of previously performed in vivo competition experiments (24), suggest that the MREs act as modulators of BLE activity. A similiar mode of action was recently shown for the interferon response sequence of class I transplantation antigen genes, which is active only in combination with a basal-level enhancer element, also present within the class I gene control region (12).

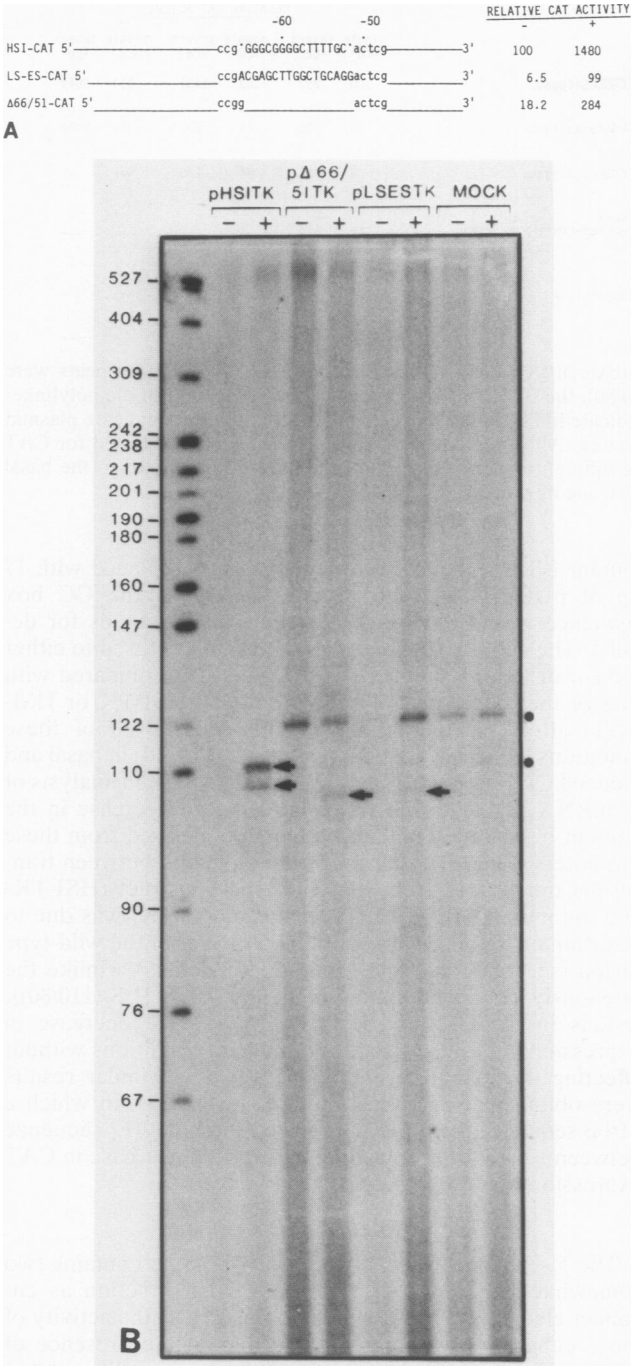

FIG. 6. Analysis of GC box mutants. (A) Sequences of two GC box mutants (LS-ES-CAT and Δ65/51 CAT) constructed as described in the text are compared with that of the wild-type promoter (HSI-CAT). Capital letters denote the natural hMT-II_A sequence at the region of the GC box, and the new sequence in this region is LE-ES-CAT. These nucleotides have been deleted from Δ66/51, as indicated by the internal line. Lowercase letters indicate the unchanged immediately flanking bases. The average and relative values (averages of three different experiments) (100% HSI-CAT uninduced activity, 2,437 U/mg of protein) of CAT expression before (−) and after (+) induction with μM Cd are shown. (B) Primer extension analysis of *tk* mRNA derived from wild-type (HSI-TK) and GC box mutants (LS-ES-TK and Δ65/51-TK) hMT-II_A-*tk* fusion genes transfected into HeLa cells. Poly(A)$^+$ RNAextracted from cells transfected with the wild-type and mutant genes (5 and 10 μg, respectively) was hybridized to end-labeled oligonucleotide *tk* primer. The hybrids were extended with reverse transcriptase, and the cDNA products were separated on an 8% denaturing gel. The two dots indicated artifacts of the primer extension method, i.e., cDNA products found in RNA samples from mock-transfected cells. The arrows indicate *tk* transcripts which were correctly initiated from the hMT-II_A promoter. Due to a slight difference in its 5′ leader, the wild-type fusion (HS1-TK) generated TK transcripts which were 10 nucleotides longer than those initiated from the mutants, but were initiated at the same position relative to the TATA box. Numbers at the left indicate sizes (in bases).

While the MREs are not capable of activating a heterologous promoter from a distance by themselves, even if present in multiple copies (Fig. 5) (26), they can impart metal induction from a distance when present next to the BLE. Two models were proposed earlier (24) to account for modulation of the BLE activity by Cd^{2+}. According to the first model, based on positive interaction between the two elements, a putative factor which recognizes the MRE, referred to as the MRF, stabilizes the binding of a rate-limiting enhancer recognition factor(s) to the BLE. The MRF requires heavy-metal ions for its activity. The second model is based on negative interaction, and according to it in the absence of Cd^{2+} the MRF acts as a repressor which binds to the MRE and sterically hinders the binding of the enhancer recognition factors to the BLE. In the presence of Cd^{2+} the MRF no longer binds to DNA, and repression is relieved. The results of the competition experiments were consistent with both models, and therefore the present study was undertaken. The most important result of the current study is that deletion of the MREs did not lead to increased basal activity of the enhancer, a result that contradicts the second model based on negative interaction between the two elements. On the other hand, the first model, based on positive interaction between the MRE and the BLE, is consistent with all the results obtained thus far. The exact nature of this interaction is not clear, but by carefully changing the distance between the two elements it might be possible to determine whether direct protein-protein interaction is taking place between the putative MRF and the enhancer recognition factor. Protein "footprinting" experiments have recently identified at least three factors which bind to the proximal and distal BLEs (Lee et al., in press; M. Imagawa and M. Karin, unpublished results). However, a factor which binds to the MREs has not been detected yet.

In addition to the two enhancers, one additional element, a GC box (13), was required for optimal activity of the hMT-II_A promoter. However, unlike the BLEs, inactivation of the GC box did not affect the ability of the promoter to be induced by heavy-metal ions. Protein footprinting experiments (Lee et al., in press) have shown that the GC box is recognized by the previously characterized transcription factor SP1 (7). In summary, the hMT-II_A control region turns out to be rather complex, and in addition to the canonical TATA box (6) it contains three other elements responsible for its basal activity, a GC box and two enhancer elements (BLEs). The activity of this promoter can be further induced by heavy-metal ions acting through the four MREs, which are interspersed among the elements important for basal expression. This unique location may be relevant to the modulatory functions of the MREs proposed above.

Another responsive element, the GRE, present further upstream, was responsible for steroid hormone induction of hMT-II_A. The GRE, which coincides with a binding site for the glucocorticoid receptor (16), acts like a hormone-dependent enhancer element (A. Haslinger and M. Karin,

unpublished results), very much like the hormone-dependent enhancer of murine mammary tumor virus (4, 21). Unlike the MRE, the GREs were capable of operating like enhancer elements on their own and did not seem to require interaction with additional elements, suggesting the existence of two basically different modes of transcriptional control.

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