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Cadmium-Induced Gene Expression is Regulated by MTF-1, a Key Metal-Responsive Transcription Factor

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The transition metal cadmium is a serious occupational and environmental toxin. To inhibit cadmium-induced damage, cells respond by increasing the expression of genes that encode stress-responsive proteins. The metal-regulatory transcription factor 1 (MTF-1) is a key regulator of heavy-metal induced transcription of metallothionein-I and II and other genes in mammals and other metazoans. Transcriptional activation of genes by MTF-1 is mediated through binding to metal-responsive elements in the target gene promoters. Phosphorylation of MTF-1 plays a critical role in the cadmium-inducible transcriptional activation of metallothionein and other responses. Studies using inhibitors indicate that multiple kinases and signal transduction cascades, including those mediated by protein kinase C, tyrosine kinase and casein kinase II, are essential for cadmium-mediated transcriptional activation. In addition, calcium signaling is also involved in regulating metal-activated transcription. In several species, cadmium induces heat shock genes. Recently much progress has been made in elucidating the cellular machinery that regulates this metal-inducible gene expression. This review summarizes these recent advances in understanding the role of some known cadmium-responsive genes and the molecular mechanisms that activate metal-responsive transcription factor, MTF-1.

Cadmium is an environmental pollutant that is considered as human toxicant as well as a potent carcinogen. Cadmium was ranked number 7 among "Top 20 Hazardous Substances Priority List" in 1997 simultaneously by the Agency for Toxic Substances and Disease Registry and by the Environmental Protection Agency (Fay and Mumtaz, 1996). It is continuously introduced into atmosphere through the smelting of ores and the burning of fossil fuels (Aylett, 1979; Friberg et al., 1986; Fay and Mumtaz, 1996). Humans are exposed to cadmium primarily via inhalation and ingestion of cadmium-containing foods (Waalkes et al., 1992). It has been suggested that increased industrialization has resulted in higher accumulation of cadmium in humans (Fortoul et al., 1996). Toxicological consesequences of cadmium exposure include kidney damage, respiratory diseases, neurological disorders, and lung, kidney, prostate, as well as testicular cancers in rats and mice (Waalkes et al., 1992). Human epidemiological data suggest that it causes tumors in the male reproductive and respiratory systems (Waalkes et al., 1992; Oberdorster, 1986). Cadmium induces intracellular damage, via the (a) nonspecific inactivation/denaturation of proteins, by binding to free sulfhydryl residues; (b) displacement of zinc co-factors from a variety of proteins, including transcription factors; and generation of reactive oxygen species, which ultimately oxidize DNA, protein, and lipids (Stohs and Bagchi, 1995; Koizumi and Li, 1992).

Exposure of tumor cell lines and animals to cadmium induces the expression of various types of defense and repair proteins. These changes in gene expression are presumed to be related to the cellular responses to cadmium toxicity. These defense-related proteins (a) chelate the metal to prevent further damage, (b) remove reactive oxygen species, (c) repair membrane and DNA damage, and (d) renature or degrade unfolded proteins (Liao and Freedman, 1998). Cadmium has been shown to affect the steady-state levels of the mRNAs encoding metallothionein (Hamer, 1986), heme oxygenase (Alam et al., 1989), γ-glutamylcysteine synthetase (Hatcher et al., 1995), low and high molecular weight heat shock proteins (Wiegant et al., 1994) and ubiquitin (Muller-Taubenberger et al., 1988). In addition, increment of in superoxide dismutase, catalase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase activities are observed following cadmium exposure in cultured cells and whole

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animals (Kostic et al., 1993; Salovsky et al., 1992). Using suppression subtractive hybridization (SSH) technique to screen the genes expressed upon cadmium exposure in COS-7 cells, five members of the heat shock protein (hsp) family - hsp10, 40, 60, 70 and 89α were determined (Lee et al., 2002).

Cadmium-activated transcription may occur through specific metal-responsive upstream regulatory elements found in the promoters of cadmium-responsive genes. These may include metallothionein-responsive element sequences, found in metallothionein genes (Stuart et al., 1984; Searle, 1990; Culotta and Hamer, 1989), or cadmium-responsive elements, as found in human heme oxygenase gene (Takeda et al., 1994). Cadmium may also affect gene expression by influencing signal transduction pathways. Cadmium affects the activities of protein kinase C, cAMP-dependent protein kinase, and calmodulin (Wang and Templeton, 1988; Beyersmann and Hechtenberg, 1997). It has been suggested that cadmium-induced transcription of the proto-oncogenes *c-jun* and *c-fos* is mediated via protein kinase C and calmodulin (Beyersmann and Hechtenberg, 1997). By modulating the activities of complex signal transduction pathways, cadmium can influence the expression of a myriad of genes. However, relatively few cadmium-responsive genes have been identified so far.

The nonparasitic nematode *C. elegans* provides an excellent model system for obtaining an integrated picture of cellular, developmental, and molecular aspects of the regulation of cadmium-responsive gene expression. The developmental and cellular biology of *C. elegans* is thoroughly understood in exceptional detail (Sulston, 1988; Kenyon, 1988). High levels of evolutionary conservation between *C. elegans* and higher organisms are observed in many signal transduction, gene regulatory, and developmental pathways (McGhee and Krause, 1997; Han and Sternberg, 1990; Clark et al., 1992). *C. elegans* also contains homologues of many of the signal transduction proteins that have been implicated in modulating the cellular/molecular response to metal exposure (Gross et al., 1990; Lu et al., 1990; Land et al., 1994a; Land et al., 1994b).

Although the mechanism(s) by which this metal modulates the levels of expression of most of these genes remain to be elucidated, partial answer is now emerging. Elucidation of these mechanisms will enable researchers to create new, more effective methods to counteract the toxic effects of cadmium on biological system. In this review, we summarize the role of some known cadmium-responsive genes and explore the regulatory mechanisms involved in cadmium-inducible gene expression.

Cadmium-responsive Genes

Cadmium is a toxic transition metal with no known physiological function, which activates the expression of several genes such as those encoding heat shock proteins (hsp), heme oxygenase, *c-fos*, *c-jun*, *Erg-1* and *c-myc* (Lee et al., 2002; Liao and Freedman, 1998; Adam et al., 1989; Garrett et al., 2002). In mammalian cells, several different sequence-specific DNA-binding proteins can function as "cadmium-responsive" factors (e.g. MTF-1). By differntial display analysis, Liao and Freedman (1998) identified 49 cDNA, which are the products of 32 different genes whose steady-state levels of expression are modulated by cadmium treatment in vivo in *C. elegans*. Although the intracellular damages elicited by cadmium exposure is similar to that induced by oxidative stress, the nucleotide and amino acid sequences of paraguat-inducible gene products, glutathione *S*-transferase, a zinc-finger/leucine zipper protein, are not homologous to the cadmium-responsive products (Liao and Freedman, 1998).

