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Eucaryotic metallothioneins: proteins, gene regulation and copper homeostasis.

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Abstract: Heavy metals such as copper, iron and zinc are essential for eucaryotic cell viability and they are required only in trace amounts. High concentrations of these metals are toxic for the cells and they trigger different molecular response mechanisms. One of the best studied of such responses involves the synthesis of metallothioneins (MTs) which are low molecular weight, cysteine-rich proteins that bind heavy metals by means of their cysteine residues. MTs have been purified from different eucaryotic cells and their structural and heavy metal binding properties have been determined. MT genes have been cloned from animal cells, fungi, plants and algae and their transcriptional activation by heavy metals has been characterized. The overexpression of MTs results in the accumulation of heavy metals in the cells. The best studied model for copper tolerance and homeostasis is the yeast *Saccharomyces cerevisiae*. In this fungus, high concentrations of copper activate transcription of the gene coding for CUP1 MT. This process involves the binding of ACE1 transcription factor to the promoter of cup-1 gene. ACE1 directly binds copper ions and undergoes a conformational change that allows its binding to the promoter region. On the other hand, copper starvation triggers the transcriptional activation of at least two copper transporter genes and a copper reductase gene. This activation involves the binding of MAC1 factor to the promoter region of these genes. MAC1 is stable in the absence of copper but is degraded when it binds copper ions via its carboxy terminal domain. Therefore, ACE1 and MAC1 are transcription factors which are able to sense intracellular concentrations of copper and they are crucial for copper homeostasis mechanisms. The above mentioned are probably not the only mechanisms involved in copper tolerance and homeostasis. Therefore, additional effort will be required to elucidate other complementary mechanisms involved in these processes.

Résumé : Les métaux lourds comme le cuivre, le fer et le zinc sont essentiels pour la viabilité de cellules eucaryotes. Ces métaux doivent être à l'état de traces sinon ils deviennent toxiques pour les cellules et déclenchent différentes réponses moléculaires. L'un des mécanismes les mieux étudiés est la synthèse de métallothionéines (MTs) qui sont des petites protéines contenant de nombreux résidus de cystéine et qui se lient à divers métaux lourds. Les MTs ont été purifiées à partir de différentes cellules eucaryotes et leur structure et propriétés d'union aux métaux lourds ont été determinées. Des gènes de MTs ont été clonés à partir de cellules animales, champignons, plantes et algues et l'activation transcriptionelle de ces gènes induite par différents métaux lourds a été caracterisée. La surexpression de MTs résulte dans l'accumulation de métaux lourds chez les eucaryotes. Le modèle le mieux étudié en ce qui concerne la tolérance et l'homéostasie du cuivre est la levure *Saccharomyces cerevisiae*. Chez ce champignon, des hautes concentrations de cuivre produisent une activation transcriptionelle du gène de la MT CUP1. Cette activation est induite par la fixation du facteur de transcription ACE1 au promoteur du gène cup-1. ACE1 est capable de fixer directement des ions cuivre, provoquant un changement de conformation qui permet sa fixation au promoteur. Par ailleurs, l'absence de cuivre induit l'activation des gènes qui codent pour au moins deux transporteurs et une réductase de cuivre. Cette activation est induite par la fixation du facteur MAC1 au promoteur de ces gènes. MAC1 est stable en absence de cuivre mais il est dégradé après la fixation de ions cuivre à son

domaine carboxy terminal. Donc, les facteurs de transcription ACE1 et MAC1 sont capables de senser la concentration intracellulaire de cuivre et sont ainsi essentiels pour les mécanismes d'homéostasie du cuivre chez la levure. Les mécanismes cités ci-dessus ne sont certainement pas les seuls à participer aux processus de tolérance et à l'homéostasie du cuivre. Des études supplémentaires sont encore nécessaires pour élucider les autres mécanismes impliqués dans ces phénomènes.

Keywords : heavy metals, metallothioneins, transcriptional regulation, copper homeostasis.

Introduction

Heavy metals such as copper, iron and zinc are essential nutrients for living cells since they act as cofactors for several enzymes such as cytochrome oxidase, catalases, peroxidases, cytochrome c, superoxide dismutases, RNA polymerases, DNA polymerases and some transcription factors. These essential metal ions are required only in trace amounts and higher concentrations are toxic for the cells. Other heavy metals like cadmium, mercury, lead and aluminium are not essential for cell viability and they are toxic even in very low amounts. It is well established that toxic concentrations of heavy metal ions produce cytotoxic reactive oxygen species that cause direct protein oxidation, DNA and RNA cleavage, lipid peroxidation and, in some cases, substitution of essential cofactors in several enzymes.

