# Differential Expression of Human Metallothionein Isoform I mRNA in Human Proximal Tubule Cells Exposed to Metals

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In contrast to the single metallothionein (MT)-1 gene of the mouse, the human MT-1 gene family is composed of seven active genes and six pseudogenes. In this study, the expression of mRNA representing the seven active human MT-1 genes was determined in cultured human proximal tubule (HPT) cells under basal conditions and after exposure to the metals $Cd^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Hg^{2+}$, $Ag^{2+}$, and $Pb^{2+}$. Basal expression of MT-1X and MT-1E mRNA in HPT cells was similar to expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase. In contrast, mRNAs representing the basal expression of MT-1A and MT-1F were a minor transcript in HPT cells. Treatment of HPT cells with $Cd^{2+}$, $Zn^{2+}$, or $Cu^{2+}$ increased the levels of MT-1E and MT-1A mRNA, but not the levels of MT-1X or MT-1F mRNA. The increase in MT-1E mRNA appeared to be influenced mainly by exposure to the various metals, whereas the increase in MT-1A mRNA was influenced more by exposure to a metal concentration eliciting a loss of cell viability. Treatment of HPT cells with the metals $Hg^{2+}$, $Ag^{2+}$, and $Pb^{2+}$ was found to have no effect on the level of MT-1 mRNA at either sublethal or lethal concentrations. Using HPT cells as a model, these results suggest that new features of MT gene expression have been acquired in the human due to the duplication of the MT-1 gene. *Key words*: cadmium, copper, gene expression, heavy metals, lead, mercury, metallothionein, mRNA, proximal tubule, RT-PCR, reverse transcriptase-polymerase chain reaction, silver, zinc. *Environ Health Perspect* 106:825–832 (1998). [Online 17 November 1998]
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The extrapolation of environmental studies from animal models to humans is potentially compromised when areas of examination involve human gene families with increased organizational complexity. A prime example is the human metallothionein (MT) gene family, in which gene organization is more complex in humans than in commonly used model systems. In both mice and humans, there are four classes of similar MT proteins, designated MT-1 through 4, defined on the basis of small differences in sequence and charge characteristics (*1–3*). For both species, the MT-1 and MT-2 proteins exhibit a ubiquitous pattern of tissue expression, whereas expression of the MT-3 and MT-4 family members is highly restricted (*3–5*). However, the gene organizations underlying the expression of the protein isoforms are quite different. In the mouse, the genes encoding the four MT isoforms are single-copy genes, and no MT pseudogenes are known to exist. The mouse MT-1 and MT-2 genes are separated by approximately 6 kb on chromosome 8 and appear to be coordinately regulated with functionally equivalent protein products (*3,6*). The MT-3 and MT-4 genes are closely linked to but not coordinately regulated with the other MT genes on mouse chromosome 8 (*4,5*). In contrast to the four single genes of the mouse, the human MT gene family is represented by 10 functional and 7 nonfunctional genes located at 16q13 (*4,5,7,8*). The genes encoding the MT-2, 3, and 4 isoforms are similar in number to the rodent (there is an MT-2 processed pseudogene on chromosome 2), but the human MT-1 locus possesses numerous MT-1 isoforms that are not present in the mouse (*7,8*). The human MT-1 gene locus is composed of seven functional genes and six pseudogenes. Complete genomic sequences are available for the seven active genes: MT-1A (*9*), MT-1B (*10*), MT-1E, MT-1F, MT-1G (*11,12*), MT-1H, and MT-1X (*8*). The potential significance of this duplication event is underscored by the fact that the human MT-1 isoforms genes have been shown to exhibit unique expression profiles with examples of inducer-specific, tissue-specific, and developmental-specific regulation (*9,13–17*).

The MTs are widely recognized and accepted as a major weapon in the cell's armamentarium for protection against and recovery from environmental insult. They are a class of low molecular weight ($M_r$ = 6,000–7,000), cysteine-rich, inducible, intracellular stress proteins that are best known for their high affinity for binding heavy metals. They are believed to serve an important role in the homeostasis of essential metals such as $Zn^{2+}$ or $Cu^{2+}$ during growth and development as well as in the detoxification of heavy metals such as $Cd^{2+}$ and $Hg^{2+}$; rendering the MTs important mediators/attenuators of heavy metal-induced toxicity, particularly hepato- and nephrotoxicity (*1,3,18–20*). To begin to define the regulation of expression of the MT gene family in human tissues and cells exposed to metals, we have undertaken an analysis of the metal-induced expression of the MT gene family in cultures of human proximal tubule (HPT) cells. This cell culture system was chosen because the kidney, and the proximal tubule in particular, represent an organ and cell type that are critically affected by chronic $Cd^{2+}$ exposure in both animals and humans (*21–23*). We report here the expression of mRNA for each active MT-1 isoform in HPT cells as a function of exposure to $Cd^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Ag^{2+}$, $Hg^{2+}$, and $Pb^{2+}$ at both lethal and sublethal concentrations.

## Materials and Methods

***Cell culture, metal treatments, and viability determinations.*** Total RNA samples for the analysis of MT-1 isoforms expression were obtained from a previous study that examined the viability and MT protein accumulation of HPT cells when exposed to sublethal and lethal concentrations of $Cd^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Ag^{2+}$, $Hg^{2+}$, and $Pb^{2+}$ (*24*). Stock cultures of HPT cells were grown in 75-$cm^2$ T-flasks with a serum-free growth medium and a collagen matrix using procedures recently detailed (*25*). For use in experimental protocols, the cells were subcultured at a 1:2 ratio, allowed to reach confluence (6 days following subculture), and then exposed to media containing the various concentrations of metals. Thereafter, the cells were fed every 3 days with media containing the various concentrations of metals. We chose metal concentrations so that over a 16-day time course there would be situations where a loss of cell viability occurred and situations where exposure elicited no loss of cell viability. Metal concentrations eliciting these various levels of toxicity were determined in preliminary experiments. The effect of the individual components of the serum-free medium on the level of metal needed to elicit HPT cell toxicity has not been determined. These concentrations were determined to be 9, 27, and 45 µM for $Cd^{2+}$; 20, 40, and 60 µM for $Hg^{2+}$; 50, 100,

