Biol Res 39: 125-142, 2006

Gene expression profiling in wild-type and metallothionein mutant fibroblast cell lines

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ABSTRACT

The role of metallothioneins (MT) in copper homeostasis is of great interest, as it appears to be partially responsible for the regulation of intracellular copper levels during adaptation to extracellular excess of the metal. To further investigate a possible role of MTs in copper metabolism, a genomics approach was utilized to evaluate the role of MT on gene expression. Microarray analysis was used to examine the effects of copper overload in fibroblast cells from normal and MT I and II double knock-out mice (MT-/-). As a first step, we compared genes that were significantly upregulated in wild-type and MT-/- cells exposed to copper. Even though wild-type and mutant cells are undistinguishable in terms of their morphological features and rates of growth, our results show that MT-/- cells do not respond with induction of typical markers of cellular stress under copper excess conditions, as observed in the wild-type cell line, suggesting that the transcription initiation rate or the mRNA stability of stress genes is affected when there is an alteration in the copper store capacity. The functional classification of other up-regulated genes in both cell lines indicates that a large proportion (>80%) belong to two major categories: 1) metabolism; and 2) cellular physiological processes, suggesting that at the transcriptional level copper overload induces the expression of genes associated with diverse molecular functions. These results open the possibility to understand how copper homeostasis is being coordinated with other metabolic pathways.

Key terms: copper homeostasis, metallothionein, microarray

INTRODUCTION

Copper is an essential nutrient that is a structural part in some proteins and part of the electron transfer system in many redox-enzymes involved in processes such as respiration, iron metabolism, and neurotransmitter biosynthesis (Uauy et al., 1999). Although essential metals are normally present in trace amounts in the cell, their levels can increase following environmental or nutritional changes. Metal overload can be toxic to the cell, causing a range of effects and leading to cell death when concentrations are extremely high (Pulido and Parrish, 2003; Oteíza et al., 2004; Leonard et al., 2004). To avoid metal-induced toxicity, most organisms have developed several cellular mechanisms of protection. Three general mechanisms, which typically work in combination for effective detoxification, include: reduction of metal uptake; enhanced metal export; and metal sequestration mechanisms (Dameron and Harrison, 1998). The third mechanism, the intracellular chelation or sequestration of metals into less reactive complexes or organelles to limit their toxicity, is a commonly used mechanism. In mammalian systems, excess metals, in particular

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Received: February 15, 2005. Accepted June, 25 2005.

copper, are partially detoxified by sequestration in the metal-binding metallothioneins (Dameron and Harrison, 1998; Bremner and Beattie, 1990; Nordberg, 1989).

Metallothioneins (MTs) are a class of low molecular weight, intracellular and cysteine-rich proteins that have high affinity for metal ions (Cousins, 1983; Park et al., 2001; Coyle et al., 2002). MT gene sequences are highly conserved in a wide range of species from bacteria to humans, which is a suggestive feature of a protein with a high biological importance. MTs have unique structural characteristics for their potent metal-binding and redox capabilities. Although the members of this family were discovered nearly 40 years ago, a primary role has not been identified, and new functions continue to be discovered (Palmiter, 1998). Currently, MTs are known to be involved in metal ion homeostasis and detoxification, protection against oxidative damage, cell proliferation and apoptosis, chemoresistance, and radiotherapy resistance (Palmiter, 1998; Coyle et al., 2002). MT expression has been implicated as a transient response to any form of stress or injury and may provide cytoprotective action. Four major MT isoforms – MT I, MT II, MT III, and MT IV – have been identified in mammals (Coyle et al., 2002; Theocharis et al., 2003). MT I and MT II are the two major forms in mammals; they are expressed in most tissues and stages of development. The analysis of mice with altered gene expression of MT I and II has enhanced our understanding of the multifaceted role of MT. MT I/II double knockout (MT-/-) mice demonstrated that MT expression is not essential for the normal development, growth, or reproductive capacity of these mice (Klaassen and Liu, 1998). These mutant mice and the cell lines derived from them have been used extensively in recent years to investigate the role of MT. Most results show that animals or cells lacking MT I/II are more sensitive to a wide range of stressors, such as oxidative stress; excess metals, such as cadmium, lead, copper and zinc; and infectious and inflammatory agents (Zheng et al., 1996; Kelly et al., 1996; Kelly and Palmiter, 1996; Liu et al., 1999; Rojas and Klaassen, 1999; Liu et al., 2000; Qu et al., 2002).

The role of MT in copper homeostasis is of great interest, as it appears to be responsible for the regulation of intracellular copper levels during adaptation to extracellular copper excess. It has been shown that MT protects cells against elevated levels of extracellular copper (Freedman and Peisach, 1989; Kawai et al., 2000). As copper toxicity involves its ability to catalyze the generation of free radicals and/or to directly interact with essential biomolecules, copper sequestering by MT is of critical importance for cell protection. Besides serving to detoxify excess copper, MT may play a role in normal copper metabolism, although currently this role is unknown.

In a recent study, we demonstrated notable changes in copper metabolism in a MT I/II mutant (MT-/-) fibroblast cell line (Tapia et al., 2004). We showed that MT-/- cells were more sensitive to increasing amounts of copper in the media than wild-type cells exposed to the same concentrations. Also, by measuring intracellular copper levels and by conducting uptake studies, we demonstrated that although both mutant and wild type cell lines accumulate copper upon treatment with copper, the MT-/- cells took up considerably less copper than the wild-type cells. Interestingly, in this condition, we observed that mutant cells died with lower intracellular copper content (2-fold lower), supporting a protective role for MT in the response to Cu excess. These results permitted us to deduce that copper toxicity does not rely on the increased amount of intracellular metal, but rather on the cellular ability to manage the metal, therefore, in the molecular interactions that copper establishes inside the cell. In this context, we showed that in the absence of MTs, the capacity of copper to induce gene expression of MT, SOD1 and its chaperone, Ccs, is lost. This observation could partially explain the increased vulnerability of MT null cells to lower intracellular copper concentrations and suggest that MT may be a modulator of gene expression (Tapia et al., 2004).

In the present study, we show that wild-type and mutant cells are undistinguishable in terms of their morphological features and rates of growth and define a minimal copper dose that provided a significant increment in the copper content of both cell lines. Using these conditions, we applied a genomic approach to further investigate a possible role of MTs as part of a pathway that induces gene expression in response to copper. As a first step in exploring the effects that loss of this important copper sequestering protein had on gene expression, we carried out microarray analysis to identify genes significantly up-regulated in wild-type and MT-/- cells exposed to an excess of copper and to determine which genes failed to be upregulated in MT mutant cells as compared to wild-type cells. The results of these analyses are presented here.

