Annu. Rev. Nutr. 14:449–69

METAL ELEMENTS AND GENE EXPRESSION

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KEY WORDS: regulation, zinc, transgenic, transcription

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INTRODUCTION

The flow of nutrients absorbed from the gastrointestinal (GI) tract to cells enables individual nutrient molecules, i.e. micronutrients, to exercise unique regulation of cellular control mechanisms. Through biologically active metabolites, micronutrients Vitamins A and D regulate transcription of specific families of genes that characterize functions of these vitamins. Trace metal elements represent other micronutrients that influence expression of specific genes.

The concept of gene control via trace metals was advanced three decades ago, but the scope and potential of this form of biological signaling are only now gaining full appreciation. A number of reviews on this topic have been published (26, 40, 66). The purpose of this review is to provide a nutritional

0199-9885/94/0715-0449$05.00

perspective of more recent findings on transcriptional regulation of gene expression by trace elements.

METALS INVOLVED IN GENE REGULATION

All organisms require an external supply of metals in order to satisfy a variety of cellular needs. Over time, organisms have evolved the ability to use the unique chemistries of these metals to achieve special goals. For example, calcium plays a role in activation of blood clotting via binding to γ-carboxyglutamic acid residues of prothrombin. The literature contains several discussions of these metal-dependent functions (reviewed in 14). In contrast, although the literature on metals that influence gene expression is expanding rapidly, it remains limited. Much of the currently available information is based on experiments with microbial systems. Our knowledge of metals involved in mammalian gene expression is derived from evidence with established micronutrients, i.e. copper, iron, manganese, and zinc, and with a few toxic metals with no known nutritional role, e.g. cadmium and mercury. The mechanisms of action of these metals are largely similar, but they exhibit marked differences as well.

Specialized transport systems regulate intracellular metal concentrations. Although the delineation of these acquisition and retention systems is still in a developmental period, a knowledge of their physiologic roles is critical to understand the influence of natural abundance and thermodynamic properties in determining which metals participate in regulating specific cellular functions. Metals participate in gene regulation through control of intracellular metal availability to appropriate ligands within specific cellular compartments. Cellular constituents, particularly proteins, provide metal-binding sites via amine, thiolate, carboxyl, and other ligands with metal binding that follows (approximately) the Irving-Williams order of divalent ions: Cu(II) > Zn(II) ≥ Ni(II) > Co(II) > Fe(II) > Mn(II) > Mg(II) > Ca(II). If binding ligands and the cellular concentrations of these metals were not controlled, copper would be the primary metal bound to all sites (73). Fortunately, copper concentrations within cells are regulated in part by effective efflux mechanisms (35, 68). Therefore, it is not surprising that zinc has the most collective influence in molecular biology compared with other metals. Cells have a higher concentration of zinc than copper. Furthermore, zinc does not have redox properties and can produce important structural configurations through tetrahedral coordination chemistry. Detailed discussions of the unique chemistry of each metal and its potential role in these processes are available (14, 40).

The roles of specific dietary metals in regulating specific genes must be separated from the more generalized roles of metals in gene expression. In the broadest sense, all essential metals in the diet can be viewed as determinants

of expression because they help maximize cellular potential. Metals can be divided into three general classes based on their involvement in processes required for gene expression. The first class is structural, i.e. metals facilitate conformations necessary to achieve unique opportunities for specific interactions among various binding groups. The zinc-finger proteins, which comprise far more than 1% of the genome, fall into this class. These proteins are involved in many different cellular functions (9, 50). Originally recognized as motifs of DNA-binding transcription factors, zinc-finger proteins of nutritional interest include receptors for the retinoic acid, calcitriol [1,25-$(OH)_2$ vitamin D_3], glucocorticoid hormone, and thyroid hormone. However, not all proteins with zinc-finger motifs are directly involved in gene expression. Zinc-finger proteins have been reviewed in detail (9, 10, 75). Although zinc appears to be the favored metal by these proteins in vivo, other metals may substitute for zinc under certain in vitro conditions. For example, the zinc-finger-containing estrogen receptor has the following affinity for DNA binding: Cu(II) > Cd(II) > Zn(II) > Co(II) > Ni(II) (45). The role of diet as a determinant of zinc-finger protein functions has not received much attention.

Metalloenzymes constitute the second class of effects that metals provide in gene expression. RNA polymerases I, II, and III (RNA nucleotide transferases) are zinc metalloenzymes essential for the synthesis of ribosomal, messenger, and transfer RNAs, respectively, and are the most well studied of this functional group (17). Conclusive experiments of a nutritional nature that clearly define a link between metals and the activity of these enzymes in animals have not been forthcoming.

The trace metals of nutritional significance, which play a regulatory role for specific genes, comprise the third class of involvement for metals in gene expression. These metals provide signals to the transcription activation process or for translational control of mRNAs. I do not discuss translational control by metals because the only recognized example of such control, i.e. of transferrin receptor and ferritin mRNAs, has been covered previously (28, 32).