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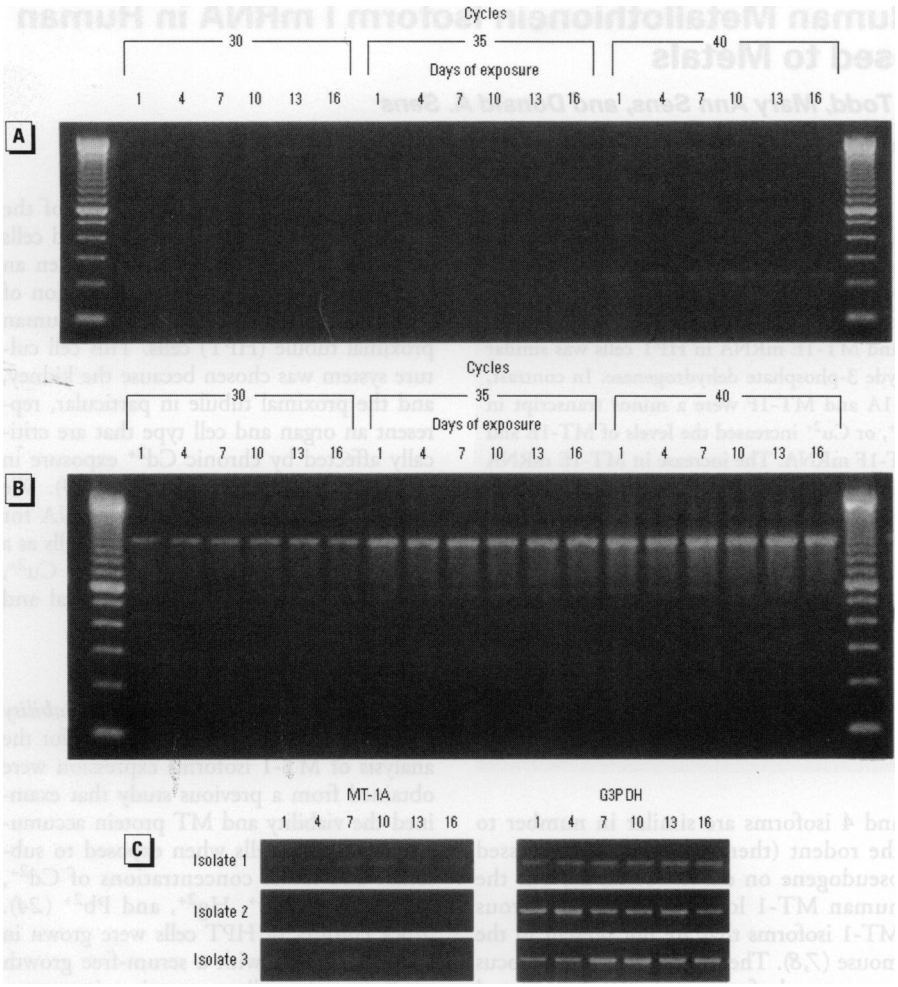


**Figure 1.** Expression of MT-1A and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA in three HPT cell isolates. Reverse transcriptase-polymerase chain reaction (RT-PCR) products were electrophoresed on 2% agarose gels containing 0.5 µg/ml ethidium bromide. (A) Full gel showing reaction products for MT-1A from one cell isolate sampled at 30, 35, and 40 PCR cycles. (B) Full gel showing reaction products for G3PDH from the same isolate sampled at 30, 35, and 40 PCR cycles. (C) Expression of MT-1A (cycle 40) and G3PDH (cycle 30) for three cell isolates.

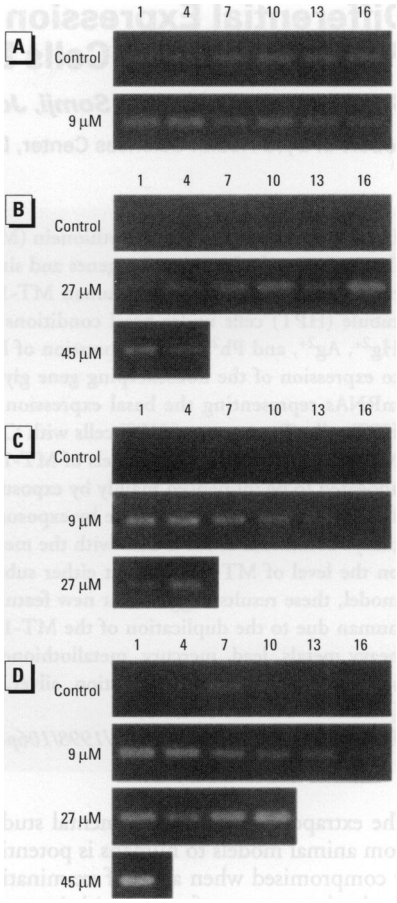


**Figure 2.** Expression of MT-1A mRNA in human proximal tubule cells exposed to cadmium. Reverse transcriptase-polymerase chain reaction for MT-1A was performed as described using RNA isolated on days 1, 4, 7, 10, 13, and 16 of Cd exposure. Reaction products sampled at 40 cycles are shown for the control and 9 µM concentration for isolate 1 (A) and for controls and three concentrations of Cd for isolates 2 (C) and 3 (D). Samples representing higher levels of exposure for isolate 1 were removed at 35 cycles (B). In all three isolates the expression of glyceraldehyde 3-phosphate dehydrogenase was constant (data not shown).

and 150 µM for $Ag^{2+}$; 50, 100, and 200 µM for $Pb^{2+}$; 100, 200, and 300 µM for $Zn^{2+}$; and 250, 500, and 750 µM for $Cu^{2+}$ (*24*). Total RNA samples were obtained after 1, 4, 7, 10, 13, and 16 days of metal exposure and were used for the analysis of MT-1 isoform specific mRNA expression. Three isolates of HPT cells were used to control for possible variations in metal susceptibility. These isolates were derived from normal cortical tissue obtained from kidneys removed for renal cell carcinoma. All cultures displayed senescence by subculture 25. We used HPT cells between passages 5 and 8. Subcultured HPT cells have been shown to retain original light and ultrastructural morphology, active transport capability as assessed by dome formation and electrical properties, and the stimulation of cAMP by angiotensin but not by vasopressin (*26*).

