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-1 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-1 promoter demonstrated that at least two other MREs (mMRE-d and mMRE-c) are responsive to hypoxia.

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 Cys2His2 family of zinc finger transcription factors (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-neoplastic agents, various mutagens, ionizing radiation, and nitric oxide (4, 5). Other studies indicate roles for MTs in the regulation of 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-1 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-1 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 alleles (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 cotransflecting 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.

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3 The abbreviations used are: MT, metallothionein; MTF, metal transcription factor; GSH, glutathione; USF, upstream stimulatory factor; ARE, antioxidative response element; USF, upstream stimulatory element; MEF, mouse embryo fibroblast; Luc, luciferase; HIF, hypoxia-inducible factor; HRE, hypoxia response element; CMV, cytomegalovirus.
(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 transcriptional factor that regulates coordinate gene expression in hypoxic and reoxygenated tumor microenvironments.

MATERIALS AND METHODS

Cell Culture and Hypoxia. The culture of C512 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. The final O2 tension at the end of the pump/gas period was ±0.1% of atmospheric O2. After incubation at 37°C for periods up to 14 h, the chambers were opened under N2 in a humidified anaerobic chamber (Anaerobe Systems, Santa Clara, CA) and harvested. Acrobic controls were incubated in 5% CO2/air at 37°C.

Northern Blots. Our procedure for Northern analysis is described elsewhere (17). Blots were probed with a EcoRI/HindIII (1.85-kb) fragment of the mMT-I genomic DNA (American Type Culture Collection, Rockville, MD) labeled by the random primer method. Signals were quantified by video densitometry using a Lymx 4000 image analyzer and normalized to β-actin mRNA levels detected by using a 32P-labeled, 200-bp oligomer of human β-actin (Clontech, Palo Alto, CA).

MT Plasmid Constructs. The vector pH51, containing −764 to 76 bp relative to the transcription start site of the hMT-IIA gene, was a generous gift of T. Mulcahy (University of Wisconsin-Madison, Madison, WI). Constructs containing successive deletions of the proximal promoter (from −167 bp) were generated by the PCR using the primers described below and pH51 as the template. Primers were designed from the published hMT-IIA sequence (30), and the amplified fragments were cloned into the XhoI/HindIII site of the pGL2 Basic luciferase reporter plasmid (Promega Corp., Madison, WI). All resulting constructs were confirmed by double-stranded sequencing using the PCR primers. The following mMT-I promoter constructs have been described elsewhere (22): pmMT-I(-153)-Luc containing the −153 to 62-bp fragment; pmMT-I(-153) containing the −153-bp deletion mutant (−100 to −89 deleted); pmMT-I(-42)-Luc containing the −42 to 62 minimal promoter, and pmMT-I(-MRE-)-Luc containing five tandem copies of MRE repeats cloned upstream of the minimal promoter from pmMT-I(-42)-Luc. The primers used were as follows (mutant [mut] bases are shown in lowercase): universal 3′-primer (to 23 of hMT-IIA), TTATAAAGTTTGGATCTTGGAGGAGGGCGTGTTGAGTTCGAC; 5′-primers to −167 bp [phMT-IIA(-167)-Luc], TTTCCTCAGAGTCAAGACCGGCGT; 5′-primers to −90 bp [phMT-IIA(-90)-Luc], TTTCCTCAGAGGCGGGCTGTCGACGACGCGGCGCGCGCC; 5′-primers to −70 bp [phMT-IIA(-70)-Luc], TTTCCTCAGAGGCGGGCGGTTTGGTGACTCTGCT; 5′-primers to −57 bp [phMT-IIA(-57)-Luc], TTTCCTCAGAGGCGGGCGGTTTGGTGACTCTGCT; and 5′-primers to −70 bp (hMRE-a mut; phMT-IIA(-70 mut)-Luc), TTTCCTCAGAGGCGGGCGGTTTGGTGACTCTGCT.

