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Transcriptional Regulation by Heavy Metals, Exemplified at the Metallothionein Genes

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Introduction

Seventeen of the thirty elements known to be essential for life are metals (Cotton and Wilkinson, 1980). They can function as structural or catalytic components of bioorganic molecules or even as signal transducers. (Lippard, 1993). The so-called transition metals are found in the groups IIIB to IIB of the periodic system. Of these, zinc (Group IIB) is the most widely used in living systems. In 1869 it was discovered that zinc is an essential trace element for higher organisms, and in 1940, it was the first trace element to be recognized as a component of an enzyme, namely carbonic anhydrase (Raulin, 1869; Keilin and Mann, 1940). To date, there are more than 300 enzymes known to require zinc for proper functioning (Vallee and Auld, 1990). Pathological zinc deficiency, due to greatly reduced intestinal zinc uptake as in the recessive, autosomal disorder Acrodermatitis enteropathica, leads to death unless treated by high oral zinc doses (Vallee and Falchuk, 1993). Among the zinc dependent enzymes several are involved in nucleic acid metabolism such as the prokaryotic and eukaryotic RNA polymerases (Vallee and Falchuk, 1993). It has been discovered only recently that zinc is also an integral constituent of proteins that regulate the activity of eukaryotic RNA polymerases. These factors, termed zinc finger transcription factors use zinc ions as structural components of those protein subdomains that bind to regulatory DNA sequences. The first representative which was found of this class of proteins is the *Xenopus laevis* RNA polymerase III transcription factor TFIIIA, which binds to the internal 5S RNA gene promoter and can also bind to the 5S RNA gene product itself (Hanas et al, 1983; Miller et al, 1985; Theunissen et al, 1992). Since then, dozens of proteins containing structures reminiscent of zinc fingers or other zinc-binding structures,

INDUCIBLE GENE EXPRESSION, VOLUME 1
P.A. Baeuerle, Editor

termed zinc clusters or zinc twists, have been found (Kaptain, 1991; Vallee et al, 1991). Other metals essential for life include cobalt (Co^{2+}), nickel (Ni^{2+}), copper ($Cu^{1/2+}$) and iron (Fe^{3+}). Nature has developed at least two ways, to provide for cellular availability of these important components, namely, specific import systems and unspecific cotransport systems. Especially in the latter case, cells need mechanisms to ensure that concentrations of otherwise essential metals do not become too high and that generally toxic metals, taken up fortuitously, are removed from the cell. In prokaryotes this is mainly achieved by specific efflux systems or sequestration of the compounds in question within the cell wall. In eukaryotes the best known mechanism is the intracellular sequestration of essential and toxic metals by a group of proteins called metallothioneins. In a few selected cases the control loops of effector and regulator molecules are reasonably well understood. This review will present our current knowledge regarding the transcriptional regulation of genes involved in heavy metal homeostasis and heavy metal detoxification with a main emphasis on metallothionein genes.

Metallothionein, a Protein Meant to Bind Heavy Metals

Sometime ago a protein responsible for the natural accumulation of cadmium in equine kidney cortex was described (Margoshes and Vallee, 1957). Due to its remarkably high content in sulfur, in the form of cysteine, and its ability to bind heavy metal it was named metallothionein (MT). Since then, it has been shown that metallothioneins comprise a class of highly conserved isoproteins found in organisms as different as fungi and man (Figure 7.1) (Kaegi and Kojima, 1987). For example, the *Neurospora crassa* MT has its cysteine residues at exactly the same positions as

Mouse	MT-I	Ac-MDPN**C**S**C**STGGS**C**T**C**TSS**C**A**C**KN**C**K**C**TS**C**KKS**CC**S**CC**PVG**C**SK**C**AQG**C**V**C**KGAADK**C**T**CC**A
Mouse	MT-II	Ac-MDPN**C**S**C**ASDGS**C**S**C**AGA**C**K**C**KQ**C**K**C**TS**C**KKS**CC**S**CC**PVG**C**AK**C**SQG**C**I**C**KQASDK**C**S**CC**A
Rat	MT-I	X-MDPN**C**S**C**STGGS**C**T**C**SSS**C**G**C**KN**C**K**C**TS**C**KKS**CC**S**CC**PVG**C**SK**C**AQG**C**V**C**KGASDK**C**T**CC**A
Rabbit	MT-I	Ac-MDPN**C**S**C**AABGS**C**T**C**ATS**C**R**C**KE**C**K**C**TS**C**KKS**CC**S**CC**PAG**C**TK**C**AQG**C**I**C**KGASDK**C**S**CC**A
Monkey	MT-I	MDPN**C**S**C**ATGVS**C**T**C**ADS**C**K**C**KE**C**K**C**TS**C**KKS**CC**S**CC**PVG**C**AK**C**AQG**C**V**C**KGASEK**C**N**CC**A
Human	MT-Ia	MDPN**C**S**C**ATGGS**C**T**C**TGS**C**K**C**KE**C**K**C**NS**C**KKS**CC**S**CC**PMS**C**AK**C**AQG**C**I**C**KGASEK**C**S**CC**A
N.Crassa	MT	MGD**C**G**C**SGASS**C**N**C**GSG**C**S**C**SN**C**GSK

Figure 7.1 Amino acid sequences of class I metallothioneins (mouse, rat, rabbit, monkey, human and *Neurospora crassa*). MT-I and MT-II designate two closely related isoforms of the class I metallothionein proteins which can be readily distinguished by reversed phase HPLC, due to the presence of an acidic amino acid residue in position 10 or 11 of the standard sequence of MT-II compared to the neutral residue found in MT-I isoforms (Kaegi and Kojima, 1987). The cysteines of the characteristic Cys-Xaa-Cys and Cys-Xaa-Xaa-Cys motifs (Xaa = any amino acid except cysteine) are in bold. X indicates an undetermined blocked aminoterminus, Ac indicates an acetylated aminoterminus.

mammalian metallothioneins, however, it is only half the size of a mammalian metallothionein, and thus corresponds to their N-terminal β-domain (described below) (Münger et al, 1985). In the mouse, there are four metallothionein isogenes (MT-I to MT-IV) clustered within some 50 kb on chromosome 8, and in human there is one MT II gene and a cluster of closely linked MT I genes on chromosome 16 (Searle et al, 1984; West et al, 1990; Quaife et al, 1994). Among the human metallothionein genes there are also several pseudogenes.

Mammalian metallothioneins generally contain 61 amino acids, 20 of which are cysteines. These cysteines are organized in characteristic Cys-Xaa-Cys or Cys-Xaa-Xaa-Cys motifs (where Xaa is any amino acid except Cys) at conserved positions within the metallothionein (see Figure 7.1). Generally, metallothioneins do not contain aromatic amino acids or histidine. All cysteines are involved in the binding of seven equivalents of bivalent transition metal ions, which in most cells are predominantly zinc, copper and cadmium (Table 1).

The metal ions are exclusively bound via thiolate clusters in the alpha-domain and beta-domain of metallothionein (Figure 7.2 A, B) (Andrews, 1990; Hamer, 1986). Interestingly, the highly toxic cadmium is bound with a 10,000-fold higher avidity than zinc, underlining the possible role of metallothionein in heavy metal detoxification (Durnam and Palmiter, 1987). Indeed, it has been shown recently that organisms deficient for their metal-inducible metallothionein genes are much more sensitive to heavy metals than their wild-type counterparts (Hamer et al, 1985; Ecker et al, 1986; Michalska and Choo 1993; Masters et al, 1994). Other

Table 1. Occurrence and Metal Composition of Metallothionein

Species	Organ	Metal composition			
		Zn	Cd	Cu	Hg
Man	Liver	++++++	+/−	+/−	
	Fetal liver	++++		++	
	Neonatal liver	++++		++	
	Kidney	+++	+++	+	+/−
Horse	Liver	++++++	+	+/−	
	Kidney	+++	+++	+/−	
	Intestine	++++	++	+/−	
Rat	Neonatal liver	+++++		+	
	Neonatal kidney	+++++			
	Adult kidney	++		++++	
	Adult testis	+++++		+	

All data refer to metallothioneins obtained from organisms not subjected to experimental pretreatment with metals (Reprinted with permission of Birkhäuser Verlag, from Kaegi and Kojima, 1987).

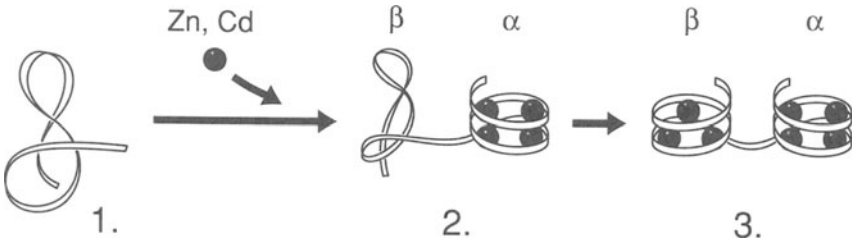

Figure 7.2A Model for binding of heavy metals to the metal-free apometallothionein. Apometallothionein exists as a random coil (1) which binds metal ions shown in black spheres in a sequential and ordered fashion. It first forms a carboxyterminal alpha-domain (2), containing four equivalents of a divalent metal ion, and then an amino-terminal beta-domain (3), containing three equivalents of a divalent metal ion (Pande et al, 1985; Stillman et al, 1987).

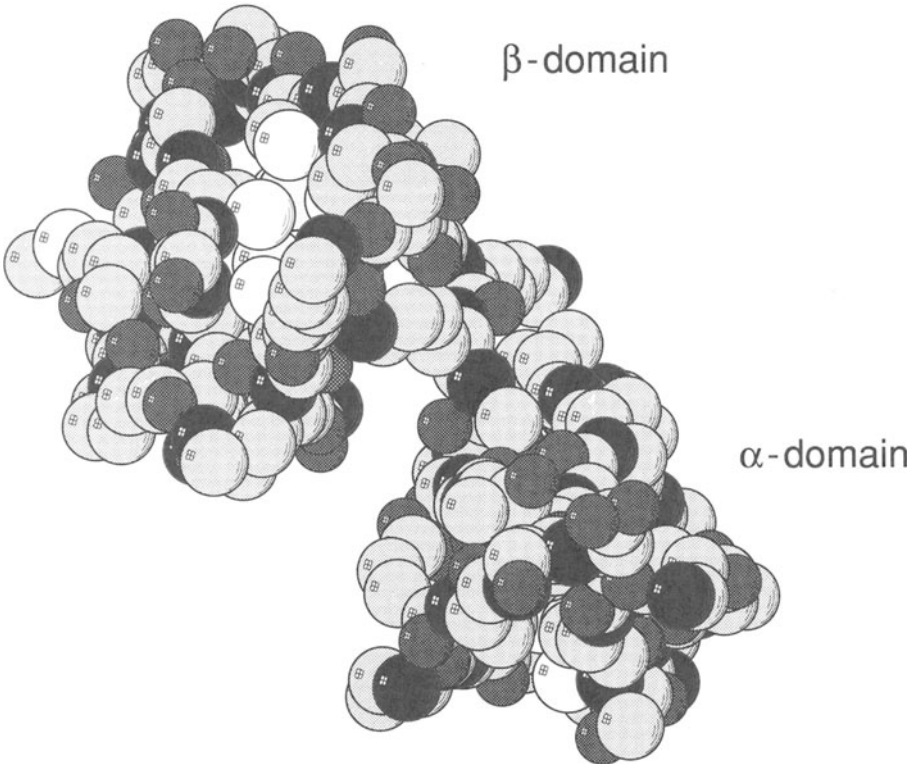

Figure 7.2B Space filling model of Zn_2Cd_5-metallothionein. The beta-domain contains a cluster of three metal ions, respectively. White spheres denote sulfur atoms; the white spheres appearing in a vertical line from the bottom to top represent cysteines 13, 7 and 5 of the beta-cluster. Stippled spheres, carbon; grey spheres,-oxygen; black spheres, nitrogen atoms. (Reprinted with permission of Academic Press LTD from Robbins et al, 1991.)

possible functions discussed for metallothioneins I and II are homeostasis of essential metals, i.e. zinc and copper, in vivo (Bremner and Beatti, 1990), and generalized cellular stress responses as deduced from their inducibility by such diverse stimuli as heavy metals, different forms of irradiation, high oxygen tension, hormones, interleukins, tumor promoters, infection, inflammation and many more (Table 2).

