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Human Metallothionein Isoform Gene Expression in Cisplatin-Sensitive and Resistant Cells

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SUMMARY

Overexpression of metallothioneins (MTs) has been observed in some *cis*-diamminedichloroplatinum (CDDP)-resistant cells. We have developed oligonucleotide probes for each of the six non-neuronal human MT (hMT) isoforms and used them to assay hMT isoform expression in three pairs of CDDP-resistant and -sensitive human carcinoma cell lines, i.e., SCC25/CP versus SCC25 cells, H69/CP versus H69 cells, and SW2/CP versus SW2 cells. We found a 9-fold increase in basal hMT-IIa mRNA levels and a 5-fold increase in hMT-Ie mRNA levels in SCC25/CP cells, compared with SCC25 cells. Nuclear run-on studies also revealed a 3-fold increase in hMT-IIa transcription rate. Basal hMT-IIa steady state mRNA levels were 2–3.6-fold greater in H69/CP and SW2/CP cells, compared with their parental cells. No significant basal expression of hMT-Ia, -Ib, -If, or -Ig was detected in any cells, suggesting that overexpression of these isoforms was not commonly associated with the CDDP-resistant phenotype. Levels of constitutively expressed hMT isoforms, as well as hMT-If, could be elevated by treatment of all cells with 100 μM zinc. The universal overexpression of hMT-IIa suggests a role of this particular isoform in CDDP resistance. Using our isoform-specific hMT-IIa probe and the demethylating agent 5′-azacytidine (AZC), we found that AZC pretreatment increased basal hMT-IIa mRNA levels in SCC25 but not SCC25/CP cells, suggesting that DNA hypomethylation was responsible for higher basal hMT-IIa mRNA levels in SCC25/CP cells. AZC had little or no effect on hMT-If or -Ig expression. Limited restriction analysis by methylation-sensitive enzymes, however, revealed no obvious differences in the methylation status of the hMT-IIa promoter in either SCC25 or SCC25/CP cells.

CDDP is effective in the treatment of testicular, ovarian, bladder, and head and neck carcinomas and has an expanding role in the therapy of small cell carcinoma of the lung (1). Nonetheless, both acquired and intrinsic tumor cell resistance limits its usefulness, as well as that of new analogues. Several mechanisms have been proposed as contributors to the CDDP resistance phenotype. These include decreased drug accumulation (2), increased DNA repair (3), elevated glutathione levels (4), and overexpression of thiol-rich MTs (5). Controversy exists, however, concerning the relative importance of each of these putative mechanisms, as well as precisely how such mechanisms are activated.

The focus of our work has been to characterize further the nature of MT overexpression in a limited selection of CDDP-resistant human tumor cell lines. MTs are induced by a variety of pharmacological and environmental substances, including heavy metals such as cadmium, zinc, and copper (6). To our knowledge, all cell lines examined to date that both are resistant to heavy metals and overexpress MT are cross-resistant to CDDP. Elevated levels of MT have also been reported in some CDDP-resistant tumor cells (7, 8). We have been unable, however, to demonstrate reproducibly that acute CDDP treatment induces MT in cultured cells, suggesting that the elevated levels of MT may reflect a cell selection process. MT mRNA content in some human tumors is markedly greater than in normal tissues, but initial attempts to correlate MT levels with patient tumor response to CDDP have not been successful (9). Gene transfer approaches to increase MT expression indicate that some but not all cells become resistant to alkylating and platinating agents (8, 10–12).

All eukaryotes have the potential to synthesize multiple isoforms of MT, but there is no information on the specific isoforms expressed by anticancer drug-resistant cells. In human cells, there are seven known MT isoforms (13), one of which is thought to be specifically neuronal (14). Previous gene transfer studies (8, 10–12) have all used hMT-IIa genomic constructs.

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ABBREVIATIONS: CDDP, (*cis*)-diamminedichloroplatinum; MT, metallothionein; hMT, human metallothionein; FBS, fetal bovine serum; AZC, 5′-azacytidine; MRE, metal regulatory element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair(s).

Because MT isoforms may not be functionally equivalent, we believe it is important to examine the hMT isoform expression pattern in CDDP-resistant cells. Thus, we developed synthetic oligodeoxyribonucleic acid probes that are specific for each non-neuronal MT isoform and we examined the hMT isoform expression in several human tumor cell lines with acquired resistance to CDDP. We found that the basal mRNA levels of hMT-IIa and -Ie are increased in SCC25/CP cells, compared with parental SCC25 squamous carcinoma cells, and basal hMT-IIa mRNA levels are also increased in H69/CP and SW2/CP cells, relative to their sensitive parental small cell lung cancer cells.

We have also used these probes to begin to address the mechanistic basis for the increase in hMT isoform mRNA in CDDP-resistant cells. The elevated steady state mRNA levels for MT-IIa appeared to result from an increased transcription rate. Thus, our attention focused on epigenetic changes in drug-resistant cells, specifically DNA methylation, because previous studies revealed that DNA methylation at the 5′ position of cytosine residues, especially within regions rich in CpG (CpG islands), suppressed gene transcription (15). In addition, the demethylating agent AZC has been shown to stimulate transcription of many genes including MT (16). Thus, in the present study, we not only have assessed the native methylation patterns in the hMT-IIa promoter regions of CDDP-sensitive and -resistant cells by restriction enzyme digestion but also have used AZC to examine the expression of the hMT isoforms in CDDP-sensitive and -resistant cells.