Metals provide signals to systems that influence rates of transcription. They do not act directly on DNA, but rather through binding to specific proteins or via second messengers, as in the case of calcium. When regulatory molecules (hormones, growth factors, and cytokines) attach to a specific receptor on the plasma membrane, receptor activation results in an increased intracellular concentration of calcium or another mediator such as cAMP or tyrosine kinase. In this capacity, calcium is an intracellular mediator or second messenger rather than the actual inducer of a system that leads to increased gene expression. The role of calcium nutrition as a determinant of this second messenger activation of gene expression has not been well studied. Conversely, some transition metals can act as specific inducers of gene expression that play a direct role in the process. These activities require protein binding through

formation of coordinate covalent bonds. An increasing body of literature is devoted to this mode of gene regulation. Participation of trace metals, including both nutrient (copper, iron, and zinc) and nonnutrient metals (arsenic, cadmium, and mercury), in gene regulation has been documented. To fully appreciate metal regulation of genes, examples from both microbial and mammalian systems must be considered.

ESSENTIALS OF METAL REGULATION

In the literature, a fair consensus has developed that a minimum of three components is necessary for metal regulation of transcription. A generalized model is presented in Figure 1. First, the responsive gene must have a metal-responsive (regulatory) element (MRE) at one or more sites of the promoter sequence upstream from the transcription initiation site (start site) (63). These *cis*-acting DNA sequences are core 13–15 bp imperfect motifs. They can be found in either orientation and are usually located within the first few hundred bp of the promoter. Conclusive in vitro evidence indicates that MRE sequences provide metal-responsive regulation of a gene when placed in a non-MRE promoter construct (27, 42, 43, 62). Mammalian metallothionein (MT) promoters, the most widely studied MRE-containing promoters, have a consensus MRE sequence of CTCTGCRCNCGGCCC (Figure 2). MREs tend to be positioned near other regulatory elements such as AP-1, AP-2, and SP-1. Investigators believe that these proximities provide opportunities for synergistic or antagonistic responses (55, 66). Furthermore, multiple MREs are not necessarily equivalent, since some may become active at different metal supply levels (e.g. different dietary intakes).

A *trans*-acting metalloregulatory protein (transcription factor) is the second component required for metal regulation of transcription. Metal occupancy provides the conformational change needed for direct DNA binding or for subsequent protein-protein interactions leading to DNA binding. In either scenario, metal-induced DNA binding by the metalloregulatory protein is to the MRE motif (Figure 1). Metals bind to these DNA-binding proteins [known as metalloregulatory proteins, metalloregulatory transcription factors, or metal response element–binding proteins (MRE-BPs), as they are more appropriately called] by coordination chemistry specific for that metal. Physical characteristics of these DNA-binding proteins show that selectivity is involved in metal-binding chemistry.

The only definitive evidence concerning the mode of action of MRE-binding proteins was derived from a prokaryotic DNA-binding protein of the bacterial mercury-resistance (*mer*) operon (24, 40, 49). Transcriptional control of the mer system rests with the DNA-binding properties of mercury-resistance genes (*MerR*). Of the group IIB transition elements [Cd(II), Hg(II), and Zn(II)], the

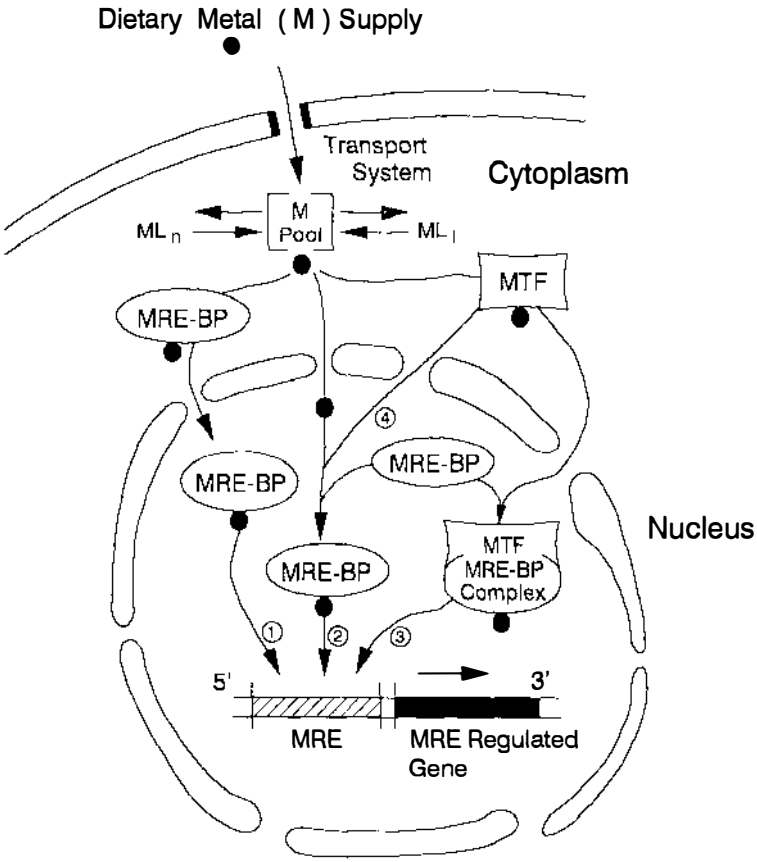

Figure 1 Potential modes of metal-binding factor interaction needed for metal-regulated gene transcription. Following transport into cells, the metal inducer (M) interacts rapidly with a variety of ligands (L). The metal enters the nucleus as a free ion or (more likely) bound to a metal response element–binding protein (MRE-BP) or to a metal transcellular factor (MTF). Binding of the MRE-BP (mode 1, 2, or 4) or of the MRE-BP/MTF complex (mode 3) to the MRE sequence of the promoter is required to initiate transcription. Mode 4 depicts intranuclear ligand exchange of M between MTF and MRE-BP. The rate of transcription is proportional to the dietary metal (M) supply and the intracellular M pool size.