In marked contrast to the ubiquitously expressed metallothionein-I and -II genes (Hamer, 1986), there are the cell type- and developmental

Table 2. Factors that induce metallothionein synthesis in cultured cells or in vivo

Heavy metal ions	Antibiotics
Cd, Zn, Cu, Au, Ag, Co,	Streptozotocin
Ni, Bi	Cycloheximide
Hormones and second messengers	Mitomycin
Glucocorticoids	Cytotoxic agents
Progesterone	Hydrocarbons
Estrogen	Ethanol
	Isopropanol
Catecholamines	Formaldehyde
Glucagon	Fatty acids
Angiotensin II	Butyrate
Arg-Vasopressin	Chloroform
Adenosine	Carbon tetrachloride
	Bromobenzene
cAMP	Iodoacetate
Diacylglycerol	Urethane
Calcium	Ethionine
Growth factors	Di(2-ethylhexyl)phthalate
Serum factors	α-Mercapto-β-(furyl)acrylate
Insulin	6-Mercaptopurine
IGF-1	Diethyldithiocarbamate
EGF	Penicillamine
Inflammatory agents and cytokines	2,3-Dimercaptopropanol
Lipopolysaccharide (LPS)	2,3-Dimercaptosuccinate
Carrageenan	EDTA
Dextran	5-Azacytidine
Endotoxin	Acetaminophen
	Indomethacin
Interleukin-1	Stress-producing conditions
Interleukin-6	Starvation
Interferon-γ	Inflammation
Tumor necrosis factor	Laparotomy
Tumor promoters and oncogenes	Physical stress
Phorbol esters	X-irradiation
ras	O_2 tension
Vitamins	Ultraviolet radiation
Ascorbic acid	
Retinoate	
1α,25-Dihydroxyvitamin D_3	

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stage-specific metallothionein-III and -IV genes. Human metallothionein-III, about 70 % homologous to human metallothionein-IIA, is expressed exclusively in the brain and has been reported to be reduced in patients with Alzheimer's disease. In cell culture, the addition of purified metallothionein-III reduces the growth of neurons, and it is speculated that the lack of metallothionein-III contributes to the increased regenerative processes that accompany the extensive degeneration of hippocampal and cortical brain structures of Alzheimer patients (Uchida et al, 1991). Metallothionein-IV is specifically expressed in stratified squamous epithelia associated with several organs such as oral epithelia and footpads (Quaife et al, 1994).

Although all four metallothionein isoproteins can confer elevated cadmium resistance when overexpressed in tissue culture, only the metallothionein-I and -II are inducible by heavy metal (Palmiter et al, 1992; Quaife et al, 1994). Therefore we will restrict our discussion to the metallothionein-I and -II, unless otherwise mentioned.

Transcriptional Regulation of the Metallothioneins

The cis Elements

The regulation of metallothionein is mostly exerted at the transcriptional level, since first, the relative rates of synthesis of rat liver metallothionein-I and -II directly correlate with the amounts of metallothionein mRNAs in these tissues and second, the induction of metallothionein mRNA synthesis is independent of the protein biosynthesis inhibitor cycloheximide (Karin et al, 1980; Durnam and Palmiter, 1981).

Initial investigations of the mouse metallothionein-I promoter including deletion and linker scanning mutation analyses lead to the conclusion that a set of similar short sequence motifs present within the first 200 bp upstream of the transcription start site are responsible for induction by heavy metals. Hence, these sequences were called MREs, for metal responsive elements (Stuart et al, 1984; Carter et al, 1984). Palmiter and colleagues have shown that each single MRE of the mouse metallothionein-I promoter can confer metal-inducible transcription to a reporter gene when cloned as tandemly repeated sequences upstream of the reporter gene's TATA box. In this assay they further note that different MREs also have a different activation potential, with MREd being the strongest inducible element (Stuart et al, 1985). By comparing all known MRE sequences and performing detailed analyses of specific point mutation-MREs, a 15 bp consensus sequence containing a mini-

mMT-I	a	-54	CTTTGCGCCCGGACT	-40
	b	-56	GTTTGCACCCAGCAG	-70
	f	-94	CTATGCGTGGGCTGG	-80
	c	-132	AAGTGCGCTCGGCTC	-118
	d	-150	CTCTGCACTCCGCCC	-136
	e	-175	CTGTGCACACTGGCG	-161
CONSENSUS			CTNTGCRCNCGGCCG	

Figure 7.3 MRE sequences of the mouse metallothionein I promoter. Numbers denote nucleotide positions relative to the transcription start site. The consensus sequence shown below is derived from a comparison of MREs present in the mouse metallothionein I promoter. The core region, containing the five virtually invariant bases is underlined. The MREs a to e were originally defined by mutational analyses, whereas MREf was detected later by in vivo footprinting studies (Stuart et al, 1985, Mueller et al, 1988).

mal core of 7 bp has been established to be necessary for functional integrity as a metal regulatory *cis* element (Figure 7.3) (Searle et al, 1987; Culotta and Hamer, 1989; Imbert et al, 1990). Additional interesting features of MREs are that they can be found, in either orientation, upstream of all heavy metal-inducible metallothionein genes analyzed to date (Karin et al, 1984; Otto et al, 1987; Harlow et al, 1989; Andersen et al, 1986; Zafarullah et al, 1988), and that both the mouse metallothionein-I promoter or a synthetic 8xMREd element can confer metal-inducible transcription to a heterologous gene from a remote position, i.e. work as inducible enhancers (Serfling et al, 1985; Westin and Schaffner, 1988a). There are other known *cis*-acting transcriptional activator sequences in metallothionein promoters, e.g. binding sites for the transcription factors Sp1, AP1, AP2, MLTF and GR, some of which have been shown to activate transcription of metallothionein promoters in vitro (Lee et al, 1987a; Lee et al, 1987b; Mitchell et al, 1987) or in vivo (Figure 7.4) (Yagle and Palmiter, 1985; Karin et al, 1984). However, metal-inducible transcription can only be mediated by MREs. This general observation has been confirmed by in vivo footprinting, a technique that uses either chemical or enzymatic modification of DNA to analyze the interaction of DNA-binding proteins and their target sites in situ (Mueller et al, 1988). In the case of the mouse metallothionein-I promoter it has been shown that from the MRE sites, only MREd is protected to some extent by a bound protein under non-inducing conditions. After the addition of zinc however, a marked increase of protection is seen over MREd and most notably over the other 5 MRE sites. The protection pattern over the Sp1 and MLTF binding sites shows constitutive protein binding, i.e. independent of metal treatment of the cells (Mueller et al, 1988).

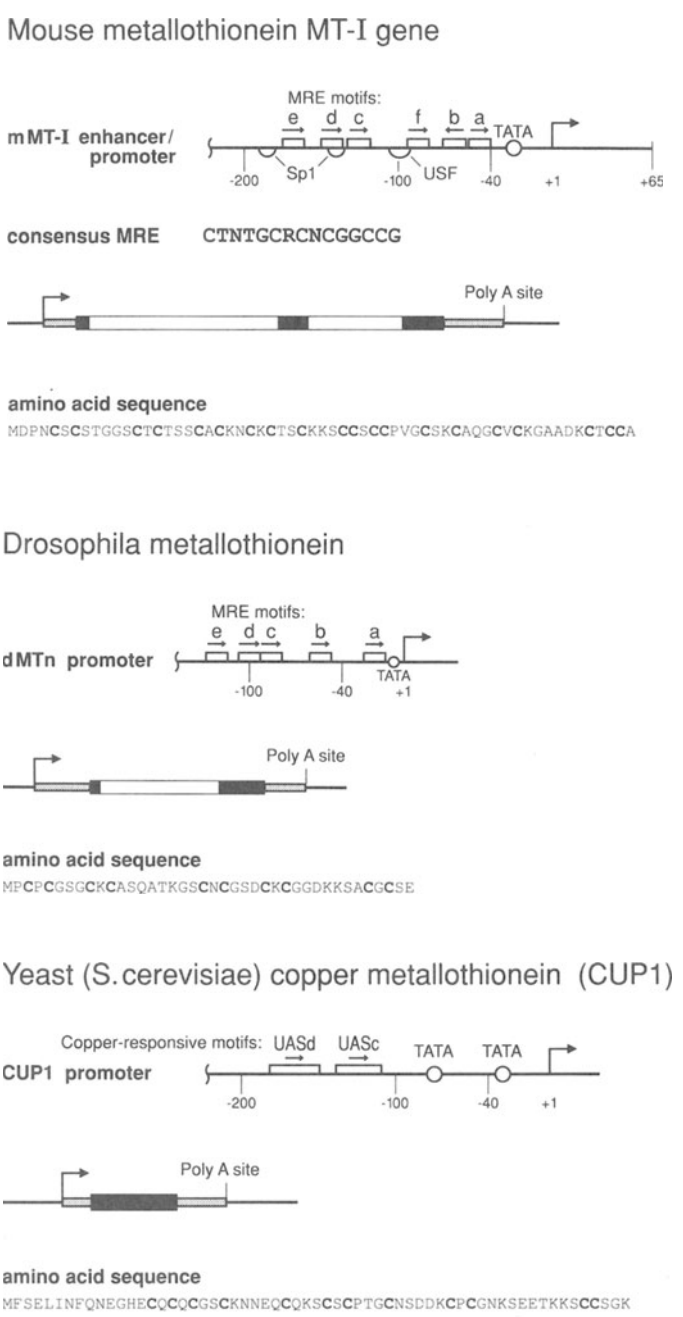


Figure 7.4 Promoter sequences from the genes of mouse metallothionein I (mMT-I), the *D. melanogaster* metallothionein (Mtn), and the yeast *S. cerevisiae* metallothionein gene (CUP1) are shown together with their respective gene structure and amino acid sequences. MRE, metal responsive element; UAS, upstream activator sequence; upstream/downstream untranslated sequences are represented as stippled boxes in the gene schema; black boxes and open boxes in the gene schema designate exon and intron sequences, respectively.

The Hunt for the Metal Inducibility Conferring Principle

After the detailed analysis of the metal-inducible promoter of the metallothionein genes, different laboratories tried to identify and ultimately clone and characterize factors interacting with the corresponding DNA sequences. First results were obtained by bandshift analyses (Westin and Schaffner, 1988a; Searle, 1990; Koizumi et al, 1992), in vitro and in vivo footprints (Mueller et al, 1988; Séguin, 1991), UV-crosslinking experiments (Andersen et al, 1990), and Southwestern blot analyses (Séguin and Prévost, 1988; Czupryn et al, 1992). Westin and Schaffner detected a specific protein-DNA interaction using the so-called bandshift or gel retardation assay. They incubated nuclear HeLa cell extracts and radioactively labelled MREd oligonucleotides from the mouse metallothionein-I promoter. These reaction mixtures were then resolved on a native (nondenaturing) polyacrylamide gel (PAGE). Labelled MREd oligonucleotides which had been specifically bound by protein showed a retarded migration (shift) compared to the free labeled DNA. Depending on the conditions for the initial binding reaction, they detected differently retarded bands. Using nuclear extracts without adding zinc to the binding reaction, only the constitutively active transcription factor Sp1 binds to MREd. Upon addition of zinc however another protein bound to MREd, resulting in a slightly faster migrating band. This DNA binding-activity has been termed MTF-1 (MRE-binding transcription factor) (Westin and Schaffner, 1988a).

Using methylation interference analyses, it has been shown that Sp1 and MTF-1 have overlapping but not identical binding sites. This in vitro technique allows the determination of individual bases that interfere with sequence specific binding of proteins due to chemical modification of the DNA prior to the binding reaction. Based on these results a model has been suggested in which elevated intracellular zinc concentrations trigger the binding of a preexisting zinc-dependent factor(s) to the MREs, resulting in metal-induced transcription. This model is also consistent with earlier experiments showing that zinc-induced metallothionein transcription in HeLa cells occurs even in the presence of protein synthesis inhibitors (Karin et al, 1980). The obvious zinc-dependence of MTF-1 for DNA-binding lead Westin and Schaffner (1988a) to propose that MTF-1 will bind to DNA with so-called zinc finger motifs as defined earlier for other transcription factors (Miller et al, 1985).

MTF-1 is a Zinc Finger Protein

MTF-1 has been cloned in our laboratory using a specifically designed MRE oligonucleotide to screen a mouse cDNA expression library of B-

cell origin (Radtke et al, 1993). This oligonucleotide has the advantage of binding very strongly to MTF-1, but unlike the original MREd oligonucleotide, it is unable to bind Sp1.

The cloned cDNA sequence of MTF-1 contains an open reading frame of 675 amino acids with a calculated molecular weight of 72.6 kDa. The amino-terminal half harbors six zinc fingers similar to the type found in the RNA polymerase III transcription factor TFIIIA (Cys_2His_2) (Brown et al, 1985; Miller et al, 1985). These structures are followed by three regions reminiscent of transcriptional activator domains, with high densities of acidic amino acids, proline and serine/threonine (Mitchell and Tjian, 1989; Seipel et al, 1992). From the three putative activation domains, the acidic one was shown to be active when fused to the GAL4 DNA binding domain (Xu et al, 1994).

