Suppression of Metallothionein-I/II Expression and Its Probable Molecular Mechanisms

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Metallothionein **(MT) promoter was methylated in rat hepatoma and in mouse lymphosarcoma cells by methylation of cytosine within the CpG dinucleotide region. After demethylation of *MT-I* promoter in mouse lymphosarcoma cells or in the transplanted rat hepatoma with 5-azacytidine, a potent inhibitor of DNA methyltransferase, the promoter was activated in response to heavy metal treatment. *MT-I* promoter was also suppressed in human prostate cancer lines PC3 and DU145, probably by promoter methylation, whereas cadmium induced MT-I in the human prostate cancer line LNCaP. In the prostate cancer lines where MT-I was suppressed, glutathione-*S*-transferase-pi (GST-pi) was expressed. On the contrary, *GST*-pi gene was repressed in the cell line where MT-I was induced, which suggests an inverse relationship between MT-I induction and GST-pi expression in some prostate cancer lines. The expressions of GST-pi and γ-glutamyl cysteine synthase were also significantly higher (5- to 12-fold) in the lymphosarcoma cells and the hepatoma relative to the parental tissues. The higher expressions of these two genes suggest a compensatory mechanism in the cells where the gene for the antioxidant MT-I/II is not induced. MT-I/II may function as a growth suppressor either alone or in concert with other factor(s), and consequently their lack of expression could facilitate the tumor growth. In addition to suppression of *MT-I/II* expression by promoter methylation, the lack of MT induction could also be brought about by nuclear factor I (NFI), probably by interaction with the metal transcription factor MTF-1. An inverse relationship was observed between the level of NFI and MT-I expression in some cells, which suggests a role for NFI in the relatively low constitutive levels of MT-I expression in these cells.** ***Key words:*** **5-azacytidine, DNA methylation, hepatoma, lymphosarcoma, metallothionein, MTF-1, NFI, transcriptional repression.** ***Environ Health Perspect*** **110(suppl 5):827–830 (2002).**
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Metallothioneins (MT) are highly conserved proteins that are expressed in all eukaryotes, including plants, yeast, worms, flies, and vertebrates. Four major isoforms, MT-I, MT-II, MT-III, and MT-IV, are known to exist. The two isoforms MT-I and MT-II are ubiquitously expressed, heavy metal–binding proteins. MT-III expression is unique to the brain and reproductive organs, whereas MT-IV expression is confined to the stratified squamous epithelium of skin, tongue, and intestinal lining. Unlike the constitutively expressed MT-III and MT-IV, MT-I and MT-II are coordinately regulated and are highly inducible by a variety of agents, including heavy metals, reactive oxygen species, ultraviolet radiation, glucocorticoids, and lipopolysaccharides [for a review, see (*1–3*)]. Significant induction of MT-I and MT-II has also been reported in the livers of Cu,Zn-superoxide dismutase knockout mice, probably as a compensatory mechanism to scavenge free radicals (*4*). Recent study has demonstrated that MT-I and MT-II can be induced robustly in the liver and lung after influenza virus infection by overlapping but distinct molecular mechanisms (*5*). Considerable efforts have been expended to elucidate the molecular mechanisms of MT induction [for a recent review, see (*3*)]. Contrary to the upregulation of MT expression, the down-regulation of its expression has not been explored to any great extent. The suppression of MT expression has been observed in Rat-1 cells that overexpress the large subunit of the protein Ku (*6*), by nuclear factor I (NFI) (*7*), and in some cancer cell lines and solid tumors (*8,9*). In this article we present a brief summary of these observations and potential molecular mechanisms for the suppression of MT expression under the different conditions.