MATERIALS AND METHODS

Cell lines and treatments

The cell lines used in the present study correspond to wild type and MT I/II mutant (MT-/-) fibroblasts obtained by trypsinization of mouse embryos from day 11 of gestation and immortalized with SV40 virus (Kelly and Palmiter, 1996). The mutation in the MT-/- cell line was achieved by introducing translation stop sequences in the MT I and II exons, thus the mutant cell line expresses a modified MT mRNA and fails to express the protein (Tapia et al., 2004). Culture and treatment conditions have been described (Tapia et al., 2004). Briefly, fibroblasts were incubated at 37 ºC in a 5% CO2 atmosphere and grown in plastic cell culture flasks (Nalge Nunc Int. Corp. IL, USA) containing Dulbecco's modification of Eagle's medium (DMEM) and 10% fetal bovine serum (SFB). The concentrations of elements in this basal culture medium were Cu 0.4, Fe 2.7 and Zn 3.8 μM (González et al., 1999). For all treatments, copper was supplemented in the cultured medium as Cu-His complex (1: 10 molar ratio) at the concentrations and times specified in the figure 2 legend. After the treatment, cells were processed for Cu, Zn and Fe quantification. To evaluate cell viability under copper treatment, cells were grown in 24-well cell culture plates (Nunc) and relative survival of the treated cells was evaluated using 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT, Sigma) reduction assays as described (Denizot and Lang, 1986). The viability percentage was determined by comparing the measurements of the average absorbance from a given treatment group with that obtained from a reference sample of the same cell line treated for identical time with a control medium (100% of viability). To determine the population doubling time, the cell number was counted under a microscope daily for at least 5 days using a hemocytometer and the trypan blue (0.4%) exclusion method.

Immunofluorescence staining

Cells were washed three times with phosphate buffered saline (PBS), fixed for 10 min in 3.7% formaldehyde in PBS and permeabilized for 5 min with 0.2% triton X-100 in 3.7% formaldehyde. The fixed cells were re-hydrated with Tris buffered saline (TBS) and incubated for 1 h in blocking solution (3% BSA in TBS). To evidence the organization of microfilaments, cells were incubated with anti-actin monoclonal antibody (SIGMA) diluted 1: 500 in 3% BSA-TBS, and with the secondary antibody, FITC-conjugated rabbit-anti-mouse IgG (Rockland, Gilbertsville, PA), at a 1: 250 dilution in 3% BSA-TBS. Cells were incubated with primary or secondary antibodies for 1 h at 37°C. Finally, cells were rinsed in TBS, mounted in DABCO/mowiol, and examined with a epifluorescence microscope (40× objective, Nikon, Labophot-2, Tokyo, Japan). As controls, experiments were performed in which the first or second antibodies were omitted.

Cu, Fe and Zn quantification

For total metal content quantification, cells were processed as previously described

(González et al., 1999; Tapia et al., 2004). Briefly, cells (2-4 x 105) at 80% of confluence were disrupted in concentrated suprapure nitric acid (Merck) in a vortex and diluted with distilled deionized water. Samples were digested at 60ºC overnight. The Cu determination was made by means of a graphite furnace atomic adsorption spectrophotometer (AAS, Perkin Elmer, SIMMA 6100). Calibration was against a standard curve made from dilutions of a Cu, Fe or Zn standard (J T Baker) and the sample values were normalized to the total protein content.

RNA isolation and cDNA labeling

Total RNA was extracted from wild-type and MT-/- mutant cell lines in the same passage number maintained under basal cell culture conditions or exposed to 100 μM Cu-His for 48 h using RNAWIZ reagent (Ambion). The indirect, or amino-allyl labeling protocol was adapted from the microarrays.org website (http: // microarrays.org/protocols.html). 25 μg total RNA and 5 μg of oligo dT were brought to a total volume of 15.5 μl and denatured for 10 min at 70ºC, then chilled on ice. An equal volume of reverse transcription reaction mix (2X first strand buffer, 20 mM DTT, 95 U SuperScript II, 1 mM dATP, 1 mM dGTP, 1 mM dCTP, 0.6 mM dTTP, and 0.4 mM amino-allyl-dUTP) was added, and the reaction was incubated at 42ºC overnight. The reaction was denatured by bringing it to 0.2 N NaOH, 0.1 M EDTA and incubating at 65ºC for 15 min, and neutralized by the addition of Tris-Cl pH 7.4 to a final concentration of 0.3 M. The buffer was removed, and the cDNA was concentrated by filtering through a Microcon-30. After drying in a speed vac, the cDNA was resuspended in 4.5 μl sterile water. The cDNA then was coupled to either monofunctional Cy3 or Cy5 dye (Amersham Pharmacia) in 0.1 M sodium bicarbonate buffer, pH 9.0, at room temperature, in the dark, for 1 h. The two dye reactions were purified separately with a Qia-Quick PCR purification kit (Qiagen). cDNA quantity and dye incorporation of each probe was assessed with a scanning spectrophotometer. The two dye probes were then combined and concentrated in a speed vac to a volume of 15-20 μl. SSC and SDS were added to a final concentration of 2.6X and 0.2% respectively. The probes were denatured for 2 min at 95ºC, allowed to cool, then placed on a post-processed microarray and incubated overnight at 60ºC. After hybridization, the microarray slides were washed at room temperature for 5 min in 1XSSC, and 0.05%SDS, then for 2 min in 0.2XSSC, and finally for 2 min in 0.1XSSC, and then were spin dried. The microarrays were fabricated at the College of Natural Resources Genomics Facility (http: //genomics.cnr.berkeley.edu). These arrays contained 11,000 mouse cDNAs, randomly selected from the Brain Molecular Anatomy Project, BMAP (http: // trans.nih.gov/bmap/) library (Research Genetics). Genes contained in this library are derived from 10 different adult mouse brain regions as well as spinal cord and retina.

Data analysis

Microarray slides were scanned on an ArrayWorx (Applied Precision) scanner. The intensity of hybridization values was determined using Genepix Pro 3.0 (Axon) image analysis software for each probe in both channels (Cy5- 695 nm, Cy3- 595 nm). All intensity values are background-subtracted, normalized by median of channel intensity (to account for dye bias), and log (base 2)- transformed. A recently developed method to identify differentially expressed genes between two mRNA samples, based on identification of outliers as candidate differentially expressed genes (Loguinov, et al., 2004), was used. The majority of data points after appropriate normalization lie in the vicinity of the line of equivalence in the scatter plot of log2(Cy5) vs. log2(Cy3) intensity values. Other data points, outliers, lie outside the vicinity of the line of equivalence and are considered to be data points of greatest interest, since they correspond to genes having noticeably different hybridization intensities. Robust scatter plot smoothers to quantify and take into account the distortion

of the data set by heteroscedasticity (if any) were applied. Outliers from this analysis are considered to represent candidate differentially expressed genes. A confidence (p value) for each outlier is assigned by calculating simultaneous prediction confidence intervals (Loguinov et al., 2004). Sequence homology searches were done against mouse/rodent sequence databases, using the WU-BLAST 2.0 program at MouseBLAST (http: // mouseblast.informatics.jax.org/). Differentially expressed genes were grouped according to their biological process using the Gene Ontology Classifications found at the Mouse Genome Informatics Website (http: // www.informatics.jax.org/) and at the Gene Ontology Consortium Website (http: // www.geneontology.org/).