Recombinant MTF-1 is Identical to the Endogenous MRE-binding Activity

Mouse recombinant MTF-1 which has been overexpressed in monkey COS cells, and the endogenous activity from mouse 3T6 cells both display identical migration behavior and binding activities in bandshift analyses. In addition, no difference in sensitivity to chelator treatment or partial proteolysis, or binding specificity and affinity to a set of mutant MRE oligonucleotide can be detected (Radtke et al, 1993).

MTF-1 is a Transcriptional Activator Protein

To test the biological activity of recombinant mouse MTF-1, human HeLa cells were cotransfected with an MTF-1 expression vector plus either natural or synthetic MRE-containing promoter/reporter gene plasmids. Recombinant MTF-1 strongly activated transcription of such reporter genes, but surprisingly, it did so even without zinc treatment of the transfected cells. These observations were not due to testing a mouse factor in human cells, since transfection of several mouse cell lines yielded the same result. There always was a clear dose-dependent relationship between the amount of transfected MTF-1 and the evoked basal, i.e. uninduced, transcriptional response. At the same time, the metal-induced transcription levels of MTF-1 transfected cells usually did not exceed the ones of cells without extra MTF-1 (Radtke et al, 1993). Therefore, some doubts remained regarding the role of MTF-1 in metallothionein gene regulation, despite the facts that recombinant MTF-1 bound to an MRE

oligonucleotide in the same zinc-dependent manner as the endogenous MRE-binding activity and that MTF-1 was the only detectable MRE-binding activity. Also, it was comforting to see that human MTF-1, recently cloned in our group, showed a pronounced zinc response in transfected cells (Brugnera et al, 1994).

Zinc Response in Higher Eukaryotes : The Role of MTF-1

To find out unambiguously whether MTF-1 is involved in metal regulated transcription of the metallothionein genes, a murine null mutant embryonic stem (ES) cell line (−/−) that lacks a functional MTF-1 gene was generated (Heuchel et al, 1994). Targeting vectors for homologous recombination were constructed in order to replace most of the first zinc finger exon with either the neomycin phosphotransferase gene or the hygromycin gene. These two replacement vectors were consecutively electroporated into ES cells. Positive clones were tested for homologous recombination by PCR and confirmed by Southern blot analysis (Figure 7.5).

In bandshift assays it has been demonstrated that nuclear extracts from −/− ES cells still contain the ubiquitous transcription factor Sp1, but no MTF-1 activity, irrespective of whether the nuclear extract is prepared

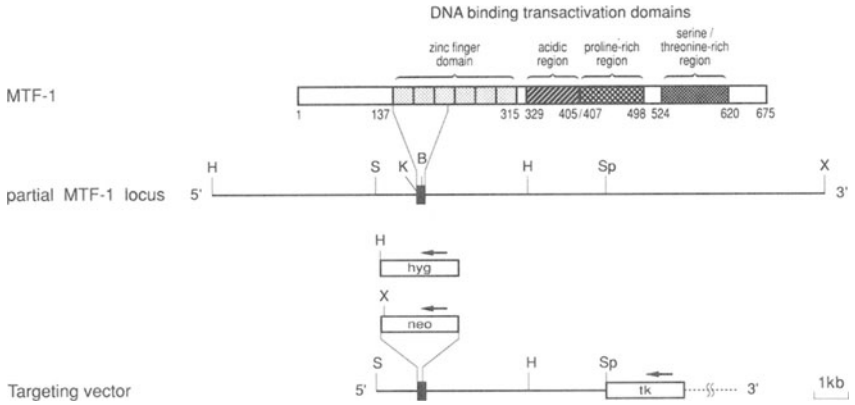


Figure 7.5 Disruption of the mouse MTF-1 locus in embryonic stem (ES) cells. A schematic diagram of the MTF-1 protein is shown together with the part of the MTF-1 gene locus containing the first zinc finger exon of 238 bp. The targeting vectors include 6.7 kb of genomic sequence. Two-thirds of the first zinc finger exon were replaced by either the neomycin cassette (neo) or hygromycin cassette (hyg) via homologous recombination. The neomycin and hygromycin genes were used for positive selection, whereas the herpes simplex virus thymidine kinase gene (tk) was used for negative selection. Sites for restriction enzymes: B, *BamHI*; H, *HindIII*; K, *KpnI*; S, *SacI*; Sp, *SpeI* (not all sites shown); X, *XbaI*; thin horizontal lines, intron sequences; black rectangle, first zinc finger exon; dotted line, pBluescript; arrows above positive and negative selection marker cassettes denote direction of their transcription (Heuchel et al, 1994).

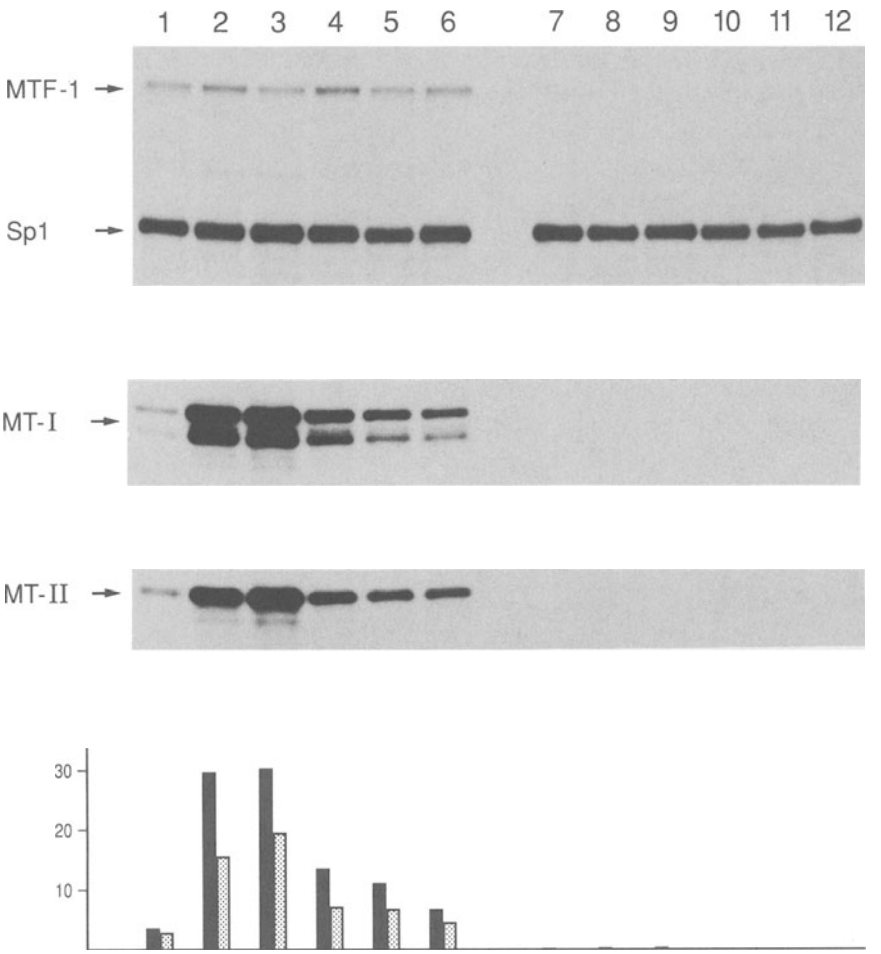
from uninduced or zinc-induced cells. +/+ ES control cells (wild-type for the MTF-1 locus but neomycin resistant due to fortuitous, nonhomologous vector integration) however, contain Sp1 and zinc-dependent MTF-1 activity.

To investigate the role of MTF-1 in metallothionein gene regulation, mRNA levels of the endogenous metallothionein-I and -II genes were measured. +/+ ES cells contain MTF-1 mRNA whose level was only marginally, if at all, changed by zinc treatment, which makes an autoregulatory loop for MTF-1 transcription rather unlikely. However, as expected, the levels of metallothionein-I/-II transcripts were significantly induced upon zinc treatment. In marked contrast, the −/− ES cells did not contain detectable amounts of MTF-1 transcripts or of metallothionein-I/-II mRNA, either before or after treatment of the cells with zinc. Similar results were obtained with other well known inducers of metallothionein genes, namely cadmium, copper, nickel, or lead (Figure 7.6). Results supporting the influence of MTF-1 on metallothionein expression came also from antisense experiments, where inducibility by a number of bivalent metals to stably integrated reporter genes driven by a 5xMREd promoter was found to be reduced (Palmiter, 1994).

In an attempt to restore metallothionein transcription by expression of additional MTF-1, we have transfected +/+ and −/− ES cells with reporter genes under the control of either 4 copies of the strong metal-responsive element MREd (4xMREd OVEC), or the complete mouse metallothionein-I promoter (MT-I OVEC). Treatment of +/+ ES cells with 400 μM zinc resulted in a 10-fold increase of transcription from a transfected 4xMREd reporter gene. By contrast, in the −/− ES cell line, reporter gene expression was barely detectable either with or without zinc treatment. After cotransfection of the cloned MTF-1 gene, transcription was restored to the same levels as observed with MTF-1 cotransfected +/+ ES cells. These results show that the metal-inducibility of natural and synthetic metal-responsive promoters is lost upon disruption of the MTF-1 gene and can be restored to a large extent by cotransfection of the MTF-1 expression vector (Heuchel et al, 1994).

Models for Heavy Metal Regulated Transcription of Metallothionein Genes

The experiments done by Heuchel et al demonstrate that normal mouse MT-I/II gene regulation is dependent on the presence of MTF-1. Interestingly, even the complete metallothionein-I/II enhancer/promo-



Inducers	–	$ZnSO_4$ 400 μM	$CdSO_4$ 80 μM	$CuSO_4$ 500 μM	$NiCl_2$ 500 μM	$PbSO_4$ 250 μM		–	$ZnSO_4$ 400 μM	$CdSO_4$ 80 μM	$CuSO_4$ 500 μM	$NiCl_2$ 500 μM	$PbSO_4$ 250 μM
relative activity of MT-I (%)	100	850	870	390	320	190		<1	<1	<1	<1	<1	<1
relative activity of MT-II (%)	100	570	710	260	240	160		<1	<1	<1	<1	<1	<1
ES cell type	+ / +							– / –					

Figure 7.6 Loss of metallothionein gene regulation in MTF-1 −/− embryonic stem cells. Transcript levels for MTF-1, metallothionein-I and metallothionein-II were determined in MTF-1 +/+ (lanes 1 to 6) and MTF-1 −/− ES cells (lanes 7 to 12). Sp1 transcript levels were used as internal controls for RNA loading. Before harvesting, cells were treated with different metal salts as indicated. In the presence of MTF-1, a several-fold induction of both the metallothionein-I and -II genes can be observed upon challenging +/+ ES cells with different heavy metals (lanes 1 to 6). The transcript levels of Sp1 and MTF-1 itself are not influenced by this treatment. However, neither basal nor heavy metal-induced transcripts of both metallothionein genes can be detected in −/− ES cells which lack MTF-1, whereas Sp1 transcript levels are unchanged as expected (lanes 7 to 12) (Heuchel et al, 1994).

ter region was not activated to any appreciable extent in the absence of MTF-1. This means that MTF-1 is not only necessary for metal-induced but also for basal transcription of the metallothionein-I and -II genes. This is surprising, since it has been shown that the binding sites for the ubiquitous transcription factors Sp1 and MLTF/USF within the mouse metallothionein-I promoter are occupied in vivo, even without heavy metal challenge, and one might thus have expected them to be responsible for basal transcription (Mueller et al, 1988). These factors may nevertheless have some auxiliary role in concert with MTF-1. Based on (1) the absolute dependence on MTF-1 for metallothionein transcription, (2) the independence of de novo protein synthesis for transcriptional induction and (3) the zinc-dependent DNA binding of MTF-1 in vivo and in vitro, several models for metal induction can be envisaged.

In the uninduced case, i.e. without heavy metal challenge, only a small percentage of MTF-1 protein is able to bind to its recognition sites, with MREd being the site with the highest binding affinity. There are several explanations for this finding. It is conceivable that the binding of nuclear MTF-1 to DNA is very sensitive to changes in intracellular zinc concentrations. Indeed, Radtke et al noticed a four-fold increase in MTF-1 binding activity in vitro after challenging the cells for four hours with zinc (Figure 7.7) (Radtke et al, 1993). At low zinc concentrations, it might be that only a fraction of the whole nuclear MTF-1 population is competent for DNA binding or that only a fraction of the six zinc fingers necessary for DNA binding are saturated with zinc. Alternatively, the nuclear factor concentration could be subcritical at low zinc conditions because, for example,

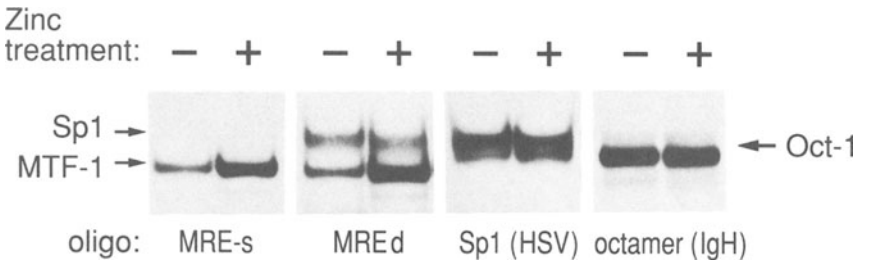


Figure 7.7 Bandshift analysis using nuclear extracts from uninduced (−) and zinc-induced (+) mouse 3T6 cells. For induction, cells were treated with 100 μM $ZnSo_4$ for 4 hr before harvesting. The positions of the protein-DNA interactions are indicated as Sp1, MTF-1 or Oct-1. MRE-s contains a single binding site for MTF-1, whereas MREd contains overlapping binding sites for both MTF-1 and Sp1. In marked contrast to the MTF-1 signal intensity, which increases about four fold upon zinc induction, the signal intensities for Sp1 and Oct-1 remain unchanged (Reprinted with permission of Oxford University Press from Radtke et al, 1993).