Materials and Methods

Cell culture. The SCC25 human head and neck squamous cell carcinoma cell line (American Type Culture Collection, Bethesda, MD) and the SCC25/CP subline, which is 5-fold resistant to CDDP (17) (a generous gift from Dr. B. Teicher, Dana-Farber Cancer Institute, Boston, MA), were grown as monolayers in Dulbecco's modified Eagle medium supplemented with 10% FBS, 400 ng/ml hydrocortisone, 100 IU/ml penicillin, and 100 μg/ml streptomycin. H69 human small cell lung cancer cells and H69/CP cells, which are 11-fold resistant to CDDP, have been characterized previously (18). Both cell lines were propagated in CDDP-free RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. SW2 small cell lung carcinoma cells and SW2/CP cells, which are 4-fold resistant to CDDP (8) (a generous gift from Dr. B. Teicher), were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. All cell lines were cultured in a humidified atmosphere of 5% CO_2 at 37° and were routinely found to be free of *Mycoplasma*. Media were purchased from GIBCO (Grand Island, NY) and FBS from HyClone (Logan, UT). Unless noted otherwise, all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

AZC and inducer treatments. Exponentially growing cells were cultured with or without 16 μM AZC for 72 hr and then treated with phosphate-buffered saline, 100 μM $ZnCl_2$, or 10 μM $CdCl_2$ for another 9 hr. The concentration of AZC and the incubation time used in these experiments were based on previously published reports (16) and our preliminary qualitative toxicity experiments. We found that treatment of cells with 16 μM AZC for 72 hr caused a ≤20% decrease in cell viability, based on trypan blue staining. The metal concentration and the exposure time for these cells were based on optimization by previous investigators (19).

RNA isolation and Northern blot analysis. Total cellular RNA was extracted by acid guanidinium isothiocyanate-phenol-chloroform extraction (20). In brief, cells were washed twice with ice-cold phosphate-buffered saline and harvested by direct addition of 4 M guanidine thiocyanate solution (containing 25 mM sodium citrate, 0.5% *n*-lauryl sarcosine, and 50 mM 2-mercaptoethanol), followed by phenol-chloroform extraction and absolute ethanol precipitation. Total RNA (10 μg/lane) was separated by electrophoresis on 1% agarose-formaldehyde gels, transferred to Nytran nylon membrane, UV-cross-linked, and then hybridized with ^{32}P-labeled hMT isoform-specific oligonucleotide probes. hMT isoform-specific oligomer probes containing a 5′-hydroxyl group were labeled with a BRL forward reaction kit, to specific activities of approximately 1.0×10^6 dpm/pmol. All radiolabeled probes were purified with Bio-Spin-30 columns (Bio-Rad, Richmond, CA). Hybridization was performed as described by Sambrook *et al.* (21). Autoradiograms were exposed for 6 days and scanned using an LKB Ultrascan XL laser densitometer, and relative signal intensity was determined using the Gelscan HL software package. In all experiments, samples were evaluated with duplicate gels. Ethidium bromide staining of 28 S and 18 S RNA was examined to ensure equal loading of samples. All blots were stripped and rehybridized with human β-actin cDNA or, in later experiments, a 28 S rRNA 40-mer probe (Oncogene Science Inc., Uniondale, NY), to ensure integrity of the RNA samples and to confirm that equal amounts of RNA had been loaded onto each lane.

Design of hMT isoform oligomer probes. There are six non-neuronal hMT isoforms, Ia, Ib, Ie, If, Ig, and IIa, each encoded by a separate gene. We synthesized 60-mer oligonucleotide probes that contained 40–50 antisense nucleotides that hybridize selectively to the 5′ nontranslated region of the six non-neuronal hMTs and 10–20-nucleotide nonhybridizing overhangs designed to allow for future monitoring by RNase digestion. Fig. 1A shows schematically the location

Fig. 1. A, General design and schematic location of six hMT isoform-specific oligomers. ▩, The 10–20-nucleotide 5′ region, which does not hybridize with any hMT sequences; ■, the 40–50-nucleotide antisense sequence of the 5′ untranslated region of the six non-neuronal hMTs. B, Sequences of hMT isoform-specific oligomers designed to hybridize with 5′ nontranslated regions of antisense hMT mRNA.

of the sites of hybridization on the different hMT genes; Fig. 1B shows the sequence of the six oligomer probes. These oligomer sequences contain >60% GC-rich sequences, do not cross-hybridize with each other, and have no significant sequence homology, as determined by PC/GENE (version 6.6), with any other mammalian DNA in the GenBank database.

Nuclear run-on assay. DNA plasmids containing either genomic or cDNA inserts, namely MT-IIa.pUC9 (genomic hMT-IIa), pHβactin-1 (human β-actin cDNA), prGAPDH (rat GAPDH cDNA), PSKS (negative vector control), pH-jun (cDNA), and pT7-fos (cDNA), were used to transform competent DH5α cells. Large-scale amplification of DNA using cesium chloride-ethidium bromide gradient ultracentrifugation was performed following the methods of Sambrook *et al.* (21). Each plasmid DNA was linearized with the appropriate restriction enzyme, slot-blotted onto a nylon membrane, and UV cross-linked. Cells (1×10^8) were harvested and nuclei were prepared using nonionic detergents, as described previously by Dignam *et al.* (22). Nuclei were incubated at 37° for 40 min with 250 μCi of [α-^{32}P]UTP (800 Ci/mmol; DuPont-NEN, Boston, MA) and 0.5 mM levels of ATP, GTP, and CTP, to elongate new RNA transcripts. The ^{32}P-labeled RNA transcripts were then purified and hybridized to the immobilized DNA at 45° for 3 or 4 days. After washing, the hybridized radioactivity on the nylon membrane was visualized by autoradiography and quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Samples were normalized to β-actin levels.

