Gene expression profiling in chronic copper overload reveals upregulation of Prnp and App

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COPPER IS AN ESSENTIAL TRACE metal that serves as an important catalytic cofactor of several enzymes (30). Copper can exist in either the oxidized, Cu(II), or reduced, Cu(I), state; the ability of copper to convert between these oxidation states accounts for its essential role in many proteins (30). These copper-containing enzymes, which include, Cu/Zn superoxide dismutase (SOD1), lysyl oxidase (LO), cytochrome c oxidase, ceruloplasmin (Cp), and tyrosinase, are involved in many important biological processes, and deficiencies or alterations in them can cause disease states (60). Recently, genetic approaches have led to the discovery of many of the mechanisms underlying copper homeostasis (48). We now have an understanding of some of the processes involved in copper uptake, transport, and removal, and we are beginning to understand the regulation of these processes (for detailed reviews, see Refs. 26, 43, 48). Diseases in which there is an established link to copper metabolism, such as Wilson disease (WD), Menkes disease, and amyotrophic lateral sclerosis, or where copper has been implicated, such as Alzheimer disease (AD) and prion disease, drive the need to better understand copper homeostatic mechanisms.

Although copper is clearly an essential nutrient, this metal can be toxic if allowed to accumulate in excess of cellular needs. The incidence of copper toxicosis in the general population is low (6, 46). Acute copper poisoning is not frequent but is usually the consequence of consumption of contaminated foodstuffs or beverages, or from accidental or deliberate ingestion of high quantities of copper salts. Acute copper poisoning is characterized with nausea, vomiting, and gastric pain. In severe cases, intravascular hemolytic anemia, acute hepatic failure, acute tubular renal failure, shock, coma, and death can occur (46).

Chronic copper toxicosis may be of greater concern (6). Nutritional studies of chronic copper overload in both mice and rats have shown copper-induced liver damage in these animals (1, 19, 38). In addition, WD, a human genetic disorder of copper overload, is characterized by a gradual hepatic accumulation of copper that eventually results in liver cirrhosis (7). Other copper overload disorders in humans include Indian childhood cirrhosis (ICC), endemic Tyrolean infantile cirrhosis (ETIC), and idiopathic copper toxicosis (ICT). These diseases result in abnormal hepatic copper accumulation and can result in chronic hepatitis and hepatic failure (36). Although the liver injury in each of these cases is copper induced, the mechanisms by which excess copper results in cellular toxicity have not been fully elucidated. The increase in copper levels, especially in WD, occurs over a period of many years, and phenotypic consequences are not reported until copper levels are extremely elevated. The lack of appreciable pathological changes from ever increasing copper levels until late in the disease process suggests that compensatory homeostatic mechanisms must exist.

Toxicity resulting from copper overload has long been hypothesized to result from the redox cycling Haber-Weiss reaction in which cuprous ions react with H2O2 to form highly reactive oxygen species (ROS), which in turn catalyze the oxidation of biomolecules such as lipids, proteins, and nucleic acids (reviewed in Refs. 22 and 15). The suspected role of ROS in copper overload is supported by observations of increased generation of lipid peroxidation products, as well as evidence of DNA damage in hepatocytes reacted with ionic copper (6, 59). In vivo studies have shown that markers of lipid peroxidation are increased in mitochondria from livers of WD patients (55). There is also evidence of lipid peroxidation in livers of rats chronically fed very high copper diets (38, 54).
The levels at which copper becomes toxic is not clear. Most studies that indicate that copper causes oxidative stress have tested very high levels of copper (38, 54). Indeed, a study in which a diet with moderately high levels of copper was fed to rats showed no lipid peroxidation (1). DNA damage, often a consequence of oxidative stress, has been observed in copper overload in vivo, however, only in the cirrhotic livers of WD patients (21). Nevertheless, the incidence of hepatocellular carcinoma (HCC) in patients with WD is not higher than that in the general population (50). This raises the question of whether copper, at moderately elevated levels, results in oxidative damage, and if not, what effects, if any, these levels have on cells. Clearly, the cellular mechanisms that protect the cell are vigorous, and only at very high levels do they become overwhelmed. We currently have only a limited understanding of the protective systems that operate in cells chronically exposed to copper. Additionally, the limits of homeostatic regulation are not known, making it difficult to define the milder effects of copper excess (3). Currently no early, biologically relevant markers of copper excess exist. A robust assay to facilitate the diagnosis of copper excess and to distinguish mild, moderate, and severe copper overload is needed.