RESULTS

The absence of MT does not modify the cellular morphology or the rate of growth of mutant fibroblasts

In order to assess whether the absence of MT affects the growth of mutant cells, the morphology and the rate of growth of both cell lines were compared. When wild-type and MT-/- cells were maintained in basal culture medium (0.4 μM Cu), they showed a monolayer growth pattern with similar sizes and morphological features (Fig. 1, upper panels), which is clearly observed by the organization of the microfilament network (Fig. 1, lower panels). After copper treatment, we did not detect changes in the morphological features of both cell lines (data not shown). In the absence of copper

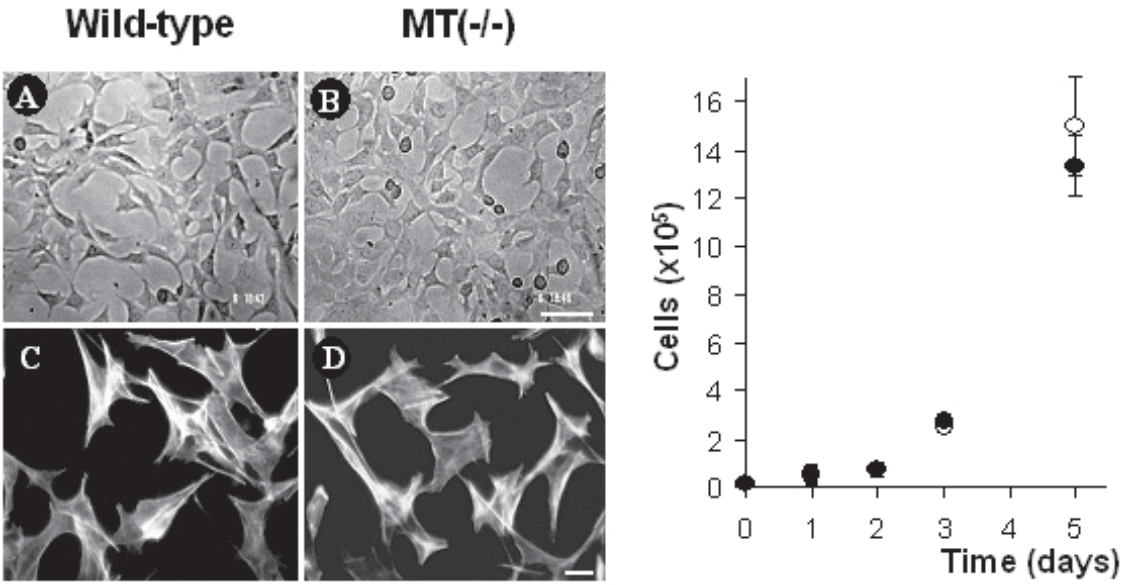

Figure 1. Comparison of morphological features and rate of growth between wild-type and MT-/- cells.

Microphotographs show the morphology of sub-confluent cultures of wild-type (A) and MT-/- (B) cells. Cells were analyzed using phase-contrast microscopy. Bar = 10 μm. Representative images of actin distribution patterns are shown for wild-type (C) and MT-/- (D). Microfilaments were stained as described in Materials and Methods. To determine the rate of growth, cells were cultured under basal conditions, and the total number of cells was quantified using trypan blue every day for 5 days. The experiment was performed in triplicate, and results are expressed as mean ± SD.

supplement, the growth curve of wild-type and MT-/- cells were nearly identical (Fig. 1). Once seeded, cells displayed a phase of slow growth (0-2 days) followed by of a phase of fast growth (3-5 days) until reaching confluence (>90% at day 5) at this time; their growth was inhibited by cellular contact. The deviation observed at day 5 was not statistically significant. From these curves, we calculated a population doubling time of 22 h. These results indicate that morphological features and proliferation rate of cells was unaffected by the absence of MT.

Differential Cu content and viability in wild type and MT-/- cells exposed to Cu-His

Wild-type and MT-/- cell lines growing in standard culture conditions showed no significant differences in the Cu, Fe and Zn content. When fibroblasts were exposed for 48 h with rising graded copper concentrations (up to 250 μM Cu-His), intracellular Cu content increased in both cell lines, but in wild-type fibroblasts, rose significantly more than in MT-/- fibroblasts (Fig. 2). Regarding the other trace metals, results show that Zn content remains unaffected and Fe content decreased about 2-fold in both cell lines. Thus, Cu exposure primarily affected intracellular Cu content, but also affected the Fe content to a lesser degree, providing further evidence for their metabolic interaction. Since we have shown that viability of wild-type and MT-/- cells exposed to an excess of Cu decreased in a time-dependent manner (Tapia et al., 2004), we selected a time of 48 hrs of copper-treatment for our gene expression analysis. The chosen extracellular copper concentration was 100 μM, a minimal dose of copper that generated a significant difference on intracellular Cu content between wild-type and MT-/- cells (Fig. 2). Under these conditions, cell viability of wild-type and MT-/- lines was 95.2% and 77.6%, respectively.

Microarray experiment design

Gene expression in copper-treated wild type and MT-/- cells was compared to their untreated counterparts. RNA from four different cell conditions was used: 1) untreated wild-type cells; 2) 100 μM copper-treated wild-type cells; 3) untreated MT mutant cells; and 4) 100 μM copper-treated MT mutant cells. All copper-treated cells were treated for 48 hours prior to harvesting; the untreated cells were collected simultaneously. Wild-type was compared to copper-treated wild-type cells in four experiments; and MT-/- was compared to copper-treated MT-/- cells in four experiments. Although a total of eight microarray experiments were carried out, six were successfully completed and analyzed. Even though the repeated experiments were technical and not biological replicates, dye swaps were incorporated into some of them. The RNA used for each cell line was isolated on the same day; however, the microarray experiments (reverse transcription, labeling, and hybridizations) were done on different days.

Detection of differentially expressed genes

We identified differentially expressed genes from each hybridization using the outlier identification method described by Loguinov et al. (2004). We were interested in those genes, which were significantly upregulated in copper-treated cells (WT, 100 μM Cu and MT-/-, 100 μM Cu) as compared to untreated cells (WT and MT-/-). As shown in figure 3, the majority of the data points lie in the vicinity of a robust linear regression line of the scatter plot of log2(Cy5) v/s log2(Cy3) intensity values. These data points correspond to genes with similar hybridization intensity values in the two samples, while other data points lie outside the scatter plots and correspond to genes having noticeably different values of intensity. We determined Log2(Cy5/Cy3)/ s(I) to estimate statistical significance (p values) for every gene to be a candidate for differential expression. s(I) is a robust scale estimator dependent on intensity and is used to build simultaneous prediction intervals for graphical representation of genes outside the intervals that are most likely outliers. Robust scatter plot smoothers are used to account

