

## A role of zinc in the regulation of gene expression

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Nutritional regulation of gene expression is recognized as an important, albeit relatively new, area of research in nutrition. There are two major routes for nutrients to influence the extent to which a specific gene is expressed. The first is the scenario in which the nutrient has a direct interaction with cellular constituents, usually transcription factors, altering the rate of transcription and the cellular abundance of a given mRNA. Retinoids, calcitriol and certain sterols and fatty acids act as ligands that bind to specific transcription factors and alter a gene or a family of genes in this way. Zn is able to alter expression by a comparable mechanism in which metal occupancy in a site on a transcription factor is necessary to increase the rate of transcription. Fe influences the abundance of specific mRNA by binding to proteins which interact with mRNA to enhance or repress their translation. The second route for nutrients to alter expression is through any of a number of indirect interactions where secondary mediators respond to the nutrient's dietary supply. Various signal transduction systems and hormonal or cytokine stimuli may be involved. These areas of gene regulation by nutrients have been reviewed (Cousins, 1998).

### Functions of zinc in gene expression

The focus of the present review is on Zn and how its abundance in the diet influences gene expression. Examples will be presented which potentially demonstrate both previously mentioned routes for gene regulation by this micronutrient. There is extensive literature which describes the effects of Zn on growth (Mills, 1989). Consequently, Zn has been viewed for decades as a factor in protein synthesis required for growth and development. Zn has functions that can be characterized in three general areas: catalytic, structural and regulatory (Cousins, 1995, 1996). Each function can be related in some way to gene expression.

RNA synthesis is an essential component of gene expression and  $Zn^{2+}$  ions are required for catalytic activity of the RNA polymerases (RNA nucleotide transferases which are Zn metallo-enzymes). Early literature is filled with examples of how Zn restriction decreases aspects of gene activity (for review, see Prasad & Oberleas, 1976). Experiments with *Euglena* showed how a lack of Zn

correlated with cessation of growth, which could be re-established with Zn repletion. This was believed to be a reflection of the limited availability of Zn for the protein synthetic mechanisms and DNA replication (Falchuk & Vallee, 1985). Similarly, experimental animals show suspended growth when their diet is severely restricted in Zn content (Mills, 1989). The resulting lack of growth could be caused by a lack of Zn required for gene expression. However, the cessation of growth is more likely to be a secondary response to a stimulus (stimuli) to restrict the energy-requiring processes needed for additional cell replication and protein synthesis.

The structural role for Zn is best demonstrated in the zinc-finger motif of proteins (Klug & Schwabe, 1995; Schwabe & Fairall, 1995). Evidence suggests that 1% of the genome codes for zinc-finger proteins. There is general agreement that the tetrahedral coordination of the  $Zn^{2+}$  involves four cysteine and/or histidine residues in various arrangements (four cysteine; two cysteine, two histidine; three cysteine, one histidine), and that the stability constants are relatively high (on the order of  $10^{12}$  M). However, there is some evidence that  $Zn^{2+}$  in the finger with three cysteine residues and one histidine residue exists at low occupancy or is easily exchanged *in vivo* (Hempe & Cousins, 1991; Kosa *et al.* 1994). While the majority of the zinc-finger proteins are transcription factors, others may participate in protein-protein interactions that provide signalling via kinase-binding domains. There has been little investigation of the effect of dietary Zn status on zinc-finger proteins, and how regulation of specific genes could be affected. As antibodies become available to study such potential changes, data should soon become available to answer these questions. Since ubiquitous transcription factors, such as Sp-1, have functional zinc-finger domains, if Zn depletion reduces function it would have major consequences on gene expression and growth.

The third function of Zn is regulatory and one that relates directly to gene expression. Also in this category is the involvement of Zn in synaptic signalling (Palmiter *et al.* 1996). That emerging area will not be discussed in the present focused review. The elucidation of the mode of regulation of the metallothionein gene is the only clear example to emerge where  $Zn^{2+}$  is able to directly regulate the transcription rate of a gene (Cousins, 1994). This is

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**Abbreviations:** MRE, metal response elements; MTF, MRE-binding transcription factor; PCR, polymerase chain reaction  
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accomplished through the interaction of a  $Zn^{2+}$ -binding transcription factor that recognizes specific sequences (metal response elements; MRE) in the metallothionein promoter.  $Zn^{2+}$  binding probably occurs in the cytosol, with translocation to the nucleus for DNA binding. There is some evidence that the core MRE sequence is present in the promoters of other genes (for review, see Cousins, 1994, 1995). Presumably, these will be regulated also by the dietary Zn supply. A scheme showing the various ways in which Zn could influence gene expression is shown in Fig. 1.

The remainder of the present review will discuss the involvement of dietary Zn in the nuclear Zn pool, dietary regulation of the metallothionein gene, and use of differential mRNA display as an approach to identify genes that are regulated directly or indirectly by dietary Zn.