Eucaryotic cells have developed sophisticated mechanisms to buffer toxic concentrations of heavy metals and to regulate the intracellular concentration of essential metal ions. During the last 25 years, mechanisms accounting for heavy metal tolerance in eucaryotic cells have been studied. One of the principal mechanisms triggered to buffer heavy metal toxicity is the synthesis of low molecular weight, cysteine-rich proteins called metallothioneins (MTs). These proteins are able to bind different heavy metals by means of their cysteine residues and their overexpression results in the accumulation of heavy metals in the cells. These proteins have been purified from different eucaryotic cells and their heavy metal binding ability has been determined. Moreover, MT genes from different species have been cloned and their transcriptional induction by heavy metals has been characterized. There are also other molecules, that are not gene encoded, that are synthesized in response to some heavy metals, principally cadmium. These molecules are obtained by condensation of glutathione and they correspond to $(\gamma\text{-glutamyl-cysteinyl})_n$glycine (n=2-11) peptides. These glutathione-derived peptides are synthesized in fungi and plants and they are called cadystins and phytochelatins, respectively. Even though the synthesis of cadystins and phytochelatins is an important mechanism of heavy metal tolerance in eucaryotes, MTs synthesis is considered the main cellular response to buffer heavy metal toxicity.

Recently, molecular mechanisms accounting for regulation of intracellular concentration of copper have been characterized in the yeast *Saccharomyces cerevisiae*. Genes coding for copper transporters and a copper reductase have been cloned and their transcriptional regulation in response to copper has been characterized. In this review, most of the aspects concerning the role of eucaryotic MTs in heavy metal tolerance and some recent advances of copper homeostasis will be discussed.

Structure of metallothionein proteins and their binding to heavy metals

Animal metallothioneins

The first metallothionein (MT) was purified from horse kidney by Margoshes and Vallee, in 1957. Animal kidney and liver are the best sources of MTs and several of these proteins have been purified from these organs. Animal MTs have been classified as MTI and MTII, depending on their chromatographic properties when purified on DEAE-cellulose. These MTs are naturally associated to zinc, cadmium and copper. It was shown that liver MTs contain predominantly zinc whereas kidney MTs contain more cadmium or copper (Kägi et al., 1974). When animals are exposed to toxic concentrations of a particular heavy metal, liver MTs preferentially bind this metal but also contain low amounts of zinc (Hamer, 1986).

Animal MTs consist of 61 or 62 amino-acids (6.5 kDa) from which 20 are cysteine residues (33%) that are arranged predominantly as Cys-X-Cys and Cys-Cys and distributed along the protein. Animal MTs show a high content of hydrophobic amino-acids, lysines and serines and no aromatic residues or histidines. They are able to bind zinc, cadmium, mercury, cobalt, lead and nickel, in which seven atoms of each of these metals are bound via cysteine residues by means of tetrahedral metal-thiolate coordination bonds, or 12 atoms of copper (Cu^{+1}), silver or gold by means of trigonal coordination bonds (Nielson et al., 1985).

Animal MTs consist of two structural domains located in the amino and carboxy terminal regions of the protein. These domains are named ß or B (amino terminal) and α or A (carboxy terminal). When metal ions fill both domains, MTs become more resistant to proteolysis. Each domain is able to bind a different number of heavy metal ions: domain

B binds three atoms of zinc or cadmium, or six atoms of copper, by means of nine cysteine residues and domain A binds four atoms of cadmium or zinc, or six atoms of copper, by means of 11 cysteine residues (Nielson et al., 1985). A cadmium-zinc metallothionein isolated from rat liver was crystallized (Furey et al., 1986). This MT naturally binds five atoms of cadmium and two of zinc: domain B binds one cadmium and two zinc atoms whereas domain A binds four cadmium atoms.

The binding of heavy metal ions to animal MTs is highly cooperative. When equimolar concentrations of MTs and metal ions are mixed, the isolated protein species show only filled domains and no partially filled domains. Cadmium and zinc ions initially bind domain A and then fill domain B in a cooperative manner. In contrast, copper initially binds domain B and then domain A (Nielson & Winge, 1983, 1984).

Animal MTs bind copper, zinc and cadmium with different affinities. The stability constant for copper is 100 times greater than that for cadmium and the cadmium constant is 1000 times greater than that for zinc. It has been postulated that MTs strongly bind copper and cadmium in order to efficiently detoxify the cell from these metals and that zinc is loosely bound to MT in order to be easily transferred to apometalloenzymes (Hamer, 1986). It has been shown that carbonic anhydrase, aldolase, thermolysine and other metalloenzymes require zinc to be active and they should capture this essential metal from a protein acting as reservoir (Li et al., 1980; Udom & Brady, 1980). Therefore, it was postulated that some MTs could act as a zinc reservoir. Then, MTs should have a dual role since they act as metal reservoirs in physiological conditions and they are involved in heavy metal accumulation and detoxification in the case of heavy metal toxicity.