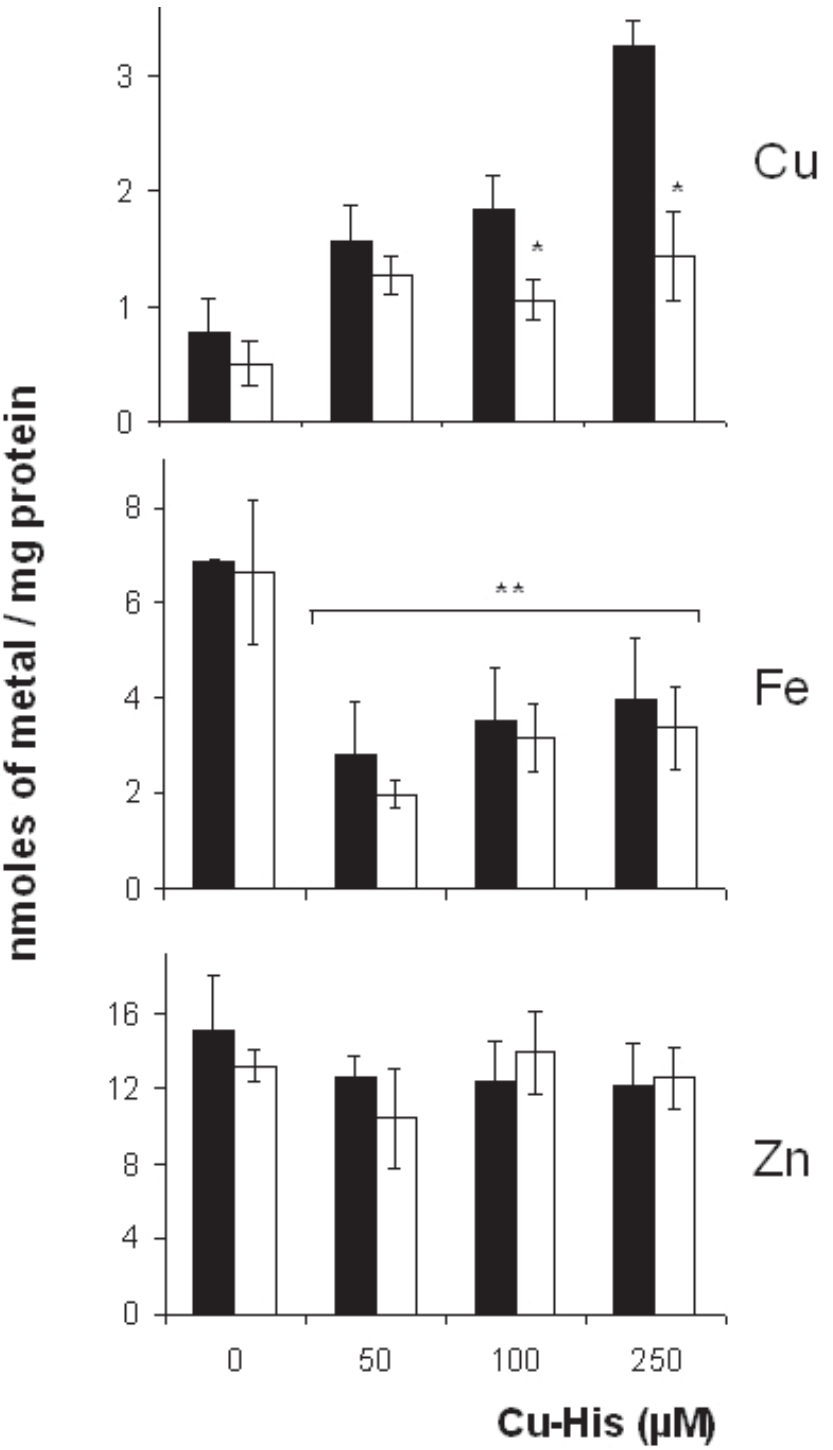

Figure 2. Cu, Fe and Zn content in wild-type and mutant MT-/- cells exposed to Cu-His. Total metal content of wild-type cells and MT mutants determined by AAS in response to graded concentrations of Cu-His at 48 h. Filled bars correspond to wild-type cells and open bars to MT-/- cells. Each bar represents the mean value of triplicate experiments and corresponding standard deviation. ANOVA analysis for both cell types demonstrated a significant effect induced by Cu exposure. (*) denotes significant difference as compared to wild-type cells, $p < 0.05$; (**) denotes level of exposure in which the concentration effect was significant, $p < 0.05$.