Thirty-one unique *C. elegans* gene products were identified by differential display whose levels of expression increase following cadmium exposure. Twenty-two of these mRNAs encode proteins whose amino acid sequences do not share significant homology with other proteins in the data base. However, several mRNAs were identified that encode homologues of protein that have been associated with cadmium-induced stress responses in cultured cells and other organisms. These include *C. elegans* HSP70F (hsp-6) and metallotionein-1 (*mtl-1*) and a *C. elegans* homologue of a protein that is associated with DNA repair, DNA gyrase (Liao and Freedman, 1998) (Table 1).

Cadmium causes DNA damage in cultured cells, inducing chromosomal aberrations and strand breaks (Hartwig, 1994). It potentates this damage by inhibiting DNA repair and thereby, enhancing oxidative stress (Hartwig, 1994; Tsuzuki et al., 1994). It has been postulated that since (a) DNA gyrase/topoisomerases are integral components in DNA repair (Hickson et al., 1990) and (b) DNA-damaging agents elevate the levels of topoisomerase in cultured cells and tumors (Kikuchi et al., 1997), any increased expression of these genes would be a likely consequence of cadmium exposure. The change observed in the level of DNA gyrase mRNA in cadmium-treated *C. elegans* might be the result of greater DNA repair activity (Liao and Freedman, 1998).

Differential display study of Liao and Freedman (1998) showed a collection of cadmium-responsive *C. elegans* ESTs (Expressed Sequence Tags) or predicted genes. Four of these, DDRT2, DDRT7, DDRT16 and DDRT26 (Table 1), which are between 217 and 240 bp in length, were derived from an identical mRNA (Liao and Feedman, 1998). The gene that was identified and characterized was later designated as *cdr-1* (cadmium-responsive gene family), from which the ESTs were derived (Liao and et al., 2002). CDR-1 encodes a novel, integral membrane protein, which is transcribed exclusively in

Table 1. Sequence analysis of cadmium-regulated, differentially expressed cDNAs

Clone name[a]	Sequence identity/homology[b]		GenBank™ accession no
	Cosmid[c]	Gene product[d]	
DDRT1	T09B4	T09B1.4, CELK00886	AF071359
DDRT2[1]	F35E8	F35E8.11	AF071362
DDRT3[2]	F35E12	F35E12.7	AF071382
DDRT4[2]	F35E12	F35E12.7	AF071391
DDRT5	C56C10	C56C10.12, CELK05910	AF071396
DDRT6	W03C9	W03C9.5, CELK06396	AF071397
DDRT7[1]	F35E8	F35E8.11	AF071398
DDRT9	C35D10	ND[e]	AF071399
DDRT10	C49C3	ND	AF071353
DDRT12	ND	ND	AF071354
DDRT15	ND	ND	AF071355
DDRT16[1]	F35E8	F35E8.11	AF071356
DDRT17	C49A9	C49A9.4, CELK02276	AF071358
DDRT18	F13G3	F13G3.4, CELK06645	AF071360
DDRT19[3]	ZK849	ND	AF071361
DDRT20[3]	ZK849	ND	AF071363
DDRT21U	Y111B2[f]	ND	AF072438
DDRT21D	ND	CELK05123	AF071364
DDRT22	F57G9	ND	AF071365
DDRT23[4]	F31C3	*C. elegans* rDNA tandem repeats	AF071376
DDRT24	C56C10	C56C10.8; CELK02788; human transcription factor BTF3[g]	AF071377
DDRT25[5]	R119	R119.5; CELK00686	AF071378
DDRT26[1]	F35E8	F35E8.11	AF071379
DDRT28[5]	R119	R119.5; CELK00686	AF071380
DDRT29	ND	ND	AF071138
DDRT30	C27H5	C27H5.5; CELK02088, *C. elegans* collagen *(col-36)*	AF071383
DDRT32[4]	F31C3	*C. elegans* rDNA tandem repeats	AF071384
DDRT33D	C34F6	CELK01885; *C. elegans* cutical collagen	AF071385
DDRT34	F20C5	F20C5.1; CELK01295	AF071386
DDRT35	R11D1	R11D1.1; CELK02809; human hypothetical protein KIAA0174	AF071387
DDRT36[6]	D2096	D2096.8; CELK01725; human nucleosome assembly protein1 LIKE-1	AF071388
DDRT37	K11H12	K11H12.2; CELK02043; rat 60 S ribosomal protein	AF071389
DDRT38[4]	F31C3	*C. elegans* rDNA tandem repeats	AF071390
DDRT40	W02B3	W02B3.2; bovine β-adrenergic receptor kinase	AF071392
DDRT41	ND	*Spiroplasma citr* DNA gyrase subunit B	AF071393
DDRT47[6]	D2096	D2096.8; CELK01725; human nucleosome assembly protein1 LIKE-1	AF071394
DDRT48[4]	F31C3	*C. elegans* rDNA tandem repeats	AF071395
VL1[7]	K11G9	K11G9.5; CELK3309; *C. elegans* metallothionein-1 *(mtl-1)*	AF073166
VL3	ND	ND	AF071374
VL5[7]	K11G9	K11G9.5; CELK3309; *C. elegans* metallothionein-1 *(mtl-1)*	AF071375
VL7[7]	K11G9	K11G9.5; CELK3309; *C. elegans* metallothionein-1 *(mtl-1)*	AF072436
VL8[7]	K11G9	K11G9.5; CELK3309; *C. elegans* metallothionein-1 *(mtl-1)*	AF072437
VL9	C50B6	ND	AF071375
VL10	K11G9	K11G9.5; CELK3309; *C. elegans* metallothionein-1 *(mtl-1)*	AF073167
VL11	C06G3	C60G3.8	AF073168
VL12[7]	K11G9	K11G9.5; CELK3309; *C. elegans* metallothionein-1 *(mtl-1)*	AF072434
VL13[7]	K11G9	K11G9.5; CELK3309; *C. elegans* metallothinein-1 *(mtl-1)*	AF073169
VL15[7]	K11G9	K11G9.5; CELK3309; *C. elegans* metallothionein-1 *(mtl-1)*	AF072435
VL19	D2023	D2023.2; CELK00011; human pyruvate carboxylase	AF073170
VL20	B0228	B0228.1	AF071371
VL21		CELK00200; *C. elegans* mitochondrial hsp70 protein F precursor	AF071372