Fungi metallothioneins

MTs have been characterized in several fungi such as the yeasts *Saccharomyces cerevisiae* and *Candida glabrata*, the mold *Neurospora crassa* and the common mushroom *Agaricus bisporus*. One of the major differences between animal and fungal MTs is that the latter are only synthesized in response to copper or silver toxicity and that their synthesis is not induced by zinc, cadmium or mercury. Moreover, MTs isolated from *Saccharomyces cerevisiae* and from other fungi only contain copper as naturally associated metal (Premakumar et al., 1975; Weser et al., 1977; Lerch, 1980; Mehra et al., 1988).

Two MTs have been found in the yeast *Saccharomyces cerevisiae*, CUP1 and CRS5 (Karin et al., 1984a; Butt et al., 1984; Cizewski-Culotta et al., 1994). The yeast *Candida glabrata* also synthesizes two metallothioneins in response to copper and silver toxicity, MT-I and MT-II, whereas it synthesizes glutathione related peptides in response to cadmium toxicity (Mehra et al., 1989). Only one MT has been isolated from *Neurospora crassa* and *Agaricus bisporus* and both are synthesized in response to copper toxicity (Lerch, 1980; Müngner & Lerch, 1985).

The yeast CUP1 MT consists of 53 amino-acids (6 kDa) from which 12 are cysteines (22%) that are arranged principally as Cys-X-Cys, Cys-X-X-Cys and Cys-Cys and they are distributed along the protein (Winge et al., 1985). Cup-1 gene encodes a protein of 61 amino-acids indicating that the initially synthesized CUP1 is processed to give a protein of 53 amino-acids (Wright et al., 1987). It has been shown that the cleaved sequence corresponds to the first eight amino terminal residues and that this sequence is not essential for protein activity since it does not contain cysteine residues (Wright et al., 1987). CUP1 binds seven atoms of copper (Cu^{+1}) or silver (Ag^{+1}) by means of its 12 cysteine residues using trigonal metal-thiolate coordination bonds (Winge et al., 1985; Narula et al., 1993). On the other hand, CUP1 is able to bind four atoms of cadmium or zinc by means of tetrahedral metal-thiolate coordination bonds. Interestingly, cadmium and zinc ions bind CUP1, even if its synthesis is not induced by these ions *in vivo* (Karin et al., 1984a; Butt et al., 1984).

The yeast CRS5 MT consists of 69 amino-acids (8 kDa) from which 19 are cysteines (27%) that are arranged as Cys-X-Cys and Cys-Cys and they are distributed along the protein (Cizewski-Culotta et al., 1994). CRS5 contains more hydrophobic amino-acids and cysteine residues than CUP1 and it binds 11 to 12 copper ions. However, binding of copper ions to CRS5 is more labile compared to CUP1 (Jensen et al., 1996). Moreover, CUP1 synthesis is induced by copper ions more efficiently than CRS5, showing an enhanced effectiveness of CUP1 MT to buffer copper ions (Jensen et al., 1996). As described for CUP1, CRS5 synthesis is only induced by copper and not by cadmium or zinc (Cizewski-Culotta et al., 1994).

Candida glabrata MT-I and MT-II consist of 62 and 51 amino-acids (7.5 and 6 kDa), respectively, from which 18 (29%) and 16 (31%) are cysteine residues that are arranged as Cys-X-Cys, Cys-X-X-Cys and Cys-Cys and they are distributed along the protein (Mehra et al., 1989). MT-I binds 11 to 12 copper ions whereas MT-II binds only 10 (Lerch, 1980). Therefore, *Candida glabrata* MTs bind more copper ions than the yeast CUP1 MT.

Neurospora crassa MT (CuMT) is a small protein consisting of 25 amino-acids (3 kDa) from which seven are cysteine residues (28%) that are arranged as those of animal MTs (Lerch, 1980). It is interesting to point out that MTs containing cysteine residues located at the same position as animal MTs are considered class I MTs and those presenting cysteine residues in different positions correspond to class II MTs. Therefore, *Neurospora crassa* MT belong to class I MTs and *Saccharomyces cerevisiae*

and *Candida glabrata* MTs are class II MTs. *Neurospora crassa* MT binds six copper ions or three zinc, cadmium, mercury, cobalt or nickel ions (Beltramini et al., 1984).

Like *Neurospra crassa* MT, *Agaricus bisporus* MT contain only 25 amino-acids (3 kDa) from which eight are cysteine residues (28%). These residues are arranged as in animals MTs, indicating that it belongs to class I MTs (Müngner & Lerch, 1985). Similar to *Neurospra crassa* MT, *Agaricus bisporus* MT binds six atoms of copper.