MTF-1 may be sequestered in the cytoplasm by (1) an RNA as in the case of the zinc finger factor TFIIIA, which can bind to the 5S RNA gene internal control region and to 5S RNA itself (Picard and Wegnez, 1979; Theunissen et al, 1992) or (2) as speculated by R.D. Palmiter, by a cytoplasmic anchoring factor as for example seen with NFκBF and IκB (Henkel et al, 1993; Palmiter, 1994). We have shown that a nuclear extract from zinc-treated cells has always a higher MTF-1 binding activity than nuclear extracts from untreated cells even though saturating amounts of zinc were added to the in vitro binding reaction (Figure 7.8), which is compatible with several possibilities.

If the intracellular zinc concentration is elevated, it could be that all six zinc fingers become saturated with zinc and bind, here in the case of the mouse metallothionein-I promoter, to all MRE sites thereby cooperating to bring about a high transcription rate (Figure 7.9A), or MTF-1 might be released from its interaction with some cytoplasmic component for transport into the nucleus (Figure 7.9B). It is also conceivable that there is a modification system that modulate the transcriptional competence of MTF-1 according to intracellular changes in the zinc concentrations. For example, the transcription factor c-Jun is differently phosphorylated upon different stimuli which modulates its transcriptional competence (Hunter and Karin, 1992). Another possibility would be the existence of a repressor which, in the absence of zinc, keeps the metallothionein promoter in a closed, inaccessible chromatin state (Figure 7.9C), or there might even be a specific coactivator involved as illustrated in

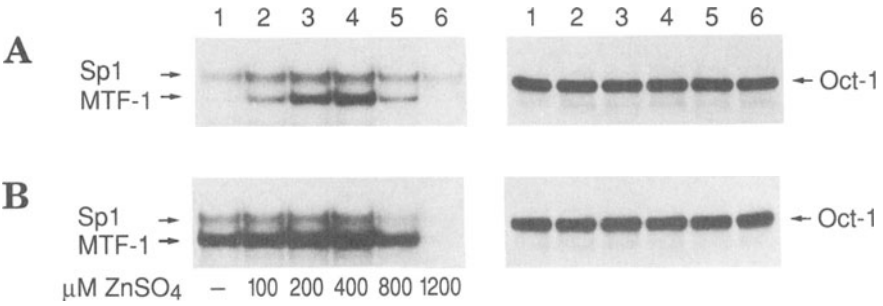


Figure 7.8 Zinc-induced DNA binding of MTF-1 from untreated and zinc-treated cells. Nuclear extracts were prepared from untreated cells (A) and cells treated with 100 μM zinc sulfate for 4 hr prior to harvest and extract preparation (B). Lanes 1-6, increasing amounts of $ZnSO_4$ as indicated were added to the binding buffer before the DNA-protein binding reaction and gel electrophoresis. The lefthand side shows bandshifts with the MREd oligonucleotide, which binds both Sp1 and MTF-1. The righthand side shows bandshifts using an octamer site as a control for the amount of protein loaded, since the Oct-1 binding activity is known to be insensitive to zinc treatment (Westin and Schaffner, 1988a). MTF-1 binding in nuclear extracts from zinc-pretreated cells is always higher than from untreated cells, even if saturating amounts of zinc are added to the binding reaction.

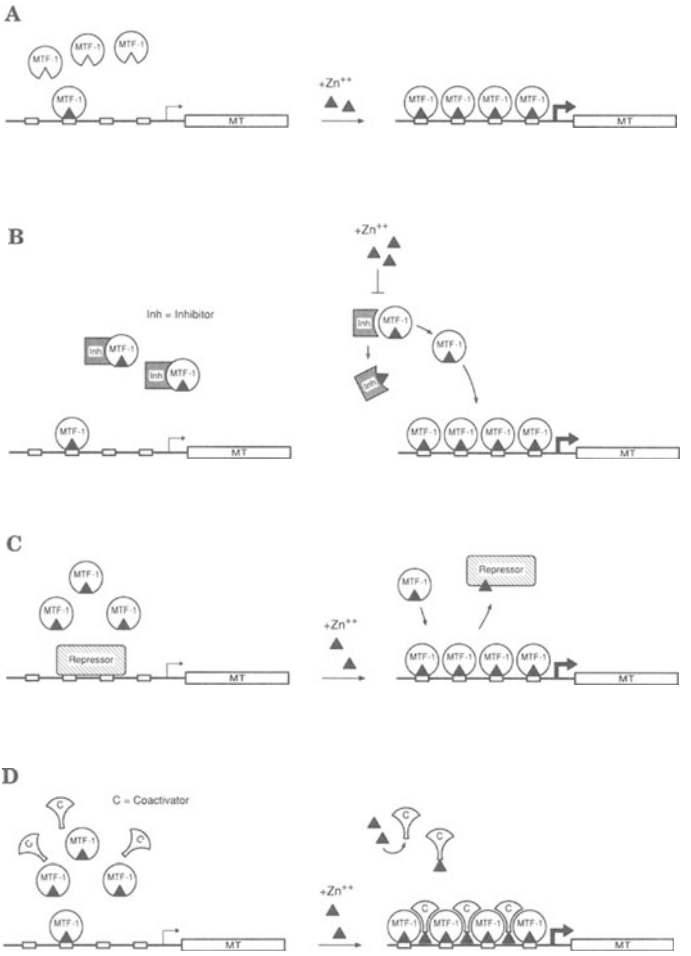


Figure 7.9 Models for metal-induced transcription of metallothionein genes by MTF-1 (A) Allosteric zinc finger model. Transcriptional induction of the metallothionein gene is solely dependent on promoter occupancy by MTF-1. Zinc acts as a coinducer in that it transforms the loose zinc finger structures of MTF-1 into a DNA binding-competent form. This model proposed for mammalian MTF-1 has been shown independently to be correct for the yeast copper metallothionein systems (Westin and Schaffner, 1988; Fuerst et al, 1988). (B) Protein/MTF-1 inhibitor model. Under normal conditions most of the MTF-1 is bound by an inhibitor that is released by intracellular increase in the zinc concentration. (C) DNA repressor model. The metallothionein promoter is kept in an inactive state by a repressor bound to the DNA. Upon an increase in the intracellular zinc concentration the repressor, which itself may have an affinity for zinc is exchanged for high affinity binding MTF-1. However, this scenario is rather unlikely in the light of the in vivo footprinting data by Mueller et al (1988). They noted that only MREd among all MREs of the mouse metallothionein-I promoter is bound by protein in untreated cells. Treating the cells with $ZnSO_4$ only increases the general intensity of the footprint over MREd, but there is no qualitative change in any of the protected guanosine bases. From this one still cannot exclude the possibility of a repressor with binding properties similar to MTF-1. (D) Coactivator model. In this model, a specific coactivator binds zinc upon increase of the intracellular zinc concentration and interacts with MTF-1 proteins to fully induce transcription of the metallothionein genes. A combination of model B, C or D with model A is possible.

Figure 7.9D. Finally, it is also possible that the heavy metal regulated transcription of the metallothionein genes is in fact a mixture of all possibilities mentioned above.

MerR, a Mercury Regulated Repressor/activator of Prokaryotic Transcription

While exciting problems remain to be solved in heavy metal-inducible gene transcription of mammals, more is known about such regulation processes in micro-organisms. Interestingly, bacteria, and yeasts, have evolved their own systems specifically tailored to cope with heavy metal load. In prokaryotes metal ions are taken up either by specific and regulated transport systems as in the case of nickel, or they are coimported by constitutive Mg^{2+} transport systems. Due to the broad specificity of the ion uptake systems, nonessential or even toxic metals such as cadmium (Cd^{2+}) can also enter the cell. In addition, even essential metals can accumulate to toxic levels within a bacterial cell (Silver and Walderhaug, 1992; Nies, 1992). Therefore resistance mechanisms, mostly encoded on plasmids, have evolved which either actively export metal ions themselves, inactivate and remove metal ions by sequestration, or reduce the ions to metallic form which can passively leave the cell. The latter is found for mercury. Resistance towards mercury is widespread in gram-negative and gram-positive bacteria (Helmann et al, 1990; Misra, 1992; O'Halloran, 1993). This complex system, encoded by several genes within the *mer* operon is a means for many bacteria to clear their microenvironment of toxic mercury. The operon comprises genes for mercury transport designated *merT* and *merP*, mercury metabolism (*merA merB*) and for the transcriptional regulation (*merR*) of the operon itself. The key enzyme in the detoxification process is the mercuric ion reductase (*merA*). This enzyme catalyses the reduction of Hg(II) to the volatile and lipophilic Hg(0), which then passively diffuses from the cell. Many *mer* operons also contain another mercury specific enzyme, organomercurit lyase (*merB*), which breaks the C-Hg bond, thereby converting a highly toxic compound into the less toxic Hg(II) which can then be metabolized by MerA. The *mer* operon is divergently expressed with the structural genes *merT, P, (C), A,* and *D* transcribed into a single polycistronic mRNA encoded by one strand and the overall regulator protein *merR* encoded by the other strand of the DNA molecule. The *merR* gene encodes a transcriptional repressor/activator of the classical helix-turn-helix protein class (Helmann, 1989). It binds to its operator sequence as a homodimer between the two

hexanucleotide polymerase recognition elements at the positions -35 and -10 of the P_T promoter. Independently of the presence of Hg, repressor binding introduces a slight bend into the operator DNA (Ansari and O'Halloran, 1992). In the absence of Hg(II), MerR represses the

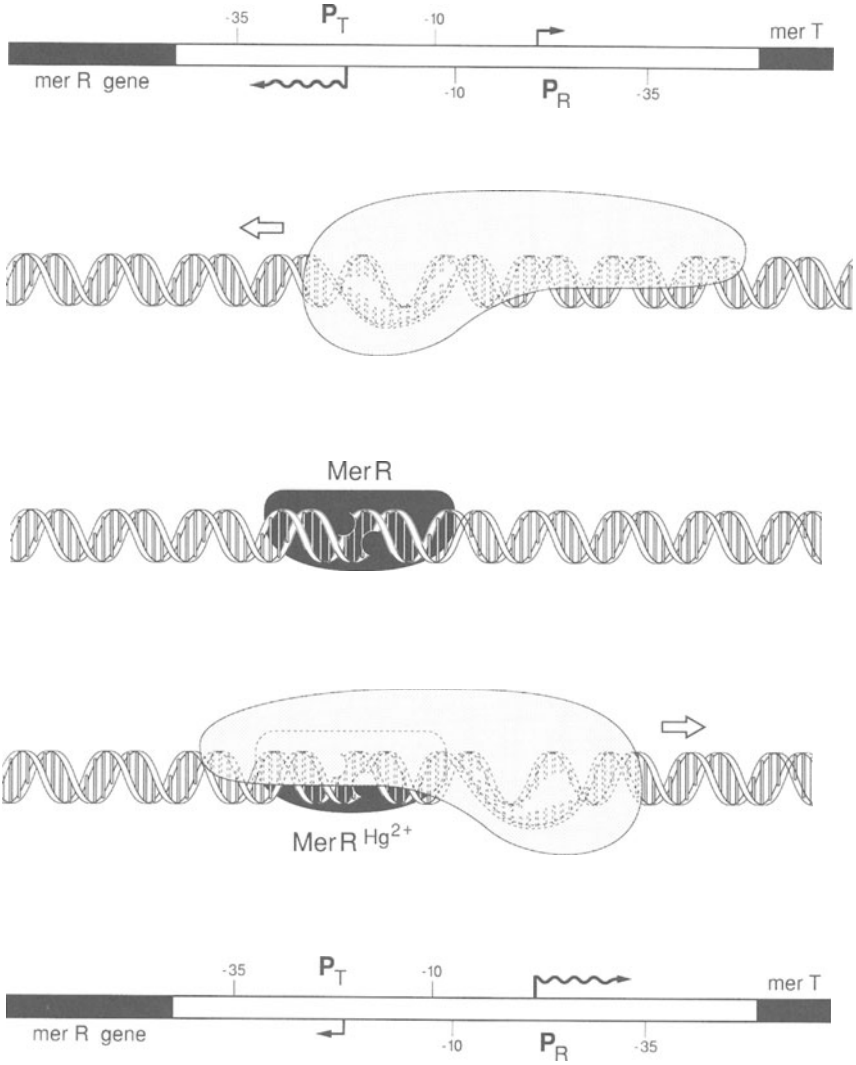


Figure 7.10 Model for the regulation of the divergently arranged promoters of the *mer* operon. Based on footprinting results with σ^{70}RNA polymerase, MerR and the *mer* promoters (P_R and P_T), the following scenario can be envisaged: in the absence of Mer R, polymerase binds to the P_R promoter and expresses *mer* R. The repressor MerR binds between the -35 and -10 RNA polymerase recognition elements of P_T, thereby sterically hindering binding of RNA polymerase to P_R. Instead, RNA polymerase binds loosely to MerR-bound P_T. In this form RNA polymerase is unable to form an open transcription initiation complex, which is only possible if MerR binds mercury. This way, MerR is transformed from a repressor to an activator protein. Adapted with permission fromO'Halloran et al (1989).

