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Metal transcription factor-1 is involved in regulation of metallothionein by hypoxia

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METAL TRANSCRIPTION FACTOR-1 IS INVOLVED IN REGULATION OF
METALLOTHIONEIN BY HYPOXIA

A Thesis

Presented to

The Faculty of the Department of
Biological Sciences
San Jose State University

In Partial Fulfillment

Of the Requirement for the Degree
Master of Science

By

Marianna Yanovsky

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ABSTRACT

METAL TRANSCRIPTION FACTOR-1 IS INVOLVED IN REGULATION OF METALLOTHIONEIN BY HYPOXIA

By Marianna Yanovsky

Metallothioneins (MTs) are small cysteine-rich proteins whose structure is conserved from fungi to man. MTs bind heavy metals such as zinc, cadmium, and copper. They may also contribute to the regulation of cellular proliferation, apoptosis, and malignant progression. This study demonstrates that the mouse metallothionein gene (MT-I) is transcriptionally activated by hypoxia. The data further demonstrate a potential involvement of the metal transcription factor-1 (MTF-1). MT-I gene expression in fibroblasts from mice with targeted deletions of both MTF-1 alleles (dko7) was not induced by zinc and showed low responsiveness to hypoxia. A transfected MT promoter was unresponsive to hypoxia or zinc in dko7 cells, but inductions were restored by cotransfecting with a mouse MTF-1 expression vector. Therefore, these results demonstrate that MT gene expression involves MTF-1.

Dedicated to my Mom, who inspired,
and to my Dad, who insisted.

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INTRODUCTION

In the 17th century when Pizzaro and his armies attacked the Incas, physicians noticed that the soldiers' blood became very viscous at high altitudes. Centuries later it was shown that the low oxygen tension caused the production of a hormone, erythropoietin, which initiated the production of red blood cells causing increased viscosity of the blood (Pahl and Baeuerle, 1994). Low oxygen tension or hypoxia is a decrease in the extracellular concentration of oxygen. Changes in oxygen tension yield the cascade of transduction reactions causing gene regulation through the common mechanism of O₂ sensing, signal transduction, and transactivation. Today it is recognized that hypoxia is involved in many pathological and physiological processes, including angiogenesis, wound healing, fibrosis, and neoplasia (Helfman and Falanga, 1993).

Hypoxic Stress

Hypoxia has been associated with malignant progression and resistance to radiotherapy and chemotherapy. Most human solid tumors contain heterogeneously oxygenated and hypoxic regions. In normal tissue, the mean oxygen concentration exceeds 40 mm Hg, whereas in malignant tumors, including breast, cervical, and squamous cell carcinoma, the oxygen concentration is 0 to 10 mm Hg (Sutherland et al., 1996).

For instance, patients with cervical carcinoma have a $pO_2 < 10$ mm Hg (measured *in vivo*); these patients have poorer disease-free survival (Zhong et al., 1998). Patients with well-oxygenated tumors have a better survival rate than those with the hypoxic tumors. Therefore, the hypoxic microenvironment affects the response to chemotherapy and promotes malignant progression (Sutherland et al., 1996; Coquellle et al., 1998).

Many cancer and normal mammalian cells respond to hypoxic stress by induction of specific proteins. These proteins are called oxygen-regulated proteins (ORPs). Several classes of hypoxic-regulated proteins have been identified. They include glucose regulated proteins involved in chaperone functions, proteins involved in oxidative stress and homeostasis regulation, transcription factors triggering immune response and signal transduction pathways (Sutherland et al., 1996). Laboratory research has identified a hypoxic stress protein, human metallothionein II (MT-II) (Murphy et al., 1994), that was also known to function in oxidative stress (Dalton et al., 1994). Metallothioneins were discovered in 1957 and after 40 frustrating years they still remain a protein in search of a function (Vallee, 1995).

Metallothioneins

Metallothionein (MT) genes encode low molecular weight proteins that are characterized by 30% cysteine content and high affinity for metals. These genes have been found in most eukaryotic organisms, and their structure has been preserved from fungi to man (Palmiter, 1986, Radke et al., 1993). MTs are involved in many cellular processes such as detoxification of heavy metals, adaptation to stress, cell proliferation, and metal homeostasis. In addition, MT may contribute to the regulation of apoptosis and malignant progression (Karin and Richards, 1982; Radke et al., 1993; Palmiter et al., 1993; Nagel and Vallee, 1995; Murphy et al., 1999).

Metallothioneins are unique proteins in which cysteine constitutes one third of the amino acids and histidine and aromatic amino acids are completely absent. The positions of 20 cysteines are conserved; these cysteines bind seven zinc atoms such that one atom of zinc is bound to four cysteines (Palmiter et al., 1993; Maret and Vallee, 1998). MTs usually bind seven zinc atoms, but they also can bind copper, cadmium and traces of other metals (Fisher and Davie, 1998). MT binds the essential metals zinc and copper under physiologic conditions and the toxic metals, cadmium and mercury, under pathologic conditions.