[a]Clones labeled with identical superscript numbers indicate that the differentially expressed cDNAs are derived from the same gene. [b]Analyzed with BLASTN using GeneBank™ and *C. elegans*-specific data bases. The sequences have > 80% nucleotide sequence identity. [c]*C. elegans* genomic cosmids that have >90% nucleotide sequence identity. [d]Predicted genes are designated by the cosmid name followed by the structural gene number (e.g. F35E8.11). *C. elegans* ESTs are denoted with the "CELK" designation. [e]ND, not detected. [f]Yeast Artificial Chromosome. [g]Homologous proteins are presented that have a >60% amino acid sequence identity, based on BLASTX analysis (adapted from Liao and Freedman, 1998).

the intestinal cells of post-embryonic *C. elegans* and is targeted to lysosomes. Lysosomes function in the intracellular storage of transition metals, including cadmium in different species (Marigomez et al., 1990; Marshall et al., 1994). *cdr-1* is found to be located near the center of chromosome V in the *C. elegans* genome. The size of *cdr-1*, including structural gene and the upstream regulatory region, is predicted to be 2475 bp. Transcription of *cdr-1* is activated in response to cadmium exposure, whereas several common environmental stressors are not effective inducers. When CDR-1 expression is inhibited, and nematodes are exposed to cadmium *in vivo*, the pseudo-coelomic space accumulates fluid. In addition, reproduction and development are inhibited (Liao et al., 2002). Following cadmium exposure, *cdr-1* promoter activity is evident exclusively in the

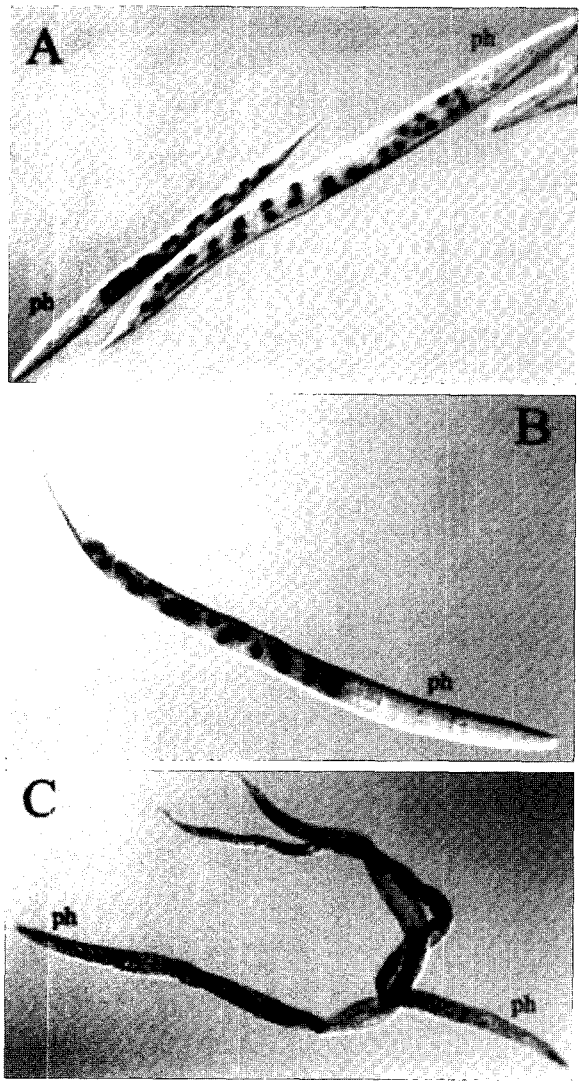

Fig. 1. Cell-specific expression of *cdr-1*. A and B, *C. elegans*, strain JF9 (mtIs7, *cdr-1/lacZ*) was exposed to 100 μM $CdCl_2$ for 24 h and then stained for β-galactosidase activity. A, Reporter gene activity in the intestinal cells of a young adult and an L3 larva. B, *cdr-1* promoter activity in the intestinal cells of an L1 *C. elegans* larvae. CDR-1 mRNA was visualized by exposing wild type nematodes to a digoxigenin-dUTP-labeled CDR-1 antisense probe as described under "Experimental Procedures." C shows that the CDR-1 transcript is expressed throughout the intestine of L1. "*ph*" marks the location of the pharynx in all panels. Nematodes were photographed using Nomarski optics (adapted from Liao and Freedman, 2002).

intestinal cells of *C. elegans*. Transcription was observed in all post-embryonic stages of development, and not in developing embryos. In absence of cadmium, reporter transgene activity was not observed. When cadmium-exposed *C. elegans* were hybridized to an antisense CDR-1 cDNA probe, CDR-1 mRNA was observed throughout the intestine in all post-embryonic developmental stages (Fig. 1). Thus *cdr-1* represents a new class of metal-responsive genes that encode novel defense/repair proteins, which is required for resistance to cadmium toxicity.

Cadmium-induced Metallothioneins

Metallothioneins (MT), which are small, cysteine-rich, metal-binding proteins, play an important role in detoxification of cadmium (Hamer, 1986; Kagi and Schaffer, 1988; Masters et al., 1994). Four families of MTs have been identified in mammals. MTI and MTII are stress-inducible proteins and are implicated in metal detoxification (Michalska and Choo, 1993; Masters et al., 1994). One of the hallmarks of the *MTI* and *MTII* genes is their rapid transcriptional induction by metals such as zinc and cadmium (Andrews, 2000). It has been found the *MT-I* and *-II* double knock-out mice were particularly sensitive to the cytotoxic effects of cadmium (Michalska and Choo, 1993; Masters et al., 1994; Liu et al., 2000). Recently, an oxidative stress response and cadmium response element was discovered in the *MT-I* promoter that maps to the -101 bp region (Andrew, 2000; Dalton et al., 1994; Dalton et al., 1996). This region contains an antioxidant response element (ARE). The ARE (also called electrophile response element) mediates induction of glutathione S-transferase Ya subunit and the quinone reductase genes in response to redox-cycling xenobiotics and H_2O_2 (Andrew et al., 2000). In the mouse *MT-I* promoter (and the hamster MT-I promoter), the ARE overlaps a previously identified upstream stimulating factor (USF)-binding site (CRCGTGRY) (Carthew et al., 1987). USF, a member of the basic-helix-loop-helix-Zip protein superfamily (Beckmann et al., 1990; Bendall and Molloy, 1994), was suggested to participate in activation of *MT-1* gene in response to cadmium and H_2O_2 by interacting with ARE-binding factors through the USF/ARE composite element (Andrews 2000, 2001).