Plant metallothioneins

It was initially believed that plants synthesize only phytochelatins in response to heavy metals (Rauser, 1990; Steffens, 1990). The first plant MT-like gene was isolated from the flowering plant *Mimulus guttatus* but its transcription was not induced by copper toxicity (de Miranda et al., 1990). Other plant MT-like genes were further isolated in pea (Evans et al., 1990a), maize (de Framond, 1991), *Brassica napus* (Buchanan-Wollaston, 1994), *Arabidopsis thaliana* (Zhou & Goldsborough, 1994), bean (Foley & Singh, 1994; Foley et al., 1997), rice (Hsieh et al., 1995), cotton (Hudspeth et al., 1996), *Nicotiana glutinosa* (Choi et al., 1996), tomato (Whitelaw et al., 1997), the conifer *Pseudotsuga menziesii* (Chattai et al., 1997) and others. Most of these MTs are synthesized in different plant organs and they are accumulated in response to some stimuli such as senescence, wounding or plant hormones. Only few plant MT genes are metal-responsive, principally to copper toxicity (Zhou & Goldsborough, 1994; Hsieh et al., 1995; Choi et al., 1996; Chattai et al., 1997).

Plants essentially contain two type of MTs: one of large size consisting of 71 to 82 amino-acids (8.5 to 10 kDa) and another of small size containing only 45 amino-acids (5.4 kDa). The large size MTs show 12 to 15 cysteine residues (around 18%) and the small size MTs have 13 cysteines (28%). In both MTs, cysteines are arranged as Cys-X-Cys, Cys-X-X-Cys or Cys-Cys. The small size MT genes have only been isolated in *Arabidopsis thaliana* and *Brassica napus* (Zhou & Goldsborough, 1994; Buchanan-Wollaston, 1994). In large size MTs, cysteine residues are clustered in the amino and carboxy terminal regions of the protein, differing from animals and fungi MTs in which cysteines are distributed along the protein. These cysteine rich domains are separated by a central region of 30 to 40 amino-acids that is rich in hydrophobic and aromatic amino-acids (Robinson et al., 1993; Whitelaw et al., 1997). In the case of small size MTs, both cysteine rich domains are linked and there is no central hydrophobic region. Plant MTs are able to bind cadmium, zinc and copper ions *in vitro* (Kille et al., 1990; Tommey et al., 1990). It was determined that pea MT binds six atoms of cadmium showing a slightly lower affinity for zinc and cadmium and a higher affinity for copper than animal MTs (Kille et al., 1990; Tommey et al., 1990).

Algae metallothioneins

Until recently, no information was available concerning algae MTs. However, a cDNA encoding a MT was isolated from the brown macroalga *Fucus vesiculosus* (Morris et al., 1999). This cDNA encodes a protein of 67 amino-acids containing 16 cysteine residues (24%) and only one aromatic residue. *Fucus* MT shows a cysteine arrangement that is similar to those of plant and invertebrate MTs. This MT has a central domain of 14 amino-acids containing no cysteine residues which is smaller than the central hydrophobic domain of plant MTs. *Fucus* MT was expressed in *Escherichia coli* and the fusion protein was able to bind cadmium and copper in vitro. It was shown that *Fucus* MT gene was transcriptionally activated by copper.

Structure, organization and expression of metallothionein genes

Animal metallothionein genes

Animal DNA genomes contain several MT genes that are chromosomally linked. These genes show two introns of 120-800 bp that are located in a conserved position. These introns define exon 1 which encodes amino-acids 1 to $8^{2/3}$, exon 2 coding for residues $8^{3/3}$ to $31^{1/3}$ and exon 3 which correspond to amino-acids $31^{2/3}$ to 62.

Human DNA genomes encode four functional MT genes, MT-I_A, MTI-$_E$, MT-I_F and MT-II_A, and five pseudogenes, MT-I_B, MT-I_C, MT-I_D, MT-I_G, MT-I_H and MT-II_B (Hamer, 1986). It was initially shown that functional MT-I_A gene is located near pseudogenes MT-I_B, MT-I_C and MT-I_D, and that functional genes MT-II_A, MT-II_E and MT-II_F lie near pseudogene MT-I_G. It was further determined that all functional human MT genes lye on chromosome 16, at locus 16qcen16q21 that is located in the proximal portion of the long arm of chromosome 16 (Karin et al., 1984b; Le Beau et al., 1985). The other pseudogenes lie on different chromosomes. Moreover, it was shown that DNA fragments containing human MT genes are amplified in cell lines chronically exposed to cadmium toxicity (Rugstadt & Nordset, 1975). This indicates that gene amplification is probably one of the major mechanisms leading to heavy metal resistance in human cells. Most animal MT genes are constitutively expressed at low levels and their transcription is activated by toxic concentrations of cadmium, zinc, copper and mercury (Hamer, 1986). It is important to point out that transcription of most human MT genes is also induced by other stimuli such as growth factors, cytokines and a variety of chemical agents (Waalkes & Goehring, 1990).