Results and Discussion

Silencing of *MT-I* Gene in Cancer Cells and Probable Molecular Mechanisms

DNA methylation in animal cells plays a critical role in developmental process, epigenetic silencing, aging, carcinogenesis, X chromosome inactivation, and certain human genetic diseases (*10,11*). The most significant methylation occurs at position 5 of cytosine in the CpG dinucleotide. Methylation within the promoter regions invariably leads to silencing of the gene. We explored the possibility that the suppression of MT-I/MT-II induction in some tumor cells may be due to promoter methylation. Our initial study showed that *MT-I* gene was not induced in mouse lymphosarcoma cell lines (P1798) and a rat hepatoma, a solid tumor transplanted into the hind legs of rats. We confirmed the methylation of *MT-I* gene first by re-activating the promoter by 5-azacytidine (5-AzaC), a DNA-demethylating agent (Figure 1), and by determining the sites of methylation using bisulfite genomic sequencing (*8,9*). The latter technique consists of treatment of genomic DNA with the bisulfite reagent that converts unmethylated cytosine to uracil while the methylated cytosine remains unaltered. After strand-specific polymerase chain reaction (PCR) of the bisulfite-treated DNA, uracils and methylcytosines are amplified as thymines and cytosines (*12,13*). Analysis of the methylation sites by this technique showed that nearly all cytosines within the CpG dinucleotides in the promoter region were methylated in lymphosarcoma cells (*9*) as well as in rat hepatoma (Figure 2) (*8*). None of these cytosines were methylated in the corresponding parental tissues thymus and liver.

Recently, we have observed that *MT-I* gene is not induced in the prostate cancer cell lines PC3 and DU145, whereas the *GST*-pi gene is expressed in these cells (Figure 3). On the contrary, *MT-I* is induced by cadmium in the prostate cancer cell line LNCaP, in which *GST*-pi is suppressed due to promoter methylation (*14*). At present we do not know whether the lack of *MT-I* expression in the first two cell lines is due to the promoter methylation or deficiency of the metal regulatory transcription factor MTF-1 or to increased activity of a repressor (e.g., NFI). Similarly, it has been reported that preneoplastic nodules formed

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in rat liver cannot induce MT-I upon treatment with cadmium. It is conceivable that the promoter methylation may be responsible for the lack of MT induction in these nodules, in contrast to its induction in the adjacent normal tissue (*15*). We are in the process of analyzing whether an inverse relationship exists between the expression of *GST*-pi and *MT-I/MT-II* genes in other tumor cells. It would be of interest to determine whether this relationship is altered when the promoter of the suppressed gene is activated or when the promoter of the active gene is suppressed.

In vivo genomic footprinting analysis showed that the *MT-I* promoter in the lymphosarcoma cells and rat hepatoma was refractory to the positive transcription factors that included MTF-1, a key factor that recognizes the metal regulatory elements (MREs) and directs *MT* transcription. By contrast, the promoter was accessible to the transcription factors in the control tissues (*8,9*) after demethylation by 5-AzaC treatment, MT induction was regained in the tumor cells. When the tumor that regressed after 5-AzaC treatment was re-transplanted into new donor rats, the *MT* promoter was re-methylated and the tumor grew back. Restriction landmark genomic scanning with the methylation-sensitive enzyme Not I (*16*) of the liver and hepatoma DNA showed that many genes in addition to the *MT-I* gene were methylated in the hepatoma. To explore the potential molecular mechanisms of promoter methylation and silencing of *MT-I* and other genes, we analyzed the activities/expression of DNA methyltransferases and the methylcytosine-binding proteins. The activities of both maintenance and *de novo* DNA methyltransferases (DNMT-3a and -3b) were 3- to 6-fold higher in the hepatoma than in the liver. Immunoblot analysis demonstrated significantly higher levels of the *de novo* methyltransferases in the tumor than in the liver. The higher activities/levels of these enzymes are probably responsible for the re-methylation of completely demethylated (both strands) *MT-I* promoter after re-transplantation because the maintenance methyltransferase requires hemimethylated DNA as substrate. Chromatin immunoprecipitation experiments revealed close association of the methylcytosine binding protein MeCP2 with the *MT-I* promoter in the hepatoma, which suggested its role in the silencing of this promoter.