The mouse *MT-I* and *MT-II* genes are actively expressed in many cell types in different organs and tissues, as well as in most cultured cells. In contrast, the *MT-III* and *MT-IV* genes show a very restricted cell type-specific pattern of expression (Liang et al., 1996). MTIII is a brain-specific, non-inducible protein identified in Alzheimer's disease-related studies. The unique biological function of MTIII is its ability to inhibit neuronal growth. MTIV is a family of MTs that is present in squamous epithelial cells. Although it has been proposed that MTIV is involved in maintaining Zn homeostasis (Quaife et al., 1994), its exact function remains unclear.

In *C. elegans*, two forms of metallothioneins have been identified. *mtl-1* (MTI) is constitutively expressed in three cells of the posterior bulb of the pharynx, which can be induced by cadmium in intestinal cells. *mtl-2* (MTII) mRNA is not expressed under basal condition (unexposed condition) and expressed in intestine only when induced by cadmium (Freedman et al., 1993).

Target Genes for the Metal-responsive Transcription Factor MTF-1

Metal-inducible MT transcription is regulated by the transcription factor, metal transcription factor-1 (MTF-1), which is an evolutionarily conserved protein that specifically binds to metal-responsive elements (MREs) and has been characterized in several species including human, mouse, pufferfish (*Fugu rubripes*), zebrafish (*Danio rerio*), trout (*Oncorhynchus mykiss*), and Drosophila (Radtke et al., 1993; Brugnera et al., 1994; Dalton et al., 2000; Auf der Maur et al., 1999; Zhang et al., 2001). MTF-1 contains six zinc finger domains and several trans-activation domains: acidic-rich, proline-rich, and serine/threonine-rich (Radtke et al., 1993). Mice lacking functional MTF-1 were generated as a result of targeted gene disruption (Gunes et al., 1998). These mice were found to die in utero around day 14 of gestation due to an acute decay of hepatocytes. To date, the *MT-I* and *MT-II gene* are the best-characterized target genes of MTF-I. Mice with disruption of both *MT-I and MT-II* genes are more sensitive to metal stress, but do not show any obvious phenotype under normal laboratory conditions (Michalska and Choo, 1993; Masters et al., 1994; Kelly et al., 1996). This finding strongly suggests that MTF-1 do have additional target genes besides *MT-I* and *MT-II* and that a failure to properly regulate these genes may be responsible for the lethal phenotype of MTF-I null mice. Using modified SABRE (selective amplification via biotin- and restriction-mediated enrichment), a novel selective amplification procedure for the detection of differentially expressed mRNAs along with Affymetrix gene chip microarray screening and computer search for metal responsive elements (MREs), α-fetoprotein (AFP), C/EBPα (liver enriched transcription factor) and tear lipocain were detected to be the possible new putative target genes of MTF-1 (Lichtlen et al., 2001). Besides the down regulation of genes in the absence of MTF-1, three more genes were identified which are expressed at higher levels in the MTF-1 knockout mice (Lichtlen et al., 2001). The first gene is a homolog of the yeast cell division control protein 3 (cdc3), which is a member of conserved eukaryotic family of septin proteins, involved in cytokinesis (Kim et al., 1991). The second gene, NF-YB codes for a subunit of a CCAAT box-binding protein (Maity and de Crombrugghe, 1998). NF-YB/CBF1 is a basal transcription factor, but nevertheless may well be relevant for the liver phenotype of MTF-1 knockout embryos (Lichtlen et al., 2001). The third gene is a receptor for hepatopoietin, a novel polypeptide mitogen specific for hepatocytes (Xanthopoulos and Mirkovitch, 1993). All three genes may be up regulated as part of any effort by the MTF-1 deficient embryo to compensate for the loss of hepatocytes at the onset of liver decay.

AFP is mainly expressed in embryonic hepatocytes and its expression is controlled at transcriptional level (Mizejewski, 1997). As a member of the albuminoid gene superfamily, it is the major embryonic protein responsible for maintenance of the colloid osmotic pressure. AFP plays various additional roles such as binding to and scavenging of heavy metals and reactive oxygen species. In addition, AFP influences developmental processes and interacts with growth factors and the apoptotic-signaling cascade (Mizejewski, 1997). C/EBPα is involved in maintenance of the differentiated, non-proliferating state of liver and other cells such as adipocytes. C/EBPα is also involved in the cellular stress response, as indicated by its induction by infectious agents in the so-called acute inflammatory response (Lichtlen et al., 2001). Tear lipocalin, another candidate target gene of MTF-1, is mainly expressed in lacrymal (tear) and lingual glands, including parotid salivary glands and sublingual von Ebner's glands (Lichtlen et al., 2001). Lipocalein is able to bind a number of lipophilic compounds such as retinol, cholesterol, fattyacids, phospholipids and fatty alcohols (Redl et al., 1992; Glasgow et al., 1995) and potentially toxic molecules as well (Lichtlen et al., 2001). In addition, it inhibits cysteine proteases, plays a role in inflammatory processes and may be part of a defense against infectious agents (Holzfeind et al., 1996; van't Hoff et al., 1997). In another studies, γ-glutamyltranspeptidase and γ-glutamylcysteine (γ-GCS_{hc}), both having MRE motifs in their promoters were also found to be potential MTF-1 target genes (Gunes et al., 1998). On the otherhand, MTF-1 knockout embryos showed decreased transcript levels of γ-GCS_{hc}, a GSH synthesizing enzyme (Gunes et al., 1998).

Regulation of Metalothionein Transcription

The expression of *MT-I* and *MT-II* genes is primarily controlled at the level of transcription (Karin et al., 1981; Durnam et al., 1984). Transcription can be induced by a variety of physiological agents and environmental stressors such as transition metals (e.g cadmium), glucocorticoids, cAMP, phorbol esters, alkalyting agents, oxidizing agents, ultra-violet and ionizing radiation and hypoxia (Durnam and Palmiter, 1984; Hamer, 1986; Nebes, 1988; Garrett et al., 1992; Rimoldi, et al., 1992; Sato and Bremner, 1993; Tamai et al., 1994; Kelly et al., 1997; Murphy et al., 1999). The activation of MT transcription by transition metals is mediated by regulatory elements, designated as metal-responsive elements (MREs). MREs contain a 7-bp core sequence (TGCRCNC) and are present in multiple copies in promoter/enhancer regions of almost all metal-inducible MTs (Searle et al., 1985; Culotta and Hammer, 1989). Inducible transcription is mediated by a variety of other regulatory elements located in promoter/ enhancer regions of MT genes, which include glucocorticoid response elements, cAMP response elements, and antioxidant response elements (Hamer, 1986; Yu and Lin, 1995; Kelly et al., 1997;

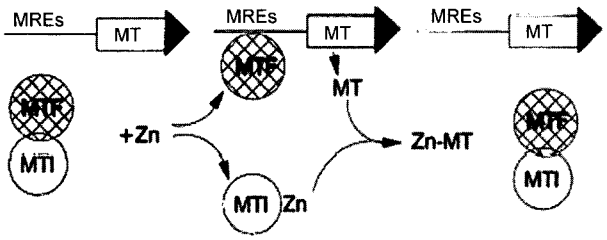

Fig. 2. Model of MT gene regulation by metals. In the absence of zinc, the transcription factor (MTF-1) is complexed with an inhibitor (MT1). MTI dissociates from MTF-1 in the presence of zinc and allows MTF-1 to interact with MREs in the MT promoter to activate transcription. The MT that is synthesized binds zinc and the MTF-1/ MTI complex reforms (adapted from Palmiter, 1994).