structural gene promoter P_T by keeping it a low affinity site for RNA polymerase, and it also represses its own promoter P_R by steric hindrance (Figure 7.10). In the presence of mercury, however, bound MerR homodimer binds one Hg(II) and by an allosteric mechanism induces an additional unwinding of its DNA recognition site of about 30° when compared to the mercury-free form (Ansari and O'Halloran, 1993). Thereby the -10 and -35 regions of P_T, which are suboptimally phased by an unusually large spacing of 19 base pairs (bp) instead of the optimal 17 bp for *E.coli* promoters (Figure 7.11A) (Harley and Reynolds, 1987; Hawley and McClure, 1983) are twisted to achieve a perfect architecture resulting in a high affinity promoter for RNA polymerase (Figure 7.11B, C) (Ansari et al, 1992). Thus MerR fulfills two tasks at the same time. It represses its own transcription independent of the presence of Hg(II) as long as the repressor concentration is high enough, and it activates the genes for Hg-metabolism/detoxification only when the specific ligand is present.

Heavy Metal Resistance in Lower Eukaryotes Conferred by Class II and Class III Metallothioneins

The class I metallothioneins of higher eukaryotes are characterized by the specific arrangement of cysteines closely related to the first characterized prototype of metallothioneins, from horse kidney. Recently, groups of clearly distinct metallothioneins have been detected, termed class II and class III metallothioneins (Fowler et al, 1987). The class II metallothioneins are characterized by an arrangement of cysteine-rich motifs which are only distantly related to those in horse metallothionein. Representative of this group were found in the cyanobacterium *Synechococcus spec.* (Olafson et al, 1988; Shi et al, 1992), in the yeasts *S. cerevisiae* and *C. glabrata* (Winge et al, 1985; Thiele, 1992), the worm *Caenorhabditis elegans* (Slice et al, 1990; Imagawa et al, 1990) and a higher plant (Kawashima et al, 1992). The atypical class III metallothioneins were isolated first from the fission yeast *S. pombe*, here called cadystins, (Murasugi et al, 1981; Kondo et al, 1984) or from plants where they are called phytochelatins (Grill et al, 1985; Grill et al, 1991; Robinson et al, 1993). Chemically, the class III metallothioneins are not proteins or peptides encoded by a specific gene but rather γ-glutamyl isopeptides (γ-glutamylcysteinyl-glycine; Figure 7.12), enzymatically synthesized from glutathione and range in M_T from 2000 to 10,000 (Figure 7.11) (Rauser, 1990; Steffens, 1990). In plants the phytochelatins are mainly involved in binding of copper and cadmium (Grill E, 1987; Robinson et al, 1987). An

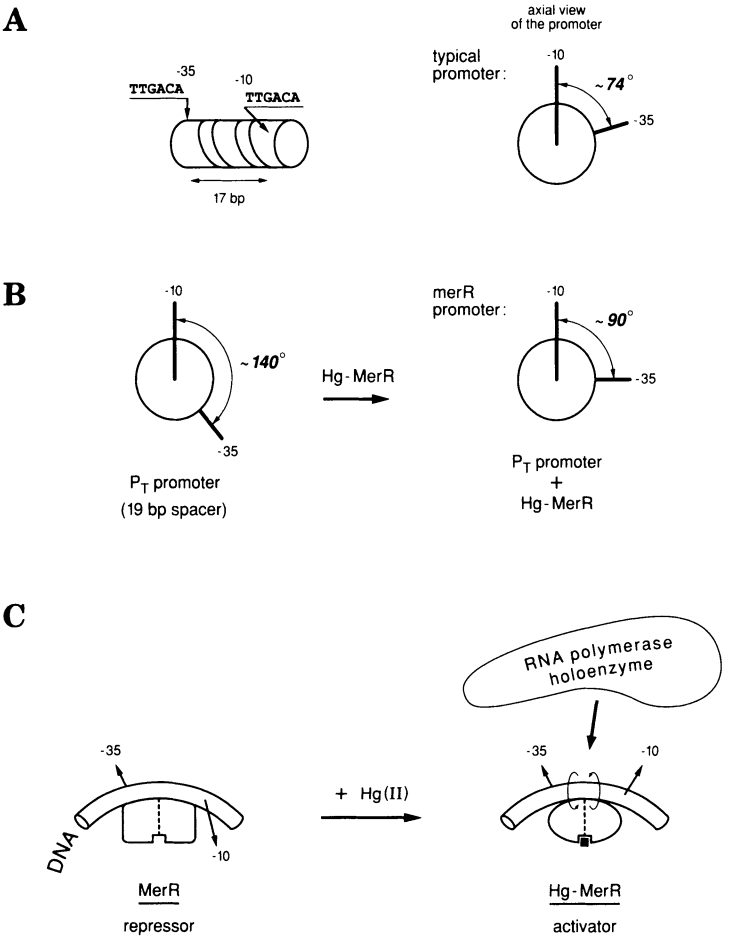


Figure 7.11 Change of promoter geometry allows transcriptional activation of the *mer* promoter P_T. (A) The relative positions of the -35 and -10 σ^{70}RNA polymerase recognition elements are schematically shown on an *E.coli* consensus promoter, here depicted as a cylinder. If we look down the helix axis, the DNA appears as a circle and the bars that are placed at the center of the -10 and the -35 regions are offset by a dihedral angle of 74°. (B) Between the -35 and -10 elements of the P_T promoter, there is a 19 bp spacing sequence. As a result of this unusual spacing, the dihedral angle is offset 140° , and consequently P_T is only a weak promoter. The underwinding introduced by binding of merR and Hg-MerR would face the -35 and -10 promoter elements such that they would fit quite closely to the dihedral angle typically seen in consensus *E. coli* promoters. (C) Local DNA distortion model for allosteric activation of the *mer* T promoter. Independently of the presence of mercury, homodimeric repressor MerR binds to the *mer* operator/promoter DNA, shown as a cylinder. This binding introduces a slight bending in the target DNA. The arrows projecting from the major groove of the DNA double helix indicate the relative positions of the -10 (projecting into the plane of the paper) and -35 (projecting out of the plane of the paper) σ^{70}RNA polymerase recognition elements. Upon binding of one mercury ion, Hg(II)-MerR twists the DNA in between the repressor monomer hands of the dimer-operator DNA complex, thereby underwinding the DNA and phasing the -10 and -35 regions for optimal interaction with the RNA polymerase (to initiate transcription). Adapted with permission from Ansari and O'Halloran (1994).

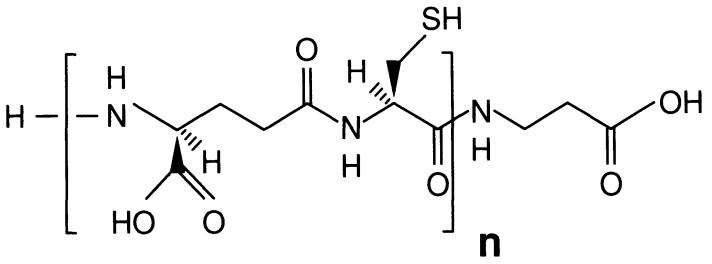


Figure 7.12 General formula of phytochelatins, the so-called class III metallothioneins. In plants the main compound to sequester cadmium ions is a group of peptides called γ-glutamyl isopeptides. The most common ones have the structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where $n = 2$ to 11, depending on the organism. In the bean *Phaseolus spec.* (order Fabales), a slightly different variant is found. In the example shown here glycine has been replaced by β-alanine (Grill et al, 1986).

interesting aspect in the regulation of the phytochelatins is that their expression is stimulated by cadmium. In keeping with this notion, the constitutively expressed enzyme, γ-glutamylcysteine dipeptidyl transferase (phytochelatin synthetase), which synthesizes phytochelatins, requires cadmium for catalytic activity (Grill et al, 1989).

Copper Resistance in Lower Eukaryotes

There are two yeasts, namely the baker's yeast *Saccharomyces cerevisiae* and the opportunistic pathogenic yeast *Candida glabrata*, which have well studied copper-inducible metallothionein genes (Thiele, 1992). Both systems have evolved from a common ancestor, but the one of *C. glabrata* is far more sophisticated, both with respect to the number of metallothionein genes and to regulation of the regulatory factor itself.

The Regulation of Metallothionein in Saccharomyces cerevisiae

S. cerevisiae has a single class II metallothionein gene, designated CUP1, which confers copper resistance on yeast cells (Brenes-Pomales et al, 1955; Butt and Ecker, 1987). CUP1 codes for a 61 amino acid long protein that has been shown to bind eight copper molecules per polypeptide, thereby preventing toxic effects of this metal (Karin et al, 1984; Butt et al, 1984; Winge et al, 1985). CUP1 gene transcription is inducible by copper and, to a lesser extent, by its electrochemical analog silver (Fuerst et al, 1988). This metal induction is mediated in *cis* by so-called upstream activator sequences (UAS_{CUP1}) within the CUP1 promoter. These confer metal-inducible transcription when fused to a

heterologous reporter gene and tested in yeast (Thiele and Hamer, 1986). However the MRE sequences regulating class I metallothioneins in higher eukaryotes do not show any sequence similarity to the yeast UAS_{cup1} sequences (Thiele, 1992). The fact that Cup1 confers copper-resistance in yeast was exploited by D. Thiele in an elegant series of experiments, applying classical yeast genetics to clone the responsible regulating factor. Towards this end he chemically mutagenized the copper-resistant yeast strain BR10 with EMS (ethylmethane sulfonate) to select for copper-sensitive survivors, which no longer accumulated CUP1 mRNA. He termed the resulting string ace1-1 for activation of CUP1 expression and used it to clone the responsible ACE1 gene by complementation (Thiele, 1988). Shortly thereafter these results were confirmed by the cloning of CUP2 by others, a gene which was later shown to be identical to ACE1 (Welch et al, 1989; Buchman et al, 1990). The transcriptional activator protein ACE1 is 225 amino acids long and its amino-terminal half exhibits striking similarity to CUP1 itself by the presence of the characteristic metallothionein Cys-Xaa-Cys and Cys-Xaa-Xaa-Cys motifs. Eleven of the twelve cysteine residues are crucial for copper dependent binding to the upstream activator sequences of the CUP1 promoter (Fuerst et al, 1988; Thiele, 1988; Huibregste et al, 1989; Buchman et al, 1990; Evans et al, 1990; Hu et al, 1990). In the absence of copper this combined metal and DNA binding domain does not have an ordered structural motif. Upon copper binding the polypeptide adopts a so-called copper fist structure, which is able to bind to the upstream activating sequences within the CUP1 promoter (Figure 7.13A). The carboxyterminal half is very rich in acidic amino acids, reminiscent of other yeast transcriptional activation domains such as those of GCN4 or GAL4 (Hope and Struhl, 1986; Ma and Ptashne, 1987). ACE1 is, however, not only required for metallothionein gene expression, but also regulates other cellular genes. It activates transcription of the yeast copper/zinc superoxide dismutase gene (Cu/Zn-SOD1), whose gene product protects the cell from oxygen toxicity (Gralla et al, 1991). Interestingly however, the ACE1 gene is not essential for the viability of *S. cerevisiae*, at least not under standard laboratory conditions (Thiele, 1988; Butler and Thiele, 1991).