To address these issues, we have investigated the effects on steady-state gene expression of chronic copper overload in a cell culture model system using cDNA microarray technology. For this study we have chosen to utilize cells from a genetic model of copper overload. We have used fibroblast cells from two mouse mutants, C57BL/6-Atp7a<sup>Mobr</sup> and C57BL/6-Atp7a<sup>Modap</sup> (henceforth to be referred to as Mo<sup>br</sup> and Mo<sup>lap</sup>, respectively, for simplicity) as genetic models of chronic copper overload. Cultured cells from mice with mutations in the Atp7a represent genetic models of moderate cellular copper overload (12, 32). Excess copper is normally exported by the ATP7A copper transporting P-type ATPase, which translocates from the trans-Golgi network (TGN) to the plasma membrane in the presence of elevated copper (45). Mutations in ATP7A in both humans (Menkes disease) and mice (mottled mouse) lead to reduced intestinal basolateral export of copper and subsequent systemic copper deficiency (33). All cultured cells tested, with the exception of hepatic cells, from mottled mice accumulate copper to abnormally high levels in normal culture media (12, 32). Our results show no evidence of oxidative stress in the copper-loaded cells and suggest remarkable cellular compensation. In addition, candidate components perhaps responsible for a copper-specific homeostatic response are identified.

**MATERIALS AND METHODS**

**Cell culture and treatments.** In this study we have used fibroblast cells from two mouse mutants with severe functional defects in ATP7A: C57BL/6-Atp7a<sup>Mobr</sup> and C57BL/6-Atp7a<sup>Modap</sup>. Fibroblasts that are wild type for ATP7A (C57BL/6-Atp7a<sup>+/+</sup>) were used as a control. The fibroblast cells were a kind gift of Dr. Seymour Packman and Dr. Jane Gitschier and were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies) containing 10% fetal bovine serum (HyClone) supplemented with penicillin and streptomycin (Life Technologies) and incubated in a humidified chamber containing 5% CO<sub>2</sub> at 37°C. Chronic copper overload of the control fibroblasts was achieved by treatment with 25 µM Cu-His in the media for 5 days. Cells were seeded such that they would reach 100% confluence at time of collection (5 days).

**Inducitively coupled plasma-atomic emission spectrometry.** To measure metal content, cells were grown to 80–90% confluence, then collected, pelleted, and washed twice with PBS. Ten percent of the pellet was used for protein concentration determination by the Bradford assay (Bio-Rad). The remainder of the pellet was digested in 1 ml nitric acid (Suprapure, Merck) overnight at room temperature. Samples were diluted twice with metal-free water and were read in triplicate on a simultaneous inductively coupled plasma-atomic emission spectrometer (ICP-AES) (Vista AX CCD, Varian) at the University of California at Davis (20). The sample values were normalized to the total protein content.

**RNA isolation and cDNA labeling.** Total RNA was isolated from cells grown to 80–90% confluence using TRIzol Reagent (Life Technologies). mRNA was subsequently isolated using oligo-dT latex beads (Qiagen). The indirect, or amino-allyl, labeling protocol, found at the Microarrays.org web site (http://www.microarrays.org/pdfs/amino-allyl-protocol.pdf), was used. For all experiments, the cDNA from the control cell lines was labeled with Cy3, while the cDNA from the mutant cell lines was labeled with Cy5. cDNA quantity and dye incorporation of each probe was assessed with a Cy3/Cy5 dual wavelength spectrophotometer. The two dye probes were then combined and concentrated to a volume of 15–20 µl. SSC and SDS were added to a final concentration of 2.6× and 0.2%, respectively. The probes were denatured for 2 min at 95°C, allowed to cool, then placed on a postprocessed microarray and incubated overnight at 60°C. Postprocessing protocol can be found at The Brown Lab web site, http://cmgm.stanford.edu/pbrown/protocols/3_post_process.html.) After hybridization, the microarray slides were washed at room temperature for 5 min in 1× SSC and 0.05% SDS, then for 2 min in 0.2× SSC, and finally for 2 min in 0.1× SSC, and then were spin dried. The microarray experiments were carried out four times for each mutant (i.e., 4 arrays for Mo<sup>br</sup> vs. control, and 4 arrays for Mo<sup>lap</sup> vs. control). The four replicates are technical replicates. Several different plates of each cell line were grown simultaneously and then pooled prior to RNA extraction. 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.

**Microarrays.** The microarrays utilized in the Mo<sup>br</sup> vs. control experiments were fabricated by a microarrayer in the laboratory of Edward Rubin at the Lawrence Berkeley National Laboratory and contained 10,000 mouse cDNAs. Microarrays used in the Mo<sup>lap</sup> vs. control experiments were fabricated in the College of Natural Resources Genomics Facility; these arrays contained 11,000 mouse cDNAs from the Brain Molecular Anatomy Project (BMAP) library (Research Genetics) (project web site, http://brainest.eng.uiowa.edu). UniGene IDs were used to determine the overlap of the two different arrays. There are 2,900 genes that appear on both arrays, the remaining genes are unique to each array.