### Nuclear zinc

Fractionation studies have shown that about 25% of the Zn content of rat liver is found in the nuclear fraction (Smeyers-Verbeke *et al.* 1977). A significant amount of  $^{65}Zn$  has been shown to be incorporated into nuclei *in vitro* (Weser & Bischoff, 1970). We approached the transcriptional regulation of genes by Zn from the perspective that  $Zn^{2+}$  from the diet would participate in ligand binding to facilitate the activation of MRE-regulated genes. The consensus sequence for the MRE of the metallothionein promoter is CTCTGCRCNCGGCC (Cousins, 1994), and was deduced in the laboratories of D. Hamer (Bethesda, MD, USA) and R. Palmiter (Seattle, WA, USA). The core sequence is underlined and R and N represent 'the purine base' and 'any base' respectively. The development of the MRE concept has been reviewed previously (Cousins, 1994).

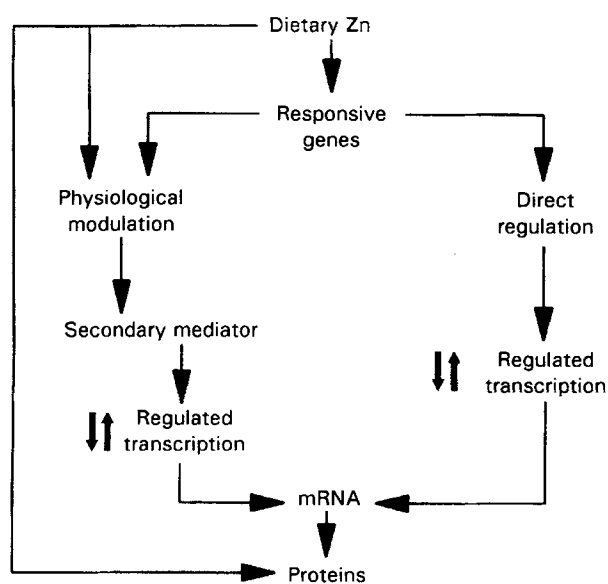


Fig. 1. A schematic representation of the ways in which zinc could influence gene expression.

The abundance of an MRE-binding transcription factor (MTF) would be expected to be very low relative to other transcription factors, which tend to be low-abundance proteins (Cousins, 1994). Attempts to isolate MTF from tissue extracts have not met with generally satisfactory results. Using molecular cloning techniques, an MTF (MTF-1) has been identified in human and animal cells (for review, see Dalton *et al.* 1997). MTF-1 may be constitutively produced, and active when an inhibitor is absent. An inhibitor molecule has not been identified. An alternative model suggests that MTF-1 binds  $Zn^{2+}$  as a lower affinity site than other zinc-finger transcription factors (Radtko *et al.* 1993). Recently, a zinc-finger domain of MTF-1 has been suggested as the site of  $Zn^{2+}$  occupancy required for a positive mode of regulation for MRE genes. Dietary regulation of MTF-1 has not been examined.

We approached the question of MRE regulation from a nutrition perspective to (a) quantify the amount of nuclear Zn arising from the diet (using  $^{65}Zn$ ), and (b) see if that dietary Zn could be identified with a specific nuclear protein(s) isolated by traditional protein-isolation methods (Cousins & Lee-Ambrose, 1992). Growing rats were given purified diets based on the AIN-76A formulation (Blalock *et al.* 1988) containing 5, 30, 180 mg Zn/kg diet. This produced the expected differential in metallothionein expression (both 1 and 2 rodent isoforms) in highly-expressing tissues, i.e. liver, kidney and intestine. Subsequently, nuclei were purified from liver, kidney and spleen using a discontinuous-sucrose-gradient method. Except for the spleen where the low Zn intake increased nuclear Zn, the nuclear Zn content of these tissues (pmol Zn/ $\mu$ g DNA as measured by atomic absorption spectrometry) was not significantly influenced.

To examine the interaction between nuclear Zn and dietary Zn, rats were dosed with a liquefied form of the same diets to which  $^{65}Zn$  was added as an extrinsic label. At 2 h after the dose, nuclei were purified from liver, kidney and spleen using the same discontinuous-gradient method. After correcting for specific activity and DNA content, the incorporation of dietary Zn, as indicated by  $^{65}Zn$ , was expressed as pmol Zn/ $\mu$ g DNA. Liver nuclei had the greatest amount of  $^{65}Zn$  from the diet. In each tissue, the value was proportional to the Zn content of the diet. In the case of the kidney, as much as 6% of Zn in the nuclei appears to have been derived from the diet in the 2 h experimental period. This suggested that there is considerable exchange of nuclear Zn with cellular Zn obtained from the dietary supply.

