Activation of Metallothionein Gene Expression by Hypoxia Involves Metal Response Elements and Metal Transcription Factor-1

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ABSTRACT

Metallothioneins (MTs) are a family of stress-induced proteins with diverse physiological functions, including protection against metal toxicity and oxidants. They may also contribute to the regulation of cellular proliferation, apoptosis, and malignant progression. We reported previously that the human (h)MT-IIA isoform is induced in carcinoma cells (A431, SiHa, and HT29) exposed to low oxygen, conditions commonly found in solid tumors. The present study demonstrates that the genes for hMT-IIA and mouse (m)MT-I are transcriptionally activated by hypoxia through metal response elements (MREs) in their proximal promoter regions. These elements bind metal transcription factor-1 (MTF-1). Deletion and mutational analyses of the hMT-IIA promoter indicated that the hMRE-a element is essential for basal promoter activity and for induction by hypoxia, but that other elements contribute to the full transcriptional response. Functional studies of the mMT-I promoter demonstrated that at least two other MREs (mMRE-d and mMRE-c) are responsive to hypoxia. Multiple copies of either hMRE-a or mMRE-d conferred hypoxia responsiveness to a minimal MT promoter. Mouse MT-I gene transcripts in fibroblasts with targeted deletions of both MTF-1 allelic (MTF-1+/−; dko7 cells) were not induced by zinc and showed low responsiveness to hypoxia. A transiently transfected MT promoter was unresponsive to hypoxia or zinc in dko7 cells, but inductions were restored by cotransfecting a mouse MTF-1 expression vector. Electrophoretic mobility shift assays detected a specific protein-DNA complex containing MTF-1 in nuclear extracts from hypoxic cells. Together, these results demonstrate that hypoxia activates MT gene expression through MREs and that this activation involves MTF-1.

INTRODUCTION

MTs are ubiquitous, low molecular weight proteins characterized by high cysteine content and high affinities for metals such as zinc and cadmium (reviewed in Ref. 1). Both the constitutive and stress-inducible expression of MT appear to be dependent on activation of MTF-1, a member of the Cys2 His2 family of zinc finger transcription factors. This family includes two polypeptides, MTF-1 and MTF-2 (2, 3). MTs have well-established roles in metal homeostasis and in the detoxification of heavy metals. Moreover, they also confer protection against reactive oxygen intermediates, electrophilic anti-factors (2, 3). MTs have diverse physiological functions, including protection against reactive oxygen intermediates, electrophilic anti-factors (2, 3). MTs have established roles in metal homeostasis, as well as roles in cell protection against reactive oxygen species generated by ionizing radiation (14). Thus, MTF-1 may be critical for modulating gene expression associated with malignant phenotypes such as resistance to therapy.

The mammalian MT family consists of two ubiquitous isoforms (MT-I and MT-II) and two tissue-specific isoforms (MT-III and MT-IV). Although only one isoform of mammalian MT-II has been identified (MT-IIA), at least seven unique functional isoforms have been described for the hMT-I gene (15). The expression and regulation of some of the hMT genes (e.g., MT-IA, MT-IB, MT-IG, and MT-IIA) have been described (15, 16). MT-IIA is the predominant human MT isoform and is expressed in most cultured human cells (15). In contrast, MT-I is the predominant MT isoform in the mouse. The mammalian MT-I and MT-II genes are transcriptionally regulated by metal ions, such as zinc and cadmium, and by a wide variety of other stimuli. These latter agonists include bacterial endotoxin (lipopolysaccharides), phorbol esters, xenobiotics, and oxidative stress (3). The hMT-IIA gene is also induced by hypoxia, growth factors, cytokines, UV radiation, glucocorticoids, X-irradiation, and some specific DNA-damaging agents (4, 15, 17). Although the regulatory mechanisms of these nonmetallic inducers are not well understood, some appear to involve redox stresses (3).

Both MT-I and MT-II promoters contain multiple copies of specific cis-acting elements that cooperate to direct metal inducibility (MREs). The MRE-associated transcription factor (MTF-1) that binds to MREs and activates MT transcription has been cloned from mouse and human cells (18, 19). The mechanism by which metals activate transcription through MTF-1 has not been well established, although it appears to involve interactions at the zinc finger domain of MTF-1 (20). We have observed that oxidative stress activates the mMT-I gene through MTF-1 binding to the MREs in the proximal promoter (21, 22). In addition to the MREs, proximal mMT-I promoters contain Sp1 binding sites, USF binding sites, AREs, glucocorticoid-responsive elements, and consensus TATA box sequences (23, 24). The hMT-IIA promoter is also complex and contains a glucocorticoid-responsive element, Sp1 and API binding sites, three putative AP2 sites, four metal response elements (MRE-a through MRE-d), and a TATA box (15).