It is noteworthy that *MT-I* and *MT-II* belong to a select group of genes that are hypermethylated and silenced in some cancer cells. The expression of human *MT-IIA* gene is drastically reduced in human colorectal tumors and cell lines derived from these tumors compared with normal tissues (*17*). Further, immortalization of human cells also causes reduction in *MT* expression (*18*). Interestingly, the common tumor suppressor genes (e.g., *p53*, *Rb*, and *p16*) are expressed in the rat hepatoma (*19*). The unabated expression of these growth suppressor genes suggests that other growth regulatory genes are modulated in this and other tumors. *MT-I/MT-II* may have fulfilled this role, probably in concert with other factors. Interestingly, overexpression of *MT-I* has been shown to protect the transgenic mice from hyperplasia in the liver induced by hepatitis B viral antigen (*20*). This notion is supported by the observation that the suppression of *MT* expression is generally confined to cancer cells. Further study is needed to establish a direct role of MT as a tumor suppressor. Recent study in our laboratory using inhibitors of DNA methyltransferases and histone deacetylases has shown that the lack of MT induction in some cancer cells is also attributable to altered chromatin structure (*21*).

Probable Compensatory Mechanism(s) to Circumvent *MT* Silencing

If MT is an antioxidant, the silencing of its gene should have an adverse effect in the cells. It was therefore of interest to explore the possibility of the existence of a compensatory mechanism. To address this issue, we measured the mRNA levels of GST-pi and γ-glutathionyl cysteinyl synthase (the first enzyme

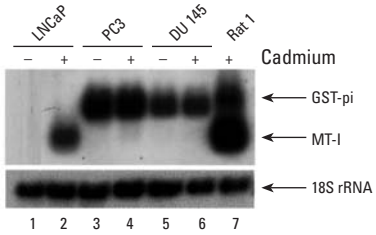

Figure 3. Lack of MT-I expression in some human prostate cancer cell lines. Total RNA (15 µg) isolated from control and 30 µM $CdSO_4$–treated prostate cancer cells was subjected to Northern blot analysis with MT-I or GST-pi cDNA or 18S rDNA. RNA from Rat-1 cells that express both MT-I and GST-pi was used as control.

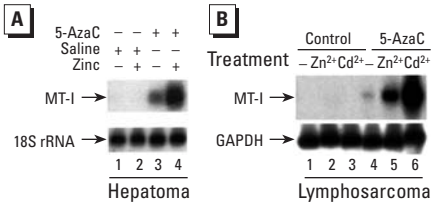

Figure 1. Northern blot analysis of *MT-I* gene expression by heavy metals in hepatoma and lymphosarcoma cells after treatment with 5-AzaC. (*A*) Hepatoma-bearing rats (3 weeks after tumor transplantation) were injected intraperitoneally with saline or 5-AzaC at a dose of 5 mg/kg body weight on alternate days for 2 weeks. The animals were then injected intraperitoneally with zinc sulfate (200 µmol/kg body weight), and sacrificed after 4 hr, and total RNA was isolated from the tumors. RNA (30 µg) was subjected to Northern blot analysis with ^{32}P-labeled random-primed MT-I cDNA or 18S rDNA. (*B*) Mouse lymphosarcoma cells (P1798) grown at a density of 1×10^6/mL were divided into four groups. One group was treated with 50 µM $ZnSO_4$ or 15 µM $CdSO_4$ for 3 hr. The other group was treated with 2.5 µM 5-AzaC for 72 hr before treatment with $ZnSO_4$ or $CdSO_4$ for 3 hr. Total RNA was isolated from the different samples, and 30 µg from each sample was subjected to Northern blot analysis with MT-I or glyceraldehyde-3-phosphate dehydrogenase cDNA.