The mammalian MT family consists of two nontissue specific isoforms (MT-I, MT-II), a brain specific MT-III and a squamous epithelium specific MT-IV isoforms, as is show in Table 1. The two isoforms, MT-I and MT-II differ by single amino acid charge at neutral pH. MT-III contains two additional amino acid insertions that distinguish this isoform from MT-I and MT-II. MT-IV also contains an insertion of one amino acid and is expressed only in differentiating squamous epithelia (Borghesi and Lynes, 1996).

Table 1. MT isoforms

Gene	Location	Reference
MT-I	Most organs, maternal deciduum	Palmiter, 1998
MT-II	Most organs, maternal deciduum	Palmiter, 1998
MT-III	Neurons, male reproductive organ, maternal deciduum	Palmiter, 1998
MT-IV	Stratified squamous epithelial cells, maternal deciduum	Palmiter, 1998

The human MT genome consists of at least 16 genes: seven or eight code for functional proteins, two are mutant genes, and the rest are nonfunctional pseudogenes (Richards et al., 1984; Palmiter, 1998). All functional MT genes are located on chromosome 16, and the mutant MT sequences are clustered at 16q22. MT sequences located on other

chromosomes are pseudogenes (Hegua et al., 1986). The MT-IIa gene is the major human MT gene and it accounts for 50% of the total metallothionein expressed in cultured human cells (Palmiter et al., 1993). Comparison of the human and murine metallothionein promoters revealed nucleotide sequence homology, suggesting that these genes are recognized by similar cellular proteins (Karin and Richards 1982). Murine MTs display less diversity than human MTs. In mice, four MT genes are arranged within a 50 kilobase (kb) segment of chromosome 8 (Quaife et al., 1998). The four MT proteins range from 61 to 68 amino acids. The positions of the 20 cysteines are conserved and they all lack aromatic amino acids (Palmiter et al., 1993). All four MT proteins appeared to bind zinc, cadmium and copper equivalently (Quaife et al., 1998).

MT Induction and Function

Metals are the most effective inducers of MT gene expression in mouse cells. MT gene expression is also controlled by glucocorticoids, cytokines, and growth factors. Increased levels of cytokines, such as interleukin-1, interleukin-6, tumor necrosis factor- α , and γ -interferon contribute to induction of MT expression (Abdel-Mageed and Agrawal, 1998). MTs are also stimulated by surgery and growing tumors (Haga et al., 1996). Transformed cells

undergoing rapid growth have high metallothionein protein levels compared to primary cells (Cherian, 1994). The concentration of metallothioneins increases 24 and 48 hours after surgery (Akintola et al., 1995). Therefore, MTs are involved in cell differentiation and proliferation (Nagel and Valle, 1995; Boghesi and Lynes, 1996).

Resistance to anticancer drug therapy is the most important factor in the failure of cancer chemotherapy. Metallothioneins are nucleophilic and they are known to protect cells against electrophilic anticancer drugs and mutagens (Kontozoglou et al., 1989; Lazo and Pitt, 1995). It has been shown that MT expression was increased in some tissues after *in vivo* treatment with DNA alkylating agents such as cisplatin, doxorubicin, melphalan, bleomycin, N-methyl-N-nitrosourea, and N-methyl-N'-nitro-N-nitrosoguanidine. Koizumi et al., (1995) demonstrated that high expression of MT was correlated with poor prognosis in human breast and lung cancer respectively. Other findings suggested that the activation of metallothioneins following cisplatin administration might be a contributing factor in the development of cisplatin resistance in renal carcinoma cells (Lazo and Pitt, 1995). Inhibiting MT synthesis with propargylglycine (cystathionase inhibitor) has been reported as a possible solution for controlling cisplatin resistance and the spread of cancer (Sato et al, 1993).

Metallothioneins are involved in protection against radiation damage, oxidative stress, and apoptosis (Sutherland et al., 1996; Lazo and Pitt, 1995; Dalton et al., 1996). MT expression is induced by a variety of stresses known to be associated with scavenging of free radicals. Ionizing radiation, hyperoxia, anticancer drugs, and toxins such as paraquat and phorbol ester, result in increased levels of oxygen reactive intermediates (ROI) and in inducing the overexpression of MTs that in turn protect cells against free radical species (Lazo and Pitt, 1995). Recent reports have shown that wild type MT cells are less susceptible to apoptotic cell death compared to null MT cells (Kondo et al., 1997).

Forty years after the discovery of metallothioneins, their function is still unclear. There are three roles proposed for MT function:

1. metal homeostasis and detoxification (Vallee, 1995; Cherian, 1994);
2. protection against oxidative damage (Satoh et al., 1993; Palmiter, 1998);
3. neuronal growth inhibition in the brain (Uchida et al., 1991);

It seems likely that the function of MTs is related to the presence of physiologically relevant metals such as zinc

and copper. MTs have the high thermodynamic stability and high kinetic ability to bind metals (Roesijadi et al., 1998; Jiang et al., 1998). Thus, MTs could function in metal homeostasis by providing zinc and copper during metal deprivation (Vallee, 1995). Thus, they might serve as chaperones for the synthesis of metalloproteins. MTs could have a function as a reservoir for the essential metals and while preventing metal detoxification, metallothioneins donate metals for biosynthesis of apometalloproteins (Palmiter, 1998). Experiments *in vitro* indicate that these reactions are possible and interaction of MTs with glutathione allow MT to serve as both a cellular reservoir for zinc and a controlled released system that supplies zinc on demand (Jiang et al., 1998). However, genetic experiments suggested that transfer of zinc and copper from MTs to other molecules is not essential (Palmiter, 1998). Yeast cells that cannot produce any MTs can grow in normal media as well as cells that make MTs.