We reported previously that low pO2 levels, similar to those measured in tumors <1 mm in diameter (25), significantly induced hMT-IIA transcript and protein expression in a variety of carcinoma cell lines (17). In this study, we present the following major observations:

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2 The abbreviations used are: MT, metallothionein; MTF, metal transcription factor; hMT, human MT; mMT, mouse MT; MRE, metal response element; GSH, glutathione; ARE, antioxidant response element; USF, upstream stimulatory element; MEF, mouse embryo fibroblast; Luc, luciferase; HIF, hypoxia-inducible factor; HRE, hypoxia response element; CMV, cytomegalovirus.

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(a) the most downstream MRE in the proximal promoter region of the hMT-IIA gene regulates MT-IIA transcriptional inductions by hypoxia, whereas at least two distal MREs are involved in the mMT-I activation; (b) MTF-1 is required for this process; and (c) it is therefore likely that MTF-1 is a redox transcription factor that regulates coordinate gene expression in hypoxic and reoxygenated tumor microenvironments.

**MATERIALS AND METHODS**

**Cell Culture and Hypoxia.** The culture of C3H12 myoblasts, NIH3T3R (Ha-ras transformed NIH3T3 cells), dko7 fibroblasts, and MEF cells and our methods for exposing cells to hypoxia are described elsewhere (2, 26–29). Briefly, culture dishes were incubated in aluminum chambers at 37°C and made hypoxic by repeated cycles of partial evacuation and gassing with 5% CO₂/air at 37°C.

9 universal 3′-primer (to 23 of hMT-IIA), TTTAAGCTTGGGACTTGGAG-

9 repeats cloned upstream of the minimal promoter from pmMT-I(-42)-Luc. The mRNA levels detected by using a 32P-labeled, 200-bp oligomer of human [phMT-IIA(90)-Luc], TTCTCGAGGCGGGGCGTGTGCAGGCACGGCC-

Luc], TTTTCTCGAGGTGCAGAGCCGGGTG; 5′ universal 3′-primers to pmMT-I (MRE-d mut), 5′-GATCCAGGAGCTCTGCACAGGCCCAAAAGTA-3′; mMRE-d mut, 5′-GATCCAAATTGAGGCGGGCTTTT-3′; Sp1, 5′-GATCCCTGAGAACTCCGTTCGCTTCG; 5′-primers to −70 bp [phMT-IIA(−70)-Luc], TTTTCTCGAGCCGGGCGGTCTTTGCTACTCGT; 5′-primers to −57 bp [phMT-IIA(−57)-Luc], TTTTCTCAGTTTGGTACACGCTTCGGGTCTCG; 5′-primers to −70 bp [hMRE-a mut; phMT-IIA(−70 mut)-Luc], TTTTCTCGAGCCGGGCGGTCTTTGCTACTCGT.

Selected mMRE double-stranded oligonucleotides (monomers) were synthesized and cloned into the XhoI/BglII sites of pmMT-I(−42)-Luc, immediately upstream of the minimal mMT-I promoter (−42 to 62; 22). The mMRE-42-Luc containing the −153 to 62-bp fragment; pmMT-I(-153)-Luc containing the −153–bp deletion mutant (−100 to −89 deleted); pmMT-I(-42)-Luc containing the −42 to 62 minimal promoter, and pmM1T-I (MRE-d, −)Luc containing five tandem copies of the hMRE-a repeats cloned upstream of the minimal promoter from pmMT-I(−42)-Luc. The primers used were as follows (mutant bases are shown in lowercase): universal 3′-primer (to 23 of hMT-IIA), TTATAGCTGGGAGGCTCGTGCC-CAACCCGAGCTTGTCCGGCCTC; 5′-primers to −167–bp [phMT-IIA(−167)-Luc], TTATACGAGAAGCCGGTGCTGACGACGCGCCGGGCGG; 5′-primers to −70 bp [phMT-IIA(−70)-Luc], TTTTCTCAGTTTGGTACACGCTTCGGGTCTCG; 5′-primers to −57 bp [phMT-IIA(−57)-Luc], TTTTCTCAGTTTGGTACACGCTTCGGGTCTCG; 5′-primers to −70 bp [hMRE-a mut; phMT-IIA(−70 mut)-Luc], TTTTCTCAGTTTGGTACACGCTTCGGGTCTCG.