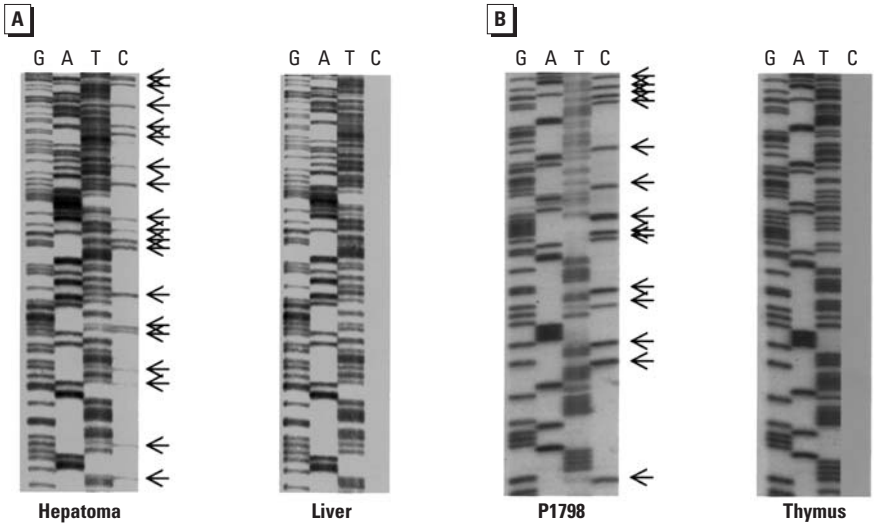

Figure 2. Bisulfite genomic sequencing of MT-I promoter in rat hepatoma and host liver (*A*) and in mouse lymphosarcoma cells and thymus (*B*). Genomic DNA isolated from the tissues or cells was subjected to bisulfite treatment after our own protocol (*13*), and the bisulfite-converted DNA was subjected to nested PCR with primers specific for rat or mouse *MT-I* gene. The amplified product was sequenced with the nested PCR primers as described (*9,13*) using ^{33}P-labeled dideoxy chain terminator kit (USB Corp., Cleveland, OH, USA). Arrows indicate the 5-methylcytosines.

involved in the biosynthesis of glutathione). The expressions of both genes were significantly higher in the lymphosarcoma cells (10- and 5-fold, respectively) and the hepatoma (8- and 12-fold, respectively) relative to the expression in the parental tissues (Figure 4).

The consequence of *MT* silencing in some cancer cells deserves comment. It is conceivable that the relative lack of *MT-I/MT-II* expression in these cells could result in greater availability of zinc for a variety of zinc finger proteins that control transcription. Another means of regulating the level of cellular zinc is via the zinc transporters (*22*). Analysis of both the plasma membrane zinc effluxers (ZnT1) and influxers (ZIP2) revealed that the zinc effluxer is constitutively expressed at a much higher level (8-fold) in the hepatoma than in the liver, whereas the basal level of influxer is 3-fold higher in the liver compared with hepatoma (*23*). The lack of *MT* expression might be beneficial when there is increased demand for zinc in rapidly proliferating hepatoma. However, because MT acts as the storage form of zinc and helps in maintaining zinc homeostasis, its absence probably results in higher basal level of zinc effluxer expression in the hepatoma. Indeed, the zinc content per gram wet weight in the hepatoma is less that in the liver, as determined by atomic absorption spectra (*24*). A direct link between *MT-I* promoter inactivation and tumorigenesis has yet to be established. Nevertheless, the potential role of zinc in tumor growth and its association with MT may play a key role in controlling the growth of at least some tumors, particularly rapidly proliferating cancer cells.