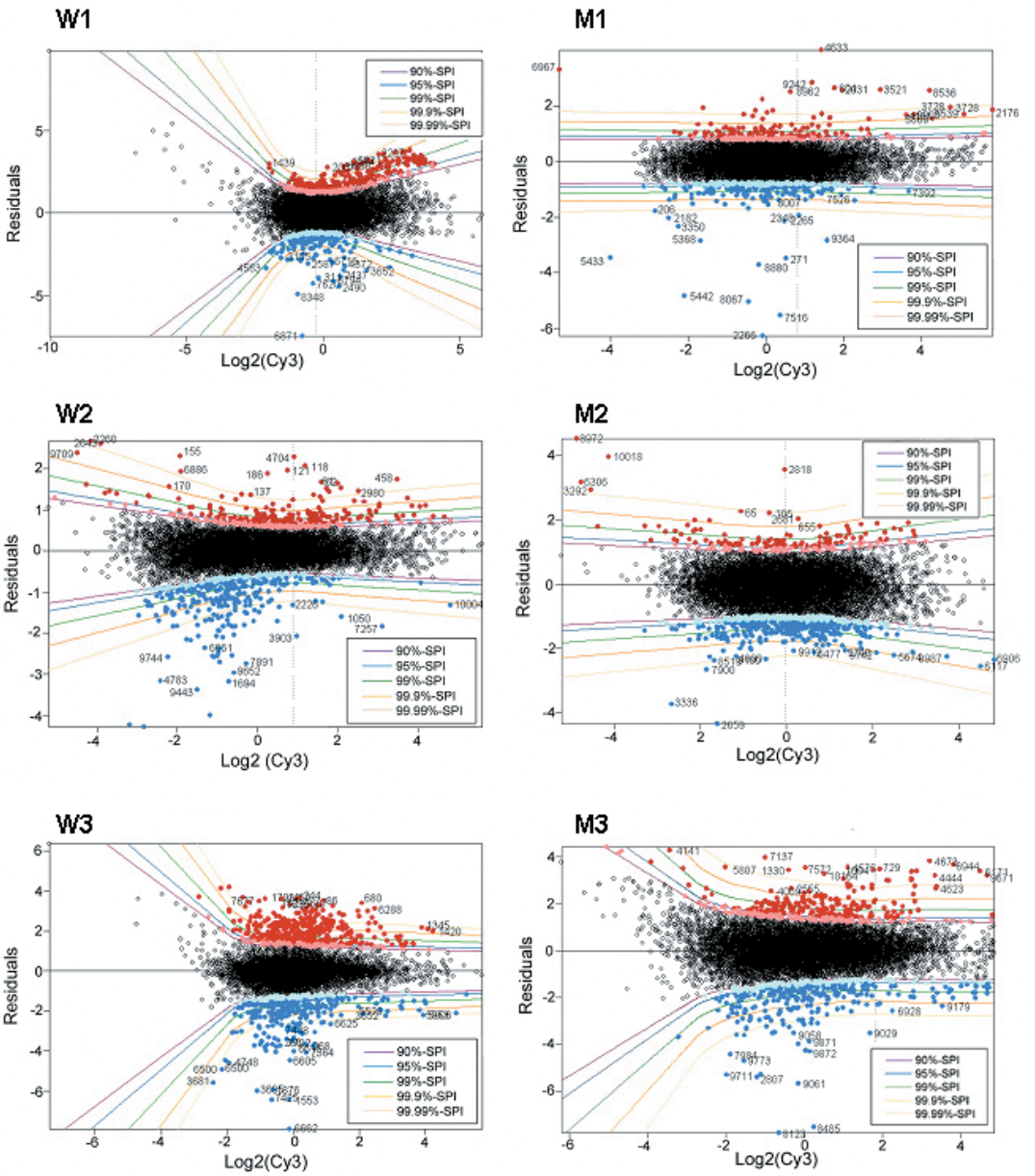

Figure 3. Detection of differentially expressed genes.
Simultaneous prediction intervals (SPI) for the three WT,100 μM Cu v/s WT and the three MT-/-, 100 μM Cu v/s. MT-/- data sets (W1, W2 and W3 and M1, M2, and M3, respectively) are shown. For each experiment, the robust and resistant linear regression line (black) was calculated using the S-plus MM-estimator. Then the data was normalized by linear regression making slope = 1, intercept = 0 and subtracting the linear trend to deal with residuals that are log2(Cy5/Cy3). SPIs are corrected with the S-plus scatter plot smoother *supsmu* applied to absolute residuals, |log2(Cy5/Cy3)|, to reveal the dependence of residual variance on intensity values. The *supsmu*-based SPIs assume residual heteroscedasticity. Pink (cyan) points lie in the interval between the upper (lower) 90% and 95% SPIs. Red (blue) points lie above the upper (lower) 95% SPI. Black points lie below the upper and lower 90% SPI. The 90%, 95%, 99%, 99.9% and 99.99% SPIs are shown. The vertical dotted line marks the location of the minima of the empirical hyperbolas. Therefore, red/pink, blue/cyan and black points represent candidate upregulated, candidate downregulated and unchanged genes respectively.

for heteroscedasticity (variation in residual variance with intensity) in each data set. Red outliers on the top of the graph represent candidate up-regulated genes while the outlying spots of blue color, on the bottom, represent candidate down regulated genes. We considered data outliers with p-value 0.05 as candidate differentially expressed genes. We compared the candidate genes of each experiment and selected genes (i.e., outliers with a p-value < 0.05) identified in two or more repeat hybridizations as likely differentially expressed genes. In the WT, 100 μM Cu v/s WT experiments, there were 104 up-regulated genes in at least two of the three experiments, 31 of them were ESTs that had not yet been annotated. In the MT-/-, 100 μM Cu v/s MT-/- experiments there were 133 up-regulated genes, 37 of them corresponded to un-annotated genes. For each of the annotated genes, tables 1, 2 and 3 display the name, symbol and their GeneBank accession number. Genes upregulated in: a) both WT and MT -/- cells; b) only WT cells; and c) only MT -/- cells are listed in tables 1, 2 and 3, respectively.

Functional classification of differentially expressed genes

Functional annotation of the differentially expressed genes was performed aiming to discover features characteristic to the sets of genes up-regulated in 100 μM copper-treated WT and MT-/- cells. We classified genes from tables 1 and 2 according to their biological process using the GO database. GO provides a controlled vocabulary organized in a hierarchical fashion that can be applied to describe gene products from eukaryotic organisms regarding molecular function, biological process, and cellular component. GO terms for the biological process ontology were found for approximately 60% of the genes differentially expressed in 100 μM copper-treated WT and MT-/- cells. Pie graphs illustrating the biological process of genes up-regulated in 100 μM copper-treated WT and MT-/- cells are shown in figure 4A and B. Approximately 50% of the genes with known biological function are involved in cellular physiological processes. This group includes genes that play roles in signal transduction, transport, cytoskeleton organization, and regulation of transcription and translation. The next larger category (≈30%) corresponds to genes involved in metabolism. Within this category, 36% of the genes up-regulated in copper treated WT cells are involved in protein modification, whereas in copper-treated MT-/- cells the greatest percentage (36%) correspond to genes that play roles in protein biosynthesis. 15% of the genes up-regulated in copper-treated WT cells are involved in response to a stimulus (highlighted in grey, Tables 1 and 2), 75% of them correspond to genes with functions in stress response to temperature. In copper-treated MT-/- cells, there is a smaller portion of genes (9%) in this category (highlighted in grey, Tables 1 and 3), and only 25% of them correspond to those involved in response to temperature.

DISCUSSION

The role of MT has been extensively studied, however it remains elusive. Part of the reason is that MT has been implicated in a variety of physiological processes, such as metal storage, metal detoxification, oxidative scavenging, cell proliferation, and inflammatory response (Palmiter, 1998; Coyle et al., 2002).

In copper homeostasis, MT plays a role in the regulation of intracellular copper levels during adaptation to excess copper. It has been well demonstrated that MT protects cells against high levels of copper by sequestering excess amounts of the metal (Palmiter et al., 1992; Quaife et al., 1994; Haq et al., 2003). In addition to serving to detoxify excess copper, MT may play a role in normal copper metabolism. In a previous work (Tapia et al., 2004), we took advantage of the simultaneous generation of two fibroblast cell lines from littermate mouse fetuses (Kelly and Palmiter, 1996): one expresses MT, while the other expresses modified mRNA isoforms of MT I and II that are not able to be translated into proteins. In these cell lines, we evaluated the role of MT in Cu metabolism, monitoring a

wide range of parameters such as viability, Cu content, Cu uptake and efflux, subcellular localization, and the expression of MT and some other Cu metabolism-related genes. Our results indicated that, within the physiological range, the role of MT is to provide a safe storage compartment for Cu. As a consequence of this function, at supra-physiological levels of Cu exposure MT has a role in detoxification. Additionally, MT is a modulator of gene expression, since in its absence, exposure to high Cu concentrations failed to induce the expression of MT and other Cu metabolism-related genes such as those encoding SOD1 and Ccs, suggesting a second mechanism by which MT may contribute to cellular resistance to Cu exposure.

In the present work, we further analyzed the effects that loss of MT had on gene expression using a genomic approach. In this study, we utilized cDNA microarray technology to evaluate the effects of copper excess on global gene expression in wild-type and MT-/- cells. In the microarray experiments, we compared transcripts from a wild-type to copper-treated wild-type cells, and from a MT-/- to copper-treated MT-/- cells. We were interested in determining genes that were upregulated in the two cell lines as a result of handling excess of copper and identifying which genes failed to be upregulated in MT null cells as compared to wild-type cells. We hypothesized that, by comparing the effects of copper overload in a normal cell to copper overload in a Mt deficient cell, insight into MT's normal role in copper homeostasis could be gained. We were interested in the differences/similarities in the handling of excess copper between the two cell types to understand what pathways are affected only in the MT deficient cells, as this might indicate areas where MT is required. Any similarities in affected pathways would likely indicate areas where MT is not important or uninvolved.