**Data analysis.** Microarray slides were scanned on either a GenePix 4000 (Axon Instruments) or 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. We have recently developed a novel approach to identify differentially expressed genes between the two mRNA samples based on identification of outliers as candidate differentially expressed genes (27). The majority of data points after appropriate normalization lie in the vicinity of the line of equivalence in the scatter plot of log<sub>2</sub>(Cy5) vs. log<sub>2</sub>(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 intensity. We apply robust scatter plot smoothers to quantify and take into account the distortion of the data set by heteroscedasticity (if any). We consider outliers from this analysis to represent candidate differentially expressed genes. We assign a confidence (P value) for...
RESULTS

Mo<sup>br</sup> and Mo<sup>dpap</sup> fibroblast cell lines are copper overloaded. We measured the intracellular copper level in the mutant cells grown in normal media and compared them to control cells to confirm copper retention in fibroblasts from the mutants. Cells to be used for microarray experiments were grown concurrently. Mo<sup>br</sup>, Mo<sup>dpap</sup>, and control fibroblast cells, growing in standard culture conditions, were collected and their intracellular copper concentration was determined as described in MATERIALS AND METHODS. Intracellular copper levels were 0.27 (±0.08) nM/mg protein (Mo<sup>br</sup>), 1.17 (±0.52) nM/mg protein (Mo<sup>dpap</sup>), indicating that Mo<sup>br</sup> and Mo<sup>dpap</sup> accumulate three to five times more copper than control cell lines. These results are similar to other studies (12, 32) and confirm a state of copper overload in both the Mo<sup>br</sup> and Mo<sup>dpap</sup> cell lines. We also measured the intracellular copper levels of the copper-treated control cell lines. Control cells in normal media were grown concurrently. Intracellular copper levels were 0.21 (±0.8) (control) and 0.72 (±0.08) nM/mg protein (control + 25 μM Cu-His), showing that the control cells lines, after 5 days of treatment with 25 μM Cu-His, contain similar intracellular copper levels as the mutant cell lines.

Exploratory microarray analysis. Gene expression in two chronically copper-overloaded fibroblast cell lines (Mo<sup>br</sup> and Mo<sup>dpap</sup>) were compared with a control fibroblast cell line (control) using cDNA microarrays containing ~10K different genes. We carried out four replicate hybridizations for each comparison. Exploratory data analysis was done to provide an assessment of the quality of the data set (27). Distortions in the data are readily apparent when using these exploratory tools but can be overlooked if not utilized. For example, a multimodal distribution suggests that the data are drawn from several populations that are behaving differently such as data arising from nonhomogeneous hybridizations. The results of four exploratory data analysis tools for one of the hybridizations (Mo<sup>dpap</sup> vs. control) are shown in Fig. 1. The results for the eight individual microarrays can be found in the Supplemental Figs. S1–S4, at the Physiological Genomics web site.1

Validation of changes in Prnp and App gene expression was carried out by semiquantitative reverse transcriptase-polymerase chain reaction (sq-RT-PCR). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3pdh) was used as a control. The G3pdh primers were obtained from a commercial source (Clontech), and the Prnp primers were designed using Primer3 software. The sequences were as follows (5'-3'): AAGAACCTTGCCCAAGGCTGA (forward), GTGATGACAATCACGGTTGC (reverse) (896-bp amplicon). The probe was radiolabeled with [32P]dCTP by a random priming method (Prime-It II, Stratagene) and hybridized to the membrane at 42°C for 2 h. After hybridization the membranes were washed under stringent conditions and exposed to BioMax film (Kodak) at −70°C. Autoradiograms were evaluated by densitometry (AlphaEase, Alpha Innotech).

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Northern blot. Total RNA from Mo<sup>br</sup>, Mo<sup>dpap</sup>, and control fibroblasts was extracted according to the TRizol reagent protocol (Invitrogen). For Northern blot analysis, 10 μg total RNA was electrophoresed in a formaldehyde-containing agarose (1%) gel, and transferred to a nylon membrane (Hybond-N, Amersham) by a standard capillary method. For the detection of App mRNA, the membranes were probed with an 896-bp fragment of App cDNA. The primers used to amplify the probe were designed using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), and the sequences were as follows (5'-3'): AAGAACCTTGCCCAAGGCTGA (forward), GTGATGACAATCACGGTTGC (reverse) (896-bp amplicon). The probe was radiolabeled with [32P]dCTP by a random priming method (Prime-It II, Stratagene) and hybridized to the membrane at 42°C for 2 h. After hybridization the membranes were washed under stringent conditions and exposed to BioMax film (Kodak) at −70°C. Autoradiograms were evaluated by densitometry (AlphaEase, Alpha Innotech).

Fig. 1. Exploratory data analysis and quality control: results of exploratory analysis of one cDNA microarray experiment (Mo<sup>dpap</sup> vs. control). Mo<sup>dpap</sup> was labeled with Cy5, and the control with Cy3. The hybridization data was examined using the following exploratory data analysis tools: box plots (A), average shifted histograms (B), quantile-quantile (QQ) normal plots (C), and scatter plots (D). Results of the exploratory data analysis for each individual microarray experiment are available in the Supplemental Figs. S1–S4, at the Physiological Genomics web site.