Mouse DNA genome contains two functional MT genes coding for MT-I and MT-II (Huang et al., 1977). MT-I and MT-II genes are separated only by 6 kb, they are oriented in

the same direction of transcription and lie on chromosome 8 (Searle et al., 1984). Mouse cell lines chronically exposed to cadmium toxicity become resistant to this metal and show the amplification of a 55 kb DNA region containing both MT genes (Beach & Palmiter, 1981; Searle et al., 1984). Mouse MT genes are constitutively expressed at low levels and their transcription is triggered by cadmium, zinc, copper and mercury (Durman & Palmiter, 1981).

Fungi metallothionein genes

The yeast *Saccharomyces cerevisiae* contains two functional genes coding for CUP1 and CRS5 MTs and they do not contain introns. Wild type yeast strains normally have five to 15 tandemly repeated copies of cup-1 gene lying on chromosome VIII (Fogel & Welch, 1982) and only one copy of CRS5 MT gene (Cizewski-Culotta et al., 1994). For this reason, wild type yeast strains are able to grow in synthetic medium containing over 1 mM Cu^{+2}. In contrast, a yeast strain carrying only one copy of cup-1 gene grows with a maximal concentration of 150 µM Cu^{+2}. Moreover, a yeast strain with a deleted cup-1 gene does not survive on micromolar concentrations of copper ions (Hamer et al., 1985). This clearly indicates that yeast cup-1 gene is responsible for copper tolerance in yeast. Cup-1 and CRS5 MT genes are constitutively expressed at low levels and their transcription is induced 10 to 50 fold and 2-3 fold, respectively, in response to copper toxicity (Karin et al., 1984a; Butt et al., 1984; Cizewski-Culotta et al., 1994). Cup-1 gene is more copper responsive than CRS5 gene and is responsible for the primary buffering action of MTs in yeast.

The yeast *Candida glabrata* contains three functional MT genes, MT-I, MT-II_A and MT-II_B which do not contain introns (Mehra et al., 1989, 1992). Wild type strains of *Candida glabrata* show several copies of MT-II_A gene and a single copy of MT-II_B and MT-I genes. MT-II genes are chromosomally linked but they lie on a different chromosome than MT-I gene (Mehra et al., 1990). Strains selected in a copper enriched medium showed an amplification of MT-II genes (30 copies) whereas MT-I gene remains as a single copy (Mehra et al., 1990). MT-I and MT-II genes are expressed constitutively at low levels but their transcription is activated by toxic concentrations of copper and silver. MT-II genes appeared to be more copper responsive than MT-I gene (Mehra et al., 1989).

The mold *Neurospora crassa* contains a single MT gene that is interrupted by a small intron of 94 bp. This intron is inserted between exon 1 coding for amino-acids 1 to $18^{1/3}$ and exon 2 which encodes residues $18^{2/3}$ to 26 (Münger et al., 1985). *Neurospora crassa* MT gene is located in chromosome VI (Münger et al., 1987). This gene is expressed constitutively at low levels and is strongly induced by copper (Münger et al., 1987).

Plant metallothionein genes

Plants contain several MT genes lying on different chromosomes (Zhou & Goldsborough, 1995; Whitelaw et al., 1997; Chattai et al., 1997). Genes coding for large and small size plant MTs are interrupted by a single intron of about 1 kb that is inserted between exon 1 (amino-acids 1-$22^{2/3}$) and exon 2 (amino-acids $22^{3/3}$-final residue) (Zhou & Goldsborough, 1995). In *Arabidopsis thaliana*, the genes coding for large size MTs are called MT_2 and those encoding small size MTs are named MT_1. It has been determined that MT_{2a} and MT_{2b} genes are present as a single copy and lie on chromosomes III and V, respectively. MT_{1a} and MT_{1c} genes are present as single copies, they are separated by 3 kb and lie on chromosome I. MT_{1b} and MT_{2b} genes were detected on chromosome V and MT_{2b} gene on the opposite arm of this chromosome (Zhou & Goldsborough, 1995). Therefore, *Arabidopsis thaliana* genome contains five MT genes that are located on four different chromosomes. In the case of *Mimulus gutattus*, at least three genes encoding a large size MT were detected (de Miranda et al., 1990). Tomato shows several genes coding for two large size MTs (Whitelaw et al., 1997), pea contains at least three genes encoding a large size MT (Evans et al., 1990a), maize has three to four genes coding for a large size MT (de Framond, 1991) and cotton contains at least six copies of a large size MT (Hudspet et al., 1996). This indicates that most plant genomes normally contain several copies of the genes coding for large size MTs.

Arabidopsis thaliana MT_{1a}, MT_{1c}, MT_{2a} and MT_{2b} genes are transcribed but only MT_{1a} and MT_{2a} transcription is induced by copper (Zhou & Goldsborough, 1995). Metal-responsive plant MT genes are expressed constitutively at low levels and their transcription is induced several folds by copper toxicity (Zhou & Goldsborough, 1994; Hsieh et al., 1995; Choi et al., 1996; Chattai et al., 1997).