It is generally accepted that copper in excess, due to its redox cycling capacity, can give rise to the generation of reactive oxygen species capable of producing oxidative stress (Halliwell and Gutteridge, 1984) and that MT protects the cell from this oxidative stress by sequestering excess copper (Dameron and Harrison, 1998; Kaway et al., 2000). Thus, we expected that stress would be high in MT-/- cells exposed to excess levels of copper. When we analyzed the genes up-regulated in 100 μM copper-treated wild-type cells, we found that seven genes encoding heat shock proteins were induced. These heat shock proteins – Hspb1, Hspca, Hsp105, Hspa1a, Hspa8, Dnajb6, and Dnajc10 – are widely conserved and over-expressed at high temperatures or other stress conditions (Lindquist and Craig, 1988). Moreover, catalase (cat) and superoxide dismutase 3 (Sod3) were also upregulated in the 100 μM copper-treated cells, as compared to untreated wild-type cells. Induction of these genes generally indicates that the cell is undergoing stress (Whitley et al., 1999; Nollen and Morimoto, 2002; Bauer and Bauer, 2002; Otterbein et al., 2003). Surprisingly, only three of the stress-related genes (Hspa1a, Dnaja1, and Hspd1) were induced in 100 μM copper-treated MT-/- cells, indicating that the MT-/- cells are not responding with induction of typical markers of cellular stress under copper excess conditions, as compared to the wild-type cell line. In a previous work, using the same experimental approach, we utilized fibroblast cell lines from two mutant mice, C57BL/6-Atp7a (Mobr) and C57BL/6-Atp7a (Modap), to evaluate the effects of chronic copper overload on cellular gene expression (Armendáriz et al., 2004). These cells showed a severe loss-of-function in the ATP7a gene with a cellular defect in copper efflux that leads to high levels of intracellular copper accumulation in standard culture media. Similar results to those obtained in MT-/- cells exposed to copper were observed for both ATP7a mutant cell lines, showing no differential expression of oxidative stress genes, besides the up-regulation of MT. When taken together, these results suggest that the transcriptional regulation of stress genes is affected when the machinery that controls copper homeostasis is altered. It is possible that: 1) the cells are unable to sense stress; or 2) the untreated MT-/- cells are already stressed, thus explaining why there is no difference as compared to copper loaded MT-/- cells.

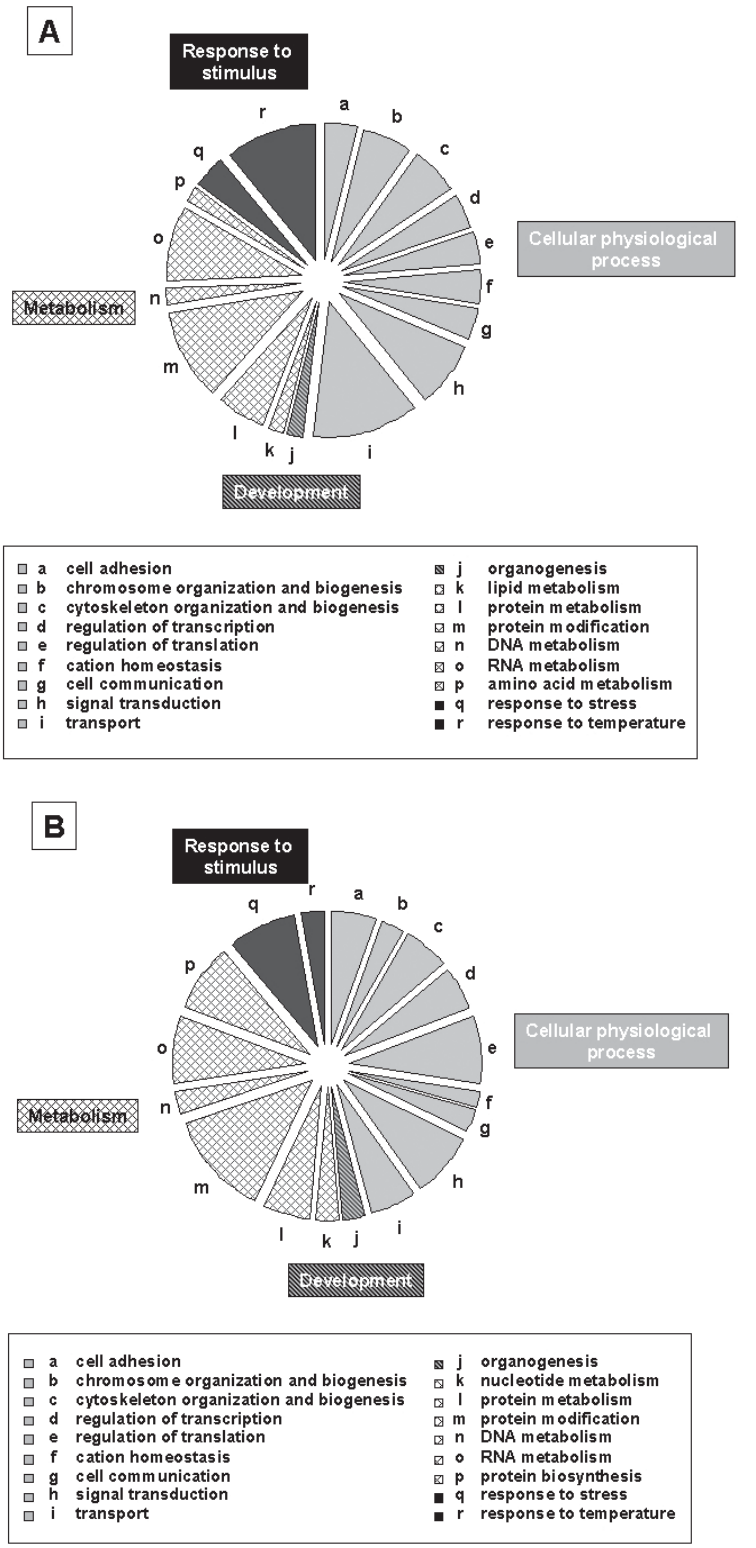

Figure 4. Functional classifications of up-regulated genes.
Up-regulated genes in 100 μM copper-treated WT (A) and MT-/- cells (B) with known functions were grouped according to their biological process in three major categories: Cellular physiological process, Metabolism, and Response to stimulus. Subcategories are labeled a to r.