Viarengo et al., 2000).

Three models have been proposed to describe the mechanism by which interaction among MREs, MTF-1, and zinc activates MT transcription. The first model proposes that MTF-1 acts as a zinc sensor that cannot bind to DNA in the absence of metal (Heuchel et al., 1994; Chen et al., 1998; Chen et al., 1999; Bittel et al., 2000; Andrews, 2000). As cells accumulate zinc, the metal binds in the finger domains, causing a conformational change in the protein and subsequent binding to the MRE (Saydam et al., 2002). It is suggested that three fingers (fingers 2-4) are essential for DNA binding and that these fingers display constitutive DNA-binding activity in the absence of finger 1 (Bittel et al., 2000). When MT levels are sufficient, it chelates the metal from the zinc finger. Now MTF-1 can no longer bind to the MREs and ultimately transcription ceases (Saydam et al., 2002).

The second model hypothesizes the existence of a zinc-sensitive inhibitor, which complexes with MTF-1 rendering it inactive (Palmiter, 1994). As cellular zinc level increases, the metal binds to the inhibitor releasing it from MTF-1, allowing the transcription factor to bind to the MRE to activate transcription. When MT levels are sufficient, the zinc is removed from the inhibitor, allowing it once again to bind to MTF-1 thereby inhibiting transcription (Fig. 2).

Typically, zinc is used as the inducer when the interactions among MREs, MTF-1, and MT transcription are investigated. A combination of mutational and transcriptional analyses clearly shows that cadmium activation of MT transcription is dependent on MTF-1 and MREs (Heuchel, 1994; Datta and Jacob, 1997). MTF-1 binding to MREs and the activation of MT transcription is also induced by oxidative stress (Dalton et al., 1996; Andrews, 2000). However, cadmium does not cause detectable changes in the DNA binding of MTF-1 *in vitro* (Bittel et al., 1998; Koizumi et al., 1999) and does so only at high concentration *in vivo* (Smirnova et al., 2000). Mutational analysis demonstrated that loss of zinc activation *in vitro* and *in vivo* was accompanied by a loss of cadmium induction *in vivo*. This finding is consistent with the concept that cadmium might displace zinc *in vivo*, making it available for activation of MTF-1 (Palmiter, 1994).

Saydam et al (2002) proposed another alternative third model in which the regulation of MT transcription, via the MTF-1/MRE interaction, is controlled by several signal transduction cascades that affect MTF-1 phosphorylation. The phosphorylated form of MTF-1 is located primarily in the cytoplasm of non-exposed cells. It has been reported that the level of MTF-1 phosphorylation is modified following exposure to cadmium or zinc *in vivo* (Adams and Freedman, 2000; LaRochelle et al., 2001). On exposure to cadmium, the level of MTF-1 phosphorylation increases significantly. This alteration is observed as a higher level of phosphorylation in the nuclear form of MTF-1 (Saydam et al., 2002). One or more metal-or stress-responsive signal transduction pathways may affect the phosphorylation of MTF-1 in vivo on serine and tyrosine residues. This model is based on several observations. First, protein motif analysis (Hofmann et al., 1999) of MTF-1 indicates the presence of several evolutionarily conserved, potential phosphorylation sites. As many as 11 PKC sites, 13 casein kinase II sites, and 1 tyrosine kinase site are predicted in the four characterized MTF-1s from different species (Saydam et al., 2002). Second, exposure of cells to activators of signal transduction cascades causes an increase in the steady-state level of MT mRNAs (Nebes et al., 1988; Garrett et al., 1992; Kelly et al., 1997; Laychock et al., 2000). Finally, many of the effectors that induce MT transcription, metals (zinc, cadmium, mercury, arsenic, chromium) as well as other environmental stressors (oxidative stress, radiation), modulate the activity of intracellular signal transduction cascades (Whisler et al., 1995; Beyersmann and Hechtenberg, 1997; Karin, 1998; Stohs et al., 2000).

The ability of cadmium to induce metallothionein gene expression in a variety of species has been documented for many years (Hamer, 1986). When *C. elegans* is exposed to $\geq$50 µM $CdCl_2$, two iso-MTs, designated CeMT1 (75 amino acid residues) and CeMT2 (63 residues), accumulate in substantial amounts (Slice et al., 1990; Imagawa et al., 1990). These two polypeptides are cysteine-rich (25-30%) and exhibit characteristic Cys-X-Cys and Cys-Cys motifs (Slice et al., 1990; Imagawa et al., 1990). However, the amino acid sequences of *C. elegans* MTs are not homologous with MTs from other species. Unusual features of CeMTs include the following: the structural divergence between CeMT1 and CeMT2 is much greater than that of vertebrate iso-MTs; each CeMT contains a Tyr residue between the two predicted metal binding domains and His residues at or near the C termini. CeMTs bind 6 mol of cadmium (or zinc)/mol of polypeptide; and CeMT1, the largest known MT, contains

