We have analyzed the human (h) metallothionein (MT)-IG proximal promoter region (−174 to +5) using a TATA box mutation (TATCA) and four trinucleotide mutants of the proximal MREa. Transient transfection of HepG2 cells was complemented by in vitro transcription with rat liver nuclear extracts. In both systems, mutations of the TATA box and conserved core of metal responsive element (MRE)a were detrimental to hMT-IG promoter activity suggesting that both elements make significant contributions to hMT-IG transcription. Although MRE binding factors were active in vitro, further metal activation of MT promoter activity was accomplished only by in vivo metal treatment rather than addition of zinc in vitro. Southwestern blotting identified nuclear proteins in rat liver and HepG2 cells which physically interact with MREa in a zinc-dependent manner and could be responsible for MREa function in each system. In addition, the functional effects of the TATCA mutation correlate with altered physical interaction with TATA box-binding protein as observed using DNase I protection.

Metallothioneins (MTs) are low molecular mass (6–7 kDa), cysteine-rich, metal-binding proteins which are thought to be important for trace metal homeostasis, as with zinc and copper, and detoxification, as with cadmium (1). Consistent with these putative functions, MTs are translocationally induced by these metals, mediated by multiple copies of metal-responsive elements (MREs) in the MT gene 5'-regulatory regions (2). MREs are 12-bp sequences which contain a heptanucleotide core sequence TGC(A/G)CNC surrounded by less conserved flanking nucleotides (2).

We are interested in the regulation of the human (h)MT genes. This gene family consists of a minimum of 12 members which include both non-functional and processed pseudogenes as well as seven functional genes (3–5). The single MT-II isoform gene hMT-IIA is responsive to a variety of inducers including cytokines, growth factors, UV light, and glucocorticoids, and its regulatory region is complicated by numerous cis-acting elements which mediate the effects of such inducers (2). However, the promoters of the five hMT-I isoform genes are comparatively simple (3, 6) only containing MREs and GC boxes, which are possible binding sites for the SP1 transcription factor (3). Because of this simplicity, the hMT-I promoters are excellent candidates to study metal-regulated gene expression with least complication from other elements as are found in hMT-IG. Although the hMT-I promoters display remarkable homology of sequence and MRE organization (3), these genes exhibit cell type-specific patterns of expression as well as distinct levels of basal and metal-induced transcription within one cell type (3, 4).

Previous studies of MRE contributions to transcription have employed synthetic MRE sequences fused to minimal promoters (7–9). However, we wished to examine MRE contributions to transcription regulation in the context of native MT promoter organization and sequences. For this purpose, we have chosen the hMT-IG gene promoter. Not only is the hMT-IG gene highly expressed compared to most other hMT-I isoforms, but the maximum metal responsive activity of MT-IG is compartmentalized into the proximal 174 bp of 5'-flanking sequence (3, 10). Since proximity to the TATA box positively affects synthetic MRE activity (8, 9), we have tested mutants of both the TATA box and the adjacent MREa region using transfection of Hep G2 cells and in vitro transcription in rat liver nuclear extracts. Our results indicate that the optimal function of the TATA box and upstream MREs is dependent on MREa. In addition, we have determined that the functional effects of the MREa and TATA mutations correlate with changes in the physical interactions of proteins with these elements.

**MATERIALS AND METHODS**

Vector Construction—The pmEV1R and pmEV3R plasmids were constructed for the convenient study of gene regulatory sequences by mutagenesis and subsequent transient transfection of eukaryotic cells without a requirement for further subcloning (11). Briefly, the plasmid pmEV1R (3) is a derivative of pGEM-2 (Promega Biotech.) which provided the multiple cloning site (MCS), an origin of replication, and an ampicillin resistance gene. The filamentous phage f1 intergenic region (f1-Ig) was obtained from pTZ18U (U.S. Biochemical Corp.) and inserted into the NaeI site of the pGEM-2 backbone to supply a single-stranded DNA replication origin for oligonucleotide-directed mutagenesis. Finally, a chloramphenicol acetyltransferase (CAT) gene with downstream SV40 polyadenylation signal was obtained from pSVCAT (12) and inserted into the MCS to act as a reporter gene. The vector pmEV3SR was derived from pmEV1R by replacing the CAT reporter gene with a firefly luciferase (LUC) gene from pCD206 (13), and the only other difference between the two vectors is that the phage T7 promoter (from pGEM-2) was removed by this substitution.