***RNA isolation and RT-PCR.*** Total RNA was isolated according to the protocol supplied with TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH) as described previously (*24,27*). The concentration and purity of the RNA samples were determined using spectrophotometer scan in the UV region and ethidium bromide (EtBr) visualization of intact 18S and 28S RNA bands following agarose gel electrophoresis. Total RNA (0.5 µg) was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) as described previously (*24,27*). The primers developed for analysis of each of the active MT genes have been previously described (*27*). The primers for mRNA analysis were upper 5′CTCGAAATGGACCCCAACT3′ and lower 5′5′ATATCTTCGAGCAGGGCTGTC3′ yielding a 219-bp product for MT-1A; upper 5′GCTTGTCTTGGCTCCACA3′ and lower 5′AGCAAACCGGTCAGGTAGTTA3′ yielding a 287-bp product for MT-1B; upper 5′GCTTGTTCGTCTCACTGGTG3′and lower 5′CAGGTTGTGCAGGTTGTTCTA3′ yielding a 284-bp product for MT-1E; upper 5′AGTCTCTCCTCGGCTTGC3′ and lower 5′ACATCTGGGAGAAAGGTTGTC3′ yielding a 232-bp product for MT-1F; upper 5′CTTCTCGCTTGGGAACTCTA3′ and lower 5′AGGGGTCAAGATTGTAGCAAA3′ yielding a 309-bp product for MT-1G; upper 5′CCTCTTCTCTTCTCGCTTGG3′ and lower 5′GCAAATGAGTCGGAGTTGTAG3′ yielding a 315-bp product for MT-1H; and upper 5′TCTCCTTGCCTCGAAATGGAC3′

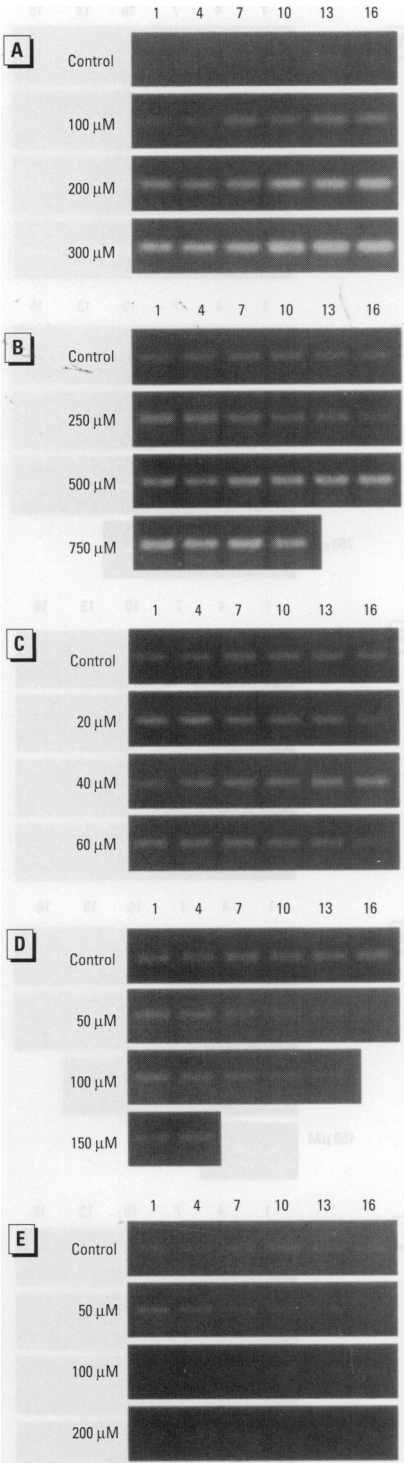


**Figure 3.** Expression of MT-1A mRNA in isolate 1 exposed to Zn, Cu, Hg, Ag, and Pb. Reverse transcriptase-polymerase chain reaction for MT-1A was performed as described on RNA obtained on days 1, 4, 7, 10, 13, and 16 of metal exposure. Isolate 1 was exposed to the following metals at the indicated concentrations: (A) Zn, (B) Cu, (C) Hg, (D) Ag, and (E) Pb. Reaction products were removed at 40 cycles for all metals except for Zn, which was removed at 30 cycles. The expression of glyceraldehyde 3-phosphate dehydrogenase was constant (data not shown).

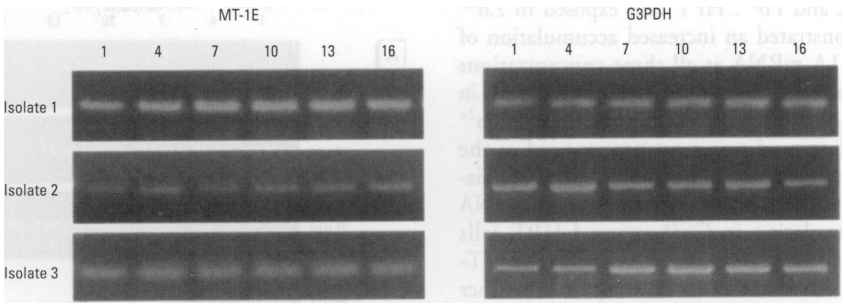


**Figure 4.** Expression of MT-1E and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA in three HPT cell isolates. Reverse transcriptase-polymerase chain reaction for MT-1E and G3PDH mRNA was performed as described on RNA obtained on days 1, 4, 7, 10, 13, and 16 of cell culture. Reaction products were removed at 30 cycles.

and lower 5′GGGCACACTTGGCA-CAGC3′ yielding a 151-bp product for MT-1X. Primers for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA were obtained commercially (Clontech, Palo Alto, CA). The thermocycler was programmed to cycle at 95°C for a 2-min initial step, then 95°C for 15 sec and 68°C for 30 sec, with a final elongation step at 68°C for 7 min. Controls for each reaction included a no-template control where water was added instead of the RNA and a no-reverse transcriptase control where water was added instead of the enzyme. Samples were removed at 30, 35, and 40 PCR cycles to ensure that the reaction remained in the linear region. The final PCR products were electrophoresed on 2% agarose gels containing EtBr along with DNA markers.