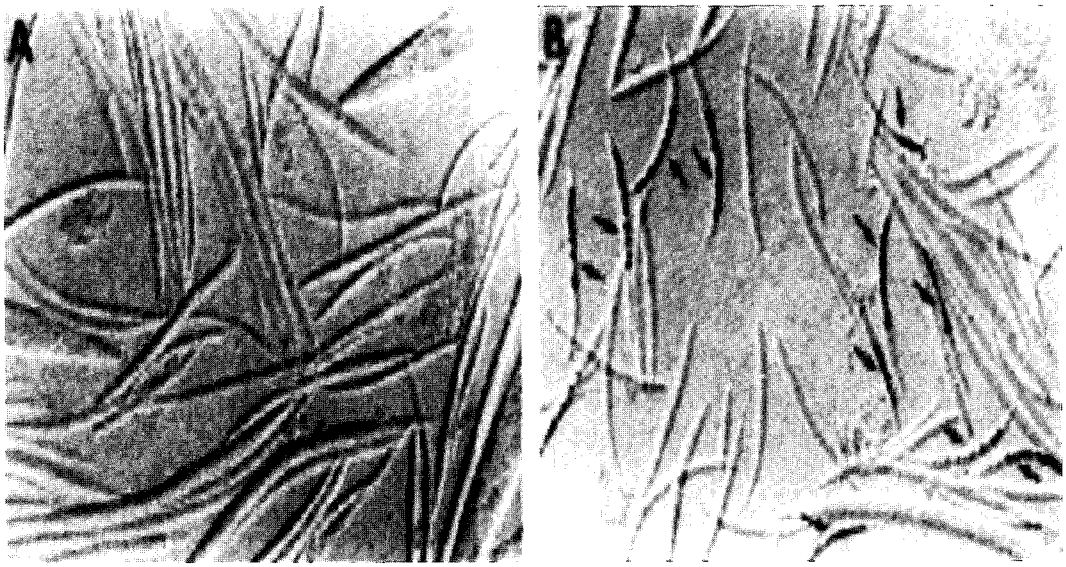

Fig. 3. Effect of cadmium on expression of the β-galactosidase reporter gene in transgenic nematodes that carry the *mtl-2*:: *lacZ* fusion gene was incubated in the absence (A) or presence (B) of 0.1 mM $CdCl_2$. After 24 h the animals were stained for β-galactosidase activity as described under "Experimental Procedures." The *arrows* in *panel B* indicate transgenic nematodes expressing cadmium-induced β-galactosidase activity. In the absence of cadmium no staining is observed (A) (adapted from Freedman et al., 1993).

a novel C-terminal extension (Slice et al., 1990; Imagawa et al., 1990).

Freedman et al. (1993) and later Liao and Freedman (1998) reported the sequences of the coding and flanking regions of the *mtl-1* and *mtl-2* genes, which encode CeMT1 and CeMT2, respectively. The promoter regions of the genes are markedly divergent and share little homology. The *mtl-2* promoter contains a TATAA box and a single proximal MRE sequence. In contrast, these elements are not evident upstream from *mtl-1* structural gene. Nevertheless, both CeMT1 and CeMT2 mRNAs are highly induced by cadmium and contain precisely initiated, short 5'-untranslated sequences. The two *mtl* genes are located on chromosome V, but they are not closely linked. Upon incubation with cadmium, larval and adult transgenic *C. elegans*, which carry an *mtl-2* promoter: *lac Z* reporter fusion gene, express β-galactosidase exclusively in intestinal cells (Fig. 3). In the absence of cadmium, expression of the *mtl-2*: *lacZ* transgene is undetectable. Cadmium also induces expression of an *mtl-1*: *lacZ* fusion gene in the intestinal cells of transgenic *C. elegans* larvae. However, expression of this transgene is markedly attenuated in the intestinal cells of cadmium-treated adult animals (Liao and Freedman, 1998). Thus, the *C. elegans mtl-1* and *mtl-2* promoters direct cell specific gene expression that is subjected to be modulation by metals and developmental factors.

The *C. elegans mtl-1* and *mtl-2* genes differ from their mammalian counterpart in their organization and the divergence of their coding and flanking sequences. Mammalian MT genes contain introns that interrupt the protein coding sequences within codon 10 and 32 (Hamer, 1986). The second intron is inserted between sequences that correspond to the end of the β domain and the beginning of the α domain in MT. The N-terminal β and C-terminal α domains bind 4 and 3 atoms of cadmium or zinc respectively (Hamer, 1986; Kagi and Schaffer, 1988). Each *C. elegans mtl* gene has a single, small intron that begins after the first nucleotide of codon 6. Conserved splice donor/acceptor sequences are evident at the junctions between the intron and the two exons. The intron does not divide the *mtl-1* and *mtl-2* genes into exons encoding two independent cadmium-binding domains (Freedman et al., 1993). Instead, the introns disrupt a conserved Cys-X-Cys-X-Cys coding sequence in the penultimate codon. Exon 1 from all mammalian (Hamer, 1986), *Drosophila melanogaster* (Maroni et al., 1986; Silar et al., 1990) and *C. elegans* (Freedman, 1993) metallothionein genes contributes the first pair of metal-binding Cys residues in a Cys-X-Cys motif.

In *Drosophila*, the two MT genes are characterized to date, designated *Mto* (or *MtnB*) and *Mtn*(or *MtnA*) metallothionein genes (Maroni et al., 1986; Silar et al., 1990) contain only one intron (at codon 8 or 9), but they encode small MTs (43 and 40 amino acid residues, respectively) that exhibit little homology with the CeMT1 and CeMT2 polypeptides (Maroni et al, 1986). *Mto*, transcribed from a TATA-less promoter, is primarily active during embryogenesis, while *Mtn*, characteristics for late embryos, larvae, and adult flies, is strongly expressed in the gut, Malpighian tubules, fat body and in hemocytes

(possibly to regulate copper supply to hemocyanin) (Bonneton and Wegnez, 1995; Durliat et al, 1995; Bonneton et al, 1996). The expression pattern of *Drosophila* MTF-1 (dMTE-1) is compatible with a role in MT gene activation/heavy metal detoxification and homeostasis (Zhang et al., 2001). Isolation of cDNAs and of genomic locus, sequence comparisons, and functional tests reveal that *Drosophila* harbors some differences in structure and properties notwithstanding, a functional homolog of vertebrate stress regulator MTF-1. It was postulated that *Drosophila Mto* is important for copper homeostasis during embryogenesis, while *Mtn* is thought to balance the toxic effects of copper and other metals, such as cadmium and mercury (Zhang et al., 2001).

Signal Transduction Pathways Controlling Metal-activated MT Transcription

The inhibitor studies (Yu et al., 1997; Saydam et al., 2002) indicate that signal transduction pathways that involved protein kinase C (PKC), casein kinase II, tyrosine kinase, and calcium control cadmium-activated MT transcription via MTF-1/ MRE interactions. The activation of the signal transduction pathway associated with these kinases by metals and other environmental stressors has been well documented (Beyersmann and Hectenberg, 1997; Pfundt et al., 2001).