TABLE 1

Genes commonly up-regulated in 100 μM copper-treated WT and MT-/- cells (p-value < 0.05)

GenBank	Gene name	Symbol
AI854152	adenomatosis polyposis coli	Apc
AI837758	capping protein, alpha 2	Capza2
AI839184	DNA segment, Chr 3, MJeffers 1	D3Jfr1
AI839363	eukaryotic translation initiation factor 3, subunit 6	Eif3s6
AI843263	eukaryotic translation initiation factor 5	Eif5
AI841289	heat shock protein 1A	Hspa1a
AI844131	heterogeneous nuclear ribonucleoprotein A2/B1	Hnrpa2b1
AI850620	matrin 3	Matr3
AI480893	metallothionein 2	Mt2
AI846930	progesterone receptor membrane component 1	Pgrmc1
AI843656	protein kinase C, delta	Prkcd
AI838689	protein tyrosine phosphatase 4a2	Ptp4a2
AI837314	RAB7, member RAS oncogene family	RAB7
AI845039	RIKEN cDNA 1110038O08 gene	1110038O08Rik
AI839993	scavenger receptor class B, member 2	Scarb2
AI840197	secreted acidic cysteine rich glycoprotein	Sparc
AI841699	ubiquitin A-52 residue ribosomal protein fusion product 1	Uba52
AI836012	t-complex-associated-testis-expressed 1-like	Tcte1l
AI838607	thrombospondin 1	Thbs1
AI847817	tight junction protein 1	Tjp1
AI836699	zinc finger, A20 domain containing 2	Za20d2

TABLE 2

Genes up-regulated in 100 μM copper-treated WT cells (p-value < 0.05)

GenBank	Gene name	Symbol
AI840802	5'-3' exoribonuclease 2	Xrn2
AI839327	activating transcription factor 7 interacting protein	Atf7ip
AI854581	advanced glycosylation end product-specific receptor	Ager
AI838490	amyloid beta (A4) precursor-like protein 1	Aplp1
AI854771	angiomotin	Amot
AI849453	arginyl-tRNA synthetase	Rars
AI835672	ATPase type 13A2 [Mus musculus]	Atp13a2
AI844698	ATP-binding cassette, sub-family A, member 2 (Abca2)	Abca2
AI527414	catalase	Cat
AI835756	dendritic cell protein GA17	Ga17
AI836002	DNA segment, Chr 13, Wayne State University 64, expressed	D13Wsu64e
AI842025	DNA segment, Chr 5, Brigham & Women's Genetics 0860 expressed	D5Bwg0860e
AI845673	DnaJ (Hsp40) homolog, subfamily B, member 6	Dnajb6
AI838060	DnaJ (Hsp40) homolog, subfamily C, member 10	Dnajc10
AI837696	erythrocyte protein band 4.1-like 2	Epb4.1l2
AI196520	ferritin heavy chain	Fth
AI852695	Growth hormone inducible transmembrane protein	Ghitm
W30103	heat shock protein 1	Hspb1
AI836122	heat shock protein 1, alpha	Hspca
AI838486	heat shock protein 105	Hsp105
AI841358	heat shock protein 8	Hspa8
AA265447	hemochromatosis	Hfe
AI851595	heterogeneous nuclear ribonucleoprotein A3	Hnrpa3
AI836554	high density lipoprotein-binding protein	Hdlbp
AI851942	kelch repeat and BTB (POZ) domain containing 2	Kbtbd2
AI847525	kinesin family member 5B	Kif5b
AI840212	leukocyte receptor cluster, member 5	Leng5
AI839417	moesin	Msn
AI842649	myosin, light polypeptide 9, regulatory	Myl9
AI847531	platelet-activating factor acetylhydrolase, isoform 1b, beta1 subunit	Pafah1b1
AI847890	proteolipid protein	Plp
AI847455	RAB10, member RAS oncogene family	Rab10
AI850275	Rap2 interacting protein	Rap2ip
AI850616	RIKEN cDNA 1700064K09 gene	1700064K09Rik
AI851276	RIKEN cDNA 2210401K01 gene	2210401K01Rik
AI849320	RIKEN cDNA 2310037P21 gene	2310037P21Rik
AI845633	RIKEN cDNA 6130401J04 gene	6130401J04Rik
AI836603	RIKEN cDNA A430005L14 gene	A430005L14Rik
AI851553	RIKEN cDNA E430034L04 gene	E430034L04Rik
AI839936	ring finger protein 13	Rnf13
AI854671	SET translocation	Set
AI843695	solute carrier family 37 (glycerol-3-phosphate transporter), member 3	Slc37a3
AI317380	superoxide dismutase 3, extracellular	Sod3
AI834833	transducin (beta)-like 1X-linked receptor 1	Tbl1xr1
AI853375	transformed mouse 3T3 cell double minute 2	Mdm2
AI848382	ubiquitin specific protease 1	Usp1
AI839048	vesicular inhibitory amino acid transporter	Viaat
AI852047	zinc finger, CCHC domain containing 6	Zcchc6

TABLE 3

Genes up-regulated in 100 μM copper-treated MT-/- cells (p-value < 0.05)

GenBank	Gene name	Symbol
AI845042	annexin A5	Anxa5
AI837594	ATP synthase, H+ transporting, mitochondrial F0 complex,b, isoform 1	Atp5f1
AI848681	ATPase, H+ transporting, lysosomal accessory protein 2	Atp6ap2
AI836191	ATP-binding cassette, sub-family C, member 5	Abcc5
AI848245	beta-2 microglobulin	B2m
AI854022	carnitine deficiency-associated gene expressed in ventricle	Cdv3
AI847251	casein kinase 1, alpha 1	Csnk1a1
AI845078	CDC42 small effector 1	Cdc42se1
AI844682	cell division cycle 42 homolog (S. cerevisiae)	Cdc42
AI841451	cut-like 1 (Drosophila)	Cutl1
AI850430	death-associated kinase 3	Dapk3
AI841974	DNA segment, Chr 11, ERATO Doi 99, expressed	D11Ertd99e
AI837235	DnaJ (Hsp40) homolog, subfamily A, member 1	Dnaja1
AI849475	enhancer of polycomb homolog 1 (Drosophila)	Epc1
AI843451	eukaryotic translation elongation factor 1 alpha 1	Eef1a1
AI851493	eukaryotic translation initiation factor 2, subunit 2 (beta)	Eif2s2
AI836099	eukaryotic translation initiation factor 4, gamma 2	Eif4g2
AI841804	expressed in non-metastatic cells 1	Nme1
AI847926	golgi apparatus protein 1	Glg1
AI838039	heat shock protein 1 (chaperonin)	Hspd1
AI841981	hepatitis B virus x interacting protein	Hbxip
AI843558	heterogeneous nuclear ribonucleoprotein K	Hnrpk
AI848331	heterogeneous nuclear ribonucleoprotein U	Hnrpu
AI836129	high mobility group nucleosomal binding domain 2	Hmgn2
AI850268	methionine adenosyltransferase II, alpha	Mat2a
AI853257	mitochondrial ribosomal protein S33	Mrps33
AI853143	mortality factor 4 like 1	Morf4l1
AI847934	neurofilament, light polypeptide	Nefl
AI848144	neuron specific gene family member 2	Nsg2
AI841664	nucleosome assembly protein 1-like 1	Nap1l1
AI838078	nudix-type motif 9	Nudt9
AI852884	Phosphatidylinositol binding clathrin assembly protein	Picalm
AI848346	polyadenylate-binding protein-interacting protein 2	Paip2
AI843948	proteasome subunit, alpha type	Psma3,
AI838874	protein phosphatase 2a, catalytic subunit, beta	Ppp2cb