Restriction analysis with methylation-sensitive enzymes. Genomic DNA was isolated from CDDP-sensitive and -resistant H69 and SCC25 cells by standard methods (21). DNA (10 μg) was digested with the methylation-sensitive enzymes *Ava*II, *Bss*HII, *Hinf*I, *Nar*I (New England Biolabs, Beverly, MA), *Hpa*II, and *Eco*RII (BRL, Grand Island, NY) according to supplier recommendations and with *Hind*III and *Bgl*II, which cut at the 5′ and 3′ extremes, respectively, of the hMT-IIa promoter region. Digests were separated by 2% NuSieve (FMC BioProducts, Rockland, ME) agarose electrophoresis, Southern (alkaline) blotted onto Zeta-probe charged nylon membranes (Bio-Rad), hybridized to a 1037-bp *Hind*III/*Bgl*II fragment specific for the hMT-IIa promoter region, and autoradiographed. Due to the high frequency of *Hpa*II sites, these digests were analyzed by 10% polyacrylamide gel electrophoresis, electrophoretically transferred to nylon membranes (Transblot; Bio-Rad), and hybridized as described above.

Results

Basal pattern of hMT isoform expression in CDDP-sensitive and -resistant cells. Elevated MT mRNA levels have been observed in cells with acquired CDDP resistance (5). To determine whether a specific isoform was overexpressed in the CDDP-resistant cells, we used our six isoform-specific oligomers to probe hMT isoform expression patterns in the following three pairs of CDDP-sensitive and -resistant carcinoma cell lines: SCC25 versus SCC25/CP, H69 versus H69/CP, and SW2 versus SW2/CP cells. In all three pairs of cell lines, the basal level of hMT-IIa was higher than that of any other isoform. As illustrated in Fig. 2, *lanes 0*, hMT-IIa was not the only basal hMT isoform expressed in SCC25 and SCC25/CP cells; hMT-Ie was also seen in untreated SCC25 and SCC25/CP cells. hMT-Ia, -Ib, and -Ig were not detected at any time in either cell type. Densitometric scanning demonstrated a 9.2-fold increase in basal hMT-IIa expression and a 5-fold increase in basal hMT-Ie expression in SCC25/CP cells. In similar studies of basal expression, only hMT-IIa was readily detected in SW2, SW2/CP, H69, and H69/CP cells. The SW2/CP and H69/CP cells displayed 3.6- and 2.0-fold more basal hMT-IIa than did their respective parental cells (Table 1).

Metal inducibility of hMT isoform expression. Our

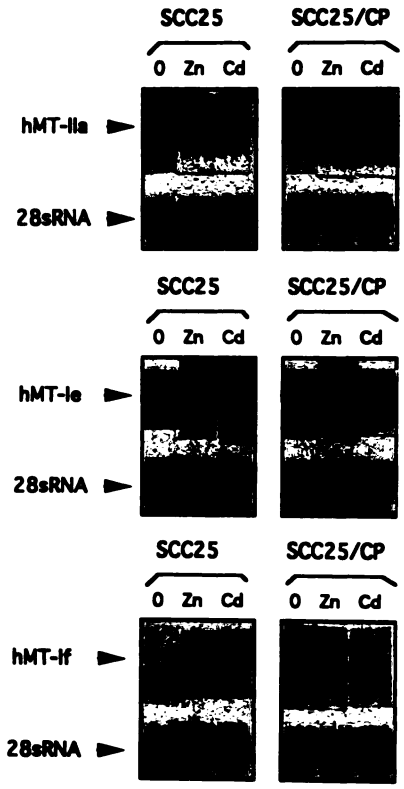

Fig. 2. Northern blot analysis of the relative expression of hMT isoform-specific mRNA in SCC25 and SCC25/CP cells. Total cellular RNA was isolated after metal treatment for 9 hr. *Lanes 0*, basal level control; *lanes Zn*, 100 μM zinc; *lanes Cd*, 10 μM cadmium. RNA samples (10 μg) were denatured, size-fractionated on 1% agarose gels, and transferred to nylon membranes. *Upper*, hMT-IIa expression; *middle*, hMT-Ie expression; *lower*, hMT-If expression. hMT-Ia, hMT-Ib, and hMT-Ig expression was undetectable (data not shown). We also show the hybridization of a 28 S RNA oligomer probe with stripped blots, to permit normalization of total RNA loading.

previous studies suggested that hMT levels were not induced in SCC25 or SCC25/CP cells by CDDP treatment. Nonetheless, MT levels can be readily induced in cells by a number of pharmacological and environmental agents, which utilize distinct 5′ *cis*-regulatory regions of MT genes (23). Therefore, we examined the hMT isoform-specific mRNA accumulation in response to a 9-hr exposure to two prototypic metal inducers (100 μM zinc or 10 μM cadmium). Northern blot analyses (Fig. 2, *lanes Zn* and *Cd*) showed that after cadmium or zinc treatment hMT-IIa levels in SCC25/CP cells were higher than those in SCC25 cells. hMT-Ie was induced by zinc in both cell types, although cadmium treatment caused induction of hMT-Ie expression only in SCC25 cells. Cadmium caused hMT-If expression in SCC25 cells but none was detectable in SCC25/CP cells. Zinc induced hMT-If in both cell types. In the presence of zinc or cadmium, hMT-Ia, -Ib, and -Ig mRNA levels remained undetectable.