Nuclear Extracts and Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared from confluent C3H12 myocytes grown in a normal aerobic environment or under hypoxia. Cells were serum starved (0.5% serum) for 4–5 days before treatments to eliminate the effects of mitogens and serum metals. For hypoxic cell extracts, cell lysis was performed with the cells still under hypoxia to avoid reoxygenation effects (34). Cells were lysed in a buffer containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.4 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 5 mM β-glycero- phosphosphate, 5 mM benzamidine, 5 mM NaF, and 10 mM sodium PPi. Nuclei were removed by centrifuging at 3000 × g for 20 min, and proteins were extracted (35). Sequences of the sense-strand oligonucleotide probes used were: hMRE-a, 5′-AGCTTCGGGGCGGTCTTTTGACTGCTGCGGCTCTA-3′; hMRE-a mut, 5′-AGCTTCGGGGCGGTCTTTTGACTGCTGCGGCTCTA-3′; HIF-1, 5′-GATCCCTGAGAACTCCGTTCGCTTCGGGTCTCG; 5′-primers to −70 bp [hMRE-a mut; phMT-IIA(−70 mut)-Luc], TTTTCTCGAGCCGGGCGGTCTTTGCTACTCGT.

Cell Transfections and Treatments. Subconfluent C3H12 myoblasts or NIH3T3R cells were used for analysis of both the hMT-IIA and mMT-I promotors and were transfected with either 10 μg of total DNA by the calcium phosphate technique as described previously (17) or with 2 μg of DNA using a standard DEAE dextran method (31). The DEAE dextran method was also used for cotransfections of the mMTF-1(−42) fibroblast cells (dko7) involving 2.0 μg of pMT-I(−153)-Luc and variable amounts of an mMTF-1 expression vector (up to 1.0 μg). Where appropriate, a Bluescript phagemid was used to maintain an equal administration of total DNA. Transfections were normalized either by cotransfection with a plasmid containing the β-galactosidase gene, driven by the mouse hydroxymethylglutaryl-CoA reductase promoter, which has a minimal hypoxia response (32), or with a Renilla luciferase construct (Dual-Luciferase Reporter Assay System; Promega). At 24 h after transfection, the medium was replenished, and the cells were subjected to hypoxia or metal treatments. Luciferase activity in cell extracts was assayed with a Promega Biotec assay kit and either a LKB BioOrbit 1250 luminometer or a Turner Designs TD-20/20 luminometer (Promega).

**MTF-1 Null Analyses.** The mouse dko7 cell line, which lacks MTF-1, was a gift of Walter Schaffner (University of Zurich, Zurich, Switzerland; Ref. 2). These fibroblast-like cells were derived from embryonic stem cells and immortalized with SV40 large T antigen (33). Wild-type MEF cells, which were also immortalized with SV40 large T antigen, were obtained from Dr. John Lazo, University of Pittsburgh (Pittsburgh, PA; Ref. 28). Both lines were maintained in high-glucose DMEM supplemented with 10% FBS (2). The expression plasmid CMV-mMTF-1 was created by inserting the mMTF-1 cDNA, isolated by reverse transcription-PCR, into the NotI site of a cytomeglovirus expression vector (20).
nondenaturing 6% polyacrylamide gels at 4°C. For competition assays, binding reactions included 100 ng of indicated unlabeled oligonucleotide. For supershift assays, polyclonal antibodies (0.5 µl) against GST-mMTF-1 fusion protein, or against Sp1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added to the binding buffer 1 h before addition of the labeled oligonucleotide. The polyclonal antiserum against GST-mMTF-1 was raised in rabbits (Covance Research Products, Inc., Denver, CO) and was enriched for IgG by protein A affinity purification.