MerR protein is most sensitive to Hg(II) at nanomolar concentrations. These three metals increase transcription rates when presented to the organism or to an isolated transcription system (49). Transcription is increased by the Hg-MerR complex through greater DNA distortion, which allows greater accessibility of RNA polymerase. At present, we have no reason to believe that a similar mode of action for other metalloregulatory proteins does not exist

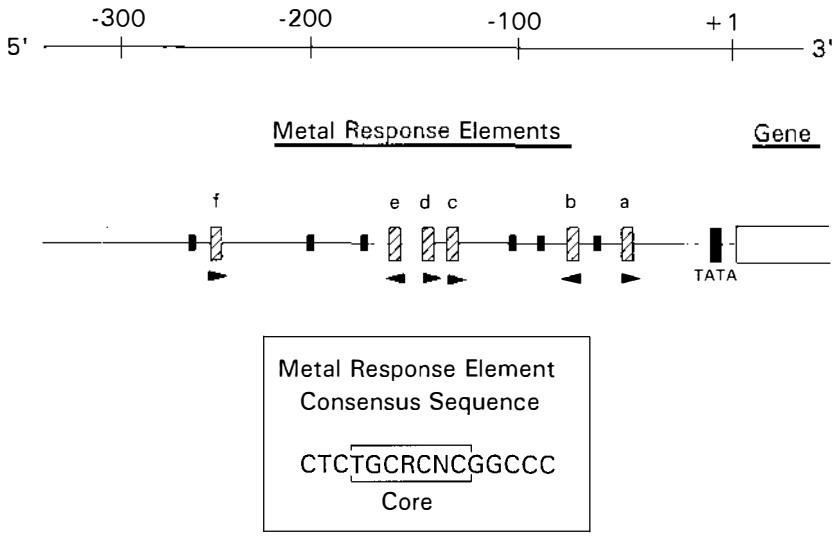

Figure 2 Orientation of MREs (a-f) in the MT promoter. Relative induction efficiency is approximately: MREd (++++), MREa or c (++ to +++), MREb (~ +), MREf (unknown), and MREe (~ 0). Orientation of MRE sequence is shown by solid arrows. Approximate relative upstream positions (from +1 to –300) of binding sites for transcription factors SP1, AP-1, AP-2, AP-2, AP-2, and glucocorticoidhormone receptor (glucocorticoid response element)–binding factor are indicated by solid rectangles. The insert shows the consensus sequence for mammalian MT promoter MREs. Derived from data presented or reviewed previously (26, 29, 40, 55, 62, 66).

for nutritionally relevant metal-regulated mammalian genes. The prokaryotic MerR system exhibits the interesting phenomenon of metal-regulated transcriptional repression as well as activation.

The third component of metal regulation of transcription is the inducer metal. Of the inducer metals with direct nutritional relevance, copper and zinc have received the most attention. Intracellular concentrations of metals are fairly closely regulated by absorptive processes and membrane transporters working together. Nutritional perturbations such as dietary restriction, food fortification, or dietary supplementation can alter this balance. We know, for example, that the flow of dietary zinc that enters cell nuclei is proportional to dietary intake (12). A similar finding would be expected for other metals. The fate of metal ions in a path leading to transcriptional regulation has not been delineated. Potential interactions have been outlined in Figure 1. The metal pool (M) that serves as a precursor pool for eventual binding to nuclear factors could result from a variety of proteins, from a specific protein, or from a chelate of low molecular mass. Dietary intake and/or physiologic stimuli could influence the availability of a metal derived from this pool for binding to a nuclear metal-

loregulatory protein. Specificity of the metalloregulatory protein(s) for individual metals is essential at some point in transcription activation. Thiele (66) has advanced the concept of multiple metalloregulatory proteins, since more than one metal may activate transcription by a single MRE. Specificity also relates to the mode of nuclear entry by the inducer metal, i.e. via a metal transcellular factor(s) or via the actual translocation of the metalloregulatory binding protein(s).

MICROBIAL MODEL SYSTEMS

Studies of transcriptional regulation of genes in prokaryotic cells have provided important insights into how metals influence gene expression. Most of this activity has focused on metal ion–resistance operons.

The *mer* operon is one such model that is very relevant to nutritional regulation in animals. Resistance to mercury in bacteria involves control of Hg(II) transport as well as reduction of Hg(II) to the less toxic Hg(O). The MerR gene product, a metalloregulatory protein, produces the resistance phenotype (24). The MerR protein is 16 kDa in size and, in its activated state, exists as a dimer that shares one Hg(II) atom. The metal-binding site of MerR protein is probably a -Cys-X_{32}-Cys-X-Cys-His-$(X)_7$-Cys domain (41) that provides trigonal coordination chemistry. Current evidence suggests that the MerR protein binds to a palindromic site of DNA near the operon's structural genes and the *mer* promoter (24, 41). The MerR protein normally represses the operon, whereas exposure to Hg(II) at concentrations of 1 x 10^{-8} M produces half-maximal activation (induction) of transcription of operon genes. Activation by Hg(II) is orders of magnitude greater than by Cd(II) or Zn(II). The mechanism proposed is that the inducer, in this case Hg(II), enters the cell prior to exercising its signaling function. As Ralston & O'Halloran pointed out (49), this finding is unexpected, because Hg(II) has deleterious effects on the cell exterior and interior. This observation is of interest because nature seemingly has retained the need for cell uptake of metals as a mode of gene regulation in eukaryotes as well. Metal-sensing membrane receptors for indirect regulation by metals could have evolved but apparently did not. Frantz & O'Halloran (19) proposed a model for transcriptional regulation by the activated MerR (Hg-MerR) protein involving stabilization of DNA distortion. This process appears to open the MerR-DNA complex, thereby allowing RNA polymerase II to initiate transcription of genes of the *mer* operon.