The work of many research groups indicated that all MTs can protect against metal toxicity. However, it is still unclear whether this is a primary function of MT or byproducts of another function (Quaife et al., 1998). Mice with simultaneous disruption of the MT-I and MT-II genes (Mishalska and Choo, 1993) grow and reproduce normally. Only high doses of zinc and cadmium can cause toxicity in these transgenic mice. Cadmium and zinc toxicity is rare and is caused by

man. Therefore, from an evolutionary point of view, cadmium and zinc detoxification could be a preadaption property of MTs rather than their evolved function (Palmiter, 1998).

MTs can protect against oxidative damage. The cysteine zinc clusters are readily oxidized *in vitro* (Maret and Vallee, 1998) and they can scavenge free radicals (Palmiter, 1998; Roesijadi et al., 1998). However, some investigators described results in which cells or mice lacking MT were more sensitive to oxidative stress caused by chemical agents, drugs or toxins, whereas other investigators found conditions where overexpression of MT protected against treatments thought to produce oxidative damage (Palmiter, 1998).

The recent discovery of MT-III and IV with more restricted expression suggested that these isoforms have distinct functions which might shed light on the more widely expressed isoforms, MT-I and MT-II (Palmiter, 1998). Indeed, MT-III has been shown to inhibit the survival of neurons cultured with brain extracts from people with Alzheimer's disease (Uchida et al., 1991). This inhibition was not observed with MT-I or MT-II isoforms. Also, MT-III competes for available zinc whereas MT-I does not (Palmiter and Findley, 1995). The metal binding properties of MT-IV have not been examined biochemically (Quaife et al., 1998). These results indicated that all these isoforms have different

biological functions; however, their physiological roles have not been revealed yet (Palmiter, 1998).

Metal Responsive Elements

As was mentioned earlier, expression of MTs can be induced by a wide ranged of stimuli, including exposure to heavy metals, steroid hormones, interleukins, cAMP, phorbol ester, and interferon (Heuchel et al., 1994; Hegua et al., 1986; Karin and Richards, 1982). The synthesis of metallothioneins is controlled at the transcriptional level and can be induced by a range of heavy metals (Chen et al., 1998). Transcriptional activation by zinc, cadmium, and copper is mediated by several copies of *cis* elements, 15 base pair consensus sequences, termed metal responsive elements (MRE). It has been reported that *cis* elements, important for basal and induced expression of human MT-I and MT-II genes, are pre-sent in the 5'-flanking regions of the promoter region (Karin and Richards, 1982). Similar elements of the MT promoter were found in mouse, sheep, *Drosophila* (Stuart et al., 1985) and Japanese pufferfish (Auf der Maur et al., 1999). The mouse MT-I promoter contains six MREs (MREa-f) within the first 200 bps of the transcriptional start site (Radke et al., 1993). The ability to activate metal-dependent transcription varies between the different MREs. It has been demonstrated that in response to heavy metals, MREd

was the strongest of the MREs of the MT-I and MT-II promoters (Radke et al., 1993; Murphy et al., 1999). In addition to the MREs, the MT-I promoter contains an Sp-1 binding site, antioxidant response element (ARE), upstream regulatory element (URE), and TATA box sequences (Dalton et al., 1996).

Metal Transcription Factor-1 (MTF-1)

Several proteins have been characterized as MRE-binding proteins such as Sp-1 and metal transcription factor-1 (MTF-1). Sp-1 and MTF-1 are zinc finger transcription factors of TFIIIIa type (Cys2 His2). Sp-1 is a transcription factor that drives expression of a wide range of vertebrate promoters (Radke et al., 1995). By deletion analysis it was demonstrated that a promoter lacking the Sp-1 binding site is still responsive to the heavy metals, suggesting that Sp-1 was not directly involved in the activation by metals. Dalton et al. (1996) demonstrated that Sp-1 was constitutively expressed in nuclear extracts treated with zinc. Dalton et al. (1997) also showed that MTF-1 was more sensitive to EDTA concentration compared to Sp-1 and *in vitro* MTF-I was more sensitive to increased zinc concentration than Sp-1 factor (Heuchel et al., 1994).