RESULTS

Induction of MT-I mRNA

We demonstrated previously that the expression of the predominant human MT isoform, hMT-IIA, is up-regulated in a number of carcinoma cell lines by hypoxia (17). Fig. 1 shows that the steady-state levels of mMT-I mRNA are increased in rodent C2C12 muscle cells by exposure to hypoxia. We confirmed this finding in a number of other rodent cell lines including NIH 3T3, ras-transformed NIH3T3 (NIH3T3R), and mouse hepatoma cells (Hepa).6 Hypoxia treatments resulted in 12.8 ± 7.0-fold inductions of state levels of mMT-I mRNA after 12 h; ZnCl2 exposures (100 µM; 4–6 h) resulted in >17-fold induction. These values are similar to those reported for MT-I mRNA in A431 human squamous carcinoma cells (17). The blot shown in Fig. 1 was reprobed with β-actin as a control gene that is not affected by hypoxia.

Mapping of MT Hypoxia-responsive Regions

hMT-IIA. To define the element(s) responsible for the induction of the hMT-II gene by hypoxia, deletions of the proximal promoter region were made and assayed under aerobic, hypoxic, or metal-treated conditions as described in “Materials and Methods.” The basal activities of the reporter constructs dropped as successive deletions were introduced downstream of −167 bp (Fig. 2). Specifically, phMT-IIA(−90)-Luc and phMT-IIA(−70)-Luc showed approximately 2- and 9-fold lower basal activities, respectively, as compared with phMT-IIA(−167)-Luc. Deletion to −57 bp caused an additional 7-fold reduction in basal transcriptional activity as compared with phMT-IIA(−70)-Luc. The deletion from −167 to −90 bp eliminates two MREs (hMRE-d and hMRE-c), an AP2 sequence and an AP1 element; deletion from −90 to −70 bp removes hMRE-b, whereas the deletion from −70 to −57 bp removes a GC (Sp1 family) binding site. The results confirm previous studies showing the importance of a number of promoter elements, including other MREs, in maintaining high basal promoter activity (for an example, see Ref. 15). Although, the basal transcriptional activities fell in these successive deletion constructs, the relative responses to hypoxia and zinc were maintained, including the smallest deleted promoter [phMT-IIA(−57)-Luc]. This −57-bp promoter fragment contains only the hMRE-a element upstream of the TATA box sequence (15). To delineate a putative contribution of hMRE-a, a mutation was introduced into the 5' core region of the hMRE-a element in phMT-IIA(−70)-Luc. This mutation eliminated both basal and inducible expression. These findings suggest a critical role for the hMT-IIA MRE-a in hypoxia and metal inducibility and in the maintenance of basal transcriptional rates.

mMT-I. The proximal promoter of mMT-I contains four MREs, an Sp1 element, an overlapping USF/ARE, and a TATA box (Fig. 3A). A construct containing this promoter region truncated to −153/67 bp (relative to the transcription start site) inserted immediately upstream of a luciferase reporter gene (pGL2-Basic), pmMT-I(−153)-Luc, was induced 7.1 ± 2.1-fold by hypoxia and 12.1 ± 5.3-fold by zinc (Fig. 3B). To analyze the contributions of mMREs to the hypoxia inducibility of mMT-I, selected MRE monomers were inserted into pmMT-I(−42)-Luc. These included mMRE-d, a mutated (mut) mMRE-d, mMRE-c, and a consensus mMRE (mMRE-s), as described above. Because single-copy mMREs were poorly expressed in C2C12 cells (data not shown; see also Refs. 36 and 37), we used the cell line NIH3T3R in which endogenous MTs are strongly induced by both

B. J. Murphy and M. Yanovsky, unpublished results.
hypoxia and zinc. Fig. 3C shows that both mMRE-d and mMRE-c conferred both hypoxia and zinc induction. A 3-bp mutation in the core region of the mMRE-d completely eliminated inducibility by both stresses. A consensus mMRE (mMRE-s), which lacks the GC-rich 5′-flanking sequence, was also responsive to hypoxia (Fig. 3C), implying that all of these mMREs may be capable of inducing transcription in response to hypoxia. Previous studies indicated that the composite USF/ARE (−100 to −89) mediated, in part, the response to H2O2 (22). In contrast, deletion of the USF/ARE did not affect the hypoxia-responsiveness of the mMT-I promoter (Fig. 3B). However, this deletion did result in significant reductions in basal activities of the promoter by 10- to 20-fold (Fig. 3A), which is consistent with other studies (22).