Repression of transcription by the ferric uptake regulation (fur) gene product is a mechanism certain bacteria use to control genes needed for iron acquisition. This subject has been studied extensively (3). The Fur protein is a 17-kDa product which, when iron is bound, acts as a transcriptional repressor of operon

genes required for the biosynthesis of siderophores. These molecules are excreted and facilitate iron uptake by the microorganism. Other divalent metals bind to Fur protein to produce repression of this operon (52).

Recently, a more circumspect role for Fur protein was proposed. Specifically, Fur-dependent proteins were found that were under both positive and negative control by iron (18). An outer-membrane constituent protein (FatA) is under iron-dependent negative transcriptional control by Fur protein (69). Iron-dependent negative transcriptional regulation was also reported for manganese-containing superoxide dismutase in *Escherichia coli* (46). Magnitude of iron repression of gene transcription is related to the specific gene and is not uniform. The SoxR protein is also a metal-dependent negative transcriptional regulator of many genes of microorganisms (47). In contrast, the positive transcriptional control of diphtheria toxin is dependent on Mn(II) binding to DtxR protein (65), which then binds to a 9-bp interrupted palindromic sequence of the diphtheria *tox* promoter (66). The gene *fliC,* which encodes flagellin in *E. coli,* is controlled by aluminum. Transcriptional control of a fusion gene containing elements of the *fliC* gene by aluminum was subsequently demonstrated (21). The *cyt c6* gene, required for photosynthesis in algae, is highly specific for copper derived from the nutrient source and is transcriptionally regulated (36).

A plethora of microbial metal-resistance operons with apparent transcriptional regulation has been identified (59). These genes are regulated by arsenic, cobalt, and zinc via the metal-binding transcription factor proteins ArsR, CzcR, and SmtB, respectively (38, 39, 53). When identified and sequenced, many microbial metal-regulated genes and genes for metal-binding proteins may help identify and characterize mammalian counterparts. The similarity of genes for cellular copper export serves as an example of this type of relationship (35, 68)

METAL REGULATION OF MAMMALIAN GENE EXPRESSION

An appreciation of the transcriptional regulation of mammalian genes by metals derived from the dietary supply requires comparisons drawn from the full spectrum of unicellular and multicellular eukaryotic systems as well as from microbial systems. This information is essential because in cases in which comparisons have been made, data from unicellular organisms, e.g. *Saccharomyces cerevisiae,* are similar to those of cells from animal tissues. The best-developed lower eukaryotic model is the copper-resistance MT (*CUP1* gene) from the yeasts *S. cerevisiae* and *Candida glabrata.* In higher eukaryotes, including humans, MT is also the best-developed model of metal regulation of transcription.

METALLOTHIONEIN

MT is a cysteine-rich, metal-binding protein of unknown function that has been well studied (11, 15, 22, 27). MT exists as multiple isoforms (usually two or three) encoded by separate structural genes. The induction of MT by Cd(II) administration as described by Piscator (44) three decades ago represents the first suggestion of metal-regulated gene expression. A decade later, experiments with actinomycin D supported a role for cadmium and zinc in MT regulation at the transcriptional level (51, 61). Although transcriptionally regulated by metals in all systems studied, mammalian cell and intact animal studies show that certain hormones and cytokines also regulate the MT genes (15). Physiologic regulation of these genes clearly has teleological implications that signify a function beyond nutritional relevance.

The components of the MT regulatory system are comparable in all eukaryotes. Specifically, multiple MREs of the MT promoter interact with a metalloregulatory protein (transcription factor) to initiate transcription of the MT gene. Metal occupancy by this factor is likely essential. These proteins have been well identified and characterized in yeast, but those of mammalian sources have not. In yeast, these proteins are called ACE1 (for activation of *CUP1* expression) or AMT1 (for activation of metallothionein transcription), and in mammals they are known as MTF-1 (for metal-dependent transcription factor), MBF-1 or MRE-BP (for metal response element–binding factor), MEP-1 (for metal element–binding protein), or ZAP (for zinc-activated protein). The metal-binding domains of ACE1 and AMT1 have the sequence Cys-X_2-Cys-X_3-His-X_4-Cys-X_2-His-X_{17}-Cys-X-His-Cys-X_{14}-Cys-X-Cys-X_{16-26}-Cys-X-Cys-X_5-Cys-X-Cys and bind six to eight Cu(II) atoms per bound molecule. This stoichiometry appears to give trigonal geometry (64, 67). These yeast proteins do not show appreciable binding of zinc. Current data suggest that Zn(II) atoms bind to the mammalian regulatory proteins in tetrahedral geometry.