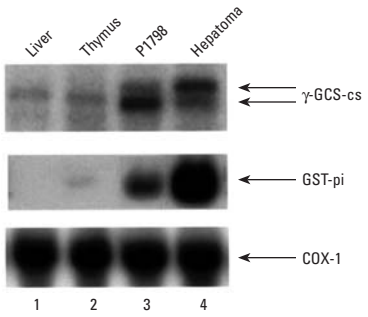

Figure 4. Northern blot analysis of γ-glutamyl cysteinyl synthase catalytic subunit (γ-GCS-cs) and GST-pi expression in Morris hepatoma 3924A. Poly(A)$^+$ RNA (5 μg) isolated from the rat liver and hepatoma as well as mouse thymus and P1798 cells was subjected to Northern blot analysis with ^{32}P-labeled cDNA for catalytic subunit of γ-glutamyl cysteinyl synthase, GST-pi, or cytochrome *c* oxidase 1 (COX-1).

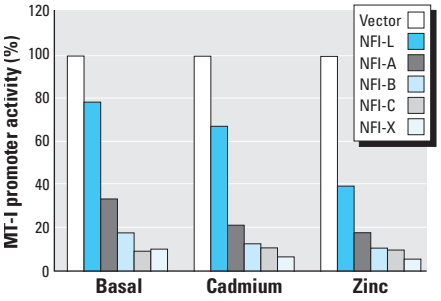

Figure 5. Effect of different NFI isoforms on MT-I promoter activity. HepG2 cells were transiently transfected with pMT-Luc (MT-I promoter–luciferase reporter) plasmid along with expression vectors for different NFI isoforms, as indicated. NFI-L is a rat liver isoform, and NFI-A, -B, -C, and -X are different mouse isoforms. The effects of NFI isoforms on basal, cadmium-, and zinc-induced MT-I promoter activity are presented as percentage of MT-I promoter activity retained in the presence of the NFI isoforms.

Role of NFI as a Repressor of the Constitutive and Induced Expression of *MT-I*

Analysis of the *MT-I* promoter revealed three half-sites for the binding of NFI, a protein expressed ubiquitously in higher eukaryotes. Distinct, highly conserved genes encode four isoforms of NFI protein (NFI-A, NFI-B, NFI-C, and NFI-X) in vertebrates (*25–28*). The DNA-binding domain is located in the N-terminal region, whereas the *trans*-activation/repression property is governed by the more heterogeneous C-terminal end. The different isoforms of NFI protein can function both as a positive and negative regulator of gene expression (*29*). We explored the possibility that NFI could function as a repressor of *MT-I* expression because this protein is known to down-regulate the expression of several genes in a cell type–specific manner.

To investigate the effect of NFI on *MT-I* expression, we performed a series of transient transfection assays using a promoter/reporter construct containing mouse *MT-I* promoter-driven luciferase reporter gene and eukaryotic vectors expressing NFI proteins. Overexpression of the different isoforms of NFI caused suppression of both constitutive and heavy metal (Cd or Zn)–induced *MT-I* promoter activity in HepG2 cells (Figure 5). The extent of the NFI-mediated inhibitory effect varied with the isoforms used in the transfection assay, although the expression levels of the five isoforms used were comparable by Western blot analysis. Electrophoretic mobility shift assays and antibody supershift analysis showed that the NFI family of proteins can interact with MRE-c′ on *MT* promoter (*7*). A mobility shift assay (Figure 6A) using three different cell lines showed different levels of NFI complex formation, depending upon the cell types, which was consistent with the concentration of NFI in these cells (Figure 6B). The most noteworthy observation was the inverse relationship between the level of NFI and *MT-I* expression (Figure 6C). Accordingly, mouse embryonic stem cells that contained the least amount of NFI expressed the highest level of MT-I. Interestingly, the NFI-mediated repression of *MT-I* promoter activity did not require NFI–DNA interaction. The lack of requirement of the NFI binding site was confirmed by the downregulation of *a*) a synthetic *MT-I* promoter deprived of the NFI binding site by NFI proteins or *b*) natural *MT-I* promoter by an NFI-C deletion mutant lacking the DNA-binding domain. Overexpression of MTF-1 could overcome the NFI-mediated inhibitory effects on the constitutive and induced expression of *MT-I* (*7*). Preliminary study did not reveal direct interaction of MTF-1 with NFI. It is reasonable to conclude that NFI suppresses *MT-I* expression, at least in part, by inhibiting the MTF-1 activity.