## Results

*MT-1A.* The initial experimental goal was to define the basal level of MT-1A mRNA expression in HPT cells not exposed to metals. We used mRNA expression for the housekeeping gene G3PDH as a comparison for MT-1A mRNA expression. The reactions were stopped and samples removed for analysis at 30, 35, and 40 PCR cycles. The results demonstrated that basal expression of MT-1A mRNA was very low in HPT cells, demonstrating only a faint band or no band for the reaction product following 40 PCR cycles for the 3 HPT cell isolates (Fig. 1A,C). This is in contrast to G3PDH, where the same total RNA input demonstrated a convincing reaction product corresponding to G3PDH mRNA at 30 PCR cycles (Fig. 1B,C). These findings indicate that HPT cells have a low basal expression of MT-1A mRNA.

The effect of $Cd^{2+}$ on the expression of MT-1A mRNA was also determined on the three independent HPT cell isolates after 1, 4, 7, 10, 13, and 16 days of exposure. Three concentrations of $Cd^{2+}$ (9, 27, and 45 µM) were used in the time course so that the cells experienced both lethal and sublethal levels of $Cd^{2+}$ exposure (*24*). Expression of MT-1A was increased in HPT cells as a result of $Cd^{2+}$ exposure, and this increase was most pronounced at $Cd^{2+}$ concentrations eliciting a loss of HPT cell viability (Fig. 2). For isolate 1, exposure to 9 µM $Cd^{2+}$ elicited no loss of cell viability over 16 days of exposure, and MT-1A mRNA levels (at 40 reaction cycles) exceeded control levels, appearing to increase early in the time course and then decrease (Fig. 2A, upper panel). After exposure to either 27 or 45 µM $Cd^{2+}$, where a loss of cell viability occurs late and early, respectively, in the time course, MT-1A mRNA levels were clearly increased over control values. This is effectively illustrated at 35 reaction cycles, where convincing product bands corresponding to MT-1A mRNA are present for $Cd^{2+}$-treated cells, but totally absent in control cells (Fig. 2A, lower panel). The second cell isolate was more susceptible to $Cd^{2+}$ and demonstrated a loss of cell viability after 1 day of exposure to 27 µM $Cd^{2+}$. At the lower concentration of $Cd^{2+}$ (9 µM), where cell loss was minimal during the time course, MT-1A mRNA levels were clearly elevated over control values (Fig. 2B). As was the case for the first isolate, at the lowest concentration of $Cd^{2+}$ MT-1A mRNA levels appeared to increase early in the time course and return to undetectable levels by day 16 of exposure. The third cell isolate had a pattern of susceptibility to $Cd^{2+}$ comparable to the first isolate and showed increased MT-1A mRNA levels and a similar pattern of MT-1A mRNA expression (Fig. 2C). These findings demonstrate that exposure to $Cd^{2+}$ results in a repeatable increase in the level of MT-1A mRNA for three independent HPT cell isolates and that this increase is most pronounced at $Cd^{2+}$ concentrations that impact cell viability.

A single HPT cell isolate was used to determine if MT-1A mRNA accumulation is increased after exposure to lethal and sublethal concentrations of $Zn^{2+}$, $Cu^{2+}$, $Hg^{2+}$,

$Ag^{2+}$, and $Pb^{2+}$. HPT cells exposed to $Zn^{2+}$ demonstrated an increased accumulation of MT-1A mRNA at all three concentrations compared to control (Fig. 3A). The increase in MT-1A mRNA correlated to the level of $Zn^{2+}$ exposure and was most pronounced at the highest concentration, where a loss of cell viability occurred. Analysis of MT-1A mRNA accumulation in $Cu^{2+}$-exposed HPT cells demonstrated increased accumulation of MT-1A mRNA similar to cells exposed to either $Zn^{2+}$ or $Cd^{2+}$ (Fig. 3B). In contrast to the increased accumulation of MT-1A mRNA demonstrated for HPT cells exposed to $Cd^{2+}$, $Zn^{2+}$, and $Cu^{2+}$, there was no increase in the accumulation of MT-1A mRNA for HPT cells exposed to $Hg^{2+}$, $Ag^{2+}$, or $Pb^{2+}$ (Fig. 3C–E). The lack of MT-1A accumulation was noted at the highest concentrations of $Hg^{2+}$, $Ag^{2+}$, and $Pb^{2+}$ that were previously determined to elicit a loss of HPT cell viability (*24*). For the six metals assessed, these findings demonstrate that only exposure to $Cd^{2+}$, $Zn^{2+}$, or $Cu^{2+}$ results in increased HPT cell accumulation of MT-1A mRNA.

***MT-1E.*** The basal expression level of MT-1E mRNA was evaluated in three HPT cell isolates using the total RNA samples described above. Results demonstrated that basal expression of MT-1E mRNA was evident after 30 PCR cycles for each HPT cell isolate. Product intensities were comparable to those noted for the G3PDH housekeeping gene at an identical cycle number (Fig. 4). Although a qualitative observation, the relative intensities of the MT-1E mRNA reaction products varied among the cell isolates, with isolate 1 demonstrating a more intense reaction product at 30 reaction cycles. This is interesting because isolate 1 has been shown to be the most resistant to the lethal effects of $Cd^{2+}$ exposure, followed by isolates 3 and 2 (*24*).

The effect of $Cd^{2+}$ on the expression of MT-1E mRNA was also determined for the HPT cell isolates. Results showed increased expression of MT-1E for all three HPT cell isolates as a result of $Cd^{2+}$ exposure (Fig. 5), as evidenced by the notable increase in MT-1E mRNA at the lowest levels of $Cd^{2+}$ exposure that produced no loss of cell viability. The $Cd^{2+}$-induced increase in MT-1E was not concentration dependent. A single HPT cell isolate was used to demonstrate that exposure to both $Zn^{2+}$ and $Cu^{2+}$ elicited an increase in MT-1E mRNA (Fig. 6A,B). As was the case for MT-1E mRNA of $Cd^{2+}$-exposed cells, the increase in expression compared to control was evident at the lowest levels of $Zn^{2+}$ and $Cu^{2+}$ exposure where there was no loss of HPT cell viability. There were only slight increases in MT-1E mRNA accumulation as dosage levels of $Zn^{2+}$ and $Cu^{2+}$ were increased. The level of MT-1E mRNA was not increased over control when cells were exposed to $Hg^{2+}$, $Ag^{2+}$, or $Pb^{2+}$ at