Data on the MT metalloregulatory protein(s) (transcription factors) of mammals vary considerably. To date, none of these proteins has been fully characterized using a homogeneous protein. This incomplete characterization is understandable in part because of the apparently low abundance of these proteins. Each cell would need relatively few molecules of these specific metalloregulatory proteins compared with factors such as Sp1, which are of more general regulatory function but are also of low abundance. Furthermore, transcription factors in nuclear extracts tend to be quite labile and susceptible to degradation and/or modifications that render them nonfunctional (20, 54, 57, 66). The apparent molecular masses of the putative mammalian metalloregulatory proteins required for MT transcription are 112 kDa for MRE-BP (29), 108 kDa for MEP-1 (56), and 39 kDa for the metalloregulatory protein

identified by Andersen et al (2). Techniques used to characterize these proteins include footprint experiments, in which the putative factor protects a MRE-containing DNA sequence from hydrolysis by DNase, and Southwestern blotting, which is used to separate putative factors by polyacrylamide electrophoresis, transfer them to nitrocellulose, and probe for binding activity using ^{32}P-labeled, MRE-containing DNA sequences (or oligonucleotides). These experiments led to the general consensus that occupancy by a specific metal is preferred by a given metalloregulatory protein (transcription factor). However, this preference is difficult to establish because in cells, multiple metalloregulatory proteins factors are present simultaneously, and nuclear extracts provide an array of these proteins that could each bind to the same MREs but in response to a specific metal.

The most successful attempts to purify a metalloregulatory protein used MBF-1 from mouse L-cells (26) and MRE-BP from HeLa cells (29). Extracts were obtained from isolated nuclei of cells and passed through heparin-agarose columns to select for DNA-binding proteins. This process was followed by affinity chromatography using MREs coupled to Sepharose®. Gel filtration and ion exchange steps were also employed. Despite the similarities of isolation approach and product homogeneity, the molecular masses of the proteins isolated were 74 kDa (26) and 112 kDa (29). These results suggest that multiple proteins may bind to MT-MRE sequences. The proposal of Czupryn et al that the isolation of two proteins (86 kDa and 28 kDa) may be responsible for basal and augmented levels of MRE binding and MT synthesis supports the presence of multiple MRE-binding proteins (13). The cDNA for a putative MRE-binding metalloregulatory protein from mouse L cells has been cloned (76), which will help identify and characterize at least one of these proteins.

Nutritional regulation of MT has been extensively studied. I published a detailed review on this subject in 1985 (11). Early methods tracked MT protein using zinc and copper content of soluble tissue extracts by gel filtration chromatography. The liver, kidney, and intestine, tissues in which MT expression is very high (12), received the most attention. In 1980, McCormick et al (34) demonstrated increased liver polyribosomal MTmRNA and increased rates of MT synthesis after a zinc-rich diet was fed to rats of low zinc status. Availability of cDNA and oligonucleotide probes extended the implications of this work. A variety of animal studies were also suggestive of a role for copper in signaling MT induction (6, 11). These experiments frequently involved large dietary intakes, some of which could have simulated endocrine responses that also increase expression. To reexamine the possible effects of metal ions on MT expression, more nearly physiological levels of copper and zinc were fed in rat diets with 5, 30, or 180 mg/kg of zinc and 1, 6, or 36 mg/kg of copper. The levels of 30 mg/kg of zinc and 6 mg/kg of copper are considered adequate intakes in the AIN-76 formulation (4). From this dietary regimen (Figure 3)

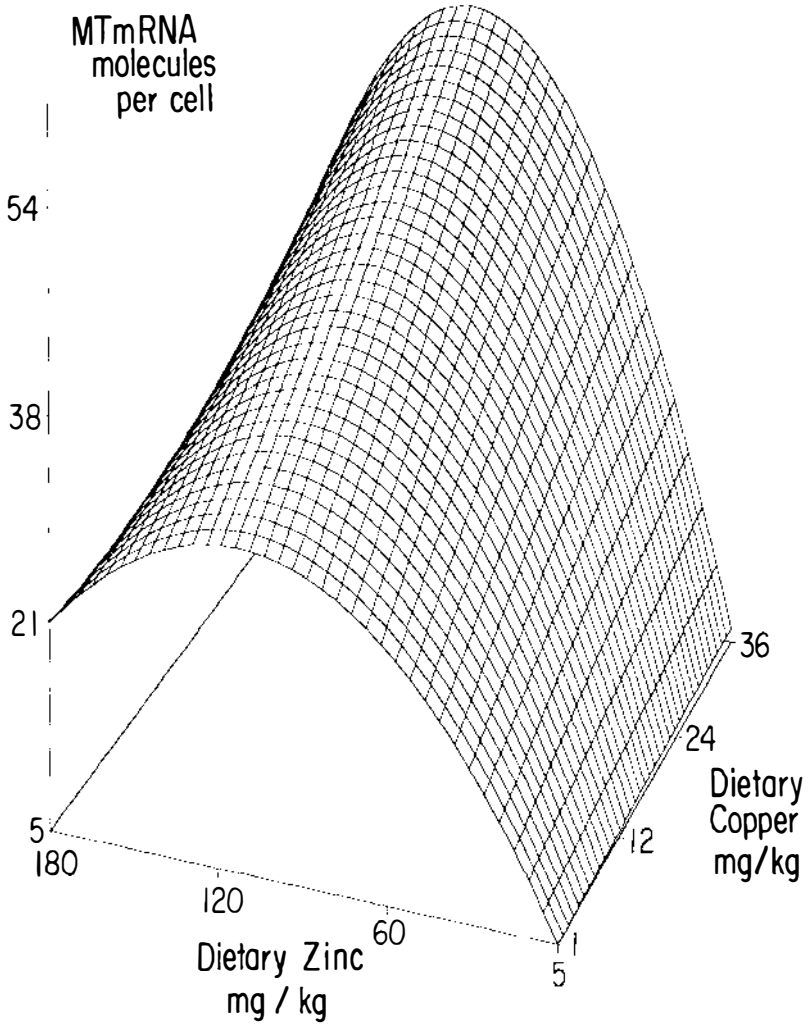

Figure 3 Surface response shows comparative differences in rat kidney MT gene expression (in molecules of mRNA per cell). High responsiveness to dietary zinc (5–180 mg/kg) and low responsiveness to dietary copper (1–36 mg/kg) are shown. Modified from Ref. 4.