Using Sephacryl S-200 column chromatography, nuclear proteins extracted by a traditional approach (Cousins & Lee-Ambrose, 1992) yielded two  $^{65}Zn$  peaks. A clearly-defined DNA-binding protein labelled with  $^{65}Zn$  was identified by further purification with heparin sepharose chromatography. Western and Southwestern blotting of these fractions showed evidence of a nuclear protein that bound both  $^{109}Cd$  and  $^{65}Zn$ , and a  $^{32}P$ -labelled multimer of the MRE sequence. Collectively, these findings suggest that the mode of action of dietary Zn in the regulation of MRE genes is via direct interaction with a MTF molecule that, on metal occupancy and entry into the nucleus, initiates transcription.

### Zinc and expression of human metallothionein

For over a decade, we have been interested in the response of human metallothionein as a model system to gain information about Zn status in human subjects. The approach is aimed at developing a 'functional' assessment of Zn status. We reasoned that the direct correlation between dietary Zn intake and metallothionein expression seen in rats (Blalock *et al.* 1988; Cousins & Lee-Ambrose, 1992) should be applicable to man.

Our initial approach was to develop an ELISA for human metallothionein (Grider *et al.* 1989, 1990). The successful use of a radioimmunoassay for rat metallothionein by Bremner and coworkers (Sato *et al.* 1984; Robertson *et al.* 1989) suggested that metallothionein protein levels in plasma and erythrocytes are responsive to dietary Zn intake. Our ELISA was used to define the general kinetics of metallothionein induction in erythrocyte-progenitor cells and the release of metallothionein-enriched reticulocytes into the circulation (Grider *et al.* 1990). Approximately 5 d after adult human subjects start to consume a daily Zn supplement (50 mg/d), the erythrocyte metallothionein concentration increases many fold. The level remains elevated until Zn supplementation has ceased. More recently, an ELISA based on the sandwich approach has yielded similar results, with comparable kinetics and level of induction (Sullivan & Cousins, 1998). Studies with rats show that Zn-induced metallothionein synthesis is observed in the reticulocyte precursor cells of the bone marrow, most probably through a Zn-dependent process involving MRE-MTF interaction (Huber & Cousins, 1993a,b). Metallothionein induction in these cells is also responsive to anaemia and acute blood loss (Robertson *et al.* 1989; Huber & Cousins, 1993b).

These studies with human subjects establish that metallothionein synthesis responds to dietary Zn intake and, as such, may provide a method to measure Zn status under controlled conditions. Furthermore, erythrocyte metallothionein synthesis may serve as a method to assess Zn bioavailability based on a function of Zn, i.e. MRE regulation of a gene.

Since erythrocyte metallothionein is subject to certain physiological stimuli, we also investigated regulated synthesis in a leucocyte population. Monocytes had been identified as the cells with the highest level of expression (Pauwels *et al.* 1994). After verifying the latter, we found that metallothionein mRNA levels by Northern blot analysis and metallothionein protein levels by ELISA were too low for evaluation with the amount of monocytes obtained with a reasonable blood sample (10 ml). To circumvent this problem, we developed a competitive reverse transcriptase-polymerase chain reaction (PCR) method to measure metallothionein mRNA via its conversion to cDNA. The latter is quantified using a cDNA of known concentration that is simultaneously amplified by PCR using the same primers. Details of the method have been reported recently (Sullivan & Cousins, 1997).

In subjects receiving a Zn supplement of 50 mg/d, monocyte metallothionein mRNA levels were elevated after only 1 d of supplementation (Sullivan & Cousins, 1998). As with erythrocyte metallothionein protein, the

mRNA levels in monocytes remained elevated for the duration of supplementation. They returned to the basal levels within 4 d of supplementation. These results reflect the rapid turnover of monocytes. The mRNA levels do not correlate with plasma Zn concentration, suggesting induction occurs while the progenitor cells are in the bone marrow rather than after these cells are released into the peripheral circulation. Again, the response of monocyte metallothionein mRNA may provide a useful approach to measure Zn bioavailability using an established function, and be useful in defining a physiological function for this protein (and Zn) in man.

### Identification of zinc-responsive genes by differential mRNA display

The basic mechanisms responsible for the clinical signs and pathology of Zn deficiency continue to be an enigma. Attempts to relate these to decreased activities of various metallo-enzymes have not delineated the biochemistry involved (Mills, 1989). Our approach to answer this question is to examine which genes are under-expressed or over-expressed in nutritional Zn deficiency. As described in the introduction to the present brief overview and shown in Fig. 1, Zn can influence gene expression either via the MRE-MTF or similar complexes to directly alter transcription rates or by a secondary mediator that is responsive to Zn nutritional status. We have used differential screening and, more recently, differential mRNA display to identify genes influenced in some way by Zn deficiency, since that information may provide a clue as to which genes are affected.