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 mMT-I MREs, including a consensus MRE (MRE-s) that lacks an Sp1-like overlapping sequence, were as follows: mMRE-d, 5′-GATCCAGGGAGCTCTGACACGGCCAAAGTA-3′ and 3′-GTCGATCGGAGCTGAGGCGGCCCTCTTTGATTTCAGT-5′; mMRE-s mut, 5′-GATCCAGGGAGCTTTAATATGACCCGGCCGGCTTCTTAC-3′ and 3′-GATCCCGAGCTGAGGCGGCCCTCTTTGATTTCAGT-5′; and mMRE-s, 5′-GATCCCGAGCTGAGGCGGCCCTCTTTGATTTCAGT-3′ and 3′-GTCGATCGGAGCTGAGGCGGCCCTCTTTGATTTCAGT-5′. 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 mMT-I MREs, including a consensus MRE (MRE-s) that lacks an Sp1-like overlapping sequence, were as follows: mMRE-d, 5′-GATCCAGGGAGCTCTGACACGGCCAAAGTA-3′ and 3′-GTCGATCGGAGCTGAGGCGGCCCTCTTTGATTTCAGT-5′; mMRE-s mut, 5′-GATCCAGGGAGCTTTAATATGACCCGGCCGGCTTCTTAC-3′ and 3′-GATCCCGAGCTGAGGCGGCCCTCTTTGATTTCAGT-5′; and mMRE-s, 5′-GATCCCGAGCTGAGGCGGCCCTCTTTGATTTCAGT-3′ and 3′-GTCGATCGGAGCTGAGGCGGCCCTCTTTGATTTCAGT-5′. A construct containing five tandem copies of the hMRE-a was assembled using a monomer containing the 15-bp hMRE-a sequence and 5-bp natural flanking sequences on both the 5′ and 3′ sides; GGGGCTTTTGGCAGTCTGCGGC. The fragment was also inserted between the XhoI/BglII sites of pmMT-I(-42)-Luc.

Cell Transfections and Treatments. Subconfluent C512 myoblasts or NIH3T3R cells were used for analysis of both the hMT-IIA and mMT-I promoters 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−/− fibroblast cells (dko7) involving 2.0 µg of pmMT-I(-153)-Luc and variable amounts of an mMT-I 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 control (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 Biotek 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 also immortalized with SV40 large T antigen, were obtained from Dr. John Lazo, University of Pittsburgh (Pittsburgh, PA; Ref. 28). Both lines were also immortalized with SV40 large T antigen, were obtained from Dr. John Lazo, University of Pittsburgh (Pittsburgh, PA; Ref. 28).

Nuclear Extracts and Electrophoretic Mobility Shift Assays. Nuclear extracts were prepared from confluent C512 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 containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.4 mM phenylmethylsulfonfluoride, 1 mM sodium vanadate, 5 mM β-glycerophosphate, 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′-AGCTTCGCGGCTTTTGGACCTGCTGGCCTCTACA-3′; hMRE-a mut, 5′-AGCTTCGCGGCTTTTGGACCTGCTGGCCTCTACA-3′; hMRE-a mut, 5′-AGCTTCGCGGCTTTTGGACCTGCTGGCCTCTACA-3′; hMRE-a mut, 5′-AGCTTCGCGGCTTTTGGACCTGCTGGCCTCTACA-3′; hMRE-a mut, 5′-AGCTTCGCGGCTTTTGGACCTGCTGGCCTCTACA-3′; hMRE-a mut, 5′-AGCTTCGCGGCTTTTGGACCTGCTGGCCTCTACA-3′; hMRE-a mut, 5′-AGCTTCGCGGCTTTTGGACCTGCTGGCCTCTACA-3′; hMRE-a mut, 5′-AGCTTCGCGGCTTTTGGACCTGCTGGCCTCTACA-3′. Compiled oligonucleotides were end-labeled using the T4 polynucleotide kinase (Promega) and [γ-32P]ATP (New England Nuclear). Equal amounts of radioactive probe (1.5–2.5 × 104 cpm) were added to binding reactions that contained 8 µg of protein in 20 µl of a buffer containing 4 mM Tris (pH 7.8), 12 mM HEPES (pH 7.9), 60 mM KCl, 30 mM NaCl, 0.1 mM EDTA, and 1 µg of poly(odeoxinosinopolydeoxycytidylic acid; Pharmacia). Reactions were incubated for 15 min at 22°C before separation on

Fig. 1. Northern analysis of mMT-I induction kinetics. C512 cells were exposed to hypoxia (≤0.1% O2) for 0–12 h, and mMT-I RNA was detected by Northern analysis using a 32P-labeled MT-I EcoRI/HindIII fragment of the mouse MT-I genomic DNA. This representative blot was reprobed for β-actin mRNAs as described in “Materials and Methods.”
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 μg) against a recombinant 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 expressed in Escherichia coli 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 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).4 Hypoxia treatments resulted in 12.8 ± 7.0-fold inductions of state levels of mMT-I mRNA after 12 h; ZnCl2 exposures (100 μM) resulted in >17-fold induction. These values are similar to those reported for MT-IIA 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 −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 ± 5.5-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, 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 hypoxia and zinc (data not shown; see also Refs. 36 and 37), we used the cell line NIH3T3R in which endogenous MTs are strongly induced by both hypoxia and zinc (data not shown; see also Refs. 36 and 37), we used the cell line NIH3T3R in which endogenous MTs are strongly induced by both hypoxia and zinc (data not shown; see also Refs. 36 and 37), we used the cell line NIH3T3R in which endogenous MTs are strongly induced by both hypoxia and zinc (data not shown; see also Refs. 36 and 37), we used the cell line NIH3T3R in which endogenous MTs are strongly induced by both