Human MT-IG Promoter Constructs—Trinucleotide mutations of the −174 to +66 fragment of the hMT-IG promoter were created in pmEV1R by the uracil strand selection method according to the methods of Zalier and Smith (14) except that reactions were performed with
only the mutagenic oligonucleotide (3, 14). The TATA box mutation, to
TATCA, was constructed previously (3). The following oligonucleotides
were employed for MREa mutagenesis with the truncated T changes
indicated in lowercase letters: MREa1, 5'-GGGGGCGGGGCGTAGC
CGGCCCGGGT-3'; MREa2, 5'-GGGGGGCTTGCGAAGGCCTGCTG
-3'; MREa3, 5'-CGTGGCGGCGCATCCCCGTCTCAG-3'; MREa4, 5'-
GCGGGGGCGACGCGCTCAG-3'. The resulting promoters were
mutated in the core or flanking sequences of MREa which is proximal to
the TATA box (see Fig. 1).

The hMT-IG wild type and mutant promoter fragments were PCR
amplified from pMEV1R using the common SP6 sequencing primer at
the 5' end of the MCS and a second primer 027, 5'-ATTATTGGAGTG-
GAACCAACAGTTCGCTCAG-3', which anneals at transcription initiation ('*'
is underlined) and contains a SspI restriction site (AATATT). The PCR
products were restricted at the 5' end using either SacI or EcoRI (from
the MCS) with SspI on the 3' end. These fragments were ligated with SacI-Smal digested pMEV1R, for transient transfection, and EcoRI-
EcoRV digested P(C2AT)19 (15) for in vitro transcription. These manip-
ulations were performed to remove the 5'-untranslated region from +6
to +66 for consistency between the promoter fragments used for tran-
sient transfection and in vitro transcription. The in vitro transcription
vector, P(C2AT)19, contains a 390-bp guanosine-free cassette which
forms a discrete transcript in the absence of GTP (15). Thus, the 3'
boundary of the hMT-IG promoter fragment is limited to +5 because of
a G at +6.

All hMT-IG constructs were completely sequenced using the T7 Se-
quencing Kit (Pharmacia Biotech Inc.). Plasmid DNA for transient
transfection was prepared using the Plasmid Maxi Purification Kit
(Pierce) with bovine serum albumin as the standard.

Cell Culture and Transfection—The liver carcinoma-derived Hep G2
cell line (17) was used for transient transfection since the human MT
genes are highly expressed in these cells (3, 4, 10). Hep G2 cells were
grown as monolayers and transfected as has been described previously
in detail (3). The DNA mixture for transient transfection contained 0.70 pmol
of test promoter in pMEV1R, 0.70 pmol of control promoter hMT-IG
(17) (Promega Biotech), and sufficient reaction DNA (pGEM2) to
adjust the total DNA mass to 10 μg for each 106 cells plated (3). Under
these transfection conditions, the control and test MT-IG promoter
constructs are not in competition (data not shown). For metal induction,
100 μM ZnCl2 was added to the media 16 h after glycerol shock, and cells
were scraped into Cell Lysis Buffer (Promega Biotech) after an addi-
tional 7 h. Cells were lysed by freeze-thawing, and the protein concen-
trations of cleared extracts were determined using the BCA assay
(Pierce) with bovine serum albumin as the standard.

LUC assays for standardization were performed in duplicate for each
transfection plate as per the protocol of de Wet et al. (13) and measured
as relative light units using a luminometer (Analytical Luminescence
Laboratory). The CAT activity of test constructs was determined by the
procedure of Chaudry et al. (18) and was quantified by densitometry.