GenBank	Gene name	Symbol
AI838463	RAS related protein 1b	Rap1b
AI849041	Ras-like without CAAX 2	Rit2
AI847923	regulator of G-protein signaling 2	Rgs2
AI844168	reticulon 4 receptor	Rtn4r
AI845529	ribonuclease P 40 subunit (human)	Rnasep1
AI851588	ribosomal protein L23	Rpl23
AI834863	ribosomal protein L26	Rpl26
AI841501	ribosomal protein S3a	Rps3a
AI853900	RIKEN cDNA 1110055L24 gene	1110055L24Rik
AI847909	RIKEN cDNA 1700030A21 gene	1700030A21Rik
AI840191	RIKEN cDNA 2210409B22 gene	2210409B22Rik
AI840046	RIKEN cDNA 2310047D13 gene	2310047D13Rik
AI854255	RIKEN cDNA 2410002O22 gene	2410002O22Rik
AI838261	RIKEN cDNA 3110001N18 gene	3110001N18Rik,
AI849886	RIKEN cDNA 4631403P03 gene	4631403P03Rik
AI843449	RIKEN cDNA 4921531G14 gene	G630041M05Rik
AI835456	RIKEN cDNA 5730454B08 gene	5730454B08Rik
AI835904	RIKEN cDNA 5730470L24 gene	5730470L24Rik
AI849033	RIKEN cDNA 7530403E16 gene	7530403E16Rik
AI850130	RIKEN cDNA 8430420C20 gene	8430420C20Rik
AI842640	RIKEN cDNA A730042J05 gene	A730042J05Rik
AI853842	RWD domain containing 1	Rwdd1
AI850227	SECIS binding protein 2	Secisbp2
AI847805	secreted phosphoprotein 1	Spp1
AI848364	SMT3 suppressor of mif two 3 homolog 1 (yeast)	Ubl1
AI845086	smu-1 suppressor of mec-8 and unc-52 homolog (C. elegans)	Smu-1
AI840676	spectrin beta 2	Spnb2
AI845051	spectrin beta 3	Spnb3
AI843287	Tax1 binding protein 1	Tax1bp1
AI853152	thymopoietin	Tmpo
AI851197	transcription factor 4	Tcf4
AI849958	transducin (beta)-like 3	Tbl3
AI849063	transforming growth factor beta 1 induced transcript 4	Tgfb1i4
AI854679	tumor differentially expressed 2	Tde2
AI844401	tumor protein D52	Tpd52
AI845961	ubiquitin-conjugating enzyme E2L 3	Ube2l3,
AI854256	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 10	Galnt10

It is relatively well established that MT-deficient cells are more sensitive (i.e., less viable) to excess copper; therefore, it seems counterintuitive that these cells did not show signs of increased stress. However, it is important to note that these MT-/- cells were shown by us (Tapia et al., 2004) to accumulate less copper when exposed to increasing levels of the metal. A similar phenomenon was seen in MT-deficient cells exposed to lead. In this study, lead-exposed cells were more sensitive to lead despite accumulating much less of the metal as compared to their wild-type counterparts (Qu et al., 2002). This might suggest that MT-/- cells are unable to sense the stress, and perhaps it is the lack of induction of the stress response pathways that make them more sensitive to these metals. This potential incapability to sense the stress might be related to impaired activation of the MTF-1 transcription factor, since the specific activation of MTF-1 requires, in addition to copper excess, a zinc-saturated MT (Zhang et al., 2003). MTF-1 is involved in several cellular responses, including oxidative stress, hypoxia, and amino acid starvation (Murphy et al., 1999), and its activation might be modulated by heat shock (Saydam et al., 2003), which place MTF-1 as an important component of the cross-talk between different anti-stress systems in the cell. The possibility that untreated MT-/- cells are already stressed is consistent with their higher sensitivity to copper exposure as compared to wild-type cells, which has been observed in cell viability studies by us and others (Kelly and Palmiter, 1996; Tapia, 2004) and with the fact that in the absence of MT, cells are unable to store copper in a safe compartment (Tapia et al., 2004). In this scenario, we might assume a high level of expression of stress genes, even when cells are exposed to low extracellular copper. Therefore, we are unable to detect significant changes in the relative expression of stress genes; in order to clarify this point, it is necessary to perform a comparison between untreated wild-type and MT-/- cells. Moreover, we cannot rule out the option that the mechanisms to induce the expression of stress genes in MT-/- cells require a longer period of time to become activated, thus it will be interesting to perform serial time-course microarray experiments to compare the gene profile in wild-type and MT-/- cell lines.

Among other genes up-regulated in 100 μM copper-treated wild-type cells, we found ferritin, an iron sequestering protein composed of 2 types of subunits – H (heavy chain) and L (light chain) – that play a key role in the maintenance of the intracellular iron balance (Torti and Torti, 2002). Although ferritin is known primarily for its critical role in cellular and organism iron homeostasis, it is unlikely that high iron levels are inducing ferritin, as intracellular iron levels in both the MT-/- and WT cells slightly decrease upon copper treatment (Fig. 2). There is evidence, however, that suggests that ferritin plays a role in protection against oxidative damage (Arosio and Levi, 2002). It has been shown that overexpression of the H subunit leads to a reduced production of reactive oxygen species (ROS) after exposure to hydrogen peroxide and that ferritin is subject to both transcriptional, as well as translational, regulation by oxidative stress (Tsuji et al., 2000; Orino et al., 2001). Further studies will be needed to understand the role of MT in the transcriptional regulation of ferritin in copper overload cells.

Another gene with potential roles in metal homeostasis is HFE, the hemochromatosis gene. Mutations in this gene cause progressive body iron overload, which leads to tissue damage and ultimately multiorgan failure (Drakesmith and Townsend, 2000; Parkkila et al., 2001). The function of HFE protein is unknown, but some evidence suggests that it acts together with beta2-microglobulin and transferrin receptor 1 to regulate iron uptake in the intestinal cells (Anderson and Powell, 2002). In the Caco2 cell line, it was shown that HFE mRNA is induced by Fe, but not by other metals (Co, Cu, and Zn) (Han et al., 1999), and there is currently no evidence to suggest that it is induced by oxidative stress (Sánchez et al., 1998; Mura et al., 2004). As with ferritin, further studies are necessary to understand the significance of HFE induction in the copper-treated cells.

A large proportion of genes (>80%) upregulated in wild-type and MT-/- cells after copper exposure were classified into two major categories: 1) metabolism; and 2) cellular physiological processes. A similar distribution was reported by Armendáriz et al. (2004) for the two cell lines that accumulate copper due to a defect in copper export, suggesting that copper overload induces the expression of genes associated with diverse molecular function. Therefore, microarray analysis seems to be useful to compare the behavior of wild-type cells and cells with mutations in different components of Cu metabolism in response to fluctuations in Cu availability. We consider that the results obtained through these experiments provide experimental support to connect copper homeostasis with other metabolic pathways.

ACKNOWLEDGEMENTS

This work was supported by grants 1050235 and 1030618 from Fondecyt (to VC and MG) and by grants from the International Copper Association, United States Department of Agriculture NRI 9900815 and a University of California Toxic Substance (to CDV) Research and Training Program traineeship (ADA).

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