Similar studies were performed with the H69 and SW2 CDDP-sensitive and -resistant cells (Fig. 3). As mentioned earlier, only hMT-IIa mRNA was detected in these cells in the absence of any treatment, but after exposure of cells to 100 μM zinc for 9 hr hMT-IIa mRNA levels in H69, H69/CP, SW2, and SW2/CP cells were increased, hMT-If was seen in all four cell lines, and hMT-Ig mRNA was detected in H69 and H69/CP cells. Interestingly, induction of hMT-Ig and -If was significantly less in H69/CP than in H69 cells. In contrast, no

TABLE 1

Basal level hMT isoform mRNA expression in CDDP-resistant cells

Each numerical value is the relative fold increase in hMT isoform expression, compared with the parental cell lines, from scanning laser densitometric quantitations of two or more determinations.

Cell lines	Fold increase					
	hMT-IIa	hMT-Ia	hMT-Ib	hMT-Ie	hMT-If	hMT-Ig
SCC25/CP	9	ND[a]	ND	5	NE	NE
SW2/CP	3.6	ND	ND	NE	NE	ND
H69/CP	2	ND	ND	NE	ND	ND

[a] ND, not detectable; NE, no basal expression in parental cells, with detectable expression only in CDDP-resistant cells.

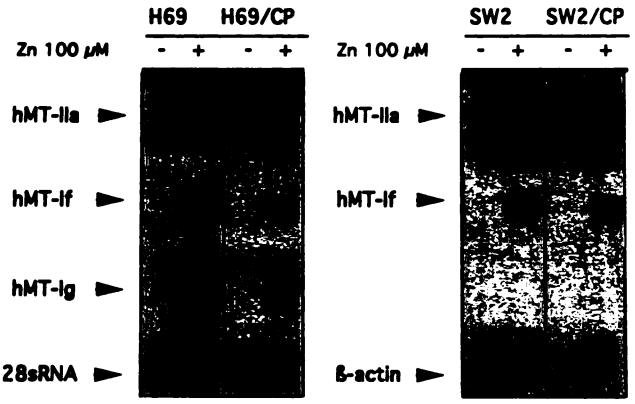

Fig. 3. Northern blot analysis showing the metal inducibility of hMT-IIa, -If, and -Ig mRNA in small cell lung cancer cells, i.e., H69, H69/CP, SW2, and SW2/CP cells. *Lanes* −, basal level control; *lanes* +, zinc (100 μM) treatment for 9 hr. *Left*, H69 and H69/CP cells. *Right*, SW2 and SW2/CP cells. Hybridizations are the same as in Fig. 2, except that we used a human β-actin probe to permit normalization of total RNA loading in SW2 and SW2/CP cells.

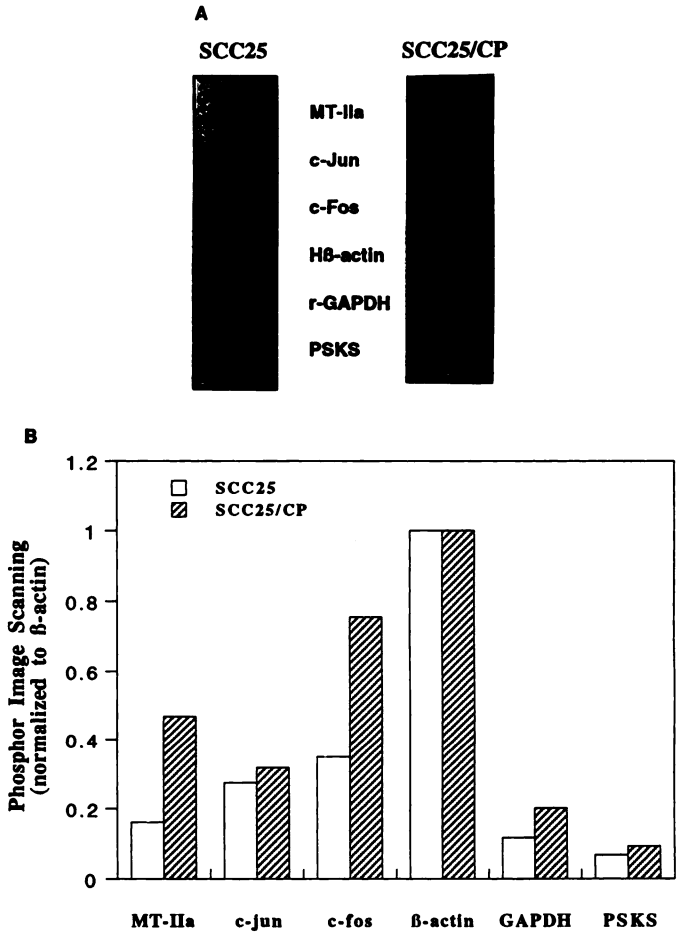

Fig. 4. Nuclear run-on analysis of hMT-IIa. Plasmid DNA (5 μg) was linearized with the appropriate restriction enzyme, slot-blotted onto nylon membranes, and immobilized by UV-cross-linking. The nuclei of SCC25 or SCC25/CP cells were prepared from 1×10^8 cells and *in vitro* transcription was performed at 37° for 40 min in the presence of [^{32}P] UTP (250 μCi/reaction) mixed with 0.5 mM ATP, GTP, and CTP. New RNA transcripts were isolated, hybridized at 47° for 72 hr, and autoradiographed. A, Nuclear run-on autoradiograph. The plasmids containing the genomic or cDNA inserts are (from *top* to *bottom*) MT-IIa.pUC9, pH-jun, pT7-fos, pHβactin-1, prGAPDH, and PSKS (negative control). B, Quantitation of nuclear run-on data by PhosphorImager scanning and normalization to β-actin.