MTF-1 is activated *in vivo* by oxidative stress and metals (Dalton et al., 1996; Radke et al., 1993). Radke et

al. (1993) and Brunega et al. (1994) cloned mouse and human MTF-1 respectively. Zinc treatment of mouse cells increased the level of the MTF-DNA complex several fold as measured by the band shift assay. In addition, it has been reported (Brunega et al., 1994) that metal induction was more pronounced with human MTF-1 as compared to mouse MTF-1. Heuchel et al. (1994) demonstrated, by targeted disruption of both MTF-1 alleles in mouse embryonic stem cells, that MTF-1 was essential for the basal and the metal induced activity. Furthermore, they presented data showing that MTF-1 responded to changes in intracellular zinc concentration. Functional domains of mouse MTF-1 have been identified by Radke et al. (1995) (Figure 1) and characterized as "a complex of interplay of different functional domains." By deletion and fusion experiments, they revealed three putative activation domains: acidic, proline-rich and serine/threonine domains. The acidic domain is capable of activation over a large distance and proline and serine /threonine domains act only from a proximal promoter.

Figure 1. Schematic diagram showing zinc finger and putative transcription domains of mouse and human MTF-1 protein (Radke et al., 1995).

Recent studies by Gunes et al. (1998) revealed the physiological role of MTF-1 by generating null mutant mice (MTF-/-) by targeted gene disruption. Their finding indicated that embryos lacking MTF-1 die *in utero* after about 14 days of gestation and these embryos failed to transcribe MT-I and MT-II genes. Although heterozygous mice (MTF+/-) developed without abnormalities under laboratory conditions, they were more susceptible cadmium treatment compared to the

wild type. Therefore, MTF-1 is an essential gene for mouse development.

Models of MT Regulation

MT regulation *in vivo* is a complex process of cellular interaction with the appropriate stimuli and it can involve the presence of appropriate transduction pathways (Palmiter and Findley, 1995). A recent study indicates that a protein kinase C dependent pathway may be involved in the regulation of metallothionein gene expression (Studer et al., 1997; Wu et al., 1996). Transcription activation of metallothionein MTF-1 is poorly understood. The first model of MT activation suggested that MTF-1 was regulated by zinc availability; only after a high zinc load binds to MTF-1, would it become a DNA-binding competent. On the other hand, all other zinc dependent transcription factors (e.g. Sp-1) would be saturated with zinc under all physiological conditions (Radke et al., 1993). This model did not take into account that activation of MTF-1 was largely constitutive when transfected into mouse null MTF-/- cells in the absence of additional zinc load (Radke et al., 1993). Palmiter and Findley (1995) reported evidence for a negative mechanism of MTF-1 activation. In this model MTF-1 was constitutively expressed in the presence of a metal-sensitive inhibitor and this inhibitor disassociated from MTF-1 after zinc binding.

How-ever, this inhibitor has never been isolated. Another model was proposed by Radke et al. (1993) where MTF-1 itself was the zinc sensor. He showed that MTF-1 binding to DNA could be reversibly inactivated and reactivated *in vitro* by addition or withdrawal of zinc. Therefore, in a high zinc concentration MTF-1 was converting from a low affinity inactive form to an active DNA binding form. Recently, a new model, which is not consistent with the previously described models of metallothionein activation via MTF-1, has been proposed by Dalton et al. (1997). In this new model, MTF-1 is not constitutively expressed in the mouse cells. It is suggested that reversible activation of MRE binding by MTF-1 involves direct interaction of zinc with the zinc finger do-main of MTF-1. Thus, MTF-1 DNA-binding activity is regulated by direct zinc interaction.

It is known that hypoxia induces a variety of physiologically important genes such as erythropoietin, vascular endothelial growth factor, tyrosine hydroxylase and nitric oxide synthase (Huang et al., 1998). Several lines of evidence have suggested that metallothioneins are transcriptionally induced by hypoxia (Murphy et al., 1994; Sutherland et al., 1996; Murphy et al., 1999). In the present study, transient transfections and Northern studies were used to elucidate MTF-1 involvement in transcription activation of mouse MT-I upon induction with hypoxia.

MATERIALS and METHODS

Cell Culture and Hypoxia

C₂C₁₂, myoblasts (ATCC, Rockville, MD), dko 7 fibroblasts (MTF-/-) (Walter.Shaffner, University of Zurich, Switzerland), and mouse embryo fibroblast (MTF+/+) (John Laze, University of Pittsburg) cells were cultured in DMEM-high glucose medium supplemented with 10% fetal bovine serum (FBS) and 25 mM HEPES buffer, pH 7.4. Fresh frozen stocks of cells were taken after 10 passages. Methods for exposing cells to hypoxia are described elsewhere (Murphy et al., 1994). Briefly, 60 mm culture dishes were incubated in an aluminum chamber at 37° C and made hypoxic by repeated cycles (5 cycles) of partial evacuation and gassing with 5% CO₂/N₂. The final O₂ tension at the last cycle was approximately 0.1% of atmospheric O₂. Plates were incubated at 37° C for 15 hours and then harvested. Aerobic controls were incubated in 5% CO₂/air at 37° C.