Blalock et al concluded that in the young male rat, copper nutrition has little effect on MT gene expression, whereas zinc nutrition has a substantial effect.

Further exploration of the dietary regulation of MT expression by zinc addressed the questions of dietary intake level, nuclear zinc, and MRE-binding proteins. The signaling event for the regulation of MT expression by the dietary

zinc supply could be either a direct nuclear interaction with zinc of dietary origin via a metalloregulatory zinc-binding protein or an indirect interaction via a signal from a zinc-sensing membrane receptor. Evidence favors a direct effect (12). To test the concept of a direct effect, rats were fed a liquid diet containing 5, 30, or 180 mg/kg of zinc labeled with ^{65}Zn. Nuclei were purified from liver, kidney, and spleen 2 h later. When expressed on a per DNA basis, as much as 6% of the total nuclear zinc was derived from—and proportional to—zinc in the diet. This finding showed that a rapid transfer of dietary zinc to the nucleus occurs. Labeled nuclear proteins from liver that bound to a MRE oligonucleotide affinity column were in the 20–2000 kDa range. This observation is consistent with metalloregulatory proteins thus identified. Further research in this area is needed, but the MT model system clearly demonstrates that dietary zinc can significantly alter expression of genes regulated through MRE sequences in their promoters.

ACUTE PHASE PROTEINS

As more genes and their upstream regulatory regions are sequenced, several genes with MREs are likely to emerge. If one assumes that the metalloregulatory proteins can initiate transcription of all MRE-containing genes, families of genes may be regulated by dietary zinc, copper, etc. In addition to the research interest that metal regulation of these gene families may stimulate, it may also as a result give rise to interesting questions about recommended daily allowances (RDAs) for minerals as well as medical and policy questions associated with mineral nutrition.

Host defense systems require trace elements to function properly. Consequently, cellular responses to injury resulting in synthesis of acute phase proteins may require metal-dependent genes. The metal regulation of murine α_1-acid glycoprotein (α-AG) and C-reactive protein (CRP) gene expression, which has been convincingly demonstrated, supports this concept (77). Intraperitoneal injections showed that expression was affected in the order $Cd^{2+} > Cu^{2+} > Zn^{2+}$. Unchanged albumin mRNA levels indicate that the induction by metals was not a response to inflammation. Adrenalectomy was also without effect, which suggests that glucocorticoid hormones were not involved. Furthermore, sequence analysis of the promoters for these genes revealed homology with MREs. When transfected into human liver cells (HepG2), a construct consisting of the α-AG promoter (–595 to +18) and a reporter gene was responsive to these metals. Experiments to test the dietary regulation of these genes have not been attempted.

Interestingly, cadmium is the strongest inducer of the α-AG and CRP acute-phase genes. The response to cadium is the same as that for the MRE-rich metallothionein promoter. Cadmium is a poorly absorbed metal (70), which is

fortuitous in view of the potentially wide distribution of MRE-regulated genes and their strong regulation by this toxic metal. Furthermore, the strong response of acute-phase gene expression by copper as noted by Yiangou et al (77) may be indicative of a group of mammalian MREs with stronger affinity for copper-binding metalloregulatory proteins.

OTHER MAMMALIAN GENES WITH PUTATIVE METAL REGULATION

Changes in the synthesis of a protein or in levels of a specific mRNA in animals may accompany alterations in the dietary intake of one metal or a combination of metals. In addition, injection of a metal into an animal may elicit endocrine and/or inflammatory responses that mediate gene expression through signals distinct from metal regulation. Similarly, changes in gene expression may occur in cells cultured in medium with additions of specific metals. However, none of these situations implies direct metal regulation requiring MREs. Proof of gene regulation by a metal ultimately requires direct involvement of the metal with the regulators of the promoter for that gene. These strategies are outlined in Figure 4. The methods used can also determine when genes are not metal regulated (30).

Several potential candidate genes for metal regulation have emerged. The phorbol esters induce expression of a series of genes in mouse cells. These primary-response genes, which do not require protein synthesis to be transcribed, can be induced by cadmium or zinc in cultured 3T3 cells (16). The metals do not stabilize these mRNAs, which suggests a direct MRE-requiring induction process.