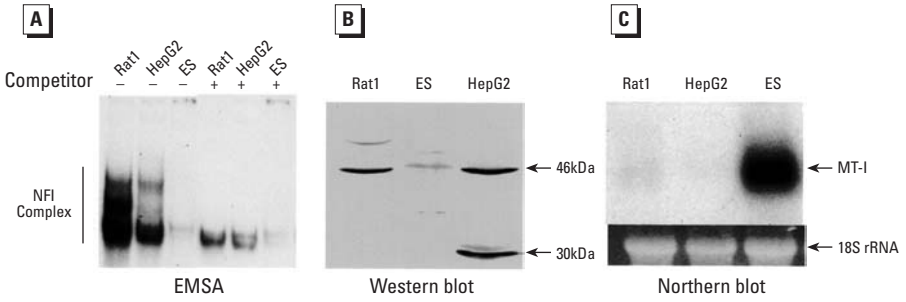

Figure 6. (*A*) Electrophoretic mobility shift assay (EMSA) of NFI complex present in different cell lines. Whole-cell extracts from Rat-1, HepG2, and mouse embryonic stem (ES) cells were allowed to bind to ^{32}P-labeled MRE-c′ oligonucleotide in appropriate binding buffer. The DNA protein complex was then separated by 4% SDS-PAGE and analyzed by autoradiography. Cold NFI consensus oligo at 100-fold molar excess was used as competitor, as indicated in the figure. (*B*) Western blot analysis of NFI protein. Whole-cell extracts from Rat-1, HepG2, and mouse ES cells were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with anti-NFI antibody. (*C*) Northern blot analysis of MT-I expression. Total RNA (30 μg) isolated from Rat-1, HepG2, and mouse ES cells was separated on 1.0% agarose–formaldehyde gel, transferred to nylon membrane, and probed with ^{32}P-labeled mouse MT-I probe. Ethidium bromide profile of 18S ribosomal RNA was used as RNA loading control.

Concluding Remarks

Despite considerable efforts to elucidate the molecular mechanisms for the constitutive and induced expression of MTs, relatively little information is available regarding the repression of *MT* expression. The present article has discussed the conditions for the suppression of MT expression and its potential molecular mechanisms. The silencing of a highly inducible protein such as MT-I/MT-II in many tumor cells is of considerable interest. The key issue is why it is not induced in some of these rapidly proliferating cells. It is convenient to discard the significance of these data by advocating that MT is not essential for the cell survival. However, this article has proposed novel ideas to explain some of these data. These include the potential role of MT in retarding the growth of rapidly proliferating cancer cells, probably in concert with other growth suppressors, or lack of MT that might facilitate the availability of free zinc for tumor growth. Clearly, further study is needed to address this important issue.

The observation that NFI can downregulate *MT* expression is of considerable interest. A potential inverse relationship between the NFI concentration in the cell and MT-I expression is evident from this study. There is indication that NFI inhibits *MT* suppression by interfering with the activity of the transcription factor MTF-1. The exact mechanism for the modification of the MTF-1 activity by NFI should now be established.

Finally, other suppressors of *MT* expression should be explored. It is known that treatment of cells or tissues with cycloheximide, a potent inhibitor of protein synthesis, can robustly enhance *MT* expression. The inhibitor of MT induction that was suppressed by inhibition of protein synthesis remains to be identified. Our preliminary observations suggest that NFI is not related to this inhibitor. It is important to investigate the physiological and pathological conditions that facilitate *MT* suppression and the functional relevance of such repression.

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