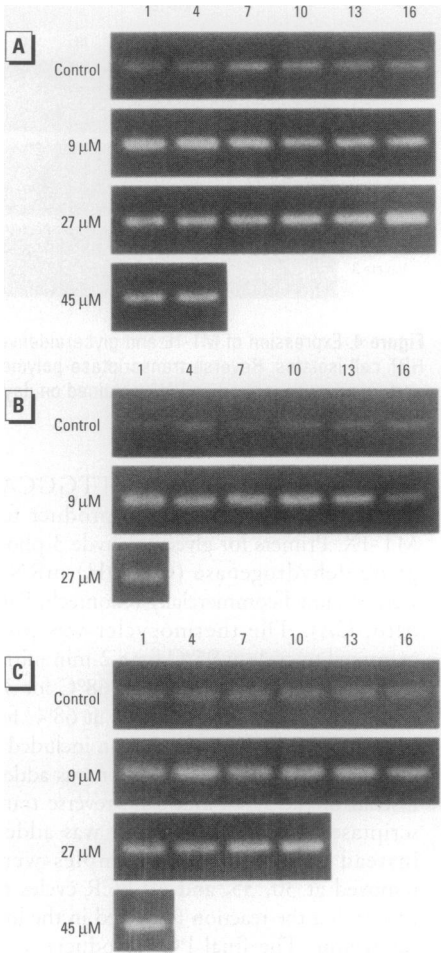


**Figure 5.** Expression of MT-1E mRNA in human proximal tubule (HPT) cells exposed to Cd. Reverse transcriptase-polymerase chain reaction for MT-1E was performed as described on RNA from three HPT cell isolates (A,B,C) obtained on days 1, 4, 7, 10, 13, and 16 of Cd exposure. Reaction products were removed at 30 cycles for the control and three levels of Cd exposure. Glyceraldehyde 3-phosphate dehydrogenase expression was constant (data not shown).

both lethal and sublethal concentrations (Fig. 6C–E). In fact, there appears to be a trend for a small decrease in MT-1E mRNA for cells exposed to $Hg^{2+}$, $Ag^{2+}$, and $Pb^{2+}$. These findings indicate that MT-1E mRNA is a common transcript in HPT cells and that accumulation is induced by both lethal and nonlethal exposure to $Cd^{2+}$, $Zn^{2+}$, and $Cu^{2+}$, but not by exposure to $Hg^{2+}$, $Ag^{2+}$, or $Pb^{2+}$.

***MT-1F.*** The basal level of MT-1F mRNA expression was also assessed in three three HPT cell isolates. MT-1F mRNA, like MT-1A mRNA, was shown to be a rare transcript in HPT cells, having only minimal basal expression in two of the three cell isolates at 40 reaction cycles (Fig. 7). This is in contrast to G3PDH, where the same total RNA input demonstrated a convincing reaction product corresponding to G3PDH mRNA at 30 PCR

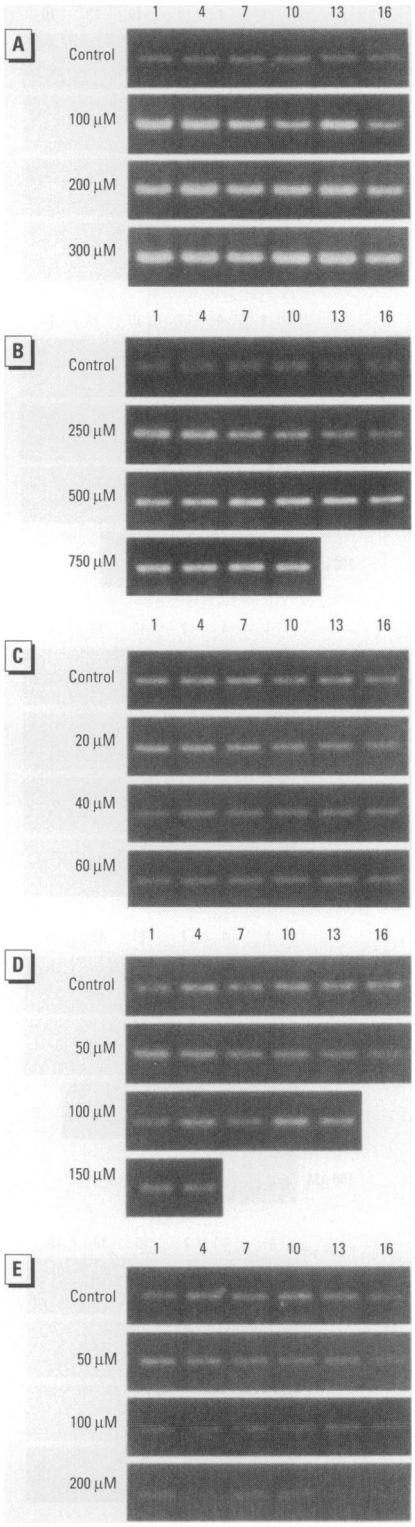


**Figure 6.** Expression of MT-1E mRNA in human proximal tubule cells exposed to Zn, Cu, Hg, Ag, and Pb. Reverse transcriptase-polymerase chain reaction for MT-1E was performed as described on RNA obtained on days 1, 4, 7, 10, 13, and 16 of metal exposure. Isolate 1 was exposed to the following metals at the indicated concentrations: (A) Zn, (B) Cu, (C) Hg, (D) Ag, and (E) Pb. Reactions products were removed at 30 cycles for all metals. The expression of glyceraldehyde 3-phosphate dehydrogenase was constant (data not shown).