1The Supplemental Material for this article (Supplemental Figs. S1–S7 and Tables S1–S6) is available online at http://physiolgenomics.physiology.org/cgi/content/full/00196.2003/DC1.
(Cy5 and Cy3) for this data set. Similarly, the average shifted histograms (Fig. 1B) provide information on the empiric distribution of a random variable and reveal that the data resembles samples from univariate normal distributions for both channels. The QQ normal plot (Fig. 1C) compares the quantiles of the empirical data distribution with the quantiles of the standard normal distribution. The majority of points in QQNP for both channels of our data conform closely to a standard normal distribution except for longer tails. These deviations may be due to random fluctuations and/or systematic variation. Finally, each point on a scatter plot (Fig. 1D) corresponds to one bivariate observation after appropriate normalization and log transformation. These results show that the red and green channel intensities in the data sets are highly correlated having approximately symmetric distributions and are appropriate for further analysis for the determination of candidates for differentially expressed genes.

Detection of differentially expressed genes. We identified differentially expressed genes from each hybridization by our outlier identification method (see MATERIALS AND METHODS and Ref. 27). We were interested in those genes that were significantly changed (either up- or downregulated) in the copper-overloaded cells compared with a control. As shown in Fig. 2, the majority of the data points lie in the vicinity of a robust linear regression line of the scatter plot of log2(Cy5) vs. log2(Cy3) intensity values. These data points signify genes with similar transcript behaviors in the two samples, whereas other data points lie outside the scatter plots and correspond to genes having noticeably different hybridization intensity values. We determine statistics 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 use it to build simultaneous prediction intervals for graphical representation that genes outside the intervals are most likely outliers. Robust scatter plot smoothers are used to account for heteroscedasticity (e.g., variation in residual variance with intensity) in each data set. Red outliers on the top of the graph in Fig. 2 represent candidate upregulated genes, whereas the outlying spots of blue color, on the bottom, represent candidate downregulated genes. Individual outlier plots for each data set can be found in the supplementary data (Supplemental Figs. S5 and S6). We considered data outliers with P < 0.05 as candidate differentially expressed genes. We then compared the candidate genes identified for each experiment and selected genes identified (i.e., outliers with a P < 0.05) in three or more repeat hybridizations as likely differentially expressed genes. In the MoBr vs. control experiments, there were 101 genes that were upregulated and 80 genes that were downregulated in, at least, three of the four experiments. In the MoDap vs. control experiments, there were 115 up- and 25 downregulated genes that were similar in at least three of the four experiments. The lists of genes can be found in the supplementary data (Supplemental Tables S1–S4). We found 11 genes to be upregulated and one gene to be downregulated in both MoDap and MoBr cell lines (Table 1). The individual P values for each experiment for these 12 genes are shown in Supplemental Table S5.

Functional classification of differentially expressed genes. Using the Gene Ontology classifications found at the Mouse Genome Informatics web site (http://www.informatics.jax.org/) and at the Gene Ontology Consortium web site (http://www.geneontology.org/), all differentially expressed genes (Supplemental Tables S1–S4) were grouped according to their biological processes (Fig. 3). In each set of differentially expressed genes, approximately 50% of the genes had unknown biological processes; thus shown in the pie graphs are the processes of the known halves. Biological processes of the genes found to be upregulated are depicted in Fig. 3 (Fig. 3A for MoDap and 3B for MoBr). A pie graph illustrating the biological processes of the downregulated genes in the MoBr cell lines is available in the Supplemental Fig. S7; too few downregulated genes for the MoDap experiment exist to illustrated by pie graph, but the list and their functions can be found in Supplemental Table S6. Over 50% of the upregulated genes with known biological function are involved in cell growth and/or maintenance processes. This group includes genes that play a role in signal transduction, cell cycle regulation, cell adhesion, etc. (see Fig. 3). Unexpectedly, only a very small portion of the differentially expressed genes are involved in the response to stress.

No evidence of oxidative stress-related gene expression. Despite having copper levels five times greater than the control cell lines, these two chronically copper-overloaded cells show no evidence of changes at the transcription level of oxidative stress genes. We expected that many of the differentially expressed genes would be those that encoded proteins involved in the response to oxidative stress. A number of oxidative stress-responsive transcription factors and genes have been identified (reviewed in Ref. 14). However, none of these genes (e.g., superoxide dismutase, catalase, cytochrome P-450s, DNA damage inducible genes) was induced, or repressed, in the either the MoDap or MoBr cells (individual lists of differen-
known metal-responsive gene, was upregulated in the Mobr and Modap cell lines, and no significant induction of either of the two genes in either the Mobr or the Modap cell lines (Fig. 4).