The Regulation of Metallothioneins in the Yeast Candida glabrata

Candida glabrata has, in contrast to *S. cerevisiae*, two classes of metallothionein genes, a single MT-I gene and two distinct MT-II genes. The MT-II genes consist of a tandemly-amplified (3 to 9) MT-IIa

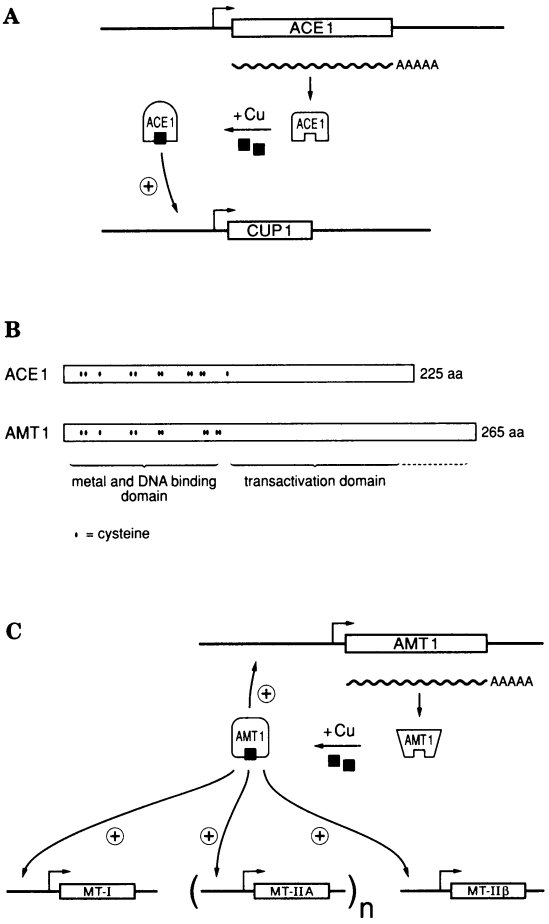


Figure 7.13 Model for the induction of the *S. cerevisiae* copper metallothionein gene CUP1 by ACE1. (A) The metal-regulatory transcription factor ACE1 is constitutively expressed and adopts a random coil structure in the absence of copper. In the presence of inducing amounts of copper the loose amino-terminal part of ACE1 changes into a so-called copper fist structure which is able to bind to the CUP1 upstream activator sequences (UAS_{CUP1}) for full transcriptional activation. (B) Schematic comparison of the yeast metallothionein gene activator proteins ACE1 and AMT1 from *S. cerevisiae* and *C. glabrata*, respectively. Relative locations of the metal and DNA-binding domains and the transactivation domains are indicated. Cysteine residues, indicated by dots in the metal and DNA binding domain, are arranged in Cys-Xaa-Cys motifs and Cys-Xaa-Xaa-Cys motifs characteristic for heavy metal binding proteins. Adapted with permission from Jungmann et al, 1993. Curiously, there are no cysteines whatsoever in the carboxyterminal transactivating portion of these factors.(C) Model for the induction of the *C. glabrata* copper metallothionein genes. Although the *C. glabrata* metallothionein system in principle functions like that of *S. cerevisiae*, namely via a copper-inducible transcriptional activator protein, there are fundamental differences. First, there are different metallothionein genes coding for isoproteins, reminiscent of the class I metallothionein gene families in vertebrates. Second, AMT1 which plays the role of the *S. cerevisiae* ACE1 in *C. glabrata*, not only induces the MT-I and MT-II genes, but also induces its own expression in a copper-dependent, autoregulatory fashion.

gene and an unlinked single copy metallothionein-IIb gene. The transcription of both classes of MT genes is activated by copper and silver but not by cadmium, unlike the class I metallothioneins of higher eukaryotes (Mehra et al, 1989; Mehra et al, 1990; Mehra et al, 1992). Again it was the group of D. Thiele and colleagues who cloned the ACE1 analog from *C. glabrata*. This time they selected for the ability to rescue a copper-sensitive *S. cerevisiae* host strain by transfecting a *C. glabrata* cDNA library which contained a *C. glabrata* MT-I cDNA. The factor responsible for the metal-induced activation was designated AMT1. As with ACE1, AMT1 acts as a copper sensor and transcriptional activator. Both factors show a high degree of structural and functional homology, an aminoterminal metallothionein-like copper and DNA binding domain, and a carboxyterminal acidic domain for transcriptional activation (Figure 7.13B) (Zhou and Thiele, 1991; Zhou et al, 1992). A significant and highly interesting difference between these proteins is, that the expression of ACE1 is constitutive, i.e. not influenced by the copper status of the cell, whereas the expression of AMT1 is more sophisticated in that it is induced by copper in an autoregulatory fashion (Figure 7.13C) (Szczypka and Thiele, 1989; Zhou and Thiele, 1993). As one would expect for a preexisting, inducible factor, AMT1 exhibits very fast activation kinetics in activating the MT-I and -II promoter as well as its own promotor within minutes (Zhou and Thiele, 1993).

Physiological Functions of Metallothioneins : Facts and Speculations

Originally, metallothionein was found as a mammalian protein responsible for the natural accumulation of cadmium, and to bind cadmium much more avidly than zinc. For this reason it has been assumed that metallothionein could have a function in heavy metal detoxification (Kaegi and Vallee, 1960; Piscator, 1964). This idea is supported by several findings: (1) a correlation has been found between the expression rate of metallothionein genes and a concomitantly increased resistance to heavy metals. This is achieved by either stable transformation of MT genes in cell culture or by selection for heavy metal-resistent cell lines which are found to have undergone amplification of the endogenous MT genes (Beach and Palmiter, 1981; Compere and Palmiter, 1981; Gick and McCarty, 1982; Karin et al, 1983; Schmidt et al, 1985); (2) a mutant strain of yeast *Saccharomyces cerevisiae* containing a functionally inert CUP1

gene, is much more sensitive to copper poisoning than the wild-type strain (Brenes-Pomales et al, 1955; Fogel and Welch, 1982; Hamer et al, 1985; Ecker et al, 1986); (3) mouse strains carrying a homozygous mutation for the metallothionein-I and -II genes are much more sensitive to cadmium toxicity than wild-type mice (Michalska and Choo, 1993; Masters et al, 1994). For unknown reasons, there is apparently a sex-specific difference in that female homozygous mutant mice are slightly more resistant to cadmium treatment than male homozygous mutant mice (Masters et al, 1994). Based on the many different ways metallothionein expression can be stimulated, a few additional possible functions for the metallothionein protein have been proposed, such as homeostatic control of zinc and copper, free radical scavenging, or as a general stress response protein.

The metallothionein of *Neurospora crassa*, which, as previously mentioned, is half the size of mammalian metallothioneins, functions as a copper storage and transfer protein (see Figure 7.1 for amino acid sequence). The copper enzyme tyrosinase is produced exclusively during sexual differentiation in *N. crassa*. Tyrosinase is a key enzyme involved in the synthesis of melanin, which gives the fruiting body its typical dark appearance. While the mycelium is growing under the surface, the fruiting body protrudes into the open, and melanin thus is needed to protect its cells from sunlight damage. There is good evidence from a combination of in vitro and in vivo studies that copper is stored during the vegetative growth phase in the form of Cu-metallothionein, which in turn can transfer copper by an as yet unknown mechanism to apotyrosinase at the time of fruiting body formation. Tyrosinase gene expression itself is independent of copper, whereas the catalytic activity of the enzyme is completely dependent on copper, as is shown when *N. crassa* is grown in copper-free medium (Lerch, 1980; Beltramini and Lerch, 1982; Huber and Lerch, 1987).

These findings argue for a general function of metallothionein in cellular metal homeostasis, ensuring that a sufficiently high intracellular concentration of essential metals are available at any particular time. However, since the metallothioneins bind their heavy metal ions with a very high affinity one can envisage several mechanisms for a redistribution of heavy metals into recipient molecules: (1) heavy metal-transfer molecules may serve as go-betweens to link metallothionein and a metal-free apoprotein; (2) (active) degradation of metallothionein releases metal that is then bound by apoproteins; (3) on the basis of the extremely high exchange rate for these metals, metallothionein may share metal ions with other proteins (Vasák and Kaegi, 1983; Nettesheim et al, 1985; Petering et al, 1987; Otvos et al, 1987; Schmid et al, 1990).

However, there are also data in support of the opposite process,

namely active removal of metal from target proteins by metallothionein whereby these target proteins would be downregulated or inactivated. In collaboration with the group of J.H.R. Kaegi and his colleagues, we have compared the activity of the zinc-dependent zinc finger transcription factor Sp1 and the zinc-independent homeo-domain transcription factor Oct-1 in HeLa cell nuclear extracts in the presence or absence of added apothionein (i.e. metal-free metallothionein). In vitro transcription studies showed that in the presence of added apothionein, only the reporter gene driven by a promoter containing binding sites for the octamer factor but not for Sp1 is transcribed. The reason for this behavior is explained by bandshift assays. Only the homeo-domain factor Oct-1, but not the zinc finger factor Sp1 is able to bind to its respective DNA recognition sequence in the presence of apothionein in the binding reaction. Similar results have been obtained for the RNA polymerase transcription factor TFIIIA (Zeng et al, 1991). These results are interesting in the light of the fact that RNA polymerases are also zinc dependent enzymes. This means that metallothionein may modulate the activity of certain metal-dependent proteins, whereas others like the vital RNA polymerases would not be affected. At first glance it seems quite unlikely that metallothionein is used for downregulation of some but not other classes of zinc-binding proteins. If a protein like Sp1, which has binding sites in the promoters of most housekeeping genes could be inhibited this way, vital functions would be affected. Zinc deficiency is rather detrimental to life, with those cells being the most sensitive to zinc deprivation that have a high proliferation rate (Vallee and Falchuk, 1993). One should also keep in mind that the expression of metallothionein is completely dependent on MTF-1, whose DNA-binding activity seems to be much more sensitive to the zinc concentration than the DNA-binding activity of Sp1 (Westin and Schaffner, 1988a; Heuchel et al, 1994). Therefore it is difficult, if not impossible, to produce enough metallothionein for a serious zinc depletion of the cell unless there would be an as yet unknown mechanism for producing metallothionein at low intracellular zinc concentration. At present, we favour a model in which MTF-1 senses the intracellular zinc concentration as follows. Under normal conditions, i.e. sufficient environmental zinc, metallothionein is only moderately expressed. If environmental zinc drops under a certain threshold level, MTF-1 is unable to bind to the MRE sequences in the metallothionein promoters and as a consequence metallothionein synthesis ceases. Metal transfer from metallothionein to a hypothetical heavy metal transfer-protein, or the decay, or active degradation of metallothionein might ensure zinc-dependent functions by releasing the last zinc reserves. Upon increase

in environmental zinc, or cytotoxic metals such as cadmium, metallothionein expression will be boosted via activation of MTF-1 for metal sequestration and/or detoxification.

Conclusions

In this review we have compared four different metal inducible gene systems. Even though the task in all of them, taken at face value, is to induce transcription of specific genes as a result of heavy metal load, in each of them the problem is solved in a specific way. In bacteria, the mercury response is best studied, MerR factor binds constitutively to DNA, but only after mercury binding twists the promoter such that it can be recognized by RNA polymerase. Like several known bacterial transcription regulators, the MerR protein has activator as well as repressor properties. In the yeast *S. cerevisiae*, ACE-1 is a copper dependent DNA binding transcription factor which activates the copper metallothionein gene. In another yeast, *C. glabrata*, a similar metallothionein regulator (AMT-1) activates not one but a multitude of metallothionein genes. In addition, AMT-1 regulates itself by positive feedback upon metal treatment. In mammals finally, both man and mouse induce metallothionein gene transcription via the zinc finger factor MTF-1. This factor can reversibly bind to DNA as a consequence of the presence or absence of zinc in the zinc fingers. Therefore, these zinc fingers are likely to have a metal sensory function in vivo. Given the amazing complexity of the mammalian transcription apparatus in general, it is to be expected that MTF-1 does not exert its effect directly on RNA polymerase II but rather in concern with cofactors. These cofactor(s) would help to activate and/or inhibit transcription in presence or absence of heavy metals, respectively. While MTF-1 is highly conserved among mammals, the greater multitude of MT genes in human as compared to mouse suggests a more complex role for metallothioneins. In spite of all these differences, in each of the systems described here, metal induced genes are activated via one key factor which binds to specific upstream sequence elements.

Of the systems described the mammalian one, due to its complexity, may yield many surprises in future studies. With the isolation and characterization of MTF-1 the first crucial player in this system has been identified. It would be interesting, for example, to find out why MTF-1 contains a proline-rich and a serine-threonine-rich domain in addition to the acidic activation domain. Of course it will be important to elucidate the signal transduction cascade for heavy metal-induced transcription, and also to find out how nonmetals induce metallothionein gene

transcription. Naturally, this will raise a question about the interrelatedness of the metal induction system with other stress inducible gene systems, such as the heat shock response or the oxidative stress (NF-κB) systems. Do these systems share cofactors? Does the phenomenon of the ready-to-go, or poised RNA polymerase II, which is binding to the promoter but not released for elongation in absence of heat shock, also exist for metal induction?