The amount of Zn induced was quantified by densitometry scanning of
autoradiograms of thin layer chromatographs. After normalization to LUC
values for each trial, the MT-IG test promoter-CAT activities were adjusted
to units of relative activity by considering that, for each trial, the metal-
induced value of the w.t. MT-IG promoter fragment was 100. Values
presented under "Results" are an average of three trials of which the
standard deviations varied from 5 to 12% of the average.

In vitro Transcription—Male Sprague-Dawley rats (standard patho-
gen free) were obtained from the Department of Biological Sciences
vivarium at the University of Calgary. Liver nuclear extracts were
prepared essentially as described by Gorski et al. (19). Metal-activated
extracts were prepared by removing the liver 3 h after intraperitoneal
injection of 25 μmol of ZnCl2 in a 0.9% solution of NaCl. In vitro
transcription reactions were performed as described previously (3)
and contained 1.5 μg of the hMT-IG test construct and 0.1 μg of the internal
control, pAdML (3, 15). Plasmid DNA was preincubated with 45 μg
(unless otherwise stated) of liver nuclear extract on ice before addition
of the transcription reaction, and incubated for 3 h at 32 °C for 4 h. Den-
sitometer scanning of autoradiograms from denaturing polyacrylamide
geis was used to quantify MT and AdML promoter-derived transcripts.

Oligonucleotide competition of MT-IG transcription in vitro was per-
formed by a 10-min preincubation of nuclear extracts on ice with dou-
ble-stranded oligonucleotides (Pharmacia LKB Gene Assembler Plus)
followed by a 10-min incubation with template DNA prior to the start of
the reaction. Binding of the transcription factor to the DNA is sensitive to
the mass of DNA present in a reaction, the amount of oligonucleotide in
cell regulations was kept constant compared to the MREa competitor by using
equal amounts of oligonucleotides containing transcription factor binding
sites not present in the MT-IG promoter. The sequences of the upper
strand of the competitive oligonucleotides are as follows: TATAA, 5'-
CCTTTGGC-3'; TATCA, 5'-TCTTTGACTA-TCAAAAGCGCCGGCT-3';
MREa, 5'-CGTCTGGCCGCCCAGCCGTG-3'; API, 5'-CTGTTGATGAGTCAGCCGGAA-3'; NF1/TF, 5'-CCTTGTGCCGT
AGCTGCCAAATAG-3'.

Southwestern Blotting—Complimentary oligonucleotides (Pharma-
cia LKB Gene Assembler Plus) corresponding to the MT-IG MREa
region (5'-GCTTCGCGGCCCGCCCGT-3') and the mutant MREa2
were annealed and double-stranded products purified by elution from
10% non-denaturing PAGE (16). Oligonucleotides and PCR products
were end labeled as per accepted protocols (16) to a specific activity of
106 counts/min/μg.

Rat liver (19) and Hep G2 cell (20) nuclear proteins (50 μg) were
separated on 6 or 8% SDS-polyacrylamide gel (291, acrylamide/acy-
lamide) using the Bio-Rad Mini-Protein II™ Cell and transferred
to nitrocellulose (Bio-Rad Mini Trans-Blot16). Blots were treated
equally as described (22, 23) with probes added to a concentration of
1027 mol of ZnCl2 in a 0.9% solution of NaCl. To test if probe binding was
zinc dependent, zinc was omitted and 500 μM p-aminothiophenol was included
in the binding buffer at all times.