Northern Analysis

Total cellular RNA was isolated from C₂C₁₂ myoblasts by the guanidinium isothiocyanate extraction method (Murphy et al., 1994). Total RNA was measured spectrophotometrically and 15 µg were electrophoresed in 1% agarose gels containing formaldehyde and transferred to nylon membranes (Magna,

0.45; Micron Separation Inc.) by blotting in 10XSSC buffer (Murphy et al., 1994). The filters were probed with restriction fragments (1.85 kbp) of the mMT-I genomic cDNA (ATCC, Rockville, MD). The cDNA fragments were labeled with [α -P³²] dCTP using a standard random primed reaction (Amersham, New Jersey) to a specific activity of 1X10⁹ cpm/ μ g. The membranes were hybridize overnight at 42° C in hybridization buffer (Murphy et al., 1994) with 1X10⁶ cpm/ml of probe and washed twice in 2XSSC containing 0.1% SDS at room temperature for 20 minutes, followed by 0.1XSSC/0.1% SDS at 55°C for 15 minutes. The membranes were then autoradiographed on film (Kodak XAR-5) with intensifying screens at 80°C. The blots were reprobbed with a 1 kbp cDNA fragment that coded for human heme oxygenase-1 (HO-1) as a positive control (Sutherland et al., 1996). The HO-1 cDNA (a kind gift of Dr. Rex Tyrrell, University of Bath, UK) cross-hybridizes with rodent HO-1 mRNA and is used as a probe for HO-1 mRNA steady state levels. A mouse cDNA β -actin (Clontech, Palo Alto, CA) was used to normalize the RNA loading. For quantitation, autoradiographs were scanned by video densitometry using a Lynx 4000 image analyzer (Applied Imaging, Santa Clara). Each northern analysis was repeated three times and the average reading from the densitometer was reported.

MTF-1 Null Analysis

The mouse dko7 cell line, which lacks MTF-1, was a gift of Walter Schaffner, University of Zurich, Switzerland. These fibroblasts were derived from embryonic stem cells and immortalized with SV40 large T antigen. Wild type mouse embryo fibroblast cells, also immortalized with SV40 large T antigen, were obtained from John Laze, University of Pittsburgh. Both lines were maintained in high glucose DMEM medium supplemented with 10% FBS and 25 mM HEPES buffer, pH 7.4. The expression plasmid, CMV-mMTF-1, (a generous gift of Glen Andrews, University of Kansas) was created by inserting the mMTF-1 cDNA, synthesized by RT-PCR, into the NotI site of a cytomegalovirus expression vector. The mMT-I promoter construct, pMT-I(-153)-Luc (created by Brian Murphy, SRI), contains the -153 to +62-bp (relative to the transcription site) fragment cloned upstream of the minimal promoter from pmMT-I(-42)-Luc. PGL-2 basic (Promega, Madison, WI) was used as a luciferase reporter gene.

Cell Transfection

Subconfluent (60%-70%) dko 7 fibroblasts (MTF-/-) were used for the analysis of the restoration of hypoxia associated induction in mouse MTF-1 null cells. MTF-/- cells were cotransfected using a standard DEAE dextran method (Murphy et al., 1999). MTF-/- cells were cotransfected with 2 µg of

pMT-I(-153)-Luc and a variable amounts of a pMTF-1 expression vector (0 μ g to 1 μ g). Bluescript phagemid (Clontech, Palo Alto) was added to equalize the amount of transfected DNA per 60 mm plate. Transfections were normalized with a *Renilla* luciferase internal control (Dual-Luciferase Reporter Assay System, Promega, Madison, WI). At 24 hours post-transfection, the medium was changed and the cells were subjected to hypoxia or metal treatment. Cells were treated with 100 μ M of zinc for 4 hours or with 16 hours of hypoxia. Luciferase activity in the cell extracts was assayed with a Promega Biotec Assay Kit and a Turner Designs TD-20/20 luminometer (Promega, Madison, WI). Assays were done on three 60 mm cultures plates for each treatment (air, hypoxia, zinc) and the average fold induction was reported. The relative fold induction refers to the ratio of average luciferase activities (standardized to *Renilla* expression) from hypoxia or zinc treated cells versus untreated cell extracts (air samples). Three independent transfections were performed to confirm reproducibility.

RESULTS

Induction of MT-I mRNA by Hypoxia.

The role of hypoxia in regulation of the MT-I gene was examined in rodent C₂C₁₂ cells under aerobic, hypoxic, or zinc treated conditions. As indicated in Figure 3, hypoxia induced the mouse MT-I gene in a time dependent manner with moderate induction occurring after 4 hours and near maximal induction after 12 hours. Beyond 16 hours, MT mRNA concentration sharply declined (unpublished observations). Overall, hypoxia treatment resulted in a 12.8 ± 7.0 fold induction of steady state levels of mMT-I after 12 hours. A six hour exposure to 100 μ M ZnCl₂, resulted in >17-fold induction. This finding was confirmed in a number of other cell lines including NIH 3T3, ras-transformed NIH 3T3 (NIH 3T3R), and mouse hepatoma (HEPA) cell lines.