Our laboratory has used the differential hybridization technique to examine genes expressed in response to dietary zinc (58). Intestinal mRNA from rats fed a diet containing 1 or 30 mg/kg of zinc were reverse transcribed. The cDNAs were used to screen for clones generated from a cDNA plasmid library derived from mRNA of rats fed the zinc-deficient (1 mg/kg) diet. When hybridized to mRNA from the zinc-adequate rats, nine clones were identified that had an apparent higher expression than found with mRNA from zinc-deficient rats. Sequences of these cDNA clones were searched with GenBank for homologies to identify the zinc-responsive genes. DNA sequences identified and percentage of induction of the respective mRNAs in rats fed a zinc-adequate diet compared with a zinc-deficient diet were as follows: apolipoprotein A-1 (85%), aldolase B (60%), cytochrome b (53%), ubiquitin (214%), 9-kDa calbindin (340%), cryptdin (290%), cytochrome c oxidase (50%), pancreatic α-amylase (710%), and fatty acid–binding protein (60%). Initial data from a search of the apolipoprotein A-1 promoter showed a DNA sequence at −170 with high homology to the MRE consensus sequence (Shay & Cousins,

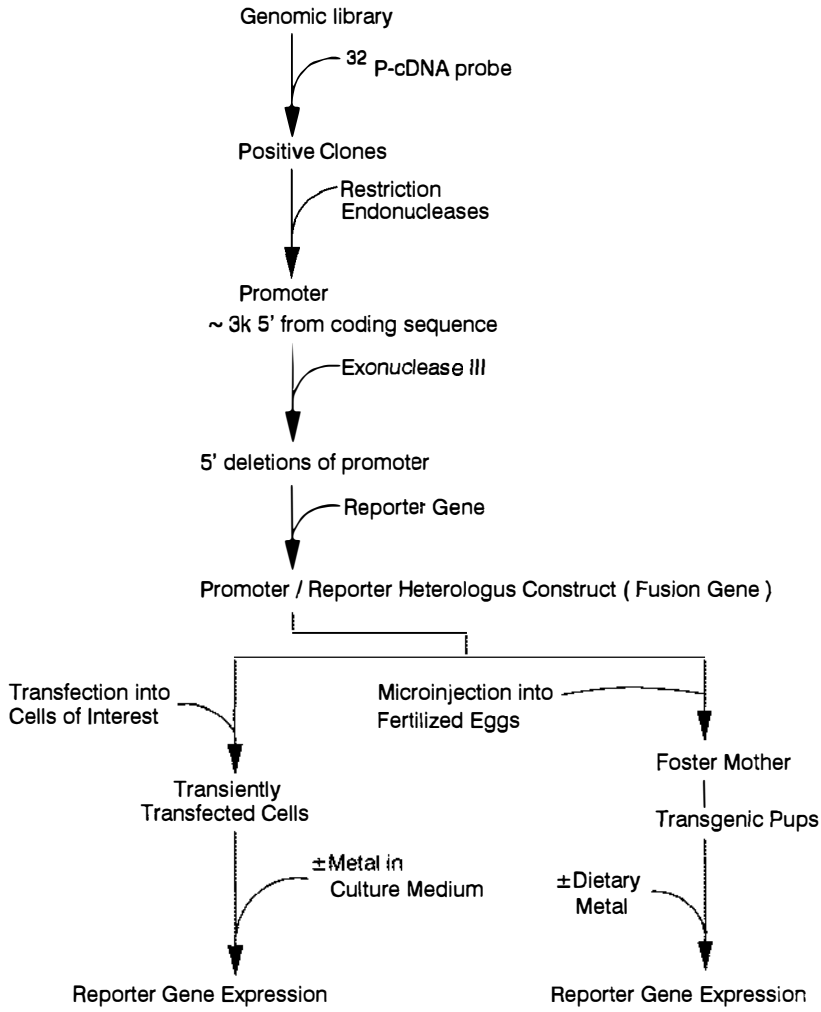

Figure 4 **Experimental strategies to establish direct metal regulation of a gene. Cells where metals increase expression of MRE-regulated genes must have MRE-binding transcription factors. Based on earlier approaches (30, 43, 55, 62, 65).**

unpublished data). This approach or one using differential display of mRNAs (31) is a powerful tool that will help identify those genes susceptible to regulation by metals in the diet.

Considerable indirect evidence supports a role for cobalt in the regulation of heme oxygenase. Cobalt may also regulate the erythropoietin gene (33).

Actinomycin D inhibition data, changes in mRNA levels for both genes, and nuclear runoff assay experiments all point to a metalloregulatory system of transcriptional control. A search of the erythropoietin promoter revealed two potential MREs upstream at –290 and –950 and one potential MRE at –575 of the heme oxygenase promoter (1). The translational regulation of liver ferritin by iron has been clearly and convincingly established (28, 32). However, iron reportedly increases transcription rates of the rat ferritin H gene, which implies some transcriptional control by iron (72).

A preliminary report shows that dietary manganese deficiency may reduce liver manganese superoxide dismutase mRNA levels (5). A sequence analysis of the rat manganese superoxide dismutase gene promoter has yet to be performed, and a model for manganese regulation of this gene has not been proposed. In contrast, nuclear runoff assays revealed that cellular glutathione peroxidase mRNA transcription rates were unaffected by dietary selenium status (7). This finding suggests that regulation by selenium is not at a transcriptional level. Conversely, expression of the c-*myc* gene (a protooncogene) may be regulated by beryllium (60), and vanadium may increase c-Ha-*ras* and c-*jun* (protooncogenes) gene expression (78) by transcriptional regulation. Neither of these metals is a dietary essential, but both are mitogenic.

APPLICATIONS OF METAL-REGULATED GENE EXPRESSION

Future developments in the area of metal-regulated gene expression will follow two major tracks. The first will be an in-depth search for metal-regulated genes and elucidation of their regulation by nutritional or other stimuli. The second will be the application of metal regulation of gene expression for a variety of novel agricultural, medical, and industrial purposes. The overall basis for these applications is the wide distribution of metalloregulatory proteins throughout plant and animal cells (although their relative abundance may be quite low) together with the amenability of MRE sequences to insertion into chimeric constructs to provide metal regulation.