To further confirm the roles of the MREs as hypoxia-responsive elements, we inserted synthetic DNA fragments containing five copies of either hMRE-a or mMRE-d in tandem immediately upstream of pmMT-I(−42)-Luc (see “Materials and Methods”). Multiple copies were used to boost expression because other studies, including this report (see Fig. 2), have demonstrated enhanced activity of multiple compared with single elements in the response to metals (36, 37). Multiple copies of either hMRE-a or mMRE-d [phMT-IIA(MRE-a′)-Luc or pmMT-I(MRE-d′)-Luc], respectively) conferred significant hypoxia inducibility to the minimal mouse promoter, pMT-I(−42)-Luc, transfected into C2C12 cells. Hypoxia and zinc inductions were similar in mMRE-d′5 transfectants (6-fold), whereas zinc activations were slightly higher in the hMRE-a′5 transfected cells (7- and 4-fold, respectively; see Fig. 3B).

**MT Regulation in Fibroblasts from MTF-1−/− dko7 Cells**

The transient expression results implicate MREs in the activation of MT expression by hypoxia. To determine whether the MRE-binding transcription factor, MTF-1, is involved in this response, we analyzed the expression of mMT-I mRNA levels and of a transfected mMT-I promoter-reporter in MTF-1−/− cells (dko7) and wild-type MEFs. Fig. 4A shows a representative study of the steady-state levels of mMT-I mRNA transcripts from untreated, hypoxia-treated, and zinc-treated cells. The induction by zinc was abolished in the MTF-1−/− cells, and the induction by hypoxia was reduced by ~60% compared with that in normal MEFs. Absolute fold inductions were impossible to compute because the steady-state levels of mMT-I mRNA were undetectable. The residual hypoxia response must be due to other transcription factors or posttranscriptional mechanisms.

Transient expression studies of the pmMT-I(−153)-Luc reporter...
Similarly, anti-MTF-1 antibody suppressed the zinc-induced band and generated a supershift. Anti-Sp1-specific antibody did not affect the specific MRE-complexes (Fig. 5C, Lane 5), whereas it strongly supershifted the low mobility complex that bound to an Sp1 consensus binding site (Fig. 5D). Therefore, Sp1 is probably not a component of the MRE-specific complex. It should be noted that the mobility of the MRE-specific protein-DNA complexes was identical in zinc-treated and hypoxic cells. However, the hypoxia-mediated increase in MTF-1 binding activity was considerably less than that detected in zinc-treated cells, and this may explain the inability to detect a supershift with the hypoxia-treated extracts (Fig. 5C). Fig. 5E shows that HIF-1-specific binding was also induced in these cells within 2 h of exposure to hypoxia. In contrast to the MRE-specific complex, HIF-1 binding was activated by cobalt treatment but not by zinc. These results indicate that HIF-1 and MTF-1 are distinct factors that are both activated by hypoxia in C2C12 cells and with similar kinetics.

**DISCUSSION**

These studies, which extend our previous findings, demonstrate that the major isoforms of the human and mouse MT family (hMT-IIA and

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**Fig. 4.** Induction of mMT-I mRNA and reporter constructs in aerobic and hypoxic MTF-1 knockout and wild-type MEFs. A, normal MEFs and MTF-1−/− (dko7) fibroblasts were exposed to either hypoxia (≤0.1% O₂) or ZnCl₂ (100 μM) and lysed, and mMT-I mRNA levels were determined by Northern analysis. The membranes were reprobed with β-actin. B, dko7 cells were transfected with either the hMT-IIA(−167)-Luc or pMT- I(−153)-Luc reporter constructs. To determine the effects of ectopic expression of the metal transcription factor MTF-1, varying amounts of the CMV-mMTF-1 vector (0–1 μg) were cotransfected with pmMT-I(−153)-Luc (1 μg) into the MTF-1−/− cells. After a 24-h recovery period, cells were exposed to hypoxia or zinc. Cells were lysed and assayed for luciferase activity as described in Fig. 2. Data represent the means of at least three independent assays; bars, SD.