The participation of PKC in MT gene regulation has been demonstrated in the stimulation of MT gene expression by several chemicals (Imbra and Karin, 1987; Angel et al., 1988; Yu et al., 1997). Recent investigations have confirmed that TPA, a PKC activator and calcium ionophore, which perturbs cytosolic calcium concentration, increases the level of MT mRNA (Angel et al., 1988; Imbra and Karin, 1987; Xiong et al., 1992; Arizona et al., 1993). On the other hand, treatment with H-7 (1-5-isoquinolinesulfonyl-2-methylpiperazine dihydrochloride), a PKC inhibitor, reduces cadmium-inducible MT transcription in COS-7 cells by 86% (Saydam et al., 2002). To further demonstrate that this inhibitory effect was due primarily to the inactivation of PKC, a more potent and selective PKC inhibitor, chelerythrine inhibits the induction of MT mRNA by cadmium (Yu et al., 1997). These findings clearly suggest that PKC is involved in the process of cadmium-induced MT gene expression.

Environmental stressors also affect the activity of casein kinase. In response to oxidative stress, casein kinase II translocates to the nucleus, and enzymatic activity of kinase increases (Sayed et al., 2000; Gerber, 2000). Mammalian MTF-1 contains more than 11 predicted casein kinase II phosphorylation sites. The effects of the casein kinase II inhibitors, DRB (5,6-dichloro-1-β-D-ribofuranosylbenose) and heparin on metal-inducible MT transcription were examined in HeLa, HEK293 and COS-7 cells (Saydam et al., 2002). In HeLa cells, treatment with DRB significantly reduced the level of cadmium-inducible reporter gene (4x MREd-Luc and CMV-LacZ) expressions. Treatment of COS-7 cells with heparin (2 μg/ml) significantly inhibited cadmium-inducible metallothionein reporter gene (CAT and psv-βGal) expression (Saydam et al., 2002). These findings suggest that casein kinase II may modulate the activity of MTF-1.

Inhibition of tyrosine kinase activity with herbimycin A (5 μM) significantly reduced the level of cadmium-inducible metallothionein reporter (4x MREd-Luc and CMV-LacZ) gene activity in both HEK293 and HeLa cells (Saydam et al., 2002). These results suggest that phosphorylation of the tyrosine residue in MTF-1 is involved in the activation of MRE/MTF-1 regulated MT transcription.

Calcium is required for the activation of many kinases (Saydam et al., 2002). Exposure of Cos-7 cells to the calcium ionophore, A-23187, markedly increases the level of cadmium-inducible metallothionein reporter gene (-153CAT and MREd'$_5$CAT) activity in the absence of cadmium. Exposure to the intracellular calcium chelator, BAPTA-AM (bisindolymaleimide), reduces the level of cadmium-inducible 4xMREd promoter activity below the level that observed in cells not exposed to metals. These observations suggest that calcium-mediated signal transduction pathways are involved in the activation of MTF-1.

Alam et al. (2000) suggested that cadmium could regulate target genes by modulating the activity of transcription factors that normally regulate the response to other physiological stimuli. In the case of MT genes, this would be by interfering with the mechanisms controlling MTF-1 activity in response to zinc. It was suggested that MTF-1 might regulate expression of zinc transporter-1 (ZnT-1), a ubiquitously expressed zinc export pump (Palmiter and Findley, 1995; Gunes et al., 1998). Cadmium is known to activate several kinases (Beyersmann and Hechtenberg, 1997; Bagchi et al., 1997; Alam et al., 2000), of which some may overlap with the MTF-1 kinase transduction pathway, thus stimulating MTF-1 transcriptional activity and MT gene expression without increasing MTF-1 DNA binding activity. While zinc is required for MTF-1 DNA binding activity, in normal physiological conditions, cells presumably contain enough endogenous zinc to keep MTF-1 in a transcriptionally inducible form that can undergo transcriptional activation through phosphorylation without further increase in DNA binding activity (LaRochelle et al., 2001) (Fig. 4). Thus, according to this hypothesis, cadmium and other metals do not simply cause the redistribution of zinc but alter the transactivation potential of MTF-1 by phosphorylation.

Transition metals and oxidative stress have been shown to modulate the activity of several members of the MAPK (mitogen-activated protein kinase)-signaling cascade (Ding and Templeton, 2000; Stohs et al., 2001).