difference in hMT-If isoform expression between SW2 and SW2/CP cells was noted after zinc treatment. These data suggest that the metal inducibility of individual hMT isoforms differs among cell lines; they also indicate that metal inducibility is differentially regulated between CDDP-sensitive and -resistant cells.

Analysis of hMT-IIa transcription rate. The nuclear run-on assay is currently the most sensitive procedure for measuring specific gene transcription. Thus, we used this assay to examine the transcription rate of hMT-IIa, the most commonly overexpressed isoform in SCC25 and SCC25/CP cells. This pair had the highest level of hMT-IIa overexpression of any we studied. The ^{32}P-labeled RNA transcripts were hybridized with plasmids immobilized on nylon membranes containing genomic DNA or cDNA for hMT-IIa as well as β-actin, GAPDH, *jun*, and *fos*. The radioactivity was visualized by autoradiography (Fig. 4A). Quantitation by PhosphorImager scanning (Fig. 4B) showed that hMT-IIa transcription normalized to that of β-actin was elevated approximately 3-fold in SCC25/CP cells, compared with SCC25 cells. No differences were seen with c-*jun* or with the "housekeeping genes" β-actin and GAPDH; the negative control plasmid PSKS was not detected in either sample. Interestingly, in light of the results of Scanlon *et al.* (24), c-*fos* was also increased in SCC25/CP cells.

Restriction analysis of hMT-IIa 5′ flanking region. Our nuclear run-on results indicated a 3-fold increase in the basal hMT-IIa transcription rate in SCC25/CP cells, compared with SCC25 cells (Fig. 4). Thus, the elevated basal levels of hMT-IIa and -Ie mRNA could be due to differences in specific *cis*-acting sequences and/or *trans*-acting factors between CDDP-sensitive and -resistant cells. Because CpG methylation within the promoter region of some genes has been implicated in the negative regulation of gene expression, we investigated the methylation status of the 5′ untranslated region of hMT-IIa in CDDP-sensitive and -resistant cell lines. Examination of this region revealed the presence of a number of sites that are recognizable only in the unmethylated state by limited

numbers of specific restriction enzymes (Fig. 5). We performed experiments using DNA from CDDP-sensitive and -resistant SCC25 and H69 cells digested with the methylation-sensitive enzymes in the presence of "bordering" enzymes *Hind*III and *Bgl*II, the latter of which excised the 1037-bp fragment of interest from the genomic DNA. Upon probing with the identical intact *Hind*III/*Bgl*II fragment, the DNA restriction pattern was compared between pairs of CDDP-sensitive and -resistant cells using *Ava*II, *Bss*HII, *Eco*RII, *Hinf*I, and *Nar*I. We found that restriction fragments of identical sizes were generated by CDDP-sensitive and -resistant cells, regardless of the enzyme (data not shown).

Similarly, polyacrylamide gel separation of the *Hpa*II digests from SCC25 and SCCC25/CP cells revealed identical patterns of separation, again suggesting no difference in the methylation status of the hMT-IIa promoter region (data not shown). Thus, differential patterns of methylation between drug-sensitive and -resistant cells were not detected, but it must be noted that only 20% of the total number of CpG dinucleotides could be analyzed within the hMT-IIa promoter sequence using the available restriction enzymes. The DNA methylation pattern did differ between the SCC25 and H69 cell lines; the major band of the *Bss*HII digest demonstrated a 0.4-kilobase shift in H69 and H69/CP, relative to the SCC25 and SCC25/CP digests, indicating that one of the two sites was affected in H69 and H69/CP cells, by either methylation or frank mutation.

AZC effects on both basal and metal-induced hMT isoform expression. Due to the large percentage of potential CpG methylation sites inaccessible to analysis by restriction enzymes, we used a pharmacological approach and treated SCC25 and SCC25/CP cells with the hypomethylating agent AZC. These cells were chosen because they exhibited the greatest overexpression of hMT-IIa. Fig. 6, *lanes control*, shows a Northern blot obtained from SCC25 and SCC25/CP cells exposed to 16 μM AZC for 72 hr; AZC treatment increased the basal hMT-IIa accumulation approximately 2.8-fold in SCC25 cells. In contrast, AZC had little effect on the basal level of hMT-IIa mRNA in SCC25/CP cells. These results are consistent with the hypothesis that hypomethylation of hMT-IIa or another regulatory gene contributes to the elevated basal expression of hMT-IIa in SCC25/CP cells, although clearly the 9.2-fold basal hMT-IIa overexpression exceeded the 2.8-fold AZC effects. AZC had little or no effect on hMT-Ie and -If basal level expression, indicating that the elevated basal level of these isoforms may be due to a mechanism other than hypomethylation.