**Fig. 5.** Effects of hypoxia and metals on nuclear protein-DNA complexes. Comparison of the effects of hypoxic and zinc exposure on proteins binding to oligonucleotides containing core sequences corresponding to hMRE-a (A), mMRE-s (B and C), Sp1 (D), and HRE (HIF-1 binding site; E). Exposure of cells to metals or hypoxia, harvesting, nuclear extraction, probe sequences, and electrophoretic mobility shift analysis are described in “Materials and Methods.” Zinc chloride (60 μM) and cobalt chloride (100 μM) were added to the cultures for the indicated time intervals. Competitor oligonucleotides (100-fold excess in A, Lanes 5–7; B, Lane 3, D, Lane 3, and E, Lane 7) or antisera (4 μg) were added to the extracts, and the mixtures were incubated for 1 h at 4°C before the addition of 32P-labeled oligonucleotides. Arrows, positions of MRE-specific (M) and nonspecific (NS) complexes, and SS refers to supershifted complexes. The binding of MTF-1, Sp1, and HIF-1, respectively, to these elements has been described previously (18, 35, 61–63).
mMT-I, respectively) are transcriptionally activated by hypoxia. Transient transfection studies using a series of constructs containing fragments of the hMT-IIA and mMT-I proximal promoters showed that hypoxia treatments caused transcriptional activations that are mediated by MREs within these promoters. DNA binding experiments, studies using MTF-1 knockout cells, and Northern analysis demonstrate the importance of MTF-1 for the transcriptional activation of MTs by hypoxia. In addition, these studies suggest that other transcription factors and/or posttranscriptional events are necessary for the full hypoxic response of MTs.

Deletion and mutational analyses of the hMT-IIA promoter demonstrated the requirement for an intact hMRE-a from the hMT-IIA promoter for both basal expression and induction by either hypoxia or zinc. MREs are highly conserved 13- to 15-bp sequences that contain a hexanucleotide core sequence T/G(T/A)C/G/CNC and a partially overlapping, less conserved GC-rich flanking region (15, 16). Mutation of the four 5′ core nucleotides of the hMT-IIA MRE-a resulted in elimination of both basal and inducible (hypoxia and zinc) activities. This result is in agreement with a previous study demonstrating a critical role for the hMRE-a in basal and zinc-inducible expression of the human MT-IG isoform (16). Our studies also confirm earlier studies (15) that more downstream elements (e.g., other MREs, Sp1, and AP1) within the proximal hMT-IIA promoter are essential for maintenance of high basal promoter activities. It is inferred that these promoter elements form a highly efficient and active enhanceosome(s) (38) with the MRE-a, allowing for maximum inductions of absolute transcription rates in response to inducers such as hypoxia and zinc.

In the functional analysis of the effects of hypoxia on the mMT-I promoter, we focused on mMRE-d and mMRE-c because previous studies have shown that the MRE-d of the mMT-I proximal promoter is involved in its regulation by zinc and oxidative stress (22), and that both mMRE-d and mMRE-c are strongly footprinted by these treatments (21). This present study extends these reports and now documents the involvement of the mMRE-d and mMRE-c in the hypoxia-associated regulation of mMT-I, suggesting a redox-sensitive characteristic of these MREs. Furthermore, transfection of a consensus mMRE, mMRE-s, clearly showed that other mMREs can contribute to the regulation of this promoter by hypoxia, indicating redundancy among these elements. These elements appear to constitute multifunctional response elements, which probably cooperate in regulating the mMT-I promoter (21, 36, 37, 39). This is further supported by the higher basal and stress-induced transcriptional activity levels of reporter constructs containing multiple copies of mMREs compared with a single copy of the element (~3–4-fold higher basal levels were observed in the multiple MREs containing plasmids compared with the single copy). It is also noteworthy that mMRE-s and mMRE-c, both of which lack any Sp1-like binding sites, remain responsive to hypoxia, suggesting that the Sp1 transcription factor is not directly involved in the activation of these elements by hypoxia. Similar to the human MT-IIA promoter, an active enhanceosome, involving elements including Sp1, may be critical for the maintenance of optimal basal and inducible activities. Indeed, sequences within the ~100 to ~89 bp of the mMT-I promoter are essential for high transcriptional activities (Fig. 3; see also Ref. 22).