DNase I Protection Assays—The MT-IG (174/5) promoter in
pMEV1R was used as a template to produce probes using PCR. Briefly,
20 pmol of SP6 sequencing primer (coding strand) or 20 pmol of primer
027 (non-coding strand) were end labeled (16), and the T4 polynucle-
tide kinase was inactivated by heating at 90 °C for 20 min. Each
labeled primer was used in a PCR reaction with the other unlabeled
primer and the PCR products were purified by elution from 10% non-
denaturing polyacrylamide gel (16). Binding reactions with human
TATA binding protein (TPB, Promega Biotech,) were performed using
manufacturer recommended conditions, and DNase I protection assays
were performed as described (23). One footprinting unit of TBP is the
amount required to completely protect 50 fmol of TATA box probe.
Probes were digested for 60 s at room temperature with 0.1 unit of
DNase I in the absence of protein and 1.0, 15.0, and 20.0 units of DNase
I for 1, 2, and 3 footprinting units of TBP, respectively.

RESULTS

Effect of MREa and TATA Box Mutations on MT-IG Pro-

mounter Activity in Hep G2 Cells—The position and sequence of
MREa suggest that it has an important role in MT-IG promoter
activity. First, MREa is adjacent to the TATA box and synthetic
MRE sequence is known to be modulated by distance from the
TATA box and the gene origin of the TATA box used as the
minimal promoter (8, 9). Second, the sequence of the MT-IG
MREa is remarkably conserved among the homologous MREs
of the MT-I genes (3), in spite of the flexibility of the MRE
consensus (2). For these reasons, we chose to determine the
functional contributions of the MT-IG MREa in the context of
native promoter sequences. Four mutants were created to com-
pletely dissect the MREa region of the MT-IG promoter (Fig. 1).

Trinucleotide mutations were employed since it was possible
that single point mutations might not affect the ability of
MREa to interact with other MREs in the promoter.

Further, trinucleotide mutations could better reveal the contributions

FIG. 1. MT-IG promoter fragments used in transfection analy-
sis. A schematic representation of the MT-IG promoter (174 to +5)
is shown with the positions of the TATA box, GC boxes, and MREs
(direction arrow conveys MRE orientation). The sequences of MREa
and the MREa mutants employed in this study are given.

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MRE 5'- and 3'-flanking nucleotides which are semiconserved but have not seemed essential for synthetic MRE function (7). A TATA box mutation, to TATCA, was also included in our examination to differentiate between general and metal regulatory contributions to MT-IG regulation. This TATCA mutant is relevant because it is naturally present in the human MT-I promoter and is a determinant of differential MT-I gene expression (3).

Transfection of human hepatoblastoma (Hep G2) cells with the MT-IG promoter-CAT fusion gene revealed that the −174/+5 fragment elicits a 31-fold response to zinc (Fig. 2). Mutation of the TATA box of a TATCA sequence abolished basal activity, but zinc-induced promoter levels were maintained (Fig. 2). When the 5' MREa-flanking nucleotides were mutated for construct MREa1 (Fig. 1) the basal activity of the promoter increased 2.5-fold, decreasing metal fold induction to 15 (Fig. 2). In contrast, mutation of the first three nucleotides within the conserved core of MREa (MREa2, Fig. 1) devastated the basal activity of the promoter while fold induction was maintained at 31. The MREa3 mutation altered the most 3' nucleotide of the conserved MRE core and two additional 3' MRE-flanking nucleotides (Fig. 1). Although not as devastating as the MREa2 mutant, the MREa3 mutation resulted in reduced basal and zinc-induced levels of activity and a nominal decrease in metal responsiveness from 31- to 26-fold. As with the MREa1-flanking mutation, the MREa4 mutant in the 3' flanking region displayed increased basal activity while metal-induced levels were nearly the same as for the native MT-IG promoter resulting in 13-fold metal induction.

It is apparent from these results that the contributions assigned to MREs are not confined to the conserved heptanucleotide core but, at minimum, involve an extended 15-bp region. Although mutations MREa1 and MREa4 decrease MREa homology to the MRE consensus (2), both increase the general activity of the promoter without reducing metal-induced levels. Perhaps these flexible nucleotides modulate the basal activity of MREs or allow increased basal activity of other nearby elements, such as SP1 sites. Within the highly conserved core of MREa, mutations are much more detrimental to promoter activity in spite of five other putative MREs (Fig. 1). The MREa2 core mutant nearly abolishes basal activity similar to mutation of the TATA box, suggesting that MREa is a basal element and required for the optimal function of the TATA box. However, unlike the TATCA sequence, this mutation also severely diminishes the metal-induced levels of promoter activity, in spite of the presence of five other putative MREs. Thus, MREa also may be required to mediate the effects of upstream MREs for efficient metal induction. This also is suggested by the core mutant MREa3 which reduces metal-induced promoter activity.