The most dramatic examples of the application of engineering metal regulation were the pioneering experiments of the Palmiter and Brinster laboratories. Chimeric constructs (fusion genes) comprised of the metallothionein-1 promoter, including the upstream regions with the MREs, as well as the structural gene for rat growth-hormone (GH) gene were produced. These constructs were microinjected into the individual nuclei of fertilized mouse eggs, which were subsequently returned to the reproductive tracts of surrogate mothers. Pups were weaned and fed a diet including drinking water with 5000 μg of zinc per liter. As early as 40 days postpartum, pups with transgenes (intact, integrated fusion genes) were clearly bigger than their litter mates and

were producing more GH, which accounted for the difference in growth (42). Similar responses were reported for constructs with the human GH gene when the transgenic mice were given zinc in the diet or cadmium by a single injection (43). It was appropriate to use zinc in these studies because dietary zinc had been shown to influence MT gene regulation (34). Moreover, this metal has low toxicity and exhibits high inducibility of MRE-regulated systems.

The fusion gene approach for production of transgenic animals has been widely adopted, and many institutions now have specialized core facilities for transgenic animal development. Alteration of phenotypic expression in livestock is an obvious application of transgenic technology. Transgenic pigs carrying the MT MRE promoter/GH gene developed to a greater extent than controls. Dietary zinc at 1000–3000 mg/kg was used to activate the transgene (48). The leaner meat produced was attributed to greater circulating levels of GH in pigs with the transgene expressed in response to the high intake of dietary zinc.

The MT promoter, with accompanying transcription initiation sequences, was used to produce constructs with the low-density lipoprotein (LDL) receptor gene (25). In transgenic mice produced with this construct, acute cadmium administration dramatically increased the disappearance of ^{125}I-labeled LDL from the circulation. Those tissues with the highest ability to produce metallothionein (12), i.e. liver, kidney, and intestine, also had the highest level of cadmium-induced LDL receptor mRNA. Presumably, availability of metallo-regulatory MRE-binding factors was the determinant of tissue-specific expression of the transgene.

The major histocompatibility (H-2) antigen (71) and large T-antigen (65) genes are among those whose expression has been regulated by metals as chimeric metallothionein promoter/structural gene constructs in transfected cells. Zinc regulation of a human H-*ras* oncogene via the MRE-containing MT promoter was shown to increase susceptibility to natural killer cells (67). Alternative technologies such as retrovirus-mediated gene transfer (74) are amenable to delivery of metal-regulated transgenes for similar applications. For a time, an Epstein-Barr virus–based expression vector with a human MT promoter was commercially available for strategies requiring inducibility.

MRE-containing promoters have been used to produce transgenic plants for the purpose of metal regulation of genes. Mett et al (37) defined the ideal characteristics of inducible gene expression in plants as follows: low expression without the inducer, high expression with the inducer, and induction that does not alter the plant's physiology or affect the expression of other genes of the plant. They developed a heterologous model system using the yeast *ace1* gene, which coded for the copper regulatable ACE1 transcription factor (described above) with a reporter gene—in this case the gene for β-glucuronidase. These investigators demonstrated copper regulation of the reporter only in

those transgenic plants that produced ACE1 protein. They stressed that this system could be used for metal-inducible expression of an enzyme in commercially important plants. Butt & Ecker proposed that metal regulation of the CUP1 locus genes in yeast could be used for a variety of applications in biotechnology (8).

Successful application of metal regulation of chimeric genes in animals also requires that the metals be relatively nontoxic, have sufficient bioavailability to promote maximum expression of transgenes, and have few long-lasting residual effects. However, application strategies must take into account the unique physiology, tissue-specific distribution, and nutritional intake of the metal to be used. Additional concerns regarding the use of metal regulation of genes are that metal-resistance genes could lead to concurrent overgrowth of endogenous microbial populations and that subsequent metal induction of some microbial proteins could cause gratuitous changes in metabolic fates of xenobiotics.

It is also becoming clear that metals support gene expression by mechanisms apart from those needed for transcriptional regulation. For example, zinc is a structural requirement in transcription factors. These metals are derived from and potentially influenced by dietary intake. These other roles for nutritionally important metals may also act as targets for novel biotechnological exploitation. Au(II), the active component of the antirheumatic drug aurothiomalate for the inhibition of RNA binding by a zinc-finger transcription factor, (23) serves as a model.

SUMMARY

The transcriptional regulation of genes by metals is a biological function separate from structural and catalytic roles for metals in gene expression. Each of these functions relies on metals that enter cells from metabolic compartments derived from and influenced by the dietary metal supply. The intracellular metal pools provide an available source for binding to metalloregulatory proteins for transcriptional regulation. These proteins bind MRE sequences found in the promoters of some genes. The distribution of MRE sequences and of metalloregulatory proteins extends from microbial to mammalian systems. The bulk of the data on metal regulation of mammalian gene expression is from the perspective of positive transcriptional regulation. Nevertheless, negative regulation by metals could potentially occur. Transcriptional regulation of genes by nutritionally important metals must be viewed in the context of the other roles of metals in cellular structure and function. Investigators are rapidly delineating the involvement of metals in molecular biology in general and in gene expression in particular.

ACKNOWLEDGMENTS

Research from the author's laboratory cited in this review was supported in part by a grant (R37 DK31127) from the National Institute of Diabetes and Digestive and Kidney Diseases.

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