The blot shown in Figure 2 was reprobed with β -actin cDNA and a documented hypoxic stress protein, human hemoxygenase-1 (HO-1) cDNA, as a positive control (Sutherland et al., 1996). The steady state levels of HO-1 mRNA were significantly increased by hypoxia, with approximately similar kinetics to that of MT-I transcript, while the levels of the β -actin transcript were not affected, indicating that all samples were loaded equally.

Figure.2 Northern analysis of mMT-I induction. C₂C₁₂ cells were exposed to hypoxia (0.1% O₂) for 0-12 hours and mMT-I RNA was detected by Northern analysis using a ³²P-labelled MT-I *EcoRI/HindIII* fragment of the mouse MT-I cDNA. This blot was reprobed for human HO-1 and for β -actin cDNA as described in Materials and Methods. Northern analysis was repeated three times to confirm reproducibility.

Involvement of MTF-1 in Induction of MT-I Gene.

To investigate the role of MTF-1 in metallothionein gene expression by hypoxia, mRNA levels of the endogenous metallothionein were measured in embryonic mouse fibroblast (MTF+/+) and dko 7 fibroblast (MTF-/-) cell lines. As shown in Figure 3, mRNA levels of MT-I gene were greatly increased after treatment with hypoxia and 100 μ M zinc chloride in MTF+/+ cells. In contrast, hypoxia treatment of MTF-/- null cells resulted in 60% reduction of the MT-I message after 8 hours and induction by zinc chloride was abolished compared with the wild-type MTF+/+.

The partial (10-15%) induction of MT-I message by hypoxia in MTF-1 null cells must be due to other transcription factors or posttranscriptional regulation. These data are in agreement with other reports (Koizumi et al., 1992; Radke et al., 1993; Inoyne et al., 1994; Remondelli and Leone, 1997). Although it has been shown that MTF-1 was essential in basal and metal induction (Radke et al., 1993; Heuchel et al., 1995), it seems likely that MTF-1 may function in cooperation with other transcription factors (Brittel et al., 1998).

Figure 3. Induction of mMT-1 mRNA in aerobic and hypoxic MTF-1 (dko 7; MTF-/-) and wild type cells (MTF+/+). Wild type cells and null cells were exposed to either hypoxia (0.1% O₂) or zinc chloride (100 μM). The membranes were reprobbed with β-actin to normalize the equal loading. Northern analysis was repeated three times.

Restoration of MT-I Expression by pmMTF-1

To restore metallothionein transcription of MT-I gene, the MTF-1 cDNA was transiently transfected into null MTF-/- cells. These cells were transfected with the pmMT-I(-153)-Luc construct and cotransfected with 0 to 2 µg of CMV-mMTF-1 vector. A construct containing the MT-I promoter region, spanning from -153 to+ 67 bp (relative to the transcription start site) was inserted upstream of a luciferase reporter gene (pGL2-basic). The proximal MT-I promoter contains five MREs, an Sp-1 binding site, an upstream stimulatory factor (USF), an antioxidant response element (ARE), and consensus TATA box sequence (Figure 4).

Figure 4. A schematic representation of the proximal promoter of the mouse MT-I gene (top) and control luciferase (bottom) constructs (Murphy et al., 1999).

As shown in Figure 5, mutant null cells transfected with only the pMT-I (-153)-Luc construct have barely detectable MT-I gene expression with either zinc or hypoxia

Figure 5. Transient transfections with pMT-I(-153)-Luc reporter construct and CMV-mMTF-1 vector in dko-7 cells. Dko-7 cells were transfected with 2 μg pMT-I(-153)-Luc reporter construct. 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 2 μg pMT-I(-153)-Luc into dko-7. After 24 hours recovery period cells were exposed to hypoxia or zinc. Cells were lysed and assayed for luciferase activity as described in the Materials and Methods section. The relative fold induction refers to the ratio of luciferase activity measured in treated cells relative to the activity observed in the untreated controls. Values represent the means of three independent transfections that were carried out in triplicates. Error bars represent the standard deviation of the mean ($\pm\text{S.D.}$).

treatments. Cotransfection with up to 1 μ g CMV-mMTF-1 restored gene expression in response to 16 hours of 0.1% hypoxia or 100 μ M zinc for 4 hours. Specifically, a titration with 0.5 μ g of mMTF-1 expression vector increased hypoxia induced expression by 3-fold and zinc induced expression by 9-fold. These data confirm that MTF-1 is involved in the response of metallothionein gene expression to hypoxia. A reporter construct, containing the HSV-thymidine kinase promoter and a *Renilla* luciferase, was cotransfected as an internal control. The minimal promoter (Figure 4) from pmMT-I(-42)-Luc was barely detectable upon transfection with either zinc or hypoxia.

DISCUSSION

Diverse mechanisms have evolved to regulate transcription in response to environmental stresses. There is growing evidence that hypoxia regulates transcription of a broad range of genes and induction of these genes confers multiple responses to maintain oxygen homeostasis (Huang et al., 1996). As generally accepted, metallothionein genes are induced by heavy metals and oxidative stress. In the work reported here, it has been shown that hypoxia is also involved in the process of metallothionein gene expression, and metal transcription factor-1 (MTF-1) alone or with cooperation of some other factors is responsible for the transcriptional activation of the MT gene.