In Vitro Activity of MT-IG Is Dependent on the TATA Box and MREa—We employed the same mutants of MT-IG in a rat liver nuclei in vitro transcription system which has been used previously to study differential hMT-I transcription (3). The activity of the w.t. and mutant MT-IG promoters in vitro are shown in Fig. 3.

Mutation of the TATA box to a TATCA sequence abolished MT-IG transcription in vitro (Fig. 3A). Although a TATAA containing oligonucleotide competed for the TATA box binding factor, TFIIID, at a 50-fold molar excess to the promoter, a TATCA sequence had no effect (Fig. 3B). This suggests that the TATCA sequence binds TFIIID with less affinity than the consensus TATAA.

All four MREa mutants decreased MT-IG transcription, and
a representative transcription assay is shown in Fig. 3C. After normalization to p(AdML)190 activity (AdML), the MREa1 mutant displayed 40% of the WT promoter activity. The MREa core mutant MREa2 had the greatest effect in numerous trials, with activity that was undetectable in most trials. The MREa3 mutation also reduced activity to only 10–15% of MT-IG activity. Finally, the MREa core mutation reduced activity to 25% of the native promoter. The results of additional assays were consistent in terms of the relative qualitative effect of each mutation with MREa > MREa1 > MREa4 > MREa3 > MREa2 in decreasing order of activity. Although the MREa core mutations were detrimental both in vitro and in vivo, the effects of the flanking mutations (MREa1 and MREa4) on MT-IG activation (45 molar excess) were detrimental both decreasing order of activity. Although the MREa core mutations were detrimental both in vitro and in vivo, the effects of the flanking mutations (MREa1 and MREa4) on MT-IG activity differed between the two systems. This could be a result of species-specific differences in MRE sequence requirements for factor binding. Alternatively, reduced contributions by more distal MREs in vitro compared to in vivo might increase promoter dependence on MREa and its flanking sequences.

To confirm the requirement for MRE binding factors in vitro, various competitive oligonucleotides were employed to titrate MRE-binding factors from the MT-IG template. Under conditions for TATA box competition and optimal MT-IG transcription (45 molar excess), significant competition effects were not observed (data not shown). This suggests that an extreme excess of MRE-binding factors is present in the extracts, which could be expected from the high level of MT expression in rat liver (24, 25). Alternatively, we have observed that MRE oligonucleotides form more nonspecific than specific complexes in rat liver extracts using mobility shift assays (data not shown). Therefore, only some of the MREa oligonucleotides could be involved in specific competition in vitro. When the amount of nuclear extract used was reduced (30 molar excess), a 100-fold molar excess (oligonucleotide:promoter) of a MT-IG MREa sequence could compete with MT-IG (Fig. 3D). Control oligonucleotides corresponding to AP1 and NF1 factor-binding sites were poor competitors of MT-IG promoter activity (Fig. 3D), but there was a nominal reduction in control AdML transcription in MREa and AP1 competitions probably due to nonspecific interference with transcription (Fig. 3D). More efficient competition of the AdML promoter activity by the NF1 sequence may be due to titration of a factor which binds the CCAAT sequence present in the promoter (27). In general, since MREa is the only sequence capable of extreme effects on MT-IG activity, these results provide further support that MRE-binding factors are active in vitro.

From the above mutagenesis and competition experiments, we conclude that the activity of the MT-IG promoter in vitro is dependent on both the TATA box and MREa as observed using transfection. These two elements can be said to functionally interact in vitro since the promoter is nearly inactive when either sequence is mutated or used for competition.