Confirmation of differential expression of App and Prnp. Two of the genes upregulated in both the Mo<br and Mo<sup>dup</sup> cells (Table 1) were amyloid-β precursor protein (App) and prion protein (Prnp). Previous research suggests that the protein products of both of these genes may play a role in copper homeostasis (61); thus we chose to confirm their differential expression. Significant changes in App and Prnp as detected by microarray analysis were validated by Northern blot analysis and sq-RT-PCR, respectively. Northern blot analysis showed induction of App in both Mo<br and Mo<sup>dup</sup> fibroblast cell lines (Fig. 5A). Equal loading of samples for the Northern blot analysis is shown in Fig. 5B; ethidium bromide-stained agarose gel shows the 28S and 18S rRNA bands from 10 μg total RNA. RT-PCR showed the induction of Prnp in both Mo<br and Mo<sup>dup</sup> cell lines (Fig. 6A). For Prnp, quantitative analysis was performed; a plot of the optical density for Prnp/G3pdh ratios revealed that Prnp was induced 3.3-fold in Mo<br and 3.5-fold in Mo<sup>dup</sup> (Fig. 6B).

To determine whether nutritional, chronic copper overload induced expression of these two genes, we analyzed, by sq-RT-PCR, the gene expression of App and Prnp in copper-treated control cell lines. Chronic copper treatment of the wild-type, control cell lines (25 μM Cu-His for 5 days) induced App gene expression 2.2-fold (Fig. 7). For comparison, App expression was also measured in the Mo<br and Mo<sup>dup</sup> cell lines and showed a 3- and 4.5-fold induction, respectively (Fig. 7). No change in Prnp gene expression was observed in the copper-treated cells (data not shown).

**DISCUSSION**

We evaluated in this study the effects of chronic copper overload on cellular gene expression. We investigated two independently derived genetic models of copper overload containing mutations in the same gene to discriminate between cell-specific effects and the effects of copper. Both of these mutations result in severe loss of function in the Atp7a gene (33). Both mutants result in a cellular defect in copper efflux (12, 32); thus we expected that genes differentially expressed in both mutant cell lines would likely represent copper-specific changes. We acknowledge that further validation of any potential biomarkers would be needed to confirm their utility as biomarkers of chronic copper overload of other etiologies. Our results suggest that it is important to evaluate the effects of multiple alleles in studies of the effects of genetic models on gene expression.

**Table 1. Genes up- and downregulated in both Mo<sup>br</sup> and Mo<sup>dup</sup> fibroblast cell lines**

<table>
<thead>
<tr>
<th>GenBank ID</th>
<th>UniGene ID</th>
<th>Name</th>
<th>Average Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mo&lt;sup&gt;br&lt;/sup&gt;</td>
</tr>
<tr>
<td>Downregulated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AI836908</td>
<td>Mm.4438</td>
<td>Hmgal, high mobility group AT-hook 1</td>
<td>4.59 (1.715)</td>
</tr>
<tr>
<td>AI843768</td>
<td>Mm.277585</td>
<td>App, amyloid beta (A4) precursor protein</td>
<td>5.93 (2.38)</td>
</tr>
<tr>
<td>AI845409</td>
<td>Mm.308134</td>
<td>Cad1, caldesmon</td>
<td>5.60 (1.31)</td>
</tr>
<tr>
<td>AI839353</td>
<td>Mm.257765</td>
<td>Clc4, chloride intracellular channel 4</td>
<td>4.93 (2.12)</td>
</tr>
<tr>
<td>AI842535</td>
<td>Mm.128733</td>
<td>D0H5S114 DNA segment</td>
<td>4.17 (0.71)</td>
</tr>
<tr>
<td>AI855088</td>
<td>Mm.182434</td>
<td>Fasl, follistatin-like</td>
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</tr>
<tr>
<td>AI835946</td>
<td>Mm.14638</td>
<td>Gabaprel1, gamma-aminobutyric acid [GABA(A)] receptor-associated protein-like 1</td>
<td>4.86 (1.43)</td>
</tr>
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<td>AI836611</td>
<td>Mm.648</td>
<td>Prn, prion protein</td>
<td>4.33 (0.99)</td>
</tr>
<tr>
<td>AI838469</td>
<td>Mm.293316</td>
<td>Ras11, Ras-like, family 11, member B</td>
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</tr>
<tr>
<td>AI847805</td>
<td>Mm.288474</td>
<td>Spp, secreted phosphoprotein</td>
<td>10.61 (3.81)</td>
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<tr>
<td>AI845381</td>
<td>Mm.20927</td>
<td>Tgfl14, transforming growth factor beta 1 induced transcript 4</td>
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<tr>
<td>AI844805</td>
<td>Mm.279361</td>
<td>Vcl, vinculin</td>
<td>2.71 (0.16)</td>
</tr>
</tbody>
</table>

Values are means, with SD in parentheses. We used fibroblast cells from two mouse mutants, C57BL/6-Atp7a<sup>Mo-br</sup> and C57BL/6-Atp7a<sup>Mo-dup</sup> (Mo<sup>br</sup> and Mo<sup>dup</sup>, respectively).
We utilized cDNA microarray technology to investigate the effects of chronic copper overload. The microarray experiments, which compared transcripts from each mutant (Mobr and Modap) to a control cell line, were carried out four times per mutant. For the analysis of the individual microarray data sets, we employed a novel, data-driven approach, recently developed in our laboratory, for identifying differentially expressed genes. The resulting lists of significantly up- and downregulated genes from each individual microarray were then compared with each other and showed high reproducibility.