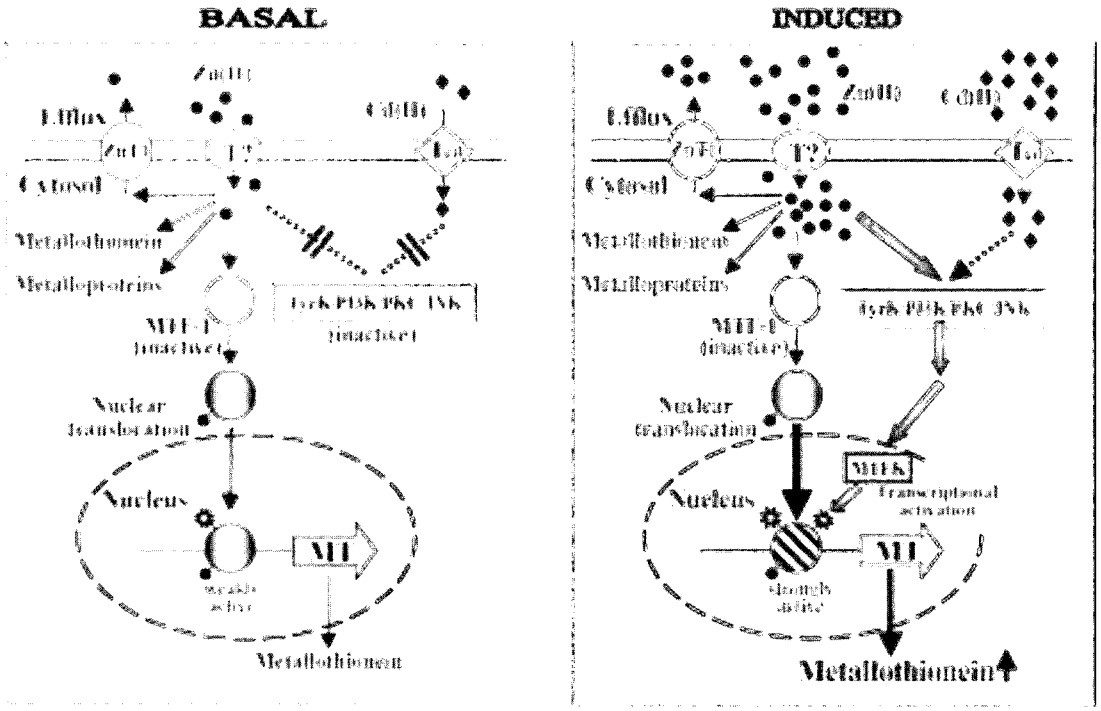

Fig. 4. Model for regulation of MTF-1 activity and MT gene expression by a metal-regulated protein kinase signal transduction cascade under basal and metal-induced conditions. Under normal physiological conditions, zinc (●) and cadmium (◆) ions enter the cell through yet unidentified transporters (*T?* and *Tcd*, respectively) and are metabolized through a variety of metallo-proteins and enzymes, including MT. ZnT1 is located at the plasma membrane and functions as an exporter for zinc efflux. In control (basal), there is presumably enough endogenous zinc to promote the transport of some MTF-1 into the nucleus and cause its conversion from an inactive (non-DNA-binding) state to an active, DNA-binding configuration. In zinc-induced cells, MTF-1 nuclear localization and transcriptional activity would be enhanced. In this model, the activity of MTFK, and ultimately the transcriptional activity of MTF-1, is controlled by a kinase transduction cascade (large arrows) involving a tyrosine-specific protein kinase (*TyrK*), PI3K, PKC, and JNK. The MTF-1 kinase signaling transduction pathway would be controlled by a yet uncharacterized zinc-sensing metallo-regulatory pathway, in which a component(s) plays the role of zinc sensor in cooperation with the metal-sensing zinc finger domain of MTF-1. The precise order in which each kinase is positioned in the cascade is still unknown. It is also possible that multiple pathways converge on MTF-1 at the same time and that more than one kinase can directly phosphorylate MTF-1 in response to metals. Because JNK and PKC can directly phosphorylate and activate transcription factors, one or both may correspond to MTFK. Cadmium and other metal ions would activate MTF-1 and induce MT gene expression by stimulating one or several kinases in the MTF-1 kinase pathway. The dashed arrow indicates putative action of cadmium on the MTF-1 kinase cascade. (¤), phosphate group (adapted from LaRochelle et al., 2001).

Recently, c-Jun N-terminal kinase, a class of MAPK has been shown to contribute to the regulation of metal-inducible MTF-1 transcription activation (LaRochelle et al., 2001). The observation that multiple signal transduction pathways contribute to the ability of MTF-1 to regulate MT transcription provides an explanation to the observation that deletion of various region in MTF-1 affects its ability to activate transcription (Saydam et al, 2000). The multiple signal transduction pathways ultimately converge at MTF-1, to activate MT transcription, which provides a mechanistic link between exposure to structurally unrelated stressors and the activation of MT transcription (Saydam et al., 2000). Potentially any stressor that can activate an MTF-1-regulating signal transduction pathway could increase MT transcription.

Regulation of Heat Shock Protein Transcription

Heat shock proteins (hsp) are molecular chaperones conserved through prokaryotic and eukaryotic cells, and are induced by hyperthermia and other environmental stresses. Hsps have been shown to increase thermal tolerance and to perform functions essential to cell-survival under these conditions (Kiang and Tsokos, 1998). Cadmium has been shown to induce some hsps (Hiranuma et al., 1993; Yamada and Koizumi, 1993; Somji et al., 2000; Lee et al., 2002) due to generation of array of abnormal or denatured proteins as a results of its reaction with vicinal thiol groups and subsitution for zinc in proteins. The occurrence of abnormal proteins is recognized as the signal for induction of hsps (Nover, 1991; Parsell and Lindquist, 1994). hsp10, 40, 60, 70, 89α mRNA have been reported to be induced by cadmium exposure (Hiranuma et al., 1993; Yamada and Koizumi, 1993; Lee et al., 2002). Lee et al. (2002) observed a marked induction of hsp70 mRNA. It is thought that cadmium-induced protein damage causes binding of hsp70 and depletion of the pool of free hsp70, which triggers stress responses including the induction of various hsps (Wiegant et al., 1997). The remarkable

increase and low threshold of induction of hsp70 mRNA may indicate that hsp70 may play a protective role upon exposure of cells to cadmium. Cadmium may activate Hsp70 transcription by several pathways. The metal can (a) directly denature proteins, which function as activators, (b) increase the levels of reactive oxygen species, or (c) modulate the levels of second messengers (Lee et al., 2002). hsp 40, a co-chaperone of hsp70, has been proposed to play an important role in promoting efficient hsp70-substrate binding by stimulating hsp70 ATPase activity (Liberek et al., 1991). hsp 60 and its co-chaperone hsp10 are reported to accelerate the activation of procaspase-3, possibly by folding/maintaining procaspase-3 in a protease-sensitive conformation (Samali et al., 1991). The fact that cadmium exposure induces apoptosis (Tanimoto et al., 1993; Ishido et al., 1995; Tsangaris and Tzortzatou Stathopoulou, 1998) suggests that hsp60 and hsp10 may participate in the activation of cadmium-induced apoptosis. Oxidative stress is reported to induce hsp89α (Bagchi et al., 1996) and hence cadmium-induced oxidative stress may be responsible for increased expression of hsp89α mRNA (Lee et al., 2002).

In *C. elegans*, *hsp-6* gene is both constitutively expressed and moderately inducible by heat shock (Heschl and Baillie, 1989). Cadmium has shown to activate the transcription of the *C. elegans hsp-16*, which encodes for a low molecular weight HSP (Stringham and Candido, 1994; David et al., 2003).

In summary, when mammalian cells are treated with heavy metal cadmium, MTF-1 is activated, which then binds to MRE's and induces transcription of target genes, notably metallothioneins (*MT-I and MT-II*). In resting cells, most MTF-1 localizes to the cytoplasm. It is translocated from the cytoplasm to the nucleus under stress conditions. MTF-1 requires an elevated concentration of zinc for strong binding to DNA, which suggests that MTF-1 is activated by allosteric regulation of DNA binding via binding of cadmium to the transcription factors itself. Zinc is replaced by cadmium in cellular and /or extracellular zinc storage proteins, leading to concomitant activation of MTF-1 by the released zinc. In addition, MTF-1 can be phosphorylated upon cadmium induction, as a result of the activation of a complex kinase signaling transduction pathway, which includes protein kinase C (PKC), casein kinase II, tyrosine kinase, c-Jun N-terminal kinase. In *C. elegans*, two genes (*mtl-1* and *mtl-2*) are found to encode metallothioneins (CeMTs) and both CeMT1 and CeMT2 mRNAs are induced by cadmium. Upon treatment with cadmium, both *mtl-1*::lac Z and mtl-2::lac Z, were expressed in intestinal cells. *cdr-1*, a member of cadmium-responsive gene family, is transcribed in intestinal cells in response to cadmium exposure. The gene encodes a novel protein that is targeted to lysosomes and is required for resistance to cadmium toxicity. Thus far, metallothionein genes are the best-characterized target genes of MTF-1 in cadmium signaling. In this view, the key to understanding the regulation of mammalian MT genes by metals will be the isolation and characterization of other target genes in different species and determination of how it interact with MTF-1 and responds to metals.

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