In Vitro Transcription Activity of MT-IG Can be Increased by Zinc—Although MT-IG transcription in this in vitro system apparently is MRE dependent, one caveat is that this activity does not require the addition of metals as would be expected if metal regulatory factors were involved. In an attempt to metal activate MT-IG transcription in vitro, the rats were given intraperitoneal injections of ZnCl2 3 h prior to sacrifice and extract preparation. This treatment is known to induce rat hepatic MT transcripts to maximum levels at 5 h (26), and we reasoned that metal regulatory factor activity would increase before this. We observed that the MT-IG promoter had increased activity in extracts from zinc-injected rats compared to control extracts while transcription from the non-metal-responsive AdML promoter remained the same (Fig. 4B). Normalization of the amount of MT-IG activity with that of the internal control revealed that, at 45 molar excess, the MT-IG activity was 3- and 4-fold higher in two separate preparations from injected rats compared to the control extracts. Although the general activity of MT-IG and AdML decreased (by 31 and 44%, respectively) when less extract was used (30 molar excess), the ratio of MT-IG activity in “metal activated” compared to control extracts was consistent (Fig. 4B). Alternative optimal amounts of extract still displayed increased activity in metal-activated extracts, although less pronounced probably because of limiting template (data not shown).

In contrast to in vivo metal activation, addition of variances of zinc directly to the in vitro reactions did not increase MT-IG activity substantially. For example, addition of 200 molar ZnCl2 directly to reactions, at best, increased transcription 1.5-fold (Fig. 4A, lanes 1 and 2). Higher Zn2+ concentrations above 500 molar inhibited transcription from both the MT-IG template and p(AdML)190, until both were inactive at 1.0 molar (data not shown). Because the internal control was similarly affected, the negative effects of ZnCl2 are nonspecific. Longer exposure to zinc by dialysis of nuclear extracts with 200 molar ZnCl2 also did not increase transcription substantially (Fig. 4A, lane 3). As well, exclusion of EDTA from dialysis and reactions did not allow a response to zinc and simply lowered the zinc concentration at which transcription was nonspecifically abolished (data not shown). In one preparation, we observed increased MT activity in extracts prepared with zinc at nuclear lysis, but attempts to repeat this have not been successful since AdML is slightly inhibited in metal-treated extracts compared to control extracts. Normalization of MT levels to AdML activity in this case results in artificially elevated MT activity which may not be biologically relevant.

The above results are significant in that they suggest that metal regulatory factors are not transcriptionally activated by simple association with metals added in vitro. Here, increased MT promoter activity in response to zinc requires an in vivo metal regulatory pathway which could increase the accessibility of MRE-binding factors to metals or cause the release of the factors from a metal sensitive inhibitor, as proposed in a recent model (28).

Southwestern Blotting—Since MREa core mutations decreased MT-IG promoter activity in vivo and in vitro, it is probable that MREa binds positive transcription factors in both systems. We attempted to identify the proteins which directly interact with MREa from both rat liver and Hep G2 cell nuclear extracts.

The technique of Southwestern blotting allows characterization of DNA-binding factors by apparent molecular mass. For our purpose, a monomer of MREa was used to probe for proteins in both extracts. In rat liver, MREa consistently bound a protein with a molecular mass of approximately 105 kDa (Fig. 4A).
TBP-induced DNA conformation changes. This might explain and it is plausible that the C:G pair could negatively affect sequence is also a factor in its tight interaction with TBP (29, 30), groove contacts (29, 30). The deformability of the TATA sequence is also a factor, in its tight interaction with TBP (29, 30), and it is plausible that the C.G pair could negatively affect TBP-induced DNA conformation changes. This might explain the reduced hypersensitivity we observe at the 5' boundaries of the TATCA footprints.