An investigation into the functions of the differentially expressed genes led us to some surprising results. Copper is thought to be toxic if allowed to accumulate in excess of cellular needs because of its ability to generate free radicals (6). Indeed, there are reports of copper-induced oxidative damage at high doses of exposure (55). Hence, we expected that many of the differentially expressed genes would be those that encoded proteins involved in the response to oxidative stress. Surprisingly, both mottled cell lines, despite being chronically copper overloaded, showed no differential expression of oxidative stress genes besides the upregulation of MT1.

MT1, long known to be induced by copper (and other heavy metals) and to be elevated in the cultured fibroblasts and other nonhepatic cell lines from both Menkes patients (25, 49) and mottled mice (41), was upregulated in the Mobr cells, (it was not on the array and, therefore, not assayed in the Modap cells). In contrast, glutathione peroxidase (GPX), one of the main intracellular antioxidants, and γ-glutamylcysteine synthetase (GCL), a regulator of glutathione synthesis, which previously has been shown to be upregulated under oxidative stress (13, 35), were not induced in either of the two cell lines. In support of our findings is a recent study that showed that fibroblasts from Menkes patients, despite having intracellular copper levels five times that of control cells, had decreased basal oxidative stress and a more efficient protection toward free radicals than fibroblasts from controls (18). Their results, which included measurements of redox metal content, antioxidant equipment, and oxidative stress markers, showed that the activity of catalase and GPX, TBARS (a marker of lipid peroxidation), and the production of hydrogen peroxide were all decreased in the Menkes (MNK) cells. Metallothionein production was also increased in the MNK cells. Together, these data suggest that chronic exposure to moderately elevated

Fig. 3. Functional classifications of upregulated genes in Mobr and Modap cell lines. Upregulated genes with known functions were grouped together in four major classes: cell growth and/or maintenance, metabolism, response to stress, and transport. Subclasses are labeled as pie slices a–s in Mobr (A) and slices a–p in Modap (B).

Fig. 4. Semiquantitative reverse transcriptase-polymerase chain reaction (sq-RT-PCR) analysis of oxidative stress genes in Mobr and Modap cell lines. Gpx and Gcl gene expression in Mobr and Modap, and control cell lines was analyzed by sq-RT-PCR as described in MATERIALS AND METHODS. The density of the amplified products (A) was calculated and normalized against the G3pdh housekeeping gene control values. B: a plot of the optical density for Gpx/G3pdh (left) and Gcl/G3pdh (right) ratios.
intracellular levels of copper does not induce oxidative damage to cells; instead, we observe evidence of an adaptive response to copper.

A large number of differentially expressed genes in both the Mo\textsuperscript{br} and Mo\textsuperscript{dap} cell lines are involved in cytoskeleton organization and cell adhesion (Table 2). The relationship between cytoskeletal organization and copper metabolism is not known.

It is possible that copper may be affecting the cytoskeleton directly. Polymerization/depolymerization of actin and tubulin is regulated in part by divalent metal-ion-binding sites, generally for Ca\textsuperscript{2+} and Mg\textsuperscript{2+}; thus other divalent metals may act as potential toxic interferers (58). Whether copper or other metal ions actually compete with or displace Ca\textsuperscript{2+} or Mg\textsuperscript{2+} in the cytoskeleton is not known, but there is evidence that zinc, arsenic, mercury, and cadmium can disrupt actin and microtubules in intact cells (58). Another possible explanation of the expression changes could be an indirect effect secondary to lysyl oxidase (LO) deficiency. LO is a copper-containing protein that is responsible for the formation of lysine-derived cross-links in connective tissue, particularly in collagen and elastin (52). LO activity has been shown to be decreased in fibroblasts from patients with Menkes disease (17, 51). Hence, alterations in the extracellular matrix could have a downstream effect on the cytoskeleton and lead to the observed changes in gene expression. Additionally, amyloid precursor protein, induced in our models of copper overload and whose function is still undetermined, has been shown to interact with collagen, sharing common binding sites with heparin, and has been proposed to be a mediator of cell-matrix and cell-cell interactions (56). The changes we observed could also be due to

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**Table 2. Cytoskeleton organization/cell adhesion-related genes up- and downregulated in Mo\textsuperscript{br} and Mo\textsuperscript{dap} fibroblast cell lines**