Metallothionein gene promoters and heavy metal-responsive factors

Eucaryotic gene promoters and general mechanisms of transcriptional regulation

Eucaryotic genes transcribed by RNA polymerase II contain promoters located in the upstream region of the transcription initiation site. These promoters consist of a TATA box sequence (TATAAA) located 30 or 40 nucleotides upstream of the transcription initiation site and several regulatory sequences located 50 to 500 bp upstream of this site. In animals, these sequences are called proximal regulatory sequences and in fungi they are named UAS (Upstream Activating Sequences). The TATA sequence binds the RNA polymerase II basal transcriptional

machinery consisting of TBP (TATA binding factor), TFIIB, RNA polymerase II-TFIIF, TFIIE and TFIIH. The proximal regulatory regions bind transcriptional regulatory factors that are involved in activation of the basal transcriptional machinery.

Eucaryotic regulatory factors are modular: they contain a DNA binding domain and an activation domain. The DNA binding domain interacts with a specific regulatory sequence and the activation domain interacts with the RNA polymerase II basal transcriptional machinery. The most common DNA binding domains of eucaryotic regulatory factors are: the zinc finger domain, of which there are two types C_2H_2 and C_4, the homeodomain, the leucine zipper-basic domain (bZip) and the helix-loop-helix-basic domain. On the other hand, the most common activation domains of eucaryotic regulatory factors are: a domain that is rich in acidic amino-acids and two others that are rich in glutamine or proline residues.

Eucaryotic regulatory factors are normally activated in response to different environmental stimuli such as mitogens, growth factors, hormones, heat-shock, heavy metals and others. The general activation mechanisms of regulatory factors are: phosphorylation, dephosphorylation, binding of a ligand, association with a monomer to form an active dimer and some others. Transcriptional regulatory factors are mostly present in the cytoplasm and after activation they are translocated to the nucleus. Once in the nucleus, activated regulatory factors bind to specific DNA sequences and interact with RNA polymerase II basal transcriptional machinery.

Some eucaryotic regulatory factors are activated via the binding to heavy metals. The DNA binding domain adopt a functional conformation after the binding to a specific heavy metal. The activation domain is rich in acidic amino-acids, glutamines or prolines. Eucaryotic DNA sequences which bind heavy metal-associated factors are found in animal, fungi and plant MT gene promoters. In animals, heavy metal responsive sequences are interdigitated with other regulatory sequences that bind regulatory factors such as sp1, AP-1 or AP-2. In the case of plants, heavy metal responsive sequences are combined with other sequences that are responsive to plant hormones, wounding, etc.

Animal MT gene promoters and metal-responsive factors

Animal MT promoters contain several copies of a single metal responsive element named MRE (*M*etal *R*esponsive *E*lement). This regulatory element shows a core consensus sequence defined by TGCRCNC (R is a purine and N is any nucleotide) flanked by pyrimidine nucleotides (Karin et al., 1984c; Thiele, 1992). Animal MREs are located 50 to 180 bp upstream of the transcription initiation site. Considering that animal MT genes are transcriptionally responsive to different metal ions, it is expected that several transcription regulatory factors may be involved in this process.

Human MTII-A gene promoter contain four MREs located at positions 50, 90, 135 and 150 bp upstream of the transcription initiation site (Karin et al., 1984c). The MRE located at position -90 is oriented in the opposite direction compared with the other MREs. Regulatory factors binding to human MREs have been purified from HeLa cells. Factor hMTF-1 (human *M*etal dependent *T*ranscription *F*actor 1) is a 90 kDa protein and its binding is stimulated by zinc ions (Westin & Schaffner, 1988). The gene coding for hMTF-1 has been cloned (Brugnera et al., 1994). This regulatory factor contains a DNA binding domain having six zinc fingers of the C_2H_2 type and an activation domain with three activating regions: an acidic region, a proline rich region and a serine-threonine rich region (Radtke et al., 1995). It has been determined that hMTF-1 is essential for constitutive expression of human MT-I and MT-II genes and for their transcriptional activation in response to zinc, cadmium and copper (Brugnera et al., 1995; Heuchel et al., 1998). Another zinc responsive factor has also been purified: ZRF (*Z*inc *R*esponsive *F*actor) is a 116 kDa protein and its binding is stimulated by zinc ions *in vitro* (Koizumi et al., 1992a; Otsuka et al., 1994). Zinc and cadmium responsive factors have also been purified from a different human cell line: MRE-BF1 and MRE-BF2 which are proteins of 86 and 28 kDa. MRE-BF1 binds to MRE in physiological conditions while MRE-BF2 binding occurs when cells are treated with zinc or cadmium (Czuprin et al., 1992). A negative regulator of MT gene transcription has also been isolated: MREBP (*MRE B*inding *P*rotein) is a protein of 112 kDa and its binding is inhibited by zinc and cadmium in vitro (Koizumi et al., 1992b).