5A). In addition, a lesser bound 40 kDa factor was observed in a few assays. In Hep G2 nuclear extracts binding a slightly larger protein (125 kDa) was observed (Fig. 5B). For both extracts, a probe corresponding to the MREA2 core mutant sequence did not detect these proteins confirming that binding was MREA specific (Fig. 5). Further, this binding was zinc dependent since addition of the chelator o-phenanthroline instead of zinc also abolished binding (Fig. 5). When equal amounts of nuclear extract from zinc-injected rats or zinc-treated Hep G2 cells were compared with their corresponding control extracts, the amount of protein bound by MREA was constant (Fig. 5). This suggests that the increase in activity observed in transfection and in vitro with metal treatment is not due to de novo synthesis of the MREA-binding proteins, but activation of pre-existing factors.

DNase I Protection of the TATA Box by TBP—Because of the functional difference between the consensus TATA box and the TATCA variant, we investigated the interaction of the TBP with both sequences. Using 1 unit of TBP, the MT-I TATA box is clearly protected on the non-coding strand from −38 to −22 with increased hypersensitivity at these boundaries as well as the −50 region at MREA (Fig. 6A, lanes 1 and 2). This protection is still clearly defined at 2 and 3 units of TBP (Fig. 6A and B, lanes 3 and 4). Similarly, the coding strand of the native promoter also is protected from −40 to −22 (Fig. 6B, lanes 1–4). However, even at 2- and 3-fold more TBP, the TATCA sequence of the non-coding strand is accessible to DNase I and displays less hypersensitivity at −41 and −50 than is observed for TATAA (Fig. 6A, lanes 5–8). The TATCA coding strand is somewhat protected but, even at 3 units of TBP, bands at −32 to −30 in the center of the footprint are still visible and the 5' boundary is not clearly defined (Fig. 6B, lanes 5–7).

In part, the reduced protection of the TATCA sequence appears to be caused by less stable interactions with TBP, as was suggested earlier from competition in vitro. Since TBP is known to hydrogen bond with both bases at the fourth position of the TATA sequence, the change to a C.G pair is potentially devastating to TBP affinity and may even interfere with minor groove contacts (29, 30). The deformability of the TATA sequence is also a factor in its tight interaction with TBP (29, 30), and it is plausible that the C.G pair could negatively affect TBP-induced DNA conformation changes. This might explain the reduced hypersensitivity we observe at the 5' boundaries of the TATCA footprints.

**DISCUSSION**

Using transfection of HepG2 cells and in vitro transcription, we have determined that the TATA box and adjacent MREs are required for optimal MT-I promoter activity. For the TATA box mutation, DNase I protection assays reveal altered physical interaction of TBP with the TATCA-containing promoter which correlate with the functional effects of this mutation. In addition, the MREA core mutation, which devastates promoter activity, also decreases MREA binding to zinc-dependent nuclear proteins from rat liver and HepG2 cell nuclei. The apparent molecular masses of the predominant rat (105 kDa) and human (125 kDa) factors bound by MREA correlate with reported values for the mouse (21) and human MTF (31) which are both zinc-finger proteins.

Previous studies have shown that MRE activity is modulated by distance from the TATA box (8, 9). However, our data suggest that the TATA box of MT promoters could be dependent on the activity of the MREs, which appear to compensate for the TATCA mutation during metal induction even though basal activity is abolished. A biologically relevant example is the hMT-I promoter which has lower absolute levels of activity due to a TATCA sequence but displays greater metal fold induction than hMT-I (3). Because we have observed a qualitative difference in the physical interaction of the TATA box-binding protein with TATCA, newly recruited MRE binding factors could alter TBP binding to this variant during metal induction or independently affect DNA conformation to compensate for the effects of the TATCA sequence.

Although the devastating effect of the TATCA mutation could suggest that the TATA box is solely responsible for basal MT-I activity, core mutation of MREA also substantially decreases basal levels. This also is consistent with a requirement for MRE effects on TATA box activity. In the past, basal contributions by MREs have not been discussed since in vivo footprinting of rat and mouse MT-I genes generally supports metal-dependent occupation of MREs (32, 33). However, in each case, one MRE is protected prior to metal treatment (32, 33) and others are somewhat protected (33) indicating that...
there is some constitutive occupancy of MREs. In our own studies, we have observed that the MREa of the rainbow trout MT-B gene makes substantial contributions to basal promoter activity in both fish and mammalian cell lines (22).