<table>
<thead>
<tr>
<th>UniGene ID</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mm.212567</td>
<td>Cald1, caldesmon</td>
</tr>
<tr>
<td>Mm.14638</td>
<td>Gabarap1, gamma-aminobutyric acid [GABA(A)] receptor-associate protein-like 1</td>
</tr>
<tr>
<td>Mm.12842</td>
<td>Vcl, vinculin</td>
</tr>
<tr>
<td>Mm.14638</td>
<td>Gabarap1, gamma-aminobutyric acid [GABA(A)] receptor-associate protein-like 1</td>
</tr>
<tr>
<td>Mm.212567</td>
<td>Tuba4, tubulin, alpha 4</td>
</tr>
<tr>
<td>Mm.1155</td>
<td>Kif5b, kinesin family member 5B</td>
</tr>
<tr>
<td>Mm.3380</td>
<td>Kif5b, kinesin family member 5B</td>
</tr>
<tr>
<td>Mm.4646</td>
<td>Krt1-13, keratin complex 1, acidic, gene 13</td>
</tr>
<tr>
<td>Mm.1012</td>
<td>Krt1-19, keratin complex 1, acidic, gene 19</td>
</tr>
<tr>
<td>Mm.4689</td>
<td>Krt2-4, keratin complex 2, basic, gene 4</td>
</tr>
<tr>
<td>Mm.2165</td>
<td>Mm.4689</td>
</tr>
<tr>
<td>Mm.22699</td>
<td>Sepp1, selenoprotein P, plasma, 1</td>
</tr>
<tr>
<td>Mm.1451</td>
<td>Mfge8, milk fat globule-EGF factor 8 protein</td>
</tr>
<tr>
<td>Mm.28598</td>
<td>Jak1, Janus kinase 1</td>
</tr>
<tr>
<td>Mm.28357</td>
<td>Map1lc3, microtubule-associated protein 1 light chain 3</td>
</tr>
<tr>
<td>Mm.1451</td>
<td>Mfge8, milk fat globule-EGF factor 8 protein</td>
</tr>
<tr>
<td>Mm.27448</td>
<td>Cdh11, cadherin 11</td>
</tr>
<tr>
<td>Mm.7257</td>
<td>Cdh2, cadherin 2</td>
</tr>
<tr>
<td>Mm.206505</td>
<td>Timp2, tissue inhibitor of metalloproteinase 2</td>
</tr>
<tr>
<td>Mm.980</td>
<td>Tnc, tenasin C</td>
</tr>
<tr>
<td>Mm.35439</td>
<td>Sparc, secreted acidic cysteine rich glycoprotein</td>
</tr>
<tr>
<td>Mm.4159</td>
<td>Tenc1, tensin-like C domain-containing phosphatase</td>
</tr>
<tr>
<td>Mm.29389</td>
<td>Tns, vimentin</td>
</tr>
<tr>
<td>Mm.27448</td>
<td>Cdh11, cadherin 11</td>
</tr>
<tr>
<td>Mm.206505</td>
<td>Timp2, tissue inhibitor of metalloproteinase 2</td>
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<tr>
<td>Mm.1155</td>
<td>Tub2a4, tubulin, alpha 4</td>
</tr>
<tr>
<td>Mm.86421</td>
<td>Cldn6, claudin 6</td>
</tr>
<tr>
<td>Mm.30142</td>
<td>D15Wsu77e DNA segment, Chr 15, Wayne State University 77, expressed (keratin 7)</td>
</tr>
<tr>
<td>Mm.3380</td>
<td>Kif5b, kinesin family member 5B</td>
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<tr>
<td>Mm.4646</td>
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</tr>
<tr>
<td>Mm.2165</td>
<td>Mm.4689</td>
</tr>
</tbody>
</table>

**Mo\textsuperscript{br} and Mo\textsuperscript{dap} indicate mouse mutants C57BL/6-Atp7a\textsuperscript{Mo\textsuperscript{br}} and C57BL/6-Atp7a\textsuperscript{Mo\textsuperscript{dap}}, respectively.**

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Fig. 5. Northern blot analysis of App in Mo\textsuperscript{br} and Mo\textsuperscript{dap} cell lines. RNA was isolated and analyzed as described in MATERIALS AND METHODS. A: Northern blot analysis shows expression levels of App in Mo\textsuperscript{br}, Mo\textsuperscript{dap}, and control fibroblast cell lines. B: ethidium bromide-stained agarose gel shows the 28S and 18S rRNA bands from 10 μg total RNA.
changes in function of this protein. Furthermore, defects in the trafficking of the ATP7A protein may be leading to changes in cytoskeletal organization. Normally, the ATP7A protein traffics between the TGN and the plasma membrane and is vesicle mediated (45, 42). The molecular mechanisms responsible for membrane trafficking of ATP7A have recently been under extensive investigation and may involve cytoskeletal factors that have yet to be identified.