Fig. 2. Mapping of the hMT-IIA proximal promoter element involved in the transcriptional response to hypoxia. A, a schematic representation of the deletion analysis of the proximal promoter of the hMT-IIA gene. Mean Luc activities relative to hMT-IIA(−167)-Luc are shown for each construct. C2C12 cells were transfected with the indicated promoter-reporter constructs as described in “Materials and Methods.” Promoter constructs containing successive deletion fragments from −167 to −57 (to 23) were generated by PCR and cloned into pGL2 (Promega); the hMRE-a mutant was constructed using pMT(−70)-Luc. B, after a recovery period of 24-48 h, cells were exposed to hypoxia or zinc for 12 and 6 h, respectively. Cells were harvested, and cell lysates were assayed for Luc activities. Reporter inductions were calculated as the ratio of normalized Luc activity in treated cells to that in control vector inductions were calculated as the ratio of normalized Luc activity in treated cells to that in control vector inductions were calculated as the ratio of normalized Luc activity in treated cells to that in control vector inductions were calculated as the ratio of normalized Luc activity in treated cells to that in control

4 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 (2100 to 289) 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 pMT-I(242)-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'5)-Luc or pmMT-I(MRE-d'5)-Luc], respectively) conferred significant hypoxia inducibility to the minimal mouse promoter, pMT-I(242)-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
Presence of MTF-1 in C2C12 cells and to determine whether hypoxia binding studies

153)-Luc (1 m^2 m^153)-Luc reporter constructs. To determine the effects of ectopic expression of the hMT-IIA hypoxia-treated cells were analyzed using the hMRE-a from the response of hypoxia or zinc. These data confirm that MTF-1 plays a role in the cotransfected as an internal control and was not responsive to either Renilla simplex virus-thymidine kinase promoter and a construct in MTF-1^−/− cells are summarized in Fig. 4B. The expression of luciferase was not significantly responsive to hypoxia or zinc. When pmMT-I^−/−)-Luc was cotransfected with CMV-mMTF-1, the response to hypoxia or zinc treatment was restored. Specifically, a titration of pmMTF-1 (0–2.0 μg) indicated that Luc activities were increased ~3-fold by hypoxia and 9-fold by zinc at a concentration of 0.5 μg of CMV-mMTF-1. A reporter construct, containing a herpes simplex virus-thymidine kinase promoter and a Renilla luciferase, was cotransfected as an internal control and was not responsive to either hypoxia or zinc. These data confirm that MTF-1 plays a role in the response of MT genes to hypoxia.

Binding Studies

Electrophoretic mobility shift assays were used to confirm the presence of MTF-1 in C2C12 cells and to determine whether hypoxia affected MTF-1 binding activity. Nuclear extracts from zinc- and hypoxia-treated cells were analyzed using the hMRE-a from the hMT-IIA promoter (Fig. 5A) and the consensus mMRE-s (Fig. 5, B and C). A specific hMRE-a-binding complex was detected in nuclear proteins from cells exposed to hypoxia for 8 or 24 h. Formation of complex M was suppressed by competition with unlabelled hMREa. Importantly, the formation of this complex was not competed by an oligonucleotide with a mutated hMRE sequence (hMRE-a mut; see “Materials and Methods”) or by an oligonucleotide containing a consensus HIF-1 binding site. Fig. 5B shows that the specific complex was induced within 2 h of exposure to hypoxia. In Fig. 5C, the effect of MTF-1-specific antibody was tested. Anti-MTF-1 antisera treatment of hypoxic extracts (8 and 24 h) eliminated the low mobility complex but did not affect the higher mobility band (Fig. 5C, NS).

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 constructs in MTF-1^−/− cells are summarized in Fig. 4B. The expression of luciferase was not significantly responsive to hypoxia or zinc. When pmMT-I^−/−)-Luc was cotransfected with CMV-mMTF-1, the response to hypoxia or zinc treatment was restored. Specifically, a titration of pmMTF-1 (0–2.0 μg) indicated that Luc activities were increased ~3-fold by hypoxia and 9-fold by zinc at a concentration of 0.5 μg of CMV-mMTF-1. A reporter construct, containing a herpes simplex virus-thymidine kinase promoter and a Renilla luciferase, was cotransfected as an internal control and was not responsive to either hypoxia or zinc. These data confirm that MTF-1 plays a role in the response of MT genes to hypoxia.
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 heptanucleotide core sequence TGC(A/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 enhancement(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 enhancerome, 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.5 The ability of ectopic expression of mMTF-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 erythropoietin development, angiogenesis, wound healing, cardiovascular-related diseases, and neo-plasia. For example, many animal tumors contain a significant fraction of hypoxic cells that can cycle between 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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