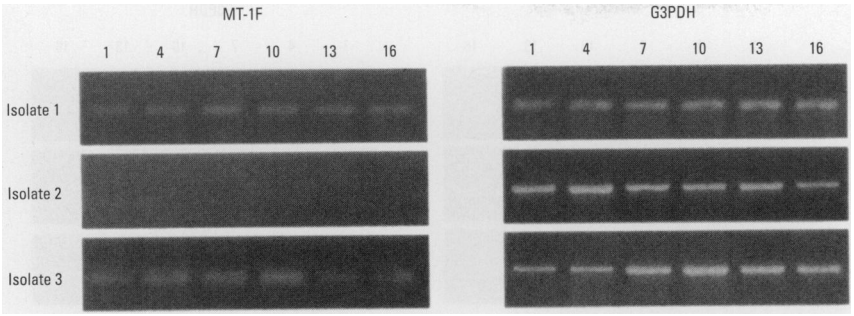


**Figure 7.** Expression of MT-1F mRNA in three human proximal tubule cell isolates. Reverse transcriptase-polymerase chain reaction for MT-1F and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA was performed as described on RNA obtained on days 1, 4, 7, 10, 13, and 16 of cell culture. Reaction products were removed at 30 cycles for G3PDH and 40 cycles for MT-1F.

cycles. In contrast to the results for MT-1A and 1E mRNA expression, exposure to $Cd^{2+}$, $Zn^{2+}$, and $Cu^{2+}$ at both lethal and sublethal concentrations did not produce any repeatable or consistent increase in the accumulation of MT-1F mRNA (Figs. 8 and 9). There was also no increase in the level of MT-1F mRNA when HPT cells were exposed to $Hg^{2+}$, $Ag^{2+}$, or $Pb^{2+}$ (Fig. 9C–E). These results indicate that MT-1F mRNA is a rare transcript in HPT cells under basal conditions and that the level of expression is not increased due to metal exposure.

***MT-1X.*** Basal expression of MT-1X mRNA was prominent and similar to expression for G3PDH for each HPT cell isolate (Fig. 10). The effect of $Cd^{2+}$ on MT-1X mRNA expression was also determined on three HPT cell isolates, and the results revealed only a marginal increase in MT-1X mRNA as a result of $Cd^{2+}$ exposure (Fig. 11). This finding was reinforced by the levels of MT-1X mRNA in HPT cells exposed to both $Zn^{2+}$ and $Cu^{2+}$, where results suggested a slight elevation in MT-1X mRNA level following metal treatment (Fig. 12A,B). However, in no instance was the increase of the magnitude found for MT-1A and MT-1E mRNA under identical conditions. There was no increase in the level of MT-1X mRNA when HPT cells were exposed to $Hg^{2+}$, $Ag^{2+}$, or $Pb^{2+}$ (Fig. 12C–E). These results demonstrate that MT-1X mRNA is a common transcript in HPT cells and that the level of expression is not greatly influenced by metal exposure.

***MT-1B, MT-1G, and MT-1H.*** Basal expression and metal-induced expression of mRNA for the MT-1B, MT-1G, and MT-1H genes were also determined using the above RNA samples. No expression of MT-1B mRNA was demonstrated under basal conditions or after treatment with any of the six metals (data not shown). MT-1G and MT-1H mRNA displayed marginal bands approximately 20% of the time at 40 reaction cycles; however, the occurrence of bands had no discernible pattern under basal conditions or when the cells were exposed to the six metals (data not shown). We conclude that the expression of MT-1B, MT-1G, and MT-1H mRNAs is at the limit of detection of the RT-PCR assay and that repeatable data regarding expression cannot be obtained.

## Discussion

One goal of this study was to determine if HPT cell cultures retained features of MT gene expression known to be present in the proximal tubule of the human kidney. Several studies using immunohistochemical analysis have shown that MT protein in the human kidney is localized exclusively in the proximal tubule (*27–29*). An analysis of MT mRNA expression in fetal and adult human kidney demonstrated the expression of mRNA representing the MT-2A, MT-1A, MT-1E, MT-1F, MT-1X, and MT-3 genes (*27,30*). For HPT cells, the present study demonstrated basal expression of MT mRNA for the MT-1A, MT-1E, MT-1F, and MT-1X genes. Earlier studies using HPT cells also demonstrated basal expression of MT protein and mRNA for the MT-2A and MT-3 gene (*24,30*). Basal levels of MT mRNA expression appear to be similar between the human kidney and cultured HPT cells. In total RNA preparation from human kidney tissue, mRNAs for the MT-2A, MT-1X, MT-1E, and MT-1F genes were demonstrated to be common transcripts, having expression levels close to the G3PDH housekeeping gene, while mRNAs for the MT-1A and MT-3 genes were demonstrated to be far less abundant (*27,30*). In the present study, basal expression of MT-1X and MT-1E mRNA was similar to expression of G3PDH mRNA in HPT cells, whereas MT-1A and MT-1F were less common transcripts. Earlier studies have shown MT-2A to be a common basal transcript and MT-3 a rarer transcript in HPT cells (*24,30*). Comparison of the findings between the HPT cells and the human kidney demonstrates that HPT cells retain the expected patterns of MT mRNA expression.

In a previous study, exposure of HPT cells to $Cd^{2+}$, $Zn^{2+}$, or $Cu^{2+}$ induced a rapid and sustained accumulation of MT protein over a 16-day time course (*24*). In this respect, HPT cells demonstrated an expected response to $Cd^{2+}$ exposure according to the large database of animal model studies (*1–3,19*). However, it was also shown in the previous study that the increase in MT protein elicited by HPT cell exposure to $Cd^{2+}$ was not accompanied by a corresponding increase in the amount of MT-2 mRNA; even though such an increase would be expected from previous studies in animal models. Prompted by these results, an additional goal of the present study was to determine if regulation of the MT-1 genes in HPT cells exposed to metals corresponds to results from animal studies. In the mouse it has been shown that the MT-1 and MT-2 genes are coordinately regulated and that mRNA is induced by treatment with the heavy metal $Cd^{2+}$ (*1–3,6*). In contrast to the increase in MT-1 mRNA predicted from mouse studies, the mRNA expression pattern of HPT cells exposed to