We next examined whether AZC had any effect on metal-induced hMT isoform expression. The Northern analysis of SCC25 and SCC25/CP cells (Fig. 6, *lanes Zn* and *Cd*) showed that AZC caused only a slight increase in the level of hMT-IIa mRNA accumulation in SCC25 cells induced by cadmium or zinc. There were no differences in the levels of hMT-IIa mRNA induced by zinc or cadmium before and after AZC treatment in SCC25/CP cells. AZC caused a moderate increase in zinc- or cadmium-induced hMT-Ie mRNA accumulation in SCC25 cells, whereas only cadmium-induced hMT-Ie was increased by AZC in SCC25/CP cells. Similarly, AZC treatment had no effect on zinc-induced hMT-If mRNA accumulation in either SCC25 or SCC25/CP cells but moderately increased the cadmium-induced hMT-If mRNA expression in both SCC25 and SCC25/CP cells. Thus, in contrast to hMT-IIa, AZC pretreatment appeared to improve the responsiveness of hMT-Ie and hMT-If to cadmium in both SCC25 and SCC25/CP cells. This metal-specific effect of AZC may reflect differential sensitivity of selective metal-binding proteins to methylation at the MREs of the hMT isoforms. Alternatively, the binding proteins, which induce MT expression, may themselves be inhibited by methylation.

We have performed similar experiments with H69 and H69/CP cells. AZC treatment had no significant effects on either basal or metal-induced MT-IIa mRNA expression between H69 and H69/CP cells (data not shown). Interestingly, after AZC pretreatment, basal levels of hMT-Ig mRNA increased significantly in H69 cells, whereas no effect was seen with the resistant H69/CP cells (Fig. 7). Zinc (100 μM) treatment had no significant effect on hMT-Ig mRNA levels in the presence or absence of AZC, but 10 μM cadmium pretreatment caused a moderate increase in hMT-Ig mRNA in H69/CP cells (and not in sensitive H69 cells).

Discussion

During the course of evolution higher eukaryotes developed the ability to express several isoforms of MT, through the creation of multiple genes and an elaborate transcriptional regulatory system. Humans, for example, have at least seven unique functional genes encoding distinct hMT isoforms (13,

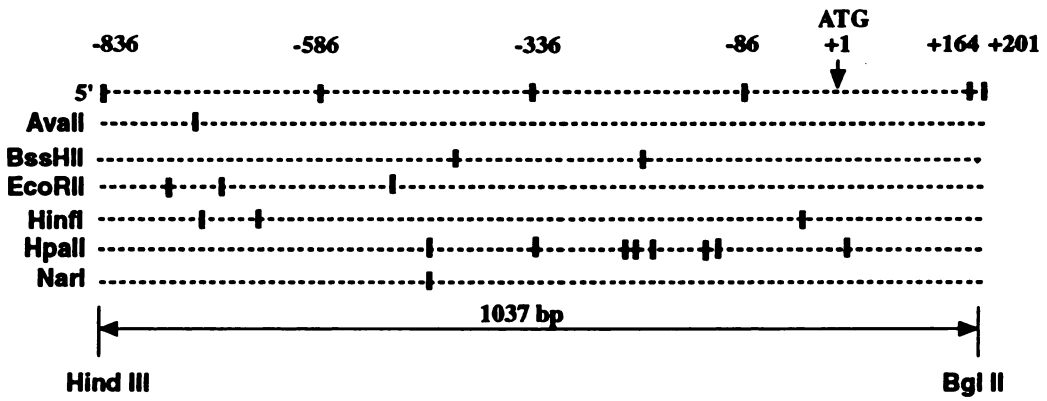

Fig. 5. Schematic diagram showing methylation-sensitive restriction sites in the 5′ flanking region of the hMT-IIa gene, i.e., *Ava*II (G′GA/T⁺CC), *Bss*HII (G′⁺CGCGC), *Eco*RII (′C⁺CA/TGG), *Hinf*I (G′⁺ANTC), *Hpa*II (C′⁺CGG), and *Nar*I (GG′⁺CGCC). The *Hind*III/*Bgl*II fragments represent the 1037-bp probe specific for the hMT-IIa promoter region and were used for Southern analysis.

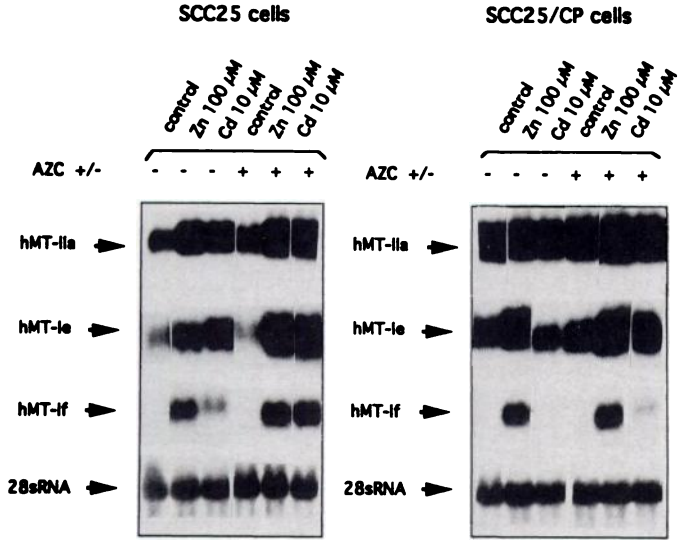

Fig. 6. Northern blots showing effects of AZC on hMT-IIa, -Ie, and -If expression in SCC25 and SCC25/CP cells. −, Without AZC treatment; +, with AZC treatment for 72 hr. *Lanes control*, basal level; *lanes Zn*, 100 μM zinc treatment for another 9 hr after AZC pretreatment for 72 hr; *lanes Cd*, 10 μM cadmium treatment for another 9 hr after AZC pretreatment for 72 hr. *Left*, SCC25 cells; *right*, SCC25/CP cells. All of these blots were stripped and reprobed simultaneously with a 28 S RNA oligomer used for normalization.