References

Andersen RD, Birren BW, Taplitz SJ, Herschman HR (1986): Rat metallothionein-I structural gene and three pseudogenes, one of which contains 5′-regulatory sequences. *Mol Cell Biol* 6: 302–314

Andersen RD, Taplitz SJ, Oberbauer AM, Calame KL, Herschman HR (1990): Metal-dependent binding of a nuclear factor to the rat metallothionein-I promoter. *Nucleic Acids Res* 18: 6049–6055

Ansari AZ, O'Halloran TV (1994): An emerging role for allosteric modulation of DNA structure in transcription. In: *Transcription Mechanisms and Regulation*, Raven Press

Ansari AZ, Chael ML, O'Halloran TV (1992): Allosteric underwinding of DNA is a critical step in positive control of transcription by Hg-MerR. *Nature* 355: 87–89

Beach LR, P, RD (1981): Amplification of the metallothionein-I gene in cadmium-resistant mouse cells. *Proc Natl Acad Sci USA* 78: 2110–2114

Beltramini M, Lerch K (1982): Copper transfer between Neurospora copper metallothionein and type 3 copper apoproteins. *Febs Lett* 142: 219–222

Bremner I, Beattie JH (1990): metallothionein and the trace minerals. *Annu Rev Nutr* 10: 63–83

Brenes-Pomales A, Lindegren G, Lindegren CC (1955): Gene control of copper-sensitivity in *Saccharomyces Nature* 176: 841–842

Brown RS, Sander C, Argos P (1985): The primary structure of transcription factor TFIIIA has 12 consecutive repeats. *FEBS Lett* 186: 271–274

Brugnera E, Georgiev O, Radtke F, Heuchel R, Baker E, Sutherland GR, Schaffner W (1994): Cloning, chromosomal mapping and characterization of the human metal-regulatory transcription factor MTF-1. *Nucl Acids Res* 22: 3167–3173

Buchman C, Skroch P, Dixon W, Tullius TD, Karin M (1990): A single amino acid change in CUP2 alters its mode of DNA binding. *Mol Cell Biol* 10: 4778–4787

Butler G, Thiele DJ (1991): ACE2, an activator of yeast metallothionein expression which is homologous to SW15. *Mol Cell Biol* 11: 476–485

Butt TR, Ecker DJ (1987): Yeast metallothionein and applications in biotechnology. *Microbiol Rev* 51: 351–364

Butt TR, Sternberg EJ, Gorman JA, Clark P, Hamer D, Rosenberg M, Crooke ST (1984): Copper metallothionein of yeast, structure of the gene, and regulation of expression. *Proc Natl Acad Sci USA* 81: 3332–3336

Carter AD, Felber BK, Walling MJ, Jubier MF, Schmidt CJ, Hamer DH (1984): Duplicated heavy metal control sequences of the mouse metallothionein-I gene. *Proc Natl Acad Sci USA* 81: 7392–7396

Compere SJ, Palmiter RD (1981): DNA methylation controls the inducibility of the mouse metallothionein-I gene in lymphoid cells. *Cell* 25: 233–240

Cotton FA, Wilkinson G (1980): *Advanced Inorganic Chemistry, A Comprehensive Text* New York: John Wiley and Sons

Culotta VC, Hamer DH (1989): Fine mapping of a mouse metallothionein gene metal response element. *Mol Cell Biol* 9: 1376–1380

Czupryn M, Brown WE, Vallee BL (1992): Zinc rapidly induces a metal response element-binding factor. *Proc Natl Acad Sci USA* 89: 10395–10399

Durnam DM, Palmiter RD (1981): Transcriptional regulation of the mouse metallothionein-I gene by heavy metals. *J Biol Chem* 256: 5712–5716

Durnam DM, Palmiter RD (1987): Analysis of the detoxification of heavy metal ions by mouse metallothionein. *Experientia Suppl*, 457–463

Ecker DJ, Butt TR, Sternberg EJ, Neeper MP, Debouck C, Gormon JA, Crooke ST (1986): Yeast metallothionein function in metal ion detoxification. *J Biol Chem* 261: 16895–16900

Evans CF, Engelke DR, Thiele DJ (1990): ACE1 transcription factor produced in Escherichia coli binds multiple regions within yeast metallothionein upstream activation sequences. *Mol Cell Biol* 10: 426–429

Fogel S, Welch JS (1982): Tandem gene amplification mediates copper resistance in yeast. *Proc Natl Acad Sci USA* 79: 5342–5346

Fowler BA, Hildebrand CE, Kojima Y, Webb M (1987): Nomenclature of metallothionein. *Experientia Suppl*, 19-22

Furst P, Hu S, Hackett R, Hamer D (1988): Copper activates metallothionein gene transcription by altering the conformation of a specific DNA binding protein [published erratum appears in Cell 1989 Jan 27;56(2):following 321]. *Cell* 55: 705–717

Gick GG, McCarty KSr (1982): Amplification of the metallothionein-I gene in cadmium- and zinc-resistant Chinese hamster ovary cells. *J Biol Chem* 257: 9049–9053

Gralla EB, Thiele DJ, Silar P, Valentine JS (1991): ACE1, a copper-dependent transcription factor, activates expression of the yeast copper, zinc superoxide dismutase gene. *Proc Natl Acad Sci USA* 88: 8558–8562

Grill E (1987): Phytochelatins, the heavy metal binding peptides of plants: characterization and sequence determination. *Experientia Suppl*, 317–322

Grill E, Loffler S, Winnacker EL, Zenk MH (1989): Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific gamma-glatmylcysteine dipeptidyl transpeptidase (phytochelatin synthetase). *Proc Natl Acad Sci USA* 86: 6838–6842

Grill E, Winnacker EL, Zenk MH (1985): Phytochelatins: The principal heavy-metal complexing peptides of higher plants. *Science* 230: 4726.

Grill E, Winnacker EL, Zenk MH (1986): Homo-phytochelatins are heavy metal-binding peptides of homo-glutathione containing *Fabales*. *FEBS Lett* 205: 47–50

Grill E, Winnacker EL, Zenk MH (1991): Phytochelatins. In: *Methods Enzymol*, New York: Academic Press

Hamer DH (1986): metallothionein. *Annu Rev Biochem* 55: 913–951

Hamer DH, Thiele DJ, Lemontt JE (1985): Function and autoregulation of yeast coppertionein. *Science* 228: 685–690

Hanas JS, Hazuda DJ, Bogenhagen DF, Wu FY, Wu CW (1983): Xenopus transcription factor A requires zinc for binding to the 5S RNA gene. *J Biol Chem* 258:14120–14125

Harley CB, Reynolds RP (1983): Analysis of *E. coli* promoter sequences. *Nucleic Acids Research* 15: 2343–2361

Harlow P, Watkins E, Thornton RD, Nemer M (1989): Structure of an ectodermally expressed sea urchin metallothionein gene and characterization of its metal-responsive region. *Mol Cell Biol* 9: 5445–5455

Hawley DK, McClure WR (1983): Compilation and analysis of Escherichia coli promoter DNA sequences. *Nucleic Acids Res* 11: 2237–2255

Helman JD, Shewchuk LM, Walsh CT (1990): *Metal-Ion Induced Regulation of Gene Expression*, New York: Elsevier

Helmann JD, Wang Y, Mahler I, Walsh CT (1989): Homologous metalloregulatory proteins from both gram-positive and gram-negative bacteria control transcription of mercury resistance operons. *J Bacteriol* 171: 222–229

Henkel T, Machleidt T, Alkalay I, Kronke M, Ben-Neriah Y, Baeuerle PA (1993): Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. *Nature* 365: 182–185

Heuchel R, Radtke F, Georgiev O, Stark G, Aguet M, Schaffner W (1994): The transcription factor MTF-1 is essential for basal and heavy metal-induced metallothionein gene expression. *EMBO J* 13: 2870–2875

Hope IA, Struhl K (1986): Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. *Cell* 46: 885–894

Hu S, Furst P, Hamer D (1990): The DNA and Cu binding functions of ACE1 are interdigitated within a single domain. *New Biol* 2: 544–555

Huber M, Lerch K (1987): The influence of copper on the induction of tyrosinase and laccase in Neurospora crassa. *FEBS Lett* 219: 335–338

Hulbregtse JM, Engelke DR, Thiele DJ (1989): Copper-induced binding of cellular factors to yeast metallothionein upstream activation sequences. *Proc Natl Acad Sci USA* 86: 65–69

Hunter T, Karin M (1992): The regulation of transcription by phosphorylation. *Cell* 70: 375–387

Imagawa M, Onozawa T, Okumura K, Osada S, Nishihara T, Kondo M (1990): Characterization of metallothionein cDNAs induced by cadmium in the nematode Caenorhabditis elegans. *Biochem J* 268: 237–240

Imbert J, Fürst P, Gedamu P, Hamer D (1990): Regulation of metallothionein gene transcription by metals. *Adv Inorg Biochem* 8: 139–164

Jungmann J, Reins H-A, Lee J, Romeo A, Hassett R, Kosman D, Jentsch S (1993): MAC1, a nuclear regulator protein related to Cu-dependent transcription factors is involved in Cu/Fe utilization and stress resistance in yeast. *EMBO J* 12: 5051–5056

Kadonaga JT, Carner KR, Masiarz FR, Tijian R (1987): Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell* 51: 1079–1090

Kaegi JHR (1991): Overview of metallothionein. In: *Methods Enzymol*, New York: Academic Press

Kaegi JHR, Kojima Y (1987): Chemistry and biochemistry of metallothionein. In: *Experientia Suppl*, 25–61

Kaegi JH, Schaffer A (1988): Biochemistry of metallothionein. *Biochemistry* 27: 8509–8515

Kaegi JHR, Vallee B (1960): metallothionein: a cadmium- and zinc-containing protein from equine renal cortex. *J Biol Chem* 235: 3460–3465

Kaptain R (1991): Zinc-finger structures. *Curr Opin Struct Biol* 2: 109–115

Karin M, Andersen RD, Slater E, Smith K, Herschman HR (1980): metallothionein mRNA induction in HeLa cells in response to zinc or dexamethasone is a primary induction response. *Nature* 286:

Karin M, Cathala G, Nguyen-Huu MC (1983): Expression and regulation of a human metallothionein gene carried on an autonomously replicating shuttle vector. *Proc Natl Acad Sci USA* 80: 4040–4044

Karin M, Haslinger A, Holtgreve H, Richards RI, Krauter P, Westphal HM, Beato M (1984): Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-IIA gene. *Nature* 308: 513–519

Karin M, Najarian R, Haslinger A, Valenzuela P, Welch J, Fogel S (1984): Primary structure and transcription of an amplified genetic locus: the CUP1 locus of yeast. *Proc Natl Acad Sci USA* 81: 337–341

Kawashima I, Kennedy TD, Chino M, Lane BG (1992): Wheat Ec metallothionein genes. Like mammalian Zn2+ metallothionein genes, wheat Zn2+ metallothionein genes are conspicuously expressed during embryogenesis. *Eur J Biochem* 209: 971–976

Keilin D, Mann T (1940): Carbonic anhydrase. Purification and nature of the enzyme. *Biochem J* 34: 1163–1176

Koizumi S, Suzuki K, Otsuka F (1992): A nuclear factor that recognizes the metal-responsive elements of human metallothionein IIA gene. *J Biol Chem* 267: 18659–18664

Kondo N, Imai K, Isobe M, Goto T, Murasugi A, Wada-Nakagawa C, Hayashi Y (1984): Cadystin A and B, major unit peptides comprising cadmium binding peptides induced in a fission yeast—Separation, revision of structures and synthesis. *Tetrahedron Lett* 25: 3869–3872

Lee W, Haslinger A, Karin M, Tjian R (1987a): Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. *Nature* 325: 368–372

Lee W, Mitchell P, Tjian R (1987b): Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* 49: 741–752

Lerch K (1980): Copper metallothionein, a copper-binding protein from *Neurospora crassa*. *Nature* 284: 368–370

Lippard SJ (1993): Bioinorganic chemistry: a maturing frontier [comment]. *Science* 261: 699–700

Ma J, Ptashne M (1987): Deletion analysis of GAL4 defines two transcriptional activating segments. *Cell* 48: 847–853

Mansour SL, Thomas KR, Capecchi MR (1988): Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature* 336: 348–352

Margoshes M, Vallee BL (1957): A cadmium protein from equine kidney cortex. *J Am Chem Soc* 79: 4813–4814

Masters BA, Kelly EJ, Quaife CJ, Brinster RL, Palmiter RD (1994): Targeted disruption of metallothionein I and II genes increases sensitivity to cadmium. *Proc Natl Acad Sci USA* 91: 584–588