Because of the basal contributions of MT-IG MREa, mutation in its core actually increases fold induction which is inconsistent with a role as a metal-responsive element. However, MREa is still essential to metal induction since it is necessary for efficient expression in spite of the presence of five other MREs. In a previous study, we have shown that these additional MREs are active since addition of the distal promoter fragment (MREs c, d, and e) to the proximal region (MREa and b) increases metal induction (10). Together, these observations suggest that the efficient function of distal MREs is dependent on the proximal promoter region and specifically MREa. Perhaps MT promoter elements are organized such that they require occupancy of a “basal” MRE (MREa) in concert with the TATA box for constitutive expression levels as well as for communication with remaining MREs during metal induction. This could explain why gene disruption of the MRE-binding factor MTF-1 in embryonic mouse cells abolishes both basal and metal-induced MT-I expression (34) in spite of the presence of other non-MRE basal elements (2).

The MT-IG TATA box and MREa are also essential for in vitro transcription. Garg et al. (35) have also employed a rat liver nuclei in vitro transcription system to study the mouse MT-I promoter and have reported that a region from 148 to -42, which contains five of the six mouse MT-I MREs, competes with promoter activity. Although MRE-binding factors appear to be active in this in vitro system, it is difficult to establish that these are functionally metal-dependent. We have employed the chelator o-phenanthroline to demonstrate the zinc dependence of MREa binding by HepG2 and rat liver nuclear proteins. However, this is not practical for functional assays since MT-IG has three potential binding sites for the zinc finger transcription factor, Sp1, (3, 36) and other non-MRE-binding transcription factors could require zinc. Instead, we have demonstrated that further activation of MT-IG transcription requires zinc to metal treatment as opposed to in vitro zinc addition suggesting that a more complicated pathway than just simple association with metals serves to activate the factors. In our experiments, it is doubtful that the 3 h in vivo zinc treatment results in synthesis of new MRE-binding proteins since metal induction of MT transcription in cell lines does not require protein synthesis (37). Also, we detect no difference in the amounts of MRE-binding proteins in regular and “metal-activated” rat liver nuclear extracts by Western blotting. Instead, it appears that in vivo zinc treatment may increase the availability or activity of exisuent MRE binding factors for greater MT transcription. Our data are consistent with a recent model proposed by Palmiter (28) in which the MRE binding activity of the mouse factor MTF is regulated through release from a zinc-sensitive inhibitor. Perhaps injection of rats with zinc causes in vivo dissociation of the inhibitor, while this factor-inhibitor interaction is “fixed” in prepared extracts and cannot be released with zinc added in vitro. However, in contrast to our findings, the rat liver nuclear factor ZAP (38) as well as mouse MTF (21) and human MTF (31) (or ZRF; 5) display increased MRE binding activity when zinc is added to nuclear extracts in vitro. With this in mind, it is possible that in vitro metal addition can release MRE-binding factors from an inhibitor for MRE binding, but MT transcription activation requires in vivo activation of additional auxiliary metalloregulatory factors not induced by in vitro treatment. In control extracts, MRE-dependent in vitro transcription could be directed by distinct basal MRE-binding factors. We control of metal regulatory factors natural release for basal expression. We doubt that the in vitro activity of control extracts is due to artificial activation of factors by metals transferred from metalloproteins during preparation as this would suggest that the factors should be activated just as easily as metal in vivo.

Our data have raised two important questions. First, why is MRE activity required for MT promoter activity outside of metal induction? Second, if simple association with metal in vitro is not sufficient to transcriptionally activate MRE binding factor activity, what is the true nature of the in vivo regulatory pathway and how does it allow cell type-specific levels of basal and metal-induced transcription? Future investigation of these areas is required to gain a full understanding of MT gene regulation.

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