Our initial studies used a differential screening method (Shay & Cousins, 1993). A cDNA library was constructed from intestinal mRNA of Zn-deficient rats (<1 mg Zn/kg). This was screened using <sup>32</sup>P-labelled cDNA probes generated from intestinal mRNA of both Zn-deficient and Zn-adequate rats. This technique can accommodate only one pair of dietary comparisons. A number of cDNA, (proportional in abundance to the parent mRNA) were decreased in abundance in Zn deficiency. These included apolipoprotein-A1, cytochrome-b, ubiquitin, cryptdin, cytochrome-c oxidase (*EC* 1.9.3.1) and fatty acid-binding protein. In contrast, a number of cDNA were increased in Zn deficiency.

While the differential screening technique demonstrated that mRNA differentially expressed by changes in the dietary Zn supply could be identified, we changed emphasis after those initial studies to capitalize on a more-powerful, newly-developed technology, differential mRNA display. This technique was developed to identify differentially-expressed genes in cancer cells (Liang & Pardee, 1992). This approach allows the comparison of three or more dietary treatment groups, i.e. Zn deficient (<1 mg Zn/kg), Zn adequate *ad libitum* (30 mg Zn/kg) and Zn adequate but pair fed to the intake of the Zn-deficient group.

The technique of differential display uses reverse transcription to quantitatively convert mRNA into cDNA. The latter are simultaneously amplified by PCR. Two sets of PCR primers are used. The first is a 3' primer that is an

oligo d(T) primer with two non-T bases at the 3' end. This is called the 'anchored primer' since it helps anchor cDNA synthesis at the end of the poly A tail and 3' untranslated region of the mRNA during reverse transcription. A set of four such anchored primers is needed for each of the four nucleotides. A 5' primer is used along with the 3' primer for cDNA amplification by PCR. Labelling of the cDNA bands is with <sup>35</sup>S-dATP. An array of twenty-four decanucleotide 5' primers (termed arbitrary primers) are used individually for the PCR reactions. Using the twenty-four antibody primers and four anchored primers require ninety-six separate reactions to cover the spectrum of possible cDNA that represent actively-transcribed genes within the genome. As with any method, the technique has limitations; for example, the length of the cDNA product generated. If the cDNA is very short, it may not provide information in the coding region essential for sequence identification. Once each reaction has been completed, the <sup>35</sup>S-labelled cDNA are separated on polyacrylamide gels. Differentially-expressed bands are excised, subcloned to increase their abundance, and sequenced. The sequence information is then analysed by searching GenBank (National Center for Biotechnology Research, Bethesda, MD, USA) or other source, to identify the genes that are differentially regulated.

We initially tested this system with kidney mRNA, since we reasoned that metallothionein gene expression is decreased in Zn deficiency, and that differential expression should be detectable using differential display. A 208 base pair cDNA fragment was generated, having the sequence of rat metallothionein-1, and was found at markedly depressed levels in the Zn-deficient rats, which also exhibited depressed weight gain and serum Zn concentrations.

Intestinal mRNA has been the principal focus in our initial studies. Some mRNA increased while others were found to decrease in the Zn-deficient rats. Occasionally, the mRNA in the pair-fed and *ad libitum* control groups differed. These were not considered further since those differences are likely to be related to differences in food intake. Two differentially-expressed genes are of particular interest, since both relate to the pathophysiology of Zn deficiency; both are over-expressed in Zn deficiency. The first is the gene for cholecystokinin, a peptide hormone that has endocrine and neurocrine effects on pancreatic secretion and satiety respectively (Blanchard & Cousins, 1996). It is, therefore, of particular interest that the compromised pancreatic function and anorexia of Zn deficiency (Mills, 1989) would appear to correlate with up-regulation of the cholecystokinin gene. The second over-expressed gene is for the precursor of the peptide hormone uroguanylin. The uroguanylin hormone is produced in enterochromaffin cells of the intestine, and acts in a paracrine fashion to activate the production of cGMP, which stimulates the cystic fibrosis transmembrane conductance regulator and leads to increased fluid secretion into the intestine (Blanchard & Cousins, 1997). This finding is in agreement with those of intervention studies in which Zn supplementation decreased the incidence of secretory diarrhoea in children (Sazawal *et al.* 1995; Rosado *et al.* 1997).

The findings made thus far, showing that cholecystokinin and uroguanylin gene expression are over-expressed, suggest that both could be used to explain aspects of the Zn deficiency syndrome. Further research using this and related technologies will certainly provide a clearer view of this nutrient deficiency, particularly as it is now being applied to genes expressed in other tissues, such as the thymus.

## Summary

Zn, without question, has important functions related to gene expression. Newer technologies applied to address these functions are providing answers relating to the importance of this micronutrient in human and animal health.

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