In search of the genes that may be involved in the putative adaptive response, we investigated the specific genes that were differentially expressed in both the Mobr and Modap cell lines. It is important to note that two different microarrays were used for each set of experiments, and it is possible that significant genes from the Mobr experiments were not assayed in the Modap experiments, and vice versa. Nevertheless, we compared the 193 and 130 reproducible genes from each mutant, and 12 genes were found to be similarly differentially expressed in the mutants (Table 1). Because of the different microarrays, the true number of similar genes may, in fact, be higher. Interestingly, 2 of the 12 genes found to be upregulated in both the Mobr and Modap mutant were App and Prnp.

The protein products of these genes are involved in the pathology of two neurodegenerative diseases, AD and prion disease, respectively. The identification of copper binding sites on both of these proteins suggests a possible role in copper homeostasis (61). APP is a cell surface transmembrane glycoprotein ubiquitously expressed in mammalian tissues and, although the function is unknown, is likely to play an important role in normal cell physiology. APP contains two copper-binding sites and is able to reduce Cu(II) to Cu(I) in vitro (37). APP is the precursor of the β-amyloid (Aβ) peptide, which constitutes the characteristic amyloid plaques of AD. The homeostasis of copper is significantly altered in the AD brain (57). Copper inhibits β-amyloid production by stimulating the non-amyloidogenic pathway of APP (5); however, in vitro, copper induces aggregation of the soluble amyloid peptide, suggesting a role in both the processing of APP and amyloid plaque assembly and may contribute to AD development (24). App−/− mice have increased copper in their livers and in the cerebral cortex, with no change in their serum (63). App−/− neurons are less sensitive to copper toxicity compared with wild-type cells (62). These observations led Strausak et al. (57) to suggest that the amyloid precursor protein could be involved in the release of copper from the cell.

Our results, demonstrating upregulation of App in both genetic models and a nutritional model of chronic copper overload, support a role for this protein in copper homeostasis. Recently the Menkes protein was overexpressed in human fibroblasts (a state of decreased intracellular copper), and the researchers showed that copper depletion significantly reduced APP protein levels and downregulated App gene expression (4). We suggest that the adaptive response to moderately elevated levels of copper may involve the amyloid precursor protein.

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), which include scrapie, bovine spongiform encephalopathy (BSE), and Creutzfeldt-Jakob disease (CJD), result in fatal neurodegenerative conditions (47). In prion disease, it is thought that the pathogenic process is related to transformation of the normal cellular prion protein (PrPc) into a protease-resistant and readily aggregated isoform.
called PrPsc (scrapie prion protein) (31). Evidence is mounting that copper may play a role in PrPsc biology (9). The Prnp−/− mouse has decreased copper levels in the brain and liver, whereas copper levels in their serum were increased (10). In addition, cell lines from Prnp−/− mice are more sensitive to copper exposure (11). The prion protein may therefore represent a potential copper-transporting or -sequestering protein that may prevent copper toxicity (9).

Our results demonstrate upregulation of Prnp in the genetic models but not the nutritional model of chronic copper overload. The copper-treated cells, although copper loaded, still have the capability to export copper, whereas the mutant cells do not. It could be that prion protein is functioning secondarily to copper export, thus protecting the cell from copper toxicity only when copper’s normal export is disrupted. Additionally, cellular distribution of copper may be different between the genetic and the nutritional models, and this may have an effect on the prion protein. Clearly, further studies on the role of this protein in copper overload are needed.

Copper exposure has been proposed to play a role in cardiovascular disease and cancer, in addition to neurodegenerative diseases (8, 16, 23, 61). Although overt copper toxicity is relatively rare, signs of moderate copper overload may be more common (6). The relationship between these conditions and the effects of chronic copper exposure has not been adequately assessed, possibly due to the inadequacy of current methodology for assessment of copper status (3, 34). Currently there is no effective measure of copper overload besides invasive liver biopsy (36). Serum ceruloplasmin (Cp) levels (53) can be decreased in copper deficiency, but since Cp is an acute phase protein, levels can be elevated in a variety of inflammatory conditions independent of copper levels (34). Clearly, there is need for a more robust assay to facilitate the diagnosis of copper excess and to distinguish mild, moderate, and severe copper overload.

Gene expression patterns could serve as biomarkers of exposure or effect (2, 40, 44). We have compared our results to all published studies (as of January 2004) on the gene expression of nutrient and xenobiotic exposure on mammalian all published studies (as of January 2004) on the gene expression of nutrient and xenobiotic exposure on mammalian conditions independent of copper levels (34). Clearly, there is need for a more robust assay to facilitate the diagnosis of copper excess and to distinguish mild, moderate, and severe copper overload.

Gene expression patterns could serve as biomarkers of exposure or effect (2, 40, 44). We have compared our results to all published studies (as of January 2004) on the gene expression of nutrient and xenobiotic exposure on mammalian cells and found no significant overlap of differentially expressed genes assessed. These include studies of several metals such as arsenic and cadmium. Thus we suggest that the genes which we have identified to be differentially expressed in both Mo-br and Mo-dap cells lines may be specific for chronic copper exposure.

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GRANTS

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REFERENCES