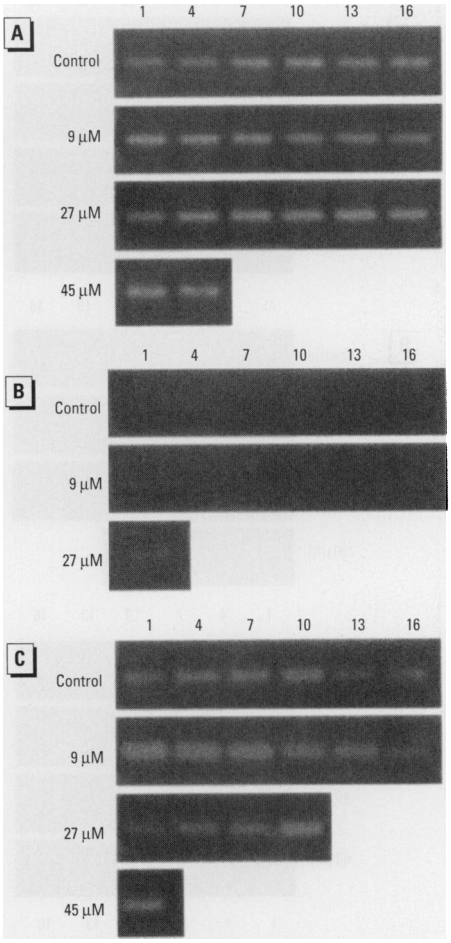


**Figure 8.** Expression of MT-1F mRNA in human proximal tubule (HPT) cells exposed to Cd. Reverse transcriptase-polymerase chain reaction for MT-1F was performed as described on RNA from three HPT cell isolates (A,B,C) obtained on days 1, 4, 7, 10, 13, and 16 of Cd exposure. Reaction products were removed at 40 cycles for the control and three levels of Cd exposure. In all three isolates the expression of glyceraldehyde 3-phosphate dehydrogenase was constant (data not shown).

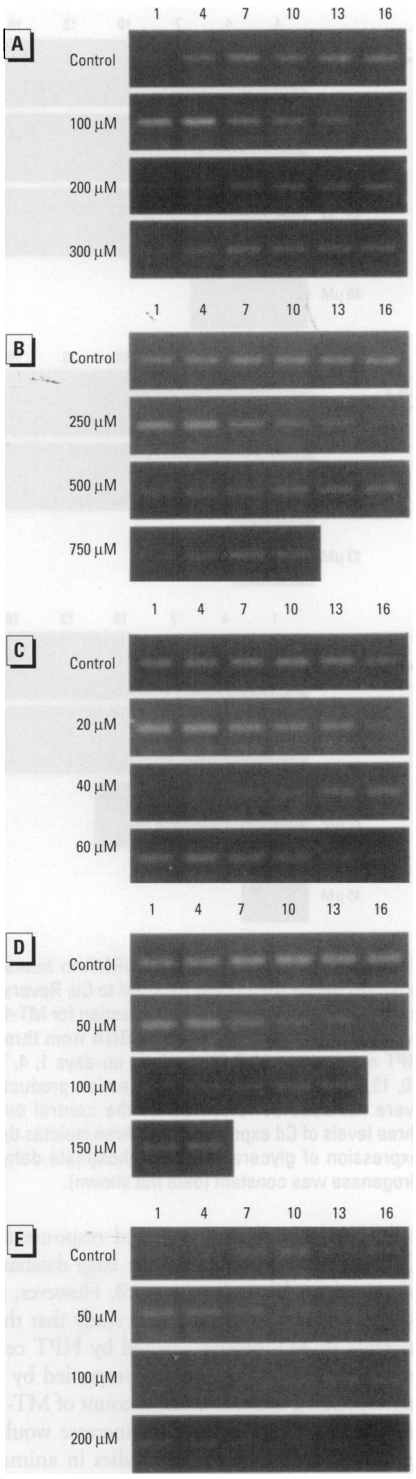


**Figure 9.** Expression of MT-1F mRNA in human proximal tubule cells exposed to Zn, Cu, Hg, Ag, and Pb. Reverse transcriptase-polymerase chain reaction for MT-1F was performed on RNA obtained following 1, 4, 7, 10, 13, and 16 days of metal exposure. Isolate 1 was exposed to the following metals at the indicated concentrations: (A) Zn, (B) Cu, (C) Hg, (D) Ag, and (E) Pb. Reaction products were removed at 40 cycles for all metals. The expression of glyceraldehyde 3-phosphate dehydrogenase was constant (data not shown).

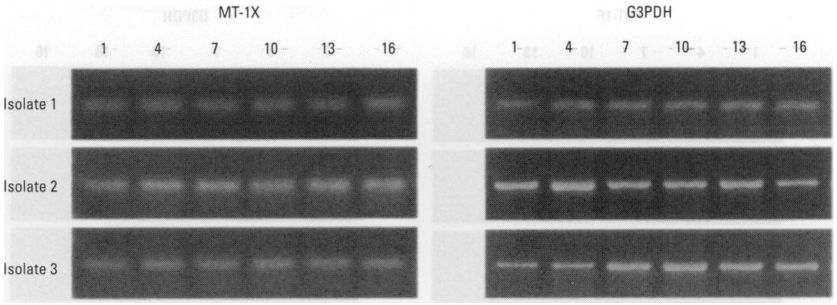


**Figure 10.** Expression of MT-1X mRNA in three human proximal tubule cell isolates. Reverse transcriptase-polymerase chain reaction for MT-1X and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA was performed using RNA obtained on days 1, 4, 7, 10, 13, and 16 of cell culture. Reactions were sampled at 30 cycles for G3PDH and 30 cycles for MT-1X.

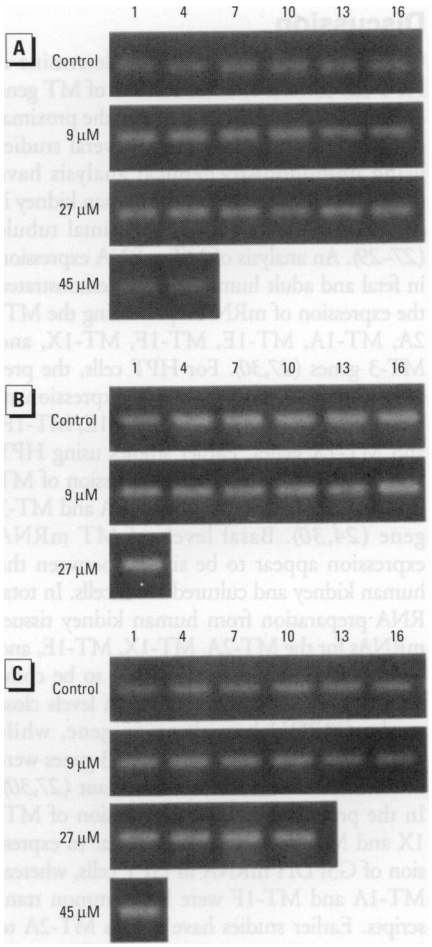


**Figure 11.** Expression of MT-1X mRNA in human proximal tubule (HPT) cells exposed to Cd. Reverse transcriptase-polymerase chain reaction for MT-1X was performed using RNA from three HPT cell isolates (A,B,C) obtained on days 1, 4, 7, 10, 13, and 16 of Cd exposure. Reaction products were removed at 30 cycles for the control and three levels of Cd exposure. The expression of glyceraldehyde 3-phosphate dehydrogenase was constant (data not shown).