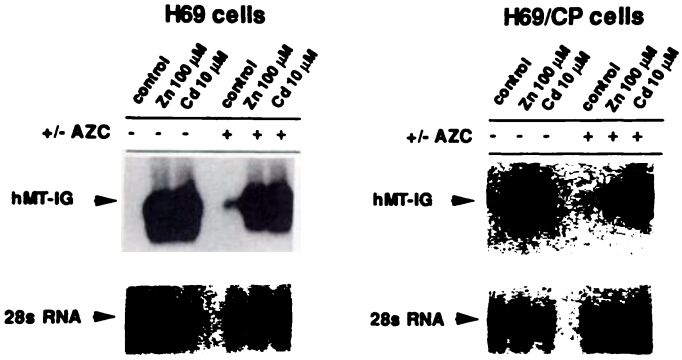

Fig. 7. Northern blots showing effects of AZC on hMT-Ig expression in H69 and H69/CP cells. −, Without AZC treatment; +, with AZC treatment for 72 hr. *Lanes control*, basal level; *lanes Zn*, 100 μM zinc treatment for another 9 hr after AZC pretreatment for 72 hr; *lanes Cd*, 10 μM cadmium treatment for another 9 hr after AZC pretreatment for 72 hr. *Left*, H69 cells; *right*, H69/CP cells. All of these blots were stripped and reprobed with a 28 S RNA oligomer used to normalize RNA loading.

14). Currently, we do not know whether multiple isoforms exist to provide expanded functionality or simply represent another example of biological redundancy. Several groups (5, 7, 25) have proposed that MT may participate in generating a phenotype resistant to CDDP and alkylating agents. Results from other investigators (9, 11, 12) argue against such a role. These differences may relate in part to variations in the isoforms expressed by cells. Thus, we have evaluated for the first time the isoform expression of hMT in cultured cells with acquired resistance to CDDP. We developed hMT isoform-specific oligonucleotide probes and found that human head and neck tumor and small cell lung carcinoma cells have different patterns of basal hMT isoform expression. It is interesting that, among the six non-neuronal hMT isoforms, the most highly overexpressed MT isoform in all three CDDP-resistant human carcinoma cell pairs is hMT-IIa and no other isoform is universally overexpressed. The hMT-IIa expression in SCC25/CP, SW2/CP, and H69/CP cells, which are 5-, 11-, and 4-fold resistant to CDDP, respectively, was 9.2-, 3.6-, and 2-fold greater than that in their respective parental cells. The ubiquity of hMT-IIa suggests a general role in cellular physiology, zinc homeostasis, and perhaps mediation of protection from CDDP cytotoxicity. Studies from our group also show an increase of hMT-IIa protein in SCC25/CP cells, indicating that the overexpressed mRNA is productive.[1] The failure to observe detectable mRNA levels for hMT-Ia, -Ib, -If, or -Ig argues against their involvement in CDDP resistance in these cells.

Because hMT is inducible by a variety of environmental and pharmacological agents, we examined MT induction by two metals, zinc and cadmium, in CDDP-sensitive and -resistant cells. As illustrated in Figs. 2 and 3, the inducibility is cell type specific. We can summarize the differences in hMT induction by the two metals according to (i) the hMT isoform pattern, (ii) the magnitude of the induction, and (iii) the sensitivity to cadmium or zinc. Of the four metal-inducible hMT genes, hMT-IIa was the most frequently induced isoform in all three CDDP-sensitive and -resistant human carcinoma cell types. Interestingly, hMT-Ig was induced greatly only in H69 cells. Richards *et al.* (26) previously showed that endogenous hMT-IIa and hMT-Ia are differentially induced by various metal ions and glucocorticoid hormones, indicating the possibility of different functional roles for these two MT genes. Those authors concluded that the differential responses were due to functional differences of the respective promoter/regulatory regions. In our studies, treatment of SCC25 and SCC25/CP with zinc also caused increases in both hMT-Ie and hMT-If; interestingly, cadmium treatment increased hMT-Ie levels only in SCC25 cells and not in SCC25/CP cells. Although the molecular basis for these differences is unclear, several possible mechanisms might explain the differential metal inducibility of hMT-Ie and hMT-If in CDDP-sensitive and -resistant SCC25 cell lines. First, the cells may synthesize different *trans*-acting factors that specifically interact with cadmium- or zinc-mediated MREs of hMT isoform genes. Recently, Koizumi *et al.* (27, 28) have identified one HeLa cell nuclear factor whose binding to MREs is enhanced only by zinc, and they also identified another MRE-binding protein whose binding affinity for different MREs varies independently in response to different heavy metal levels. It might be possible that there are differential levels of MRE-binding proteins or some metal-specific *trans* factors that contribute to the fine regulation of MT genes between CDDP-sensitive and -resistant cell lines. It is also possible, however, that the stability of induced mRNA for the MT isoform is altered during the development of CDDP resistance.

