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-aza-cytidine, 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 10-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-aza-cytidine, DNA methylation, hepatoma, lymphosarcoma, metallothionein, MTF-1, NFI, transcriptional repression. Environ Health Perspect 110(suppl 5):827–830 (2002).

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 in...
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 p1619) 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 1](https://example.com/figure1.png)

**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 32P-labeled random-primed MT-I cDNA or 18S rDNA. (B) Mouse lymphosarcoma cells (P1798) grown at a density of 1 x 10^6/ml were divided into four groups. One group was treated with 50 µM ZnSO4 or 15 µM CdSO4 for 3 hr. The other group was treated with 2.5 µM 5-AzaC for 72 hr before treatment with ZnSO4 or CdSO4 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](https://example.com/figure2.png)

**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 (8,13) using 32P-labeled dideoxy chain terminator kit (USB Corp., Cleveland, OH, USA). Arrows indicate the 5-methylcytosines.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Lack of MT-I expression in some human prostate cancer cell lines. Total RNA (15 µg) isolated from control and 30 µM CdSO4–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.
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.

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-I 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 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 P1789 cells was subjected to Northern blot analysis with 32P-labeled mouse MT-I 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-I 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 32P-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 32P-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 the retardation of 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 down-regulate 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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