Northern blotting (Figure 2) demonstrated that mouse MT-I mRNA accumulates as a function of time during 12 hours of hypoxia. Maximum levels of mMT-I mRNA were attained by 12 hours of hypoxia ($\leq 0.1\%$).

Transient transfection studies (Figure 5) with a null MTF-/- cell line show that basal, metal and hypoxic induction of MT transcription can be restored by cotransfection with the CMV-pMTF-1 expression vector. The finding that MTF-1 was also necessary for the restoration of the basal activity suggests that MTF-1 is able to bind to the MT-I promoter under non-stressed physiological conditions (Heuchel et al., 1994).

Induction of MT-I transcription by hypoxia (Figure 3) was only partially (10-15%) blocked in the MTF-/- cells, indicating the involvement of other transcription factors and mechanisms. These data are in agreement with other reports (Koizumi et al., 1992; Radke et al., 1993; Inoyne et al., 1994; Remondelli and Leone, 1997). Several groups have claimed detection of a protein with some of the features of the metal responsive factor-1, but none of these groups provided conclusive proof that the detected factor was indeed the metal transcription factor. For example, several studies described reported the isolation of the transcription factor that can bind to the MRE region of the MT promoter (Koizumi et al., 1992; Radke et al., 1993; Inoyne et al., 1994; Remondelli and Leone, 1997). This factor was called metal transcription factor-1 (MTF-1) by some laboratories or zinc regulatory factor (ZRF) by others. However, a mobility shift assay indicated that this factor was activated only by zinc and not by cadmium (Koizumi et al., 1992; Radke et al., 1993; Inoyne et al., 1994) or visa versa (Remondelli and Leone, 1997).

It could be speculated that depending on the localization of MT in the cell, different transcription factors are required for binding to the metallothionein gene to induce the transcription. Woo and Lazo (1997) postulated that the subcellular distribution of metallothionein proteins affected the functionality of these proteins. They

demonstrated that cytoplasmic metallothionein expression protected cells against oxygen reactive species (ROI) and the cytotoxicity of cadmium. On the other hand, nuclear expression of MT protected cells against the mutagenic agent, N-methyl-N'-nitro-N-nitrosoguanidine. Moreover, a recent study by Brittel et al. (1998) suggested that heavy metals other than zinc activated metallothionein gene expression by mechanisms independent of MTF-1 binding to DNA. Therefore, cellular distribution and diverse inducers of metallothionein gene expression may require different trans active elements.

It can be suggested that binding of MTF-1 to the MT-I promoter can also be stimulated by some other transcription factors. These data are in agreement with other reports (Dalton et al., 1997; Abdel-Mageed and Agrawal, 1998; Gunes et al., 1998). Transcriptional factors such as AP-1 (Jun/Fos) or NF-kB might be involved in cooperative activation of MT genes with MTF-1. It is clear that MTF-1 plays an integral part in the transduction pathway resulting in activation of MT gene transcription (Brittel et al., 1998). However, de-termination of the nature of this mechanism of MT gene regulation requires further investigation. AP-1 (Jun/Fos) or NF-kB are induced by a number of common stimuli with MTF-1, including stress signals, oxidative stress and hypoxia (Dalton et al., 1997;

Abdel-Mageed and Agrawal, 1998; Gunes et al., 1998; Britel et al., 1998). AP-1 was demonstrated to be composed of a homodimeric unit, c-Jun, or a heterodimeric complex consisting mostly of the c-Jun and c-Fos onco-proteins. In addition, many other AP-1 family members have been described (Pahl and Baeuerle, 1994). AP-1 consensus sequences are also located in the 5'-flanking region of the MT promoter (Studer et al., 1997; Dalton et al., 1996). It has been reported that exposure of cells to hypoxia causes an increase in c-Jun induction and activation of transcription, leading to increased AP-1 binding activity (Powis et al., 1995; Pahl and Baeuerle, 1994; Bunn and Poyton, 1996). Other reports showed that in SiHa SC cells severe hypoxia ($pO_2 < 0.1$ mm Hg) under normal glucose conditions caused mRNA increases of about 5-fold for c-Jun and about 3-fold for c-Fos within 6 hours. When these cells were subjected to hypoxia and low glucose, there was an induction of 30-fold in c-Jun and only a slight increase in c-Fos (Sutherland et al., 1996).