A second possibility is that the hMT isoform genes are altered by *cis*-acting epigenetic events, such as DNA methylation. Previous studies demonstrated a correlation between DNA methylation and MT cell type-specific expression (16). Lieberman *et al.* (29) found that UV radiation could induce MT-I gene activation that was associated with extensive DNA demethylation. Palmiter *et al.* (30) also correlated expression of a MT-thymidine kinase fusion gene with hypomethylation. The mechanism of AZC action has not been firmly established, but one important effect is the inhibition of DNA methylation.

[1] S.-M. Kuo and J. S. Lazo, unpublished observations.

From our studies (Fig. 5), we found that AZC increased basal levels of hMT-IIa expression in sensitive cells but not in CDDP-resistant SCC25 cells. Thus, we hypothesized that hypomethylation at CpG sites in the 5′ flanking region of hMT-IIa was involved in the elevated basal levels of hMT-IIa mRNA in SCC25/CP cells. Similar results have been reported for the MT-Ib gene isoforms. Heguy *et al.* (31) found that the 5′ flanking region of the MT-Ib gene was highly methylated in HeLa cells, which did not express MT-Ib, but unmethylated in hepatoma cells, which expressed this gene. HeLa cells could be induced to express MT-1b after AZC treatment, suggesting a strong influence of methylation in inducing *cis*-acting suppression. Thus, methylation could play a role in preferential silencing of a particular subset of genes. Although we were unable to detect differences in the methylation status of hMT-IIa among CDDP-sensitive and -resistant cells using methylation-sensitive enzymes, only a fraction of potential CpG methylation sites could be examined. Based on transient expression assays with methylated CpG-rich promoters, however, only a few sites of methylation are necessary to inhibit expression of reporter genes (32), suggesting that regional or site-specific CpG demethylation can control transcription of CpG-rich promoters. Thus, it is possible that methylation of a hMT-IIa promoter CpG site escaped detection by this method of analysis, because of the limited availability of methylation-sensitive restriction enzymes. Alternatively, the overexpression could be caused by demethylation of a regulatory region of a *trans* factor that affects hMT-IIa expression. Schmidt and Hamer (13) reported that cells transformed with the c-Ha-*ras* oncogene express atypically high basal levels of both hMT-IIa and hMT-Ie transcripts, possibly reflecting a role of normal *ras* genes in the control of MT gene regulation.

Our nuclear run-on studies demonstrate that SCC25/CP cells have an increased hMT-IIa transcription rate. We have been unable to detect any alteration in hMT-IIa mRNA stabilities between the pair of SCC25 cells and SCC25/CP cells. Recently, several groups have shown increased c-*fos* expression in CDDP-resistant cells (24), and it is interesting that we found that c-*fos* transcription was also increased in SCC25/CP cells. The c-*fos* oncogene can modulate gene expression by stimulating other genes that possess the AP-1 or AP-2 binding site, such as MT or DNA repair genes. It will be important to study further what *trans*-acting factors regulate hMT-IIa overexpression. Furthermore, we know that the 5′ flanking regulatory region of the hMT-IIa promoter contains several AP-1 and AP-2 sites. Moreover, CpG islands occur in the 5′ flanking and regulatory region of several cellular oncogenes, including the c-*myc*, c-*fos*, c-*sis*, and c-*ras* family, thus leading to speculation that DNA methylation in these regions might be intimately involved in hMT-IIa regulation. AZC pretreatment also affected induction of other hMT isoforms in response to metal treatment (Fig. 5). For example, we found that AZC increased cadmium-induced but not zinc-induced hMT-If mRNA levels in SCC25 cells. In H69 and H69/CP cells (Fig. 6), treatment with AZC had no effect on either basal level or metal-induced hMT-IIa expression. In one study with HepG2 cells, an increase in MT mRNA levels after AZC treatment was ascribed to an increase in the total cellular copper content in HepG2 cells (33); this seems an unlikely explanation for our results because it would require preferential effects on SCC25 but not SCC25/CP cells. Rather, we currently favor the hypothesis that epigenetic differences exist in a regulatory domain controlling a *trans* factor regulating the basal hMT-IIa mRNA levels in CDDP-resistant cells, compared with parental cells. We considered using AZC pretreatment to elevate endogenous MT protein, to examine CDDP sensitivity, but we found only a modest (38%) increase in MT protein content in SCC25 cells (and not in SCC25/CP cells) (data not shown). Based on previous results, this slight MT content increase would be insufficient to protect SCC25 cells from CDDP cytotoxicity. Moreover, a variety of cellular factors, such as cellular uptake, DNA repair processes, and activation of protein kinase C, can affect cellular responsiveness to CDDP, and AZC may alter expression of other genes controlling these factors, in addition to MT. Thus, we do not believe it would be useful to examine SCC25 cell sensitivity to CDDP after AZC pretreatment.

In conclusion, we designed and used six oligonucleotides to probe hMT isoform expression and we found that the six non-neuronal hMT isoform genes were differentially expressed in three pairs of CDDP-sensitive and -resistant human tumor cell lines. hMT-IIa mRNA was the only MT isoform overexpressed in all cells, consistent with a role for this isoform in CDDP resistance. In SCC25/CP cells the overexpression of hMT-IIa reflects an increase in transcription.

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