Mehra RK, Garey JR, Butt TR, Gray WR, Winge DR (1989): Candida glabrata metallothioneins. Cloning and sequence of the genes and characterization of proteins. *J Biol Chem* 264: 19747–19753

Mehra RK, Garey JR, Winge DR (1990): Selective and tandem amplification of a

member of the metallothionein gene family in Candida glabrata. *J Biol Chem* 265: 6369–6375

Mehra RK, Thorvaldsen JL, Macreadie IG, Winge DR (1992): Disruption analysis of metallothionein-encoding genes in Candida glabrata. *Gene* 114: 75–80

Michalska AE, Choo KH (1993): Targeting and germ-line transmission of a null mutation at the metallothionein I and II loci in mouse. *Proc Natl Acad Sci USA* 90: 8088–8092

Miller J, McLachlan AD, Klug A (1985): Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. *EMBO J* 4: 1609–1614

Misra TK (1992): Bacterial resistances to inorganic mercury salts and organomercurials. *Plasmid* 27: 4–16

Mitchell PJ, Tjian R (1989): Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* 245: 371–378

Mitchell PJ, Wang C, Tjian R (1987): Positive and negative regulation of transcription in vitro: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. *Cell* 50: 847–861

Mueller PR, Salser SJ, Wold B (1988): Constitutive and metal-inducible protein:DNA interactions at the mouse metallothionein I promoter examined by in vivo and in vitro footprinting. *Genes Dev* 2: 412–427

Munger K, Germann UA, Lerch K (1985): Isolation and structural organization of the Neurospora crassa copper metallothionein gene. *EMBO J* 4: 2665–2668

Murasugi A, Wada C, Hayashi Y (1981): Purification and unique properties in UV and CD spectra of Cd-binding peptide 1 from *Schizosaccharomyces pombe*. *Biochem Biophys Res Commun* 103: 1021-1028

Nettesheim DG, Engeseth HR, Otvos JD (1985): Products of metal exchange reactions of metallothionein. *Biochemistry* 24: 6744–6751

Nies DH (1992): Resistance to cadmium, cobalt, zinc, and nickel in microbes. *Plasmid* 56: 17–28

O'Halloran TV (1993): Transition metals in control of gene expression [see comments]. *Science* 261: 715–725

O'Halloran TV et al (1989): The MerR heavy metal receptor mediates positive activation in a topologically novel transcription complex. *Cell* 56: 119–129

Olafson RW, McCubbin WD, Kay CM (1988): Primary- and secondary-structural analysis of a unique prokaryotic metallothionein from a Synechococcus sp. cyanobacterium. *Biochem J* 251: 691–699

Otto E, Allen JM, Young JE, Palmiter RD, Maroni G (1987): A DNA segment controlling metal-regulated expression of the Drosophila melanogaster metallothionein gene Mtn. *Mol Cell Biol* 7: 1710–1715

Otvos JD, Engeseth HR, Nettesheim DG, Hilt CR (1987): Interprotein metal exchange reactions of metallothioneins. *Experientia Suppl*, 171–178

Palmiter RD (1994): Regulation of metallothionein genes by heavy metals appears to be mediated by a zinc-sensitive inhibitor that interacts with a constitutively active transcription factor, MTF-1. *Proc Natl Acad Sci USA* 91: 1219–1223

Palmiter RD, Findley SD, Whitmore TE, Durnam DM (1992): MT-III, a brain-specific member of the metallothionein gene family. *Proc Natl Acad Sci USA* 89: 6333–6337

Pande J, Vasak M, Kagi JH (1985): Interaction of lysine residues with the metal thiolate clusters in metallothionein. *Biochemistry* 24: 6717–6722

Petering DH, Krezoski S, Villalobos J, Shaw CF, Otvos JD (1987): Cadmium-zinc

interactions in the Ehrlich cell: metallothionein and other sites. In: *Experientia Suppl*, 573–580

Picard B, Wegnez M (1979): Isolation of a 7S particle from Xenopus laevis oocytes: A 5S RNA/protein complex. *Proc Natl Acad Sci USA* 76: 241–245

Piscator M (1964): *Nord Hyg Tidskr* 48: 76–82

Quaife CJ, Findley SD, Erickson GJ, Froelick GJ, Kelly EJ, Zambrowicz BP, Palmiter RD (1994): Induction of a new metallothionein isoform (MT–IV) occurs during differentiation of stratified squamous epithelia. *Biochemistry* 33: 7250–7259

Quaife CJ, Findley SD, Erickson GJ, Kelly EJ, Zambrowicz BW, Palmiter RD (1994): personal communication

Radtke F, Heuchel R, Georgiev O, Hergersberg M, Gariglio M, Dembic Z, Schaffner W (1993): Cloned transcription factor MTF-1 activates the mouse metallothionein I promoter. *EMBO J* 12: 1355–1362

Raulin J (1969): Etudes Cliniques sur la vegetation. *Ann Sci Nat Bot Biol Veg* 11: 93–299

Rauser WE (1990): Phytochelatins. *Annu Rev Biochem* 59: 61–86

Robbins AH, McRee DE, Williamson M, Collett SA, Xuong NH, Furey WF, Wang BC, Stout CD (1991): Refined crystal structure of Cd, Zn metallothionein at 2.0 A resolution. *J Mol Biol* 221: 1269–1293

Robinson JN, Barton K, Naranjo CM, Sillerud LO, Trewhella J, Watt K, Jackson PJ (1987): Characterization of metal binding peptides from cadmium resistent plant cells. *Experientia Suppl*, 323–327

Robinson JN, Tommey AM, Kuske C, Jackson P (1993): Plant metallothioneins. *Biochem J* 295: 1–10

Schmid R, Zeng J, Schäffer A (1990): *Experientia* 46: A36

Schmidt CJ, Jubier MF, Hamer DH (1985): Structure and expression of two human metallothionein-I isoform genes and a related pseudogene. *J Biol Chem* 260: 7731–7737

Schreiber E, Matthias P, Muller MM, Schaffner W (1988): Identification of a novel lymphoid specific octamer binding protein (OTF- 2B) by proteolytic clipping bandshift assay (PCBA). *EMBO J* 7: 4221–4229

Searle PF (1990): Zinc dependent binding of a liver nuclear factor to metal response element MRE-a of the mouse metallothionein-I gene and variant sequences. *Nucleic Acids Res* 18: 4683–4690

Searl PF, Davison BL, Stuart GW, Wilkie TM, Norstedt G, Palmiter RD (1984): Regulation, linkage and sequence of mouse metallothionein I and II genes. *Mol Cell Biol* 4: 1221–1230

Searl PF, Stuart GW, Palmiter RD (1987): Metal regulatory elements of the mouse metallothionein-I gene. *Experientia Suppl*, 407–414

Seipel K, Georgiev O, Schaffner W (1992): Different activation domains stimulate transcription from remote ('enhancer') and proximal ('promoter') positions. *EMBO J* 11: 4961–4968

Serfling E, Lubbe A, Dorsch-Hasler K, Schaffner W (1985): Metal-dependent SV40 viruses containing inducible enhancers from the upstream region of metallothionein genes. *EMBO J* 4: 3851–3859

Séguin C (1991): A nuclear factor requires Zn2+ to bind a regulatory MRE element of the mouse gene encoding metallothionein-I. *Gene* 97: 295–300

Séguin C, Prevost J (1988): Detection of a nuclear protein that interacts with a metal regulatory element of the mouse metallothionein I gene. *Nucleic Acids Res* 16: 10547–10560

Shi J, Lindsay WP, Huckle JW, Morby AP, Robinson NJ (1992): Cyanobacterial metallothionein gene expressed in Escherichia coli. Metal- binding properties of the expressed protein. *FEBS Lett* 303: 159–163

Silver S, Walderhaug M (1992): Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. *Microbiol Rev* 56: 195–228

Slice LW, Freedman JH, Rubin CS (1990): Purification, characterization, and cDNA cloning of a novel metallothionein- like, cadmium-binding protein from Caenorhabditis elegans. *J Biol Chem* 265: 256–263

Steffens JC (1990): *Ann Rev Plant Physiol Plant Mol Biol* 533–575

Stillman MJ, Cai W, Zelazowski AJ (1987): Cadmium binding to metallothioneins. Domain specificity in reactions of alpha and beta fragments, apometallothionein, and zinc metallothionein with Cd2+. *J Biol Chem* 262: 4538–4548

Stuart GW, Searl PF, Chen HY, Brinster RL, Palmiter RD (1984): A 12-base-pair DNA motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. *Proc Natl Acad Sci USA* 81: 7318–7322

Stuart GW, Searle PF, Palmiter RD (1985): Identification of multiple metal regulatory elements in mouse metallothionein-I promoter by assaying synthetic sequences. *Nature* 317: 828–831

Sczypka MS, Thiele DJ (1989): A cysteine-rich nuclear protein activates yeast metallothionein gene transcription. *Mol Cell Biol* 9: 421–429

Theunissen O, Rudt F, Guddat U, Mentzel H, Pieler T (1992): RNA and DNA binding zinc fingers in Xenopus TFIIIA. *Cell* 71: 679–690

Thiele DJ, Hamer DH (1986): Tandemly duplicated upstream control sequences mediate copper induced transcription of saccharomyces cerevisiae copper-metallothionein gene. *Mol Cell Biol* 6: 1158–1163

Thiele DJ (1988): ACE1 regulates expression of the Saccharomyces cerevisiae metallothionein gene. *Mol Cell Biol* 8: 2745–2752

Thiele DJ (1992): Metal-regulated transcription in eukaryotes. *Nucleic Acids Res* 20: 1183–1191

Uchida Y, Takio K, Titani K, Ihara Y, Tomonaga M (1991): The growth inhibitory factor that is deficient in the Alzheimer's disease brain is a 68 amino acid metallothionein-like protein. *Neuron* 7: 337–347

Vallee BL, Auld DS (1990): Zinc coordination, function, and structure of zinc enzymes and other proteins. *Biochemistry* 29: 5647–5659

Vallee BL, Falchuk KH (1993): The biochemical basis of zinc physiology. *Physiol Rev* 73: 79–118

Vallee BL, Coleman JE, Auld DS (1991): Zinc fingers, zinc clusters, and zinc twists in DNA-binding protein domains. *Proc Natl Acad Sci USA* 88: 999–1003

Vasák M, Kaegi JHR (1983): *Metal Ions In Biological Systems*, Sigel -, ed. New York: Marcel Dekker

Welch J, Fogel S, Buchman C, Karin M (1989): The CUP2 gene product regulates the expression of the CUP1 gene, coding for yeast metallothionein. *Embo J* 8: 255–260

West AK, Hildebrand CE, Karin M, Richards RI (1990): Human metallothionein genes: Structure of the functional locus at 16q13. *Genomics* 8: 513–518

Westin G, Schaffner W (1988a): A zinc-responsive factor interacts with a metal-regulated enhancer element (MRE) of the mouse metallothionein-I gene. *EMBO J* 7: 3763-3770

Westin G, Schaffner W (1988b): Heavy metal ions in transcription factors from HeLa

cells: Sp1, but not octamer transcription factor requires zinc for DNA binding and for activator function. *Nucleic Acids Res* 16: 5771–5781

Winge DR, Nielson KB, Gray WR, Hamer DH (1985): Yeast metallothionein. Sequence and metal-binding properties. *J Biol Chem* 260: 14464–14470

Xu L, Rungger D, Georgiev O, Seipel K, Schaffner W (1994): Different potential of cellular and viral activators of transcription revealed in oocytes and early embryos of *Xenopus laevis*. *Biol Chem Hoppe-Seyler* 375: 105–112

Yagle MK, Palmiter RD (1985): Coordinate regulation of mouse metallothionein I and II genes by heavy metals and glucocorticoids. *Mol Cell Biol* 5: 291–294

Zafarullah M, Bonham K, Gedamu L (1988): Structure of the rainbow trout metallothionein B gene and characterization of its metal-responsive region. *Mol Cell Biol* 8: 4469–4476

Zeng J, Heuchel R, Schaffner W, Kagi JH (1991): Thionein (apometallothionein) can modulate DNA binding and transcription activation by zinc finger containing factor Sp1. *FEBS Lett* 279: 310–312

Zeng J, Vallee BL, Kagi JH (1991): Zinc transfer from transcription factor IIIA fingers to thionein clusters. *Proc Natl Acad Sci USA* 88: 9984–9988

Zhou P, Szczypka MS, Sosinowski T, Thiele DJ (1992): Expression of a yeast metallothionein gene family is activated by a single metalloregulatory transcription factor. *Mol Cell Biol* 12: 3766–3775

Zhou P, Thiele DJ (1993): Rapid transcriptional autoregulation of a yeast metalloregulatory transcription factor is essential for high-level copper detoxification. *Genes Dev* 7: 1824–1835

Zhou P, Thiele DJ (1991): Isolation of a metal-activated transcription factor gene from Candida glabrata by complementation in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* 88: 6112–6116