Mouse MT-I gene promoter contains six MREs located at positions 40, 50, 90, 120, 140 and 170 bp upstream of the transcription initiation site (Séguin & Hamer, 1987). The MRE located at position -50 is oriented in the opposite direction compared to the other MREs. Several proteins bind to mouse MREs in a zinc dependent manner: MEP-I (*M*etal *E*lement binding *P*rotein I) of 110 kDa (Labbé et al., 1993), mMTF-1 (mouse *M*etal dependent *T*ranscription *F*actor 1) of 72.5 kDa (Radtke et al., 1993) and ZAP (*Z*inc *A*ctivated *P*rotein) of 108 kDa (Searle, 1990). On the other hand, factor MBF-1 (*M*RE *B*inding *F*actor) of 74 kDa binds to MREs but its binding is not stimulated by zinc *in vitro* (Imbert et al., 1989). Mouse MTF-1 gene has been cloned (Radtke et al., 1993) and it appears to be 93% homologous to human MTF-1 gene. Mouse MTF-1 factor also contain a DNA binding domain with six zinc fingers of the C_2H_2 type and an activation domain consisting in an acidic region, a proline rich region and a serine-threonine rich domain. Human MTF-1 activates more efficiently human MT gene transcription than its mouse homolog (Müller et al., 1995).

Fungal MT gene promoters and metal-responsive factors

Fungal MT gene promoters contain one or more UASc (Copper Responsive UAS) sequences. UASc has a core consensus sequence defined by TCY(4-6)GCTG (Y is a pyrimidine) and differs from animal MRE. Considering that fungi MT gene transcription is only activated by copper or silver, few regulatory factors are expected in this case.

The yeast *Saccharomyces cerevisiae* contains two genes coding for CUP1 and CRS5 MTs. Cup-1 gene promoter shows six UASc located at positions 120, 135, 155, 180, 210 and 250 bp upstream of the initiation site of transcription (Evans et al., 1990b). UASc sequences located at positions -120 and -155 are oriented in the opposite direction compared to the other UASc sequences. CRS5 MT gene promoter shows a single UASc located at position -175 (Cizewski-Culotta et al., 1994). The UASc elements of cup-1 and CRS5 gene promoters bind a single copper responsive regulatory factor named ACE1 (*A*ctivator of *C*up-1 *E*xpression 1). The gene coding for ACE1 was cloned and it encodes a protein of 225 amino-acids (27 kDa) (Fürst et al., 1988). ACE1 is modular and shows a DNA binding domain and an activation domain that is rich in acidic amino-acids. The DNA binding domain of ACE1 contains 12 cysteine residues arranged as Cys-X-Cys and Cys-X-X-Cys, like most eucaryotic MTs (Narula et al., 1991). From these 12 cysteinyl groups, 11 are critical to bind Cu^{+1} ions (Hu et al., 1990; Dameron et al., 1991). The 11 cysteinyl groups coordinate the binding of four Cu^{+1} ions and a single Zn^{+2} ion (Farrell et al., 1996). It was recently shown that the first 40 amino-acids of the ACE1 DNA binding domain contains three cysteines and one histidine which are essential for binding zinc. This allows the formation of a special type of zinc finger defined by C_3H_1 which interacts with the UASc element (Farrell et al., 1996). Moreover, the binding of four Cu^{+1} or Ag^{+1} ions to the DNA binding domain of ACE1 triggers a conformational change that allows its binding to UASc (Fürst et al., 1990; Dameron et al., 1991; Dameron, 1993). ACE1 is able to bind cadmium or cobalt ions using tretrahedral coordination bonds but these factors failed to bind DNA (Dameron et al., 1993). ACE1 activates transcription of MT genes by means of its acidic activation domain (Thiele, 1988; Cizewski-Culotta et al., 1989).

The yeast *Candida glabrata* contains three MT genes, MT-I, $MT\text{-}II_A$ and $MT\text{-}II_B$ (Mehra et al., 1989; Mehra et al., 1990). The UASc element of *Candida glabrata* shows the same core tetranucleotide sequence of the UASc found in *Saccharomyces cerevisiae*. The promoter region of MT-I has two UASc elements located at positions 110 and 145 upstream of the initiation site of transcription. Promoter regions of $MT\text{-}II_A$ and $MT\text{-}II_B$ contain five UASc sequences located at positions -190, -225, -265, -330, -340 and -370 (Zhou et al., 1992). The UASc of *Candida glabrata* binds the regulatory factor AMT1 (*A*ctivator of *M*etallothionein *T*ransctiption 1). The gene coding for AMT1 was cloned as it encodes a protein of 265 amino-acids (32 kDa) (Zhou and Thiele, 1991). AMT1 DNA binding domain is 50% homologous to ACE1 binding domain and its activation domain is also rich in acidic amino-acids. AMT1 DNA binding domain also contains 11 cysteine residues that coordinate the binding of four Cu^{+1} ions and one Zn^{+2} ion (Thorvaldsen et al., 1994). As described for ACE1, the structure of the first 40 amino-acids of AMT1 form a C_3H_1 zinc finger that interacts with the UASc element (Farrell et al., 1996). Moreover, AMT1 can functionally replace ACE1 since it confers coppers induced transcription of cup-1 gene *in vivo* (Thorvaldsen et al., 1993).