Another transcription factor that might be involved in activation of metallothionein genes is NF- κ B (Dalton et al., 1997; Abdel-Mageed and Agrawal, 1998). This transcription factor is induced by diverse stimuli, such as oxidative stress, interleukin-1, lipopolysaccharides (LPS), UV and γ -rays, and metals (Sutherland et al., 1996; Pahl and

Baeuerle, 1997; Abdel-Mageed and Agrawal, 1998; Powis et al., 1995). In vertebrates, the NF- κ B family is composed of five DNA-binding units, called p50, p52, p65 (RelA), c-Rel, and Rel-B. These subunits can form homo- or heterodimers in various forms. Most frequently, NF- κ B is composed of p50/p65 heterodimers (Muller et al., 1997). In mammalian cells activity of NF- κ B is regulated by its association with the inhibitory subunit, I κ B, which remains inactive in the cytoplasm. Phosphorylation of I κ B by various kinases leads to the dissociation of this subunit from the complex, allowing the active p50/ p65 heterodimer to move to the nucleus. Exposure of Jurkat T cells to hypoxic conditions resulted in increased NF- κ B binding to DNA due to tyrosine phosphorylation and dissociation of the I κ B inhibitory subunit (Powis et al., 1995; Sutherland et al., 1996). A recent study by Gunes et al. (1998) noted:

1. c-Jun mutant embryos showed a liver phenotype similar to MTF-1 null mutants. MTF-1 expression was not changed in cells derived from c-Jun null mice;

2. p65/relA mutant embryos of NF- κ B exhibited similar liver failure in fetal development as in MTF-1 null mice. No altered expression of p65/relA was found in MTF-1 null mice;

The similarity of the activation stimuli and the phenotype of MTF-1, c-Jun, and NF- κ B in the null mice suggest that all these factors can be involved in the transcription activation of metallothionein genes. This may be accomplished by interaction between all of these transcription factors; while one of the factors contacts the MT binding site, the others might act as accessory factors without binding to DNA (Abdel-Mageed and Agrawal, 1998).

In contrast, recent experiments with HeLa tumor cells confirmed that AP-1 and NF- κ B respond in different ways to hypoxia (Muller et al., 1997). Consistent with the previous reports, hypoxia strongly induced AP-1 DNA binding activity. However, NF- κ B was not activated under hypoxic conditions. Only upon reoxygenation of the hypoxic cultures was the p50/p65 complex of NF- κ B strongly induced. Therefore, it can be speculated that hypoxia blocks NF- κ B activation while inducing the AP-1 transcription factor.

Although nobody has directly demonstrated that MTs are activated by phosphorylation through the protein kinase C (PKC) dependent pathway, there is evidence that may support this possibility. It has been reported that MTF-1 contains several consensus PKC phosphorylation sites in the zinc finger domain and a serine/threonine-rich domain at the C terminal site (Wu et al., 1996; Radke et al., 1993). Therefore, it is possible that expression of MT genes is acti-

vated through the phosphorylation of MTF-1 via a PKC-dependent pathway.

AP-1 and NF- κ B are both activated by protein kinase C (Studer et al., 1997; Bunn and Poyton, 1996; Powis et al., 1995). NF- κ B induces expression of a number of genes, including defense and growth proteins. PKC activates NF- κ B by serine/threonine phosphorylation, leading to the release of the inhibitory I κ B subunit (Powis et al., 1995). The AP-1 family of leucine-zipper transcription factors has also been reported to be activated by protein kinase C. The affinity of AP-1 for the MT binding site is known to be increased by the activation of PKC; different isoforms of PKC have been suggested to be positive regulators of MT induction in rat liver tissue after hepatectomy (Studer et al., 1997). In contrast to this result, a recent study by Wu et al. (1996) reported that, although PKC regulated MT gene expression at the transcription level, AP-1 was not involved in the PKC action of the induction of MT by zinc.

Other studies suggested that full activation of AP-1 might require both calcium- and PKC-dependent pathways (Hill and Treisman, 1995). It has also been demonstrated that the influx of calcium is required for the activation of NF- κ B during oxidative stress. The calcium release precedes the formation of the reactive oxygen intermediates (ROI) in the

redox pathway (Pahl and Baeuerle, 1997). ROI production shows stimulatory effects on AP-1 and NF- κ B activity and maybe serve as activation messengers. Therefore, it can be speculated that activation of metallothionein after hypoxia is brought about by

1. formation of ROI,
2. activation of Ca²⁺ channels,
3. binding of trans-acting factors such as MTF-1, AP-1, NF- κ B via a PKC-dependent pathway.

Hypoxia is an environmental stress that induces transcription of various genes, including MTs. These genes are involved in regulation of diverse biological processes including erythroid development, angiogenesis, wound healing, cardiovascular-related diseases, neoplasia, cell interaction, proliferation and differentiation (Murphy et al., 1999; Liu et al., 1998; Helfman and Falanga, 1993).

This current study demonstrated that the mouse metallothionein gene (MT-I) was transcriptionally activated by hypoxia. The data further demonstrated a potential involvement of the metal transcription factor-1 (MTF-1). Mouse MT-I gene expression in fibroblasts from mice with targeted deletions of both MTF-1 alleles (dko 7) were not induced by zinc and showed low responsiveness to hypoxia. A transfected MT promoter was unresponsive to hypoxia or zinc in dko7 cells, but inductions were restored by cotransfecting a

mouse MTF-1 expression vector. Therefore, these results demonstrate that MT gene expression involves MTF-1.

Metallothionein is both a hypoxic and an oxidative stress protein that contributes to the resistance of mammalian cells to reactive oxygen species (ROI) generated by chemotherapy, and radiation therapy. Therefore, it is important to further elucidate metallothionein function.

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