$Cd^{2+}$ was MT-1 isoform specific. HPT cells demonstrated no basal or $Cd^{2+}$-induced expression of mRNA for the MT-1B, MT-1G, and MT-1H genes. HPT cells displayed notable basal levels of both MT-1E and MT-1X mRNA; however, only MT-1E mRNA was increased by exposure to $Cd^{2+}$. HPT cells displayed low basal levels of mRNA for both the MT-1A and MT-1F genes, although only MT-1A mRNA was increased by exposure to $Cd^{2+}$. These results, in conjunction with those for MT-2 described previously (*24*), provide convincing evidence that new features of MT gene regulation have been acquired in the human proximal tubule due to the gene duplication event in humans.

The most obvious consequence of the increased complexity and change in regulation of the human MT-1 and MT-2 genes would be to provide greater redundancy in the functional MT genes. The importance of redundancy in the MT genes can be inferred from the rodent species, in which the MT-1 and MT-2 genes are coordinately regulated and, from all examinations to date, the resulting proteins appear to be functionally equivalent. Such duplicate genes would not be expected to remain active if both were not needed for enhanced survival of the cell. Using HPT cells exposed to $Cd^{2+}$ as a model, the gene duplication event in the human would further increase the level of redundancy in the absolute number of the MT genes and also in the regulation of these genes. In HPT cells, the MT-2 and MT-1X genes were demonstrated to have basal mRNA expression levels similar to G3PDH and to be unaffected by $Cd^{2+}$ treatment. The MT-1E gene was also demonstrated to have a similar level of basal mRNA expression, but expression increased after exposure to $Cd^{2+}$. Messenger RNA for the MT-1A gene was shown to be a minor transcript in the HPT cells under basal conditions, but increased significantly as a result of $Cd^{2+}$ treatment that approached levels causing a loss of cell viability. Based on data obtained using HPT cells, the duplication event in human MT genes can be

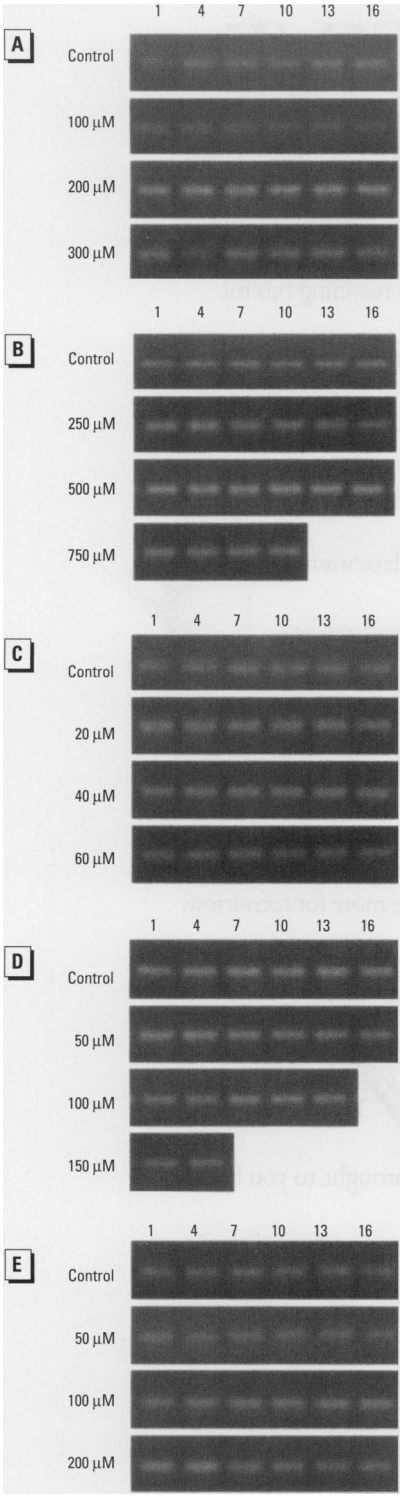


**Figure 12.** Expression of MT-1X mRNA in human proximal tubule cells exposed to Zn, Cu, Hg, Ag, and Pb. Reverse transcriptase-polymerase chain reaction for MT-1X was performed using RNA obtained on days 1, 4, 7, 10, 13, and 16 of metal exposure. Isolate 1 was exposed to the following metals at the indicated concentrations: (A) Zn, (B) Cu, (C) Hg, (D) Ag, and (E) Pb. Reaction products were removed at 30 cycles for all metals. The expression of glyceraldehyde 3-phosphate dehydrogenase was constant (data not shown).

interpreted as providing a separation in the regulatory responsibility of the MT genes. That is, one subset of the human MT gene family (MT-1X and MT-2A) is responsible for providing basal levels of MT mRNA expression to the cell, and another subset (MT-1E and MT-1A) is responsible for providing an increase in MT mRNA expression due to $Cd^{2+}$ exposure.

Further elucidation of the significance of an increased number of MT genes in humans is limited by the fact that no methods currently exist to determine the expression levels of individual MT-1 and MT-2 isoform-specific proteins. Without this ability, it is not possible to determine the relative contributions of decreased MT protein degradation and increased transcription rates on the induction of MT protein by metals. The antibodies currently available are believed to interact with all MT-1 and MT-2 isoforms, but this has never been convincingly shown, as the individual purified MT proteins are not available for cross-reactivity profiles. Recent development of the MT-1 and MT-2 null mouse allows the isolation of cell lines containing no basal MT protein levels. These should be excellent recipients for transfection by expression vectors containing each human MT isoform-specific gene, allowing the generation of homogeneous MT-isoform specific mRNA and proteins. The availability of such standards would allow the development and testing of assays capable of determining the level and functional properties of each specific MT isoform protein.

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