Plant metallothionein gene promoters

Plant MT gene promoters contain sequences with homology to animal MREs. These sequences have been identified in pea and tomato MT gene promoters (Evans et al., 1990a; Whitelaw et al., 1997). Until now, no information is available concerning heavy metal-associated factors.

Molecular mechanisms involved in copper homeostasis in yeast

Eucaryotic cells require copper only in trace amounts. Therefore, they have developed molecular mechanisms that allow the fine tuning of intracellular concentration of copper. The best studied model for copper homeostasis is the yeast *Saccharomyces cerevisiae*. It was determined that the synthesis of MTs is not required for copper uptake since kinetic parameters of copper transport were identical in a wild type yeast strain and in a mutant carrying a deletion of cup-1 gene (Lin & Kosman, 1990). Therefore, MTs act as intracellular reservoirs of copper in physiological conditions and as buffering proteins involved in copper accumulation in the case of copper toxicity.

Copper ions are found intracellularly in its reduced state (Cu^{+1}). Recent studies have determined that Cu^{+2} needs to be reduced to be efficiently transported into the cell. The reduction of Cu^{+2} ions is accomplished by at least two plasma membrane-associated Cu^{+2}/Fe^{+3} reductases called FRE1 (*F*erric *RE*ductase 1) and FRE2 (Dancis et al., 1992; Georgatsou et al., 1990). These copper reductases also reduce Fe^{+3} ions to Fe^{+2} suggesting that copper and iron uptake is coordinated. FRE1 is responsible for 50 to 70% of Cu^{+2} reduction (Hassett & Kosman, 1995). The gene coding for FRE1 reductase was cloned and it encodes a transmembrane protein having homology with cytochrome b_{558} which is an essential component of a human phagocyte oxidoreductase (Dancis et al., 1992). Moreover, copper transport into the cell is determined by at least two specific

transporters called CTR1 (*C*opper *TR*ansporter 1) and CTR3 (Dancis et al., 1994; Knight et al., 1996). The genes coding for these copper transporters have been isolated (Dancis et al., 1992; Dancis et al., 1994; Knight et al., 1996). CTR1 is a multispanning plasma membrane protein of 406 amino-acids (49 kDa) showing an amino terminal domain that is rich in methionine and serine residues (Dancis et al., 1992). CTR3 is a small cysteine-rich integral membrane protein that functions independently of CTR1 (Knight et al., 1996).

The promoter region of CTR1 and CTR3 transporters and FRE1 reductase genes contain a proximal regulatory element named CuRE (*Cu R*esponsive *E*lement) having a consensus sequence defined by TTTGCTC (Yamaguchi-Iwai et al., 1997). Two CuRE elements have been detected in FRE1, CTR1 and CTR3 gene promoters and they are located at positions -265 and -285, -310 and -330, and -180 and -230, respectively (Labbé et al., 1997). Both elements are oriented in the same direction and they bind the regulatory factor MAC1. The gene coding for MAC1 has been cloned and it encodes a protein of 417 amino-acids (47 kDa) (Jungmann et al., 1993). MAC1 DNA binding domain is similar to that of ACE1 and AMT1 since it contains three cysteines and one histidine that bind to a zinc ion and that allow the formation of a C_3H_1 zinc finger which directly interacts with DNA (Jungmann et al., 1993). The activation domain of MAC1 is rich in acidic amino-acids but, strikingly, it contains two cysteine-rich sequences. In the case of copper starvation, MAC1 binds the CuRE element and activates transcription of FRE1, CTR1 and CTR31 genes (Graden & Winge, 1997). In contrast, higher concentrations of copper inhibit MAC1 binding to CuRE. It has been determined that MAC1 carboxy-terminal cysteine-rich domains bind copper, triggering a conformational change that induces a rapid degradation of this factor (Zhu et al., 1998). Therefore, MAC1 is a novel transcriptional regulatory factor that is able to sense intracellular copper concentrations.

Perspectives

In the last 15 years, major advances have been made in our understanding of molecular mechanisms involved in heavy metal tolerance and homeostasis in eucaryotes. The best studied model is the yeast *Saccharomyces cerevisiae*. The principal mechanisms involved in heavy metal tolerance in yeast appear to be conserved in other eucaryotes. Therefore, genes determining copper homeostasis in yeast are probably going to be useful to clone their homologues in other eucaryotes. The mechanisms of heavy metal tolerance and homeostasis mentioned here are probably not the only mechanisms involved in these processes. Therefore, additional effort will be required to determine if there are complementary mechanisms governing heavy metal tolerance and homeostasis in eucaryotes.

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