Like hypoxia, the induction of mMT-I expression by oxidative stress and zinc is also mediated through MREs and is accompanied by rapid increased DNA-binding activity of MTF-1 (21, 22). Our MRE-binding studies showed that MTF-1 binding increased slightly within 2 h of the onset of hypoxia and remained active for at least 24 h. However, the changes in the binding intensities of MTF-1 were less than those induced by zinc and oxidative stress. Interestingly, the hypoxia-induced binding of MTF-1 is similar to that of cadmium, a powerful inducer of MT gene expression, which causes a relatively low increase in the amount of MTF-1 binding activity in cultured cells (40), despite its essential role in the cadmium activation of mMT-I gene expression (2). These results suggest that hypoxia and cadmium may activate MT gene expression by increasing the transactivation potential of MTF-1. This could be accomplished by interactions with coactivators (e.g., other transcription factors), removal of an inhibitor, and/or posttranslational modifications. An inhibitor of MTF-1 transactivation potential has been suggested (18, 41), and reversible phosphorylation of MTF-1 may modulate the activities of MTF-1. Previous studies have shown that protein kinases are activated by hypoxic stress and may be involved in signaling pathways that modulate gene expression (27, 42, 43). We presently have no direct evidence that hypoxia mediates the phosphorylation of MTF-1, although activated Ras potentiates the induction of MTs by low oxygen. The ability of ectopic expression of MTF-1 to restore hypoxia-associated induction of a transfected MT promoter in mMTF-1 deleted cells (Fig. 4B) supports its role in the pathway(s) of activation of MTs by hypoxia. However, the induction of endogenous MT-I transcript levels by hypoxia was only partially blocked in the MTF-1−/− cells (Fig. 4A), indicating the involvement of other transcription factors and/or posttranscriptional regulation. This is also supported by our findings that MTF-1 ectopic expression in the MTF-1 knockout cells was unable to restore comparable inducibility of the mMT-I gene by hypoxia compared with zinc. One posttranscriptional mechanism implicated in the induction of specific mRNA accumulation in hypoxic cells is message stability (43–46). Our estimates of C2C12 MT-I mRNA half-lives indicated that these were in excess of 7 h for both aerobic controls and hypoxic cells with no discernible difference (data not shown). Therefore, changes in the mRNA accumulation probably do not contribute to the hypoxia-associated increases in MT-I mRNA.

Several hypoxia-responsive transcription factors from mammalian cells have been identified, including HIF-1 (47–50), nuclear factor-κB (51), p53 (52, 53), c-Jun, and c-Fos (42, 43, 54). In addition, the ARE has been shown to be responsive to hypoxia (55). The heterodimeric complex of HIF-1, which binds to the hypoxia-sensitive HRE, is a ubiquitous hypoxia-sensitive transcription factor that regulates a variety of hypoxic stress genes (56). Our data, including functional and binding studies, demonstrated that neither HIF-1 nor HREs are involved in the regulation of MTs by hypoxia. Furthermore, the AP1 site in the hMT-IIA promoter and the ARE in the mMT-I promoter do not appear to be directly activated by hypoxia but probably cooperate with other elements to maintain high basal and inducible promoter activities. Therefore, the MREs represent a stress response activated by both hypoxia and oxidative stress signals (21, 22) and controlled, at least, by modulations of MTF-1.

Hypoxia is known to have roles in a number of physiological and pathophysiological processes, including erythroid development, angiogenesis, wound healing, cardiovascular-related diseases, and neoplasia. For example, many animal tumors contain a significant fraction of hypoxic cells that can cycle by hypoxic and reoxygenation states, and it is believed that these regions affect therapeutic responsiveness and malignant progression partially through increased synthesis of a set of hypoxic stress proteins (57, 58). As mentioned above, MT is both a hypoxic and an oxidative stress protein that contributes to the resistance of mammalian cells to reactive oxygen intermediates (generated by a variety of conditions including electrophilic and other antineoplastic drugs, radiation, and ischemia/reperfusion) and that may regulate both cellular proliferation and apoptotic pathways (5, 9, 59). We further suggest that MTF-1 is

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5 B. J. Murphy and M. Yanovsky, unpublished results.
a determinant of clinically important malignant phenotypes not only through redox control of MT expression but also through regulation of other target genes such as γ-glutamylcysteine synthetase (13, 60). For example, enhanced GSH synthesis owing to MTF-1 activation in tumor microenvironments could complement or synergize with a mechanism of drug resistance involving overexpressed cellular sulf-hydryl proteins such as MT.

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Activation of Metallothionein Gene Expression by Hypoxia Involves Metal Response Elements and Metal Transcription Factor-1

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