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Cell specificity and an effect of *ras* on human metallothionein gene expression

(heavy metals/glucocorticoids/transcription/methylation)

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ABSTRACT The expression of three human metallothionein (hMT) genes has been compared in various established cell lines and primary liver. The single gene for hMT isoform II is ubiquitously expressed in all cell types in response to cadmium. In contrast, two genes encoding hMT-I isoforms are expressed in a highly specific, reciprocal fashion that correlates with the embryonic germ layer origin of the cells. In one cell line that failed to express detectable amounts of hMT-I_E, treatment with the demethylating agent 5-azacytidine led to cadmium-inducible expression of this subtype. The genes for both MT-I isoforms are coordinately inducible by heavy metals but differ in their response to glucocorticoids. Surprisingly, cells transformed with the Ha-*ras* oncogene contain elevated basal levels of both MT-I and MT-II RNA. The implications of these results for growth-related and developmental functions of MT are discussed.

Metallothioneins (MTs) are ubiquitous, cysteine-rich proteins that bind heavy metals such as zinc, copper, and cadmium (1). They are present in a broad range of eukaryotic species and in vertebrates are expressed in many different cell types and tissues. MTs have no known enzymatic function but are thought to play a role in heavy metal homeostasis, in particular in storage and detoxification (2–5). Interest in MT genes has been stimulated by the observation that their transcription rates are increased by a variety of agents including heavy metals (6, 7), glucocorticoids (8, 9) interferon (10, 11), and an unidentified product of the inflammatory stress response (12).

All vertebrates synthesize at least two MT isoforms, MT-I and MT-II, that differ in their primary amino acid sequences. In humans the situation is complicated by the presence of multiple hMT-I isoforms. Five different hMT-I subtype proteins have been identified in liver extracts (13) and three different functional genes encoding hMT-I_A, hMT-I_E, and hMT-I_F have been cloned and sequenced (14, 15); following the rules of human gene nomenclature, the respective genes are denoted *MT1A, MT1E,* and *MT1F*. The isolation of bacteriophage λ clones containing four closely linked, tandemly arranged *MT1*-like genes suggests that these multiple subtypes have arisen by tandem duplication of an ancestral *MT1* gene (14). In contrast there appears to be a single hMT-II protein which is encoded by the *MT2A* gene (16). Chromosomal mapping studies have shown that all of the known functional *MT* genes are clustered on chromosome 16 (17, 18).

Why do humans synthesize so many different hMTs? One intriguing possibility is that these closely related yet distinct proteins play different functional roles during development or under various physiological conditions. To begin testing this possibility, we compared the expression of *MT1E, MT1F,* and *MT2A* in various cell types exposed either to heavy metals or glucocorticoids. We show that the two *MT1* genes are expressed in a differential fashion both with regard to cell type and inducing agent. We also present evidence that cells harboring the cellular Harvey ras (c-Ha-*ras*; *HRAS* in human gene nomenclature) oncogene express atypically high basal levels of both hMT-I_E and hMT-II_A transcripts, possibly reflecting a role of normal *ras* genes in the control of MT synthesis.

MATERIALS AND METHODS

Established cell lines were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 100 units of penicillin and 100 μg of streptomycin per ml. Primary human liver was obtained from a partial hepatectomy. The tissue was washed with phosphate-buffered saline, minced with scissors, and then digested with collagenase (1 mg/ml) for 15 min with constant agitation. The dispersed cells were washed three times with phosphate-buffered saline, resuspended in DMEM with 10% fetal calf serum, and incubated at 37°C for 8 hr with or without cadmium.

Metal inductions were carried out by addition of metal to the medium for 8 hr. The $CdCl_2$ concentration was 10 μM, and the concentrations of other metals are indicated in *Results*. Glucocorticoid inductions were conducted by addition of 100 nM dexamethasone to the medium for 8 hr. RNA was isolated by proteinase K/sodium dodecyl sulfate treatment followed by CsCl density gradient centrifugation (19). The RNA concentrations were determined from the absorbance at 260 nm. Single-stranded ^{32}P-labeled DNA probes specific for the 5′ ends of the genes encoding human hMT-I_E, hMT-I_F, hMT-II_A, and mouse mMT-I were prepared as described (15, 20). Nuclease S1 mapping was conducted as described by Maniatis *et al.* (21) using 10 μg of total RNA per sample. The protected fragments were analyzed by electrophoresis through thin 8% polyacrylamide/urea gels. The results were quantitated by laser densitometry of the autoradiograms at several different exposures.

RESULTS

Cell-Specific Expression in Response to Metals. Expression of the genes for hMT-I_E, hMT-I_F and hMT-II_A was studied in various established and primary cell types grown in the absence or presence of the heavy metal cadmium, a potent inducer of *MT* gene transcription. The levels of the individual transcripts were quantitated by nuclease S1 mapping of the total cell RNA by using probes specific for the 5′ ends of each of the genes. A representative experiment is shown in Fig. 1*A*, and the data for 16 different cell types are summarized in Table 1. The apparent heterogeneity of the nuclease S1-

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Abbreviations: MT, metallothionein; hMT, human MT; mMT, mouse MT.

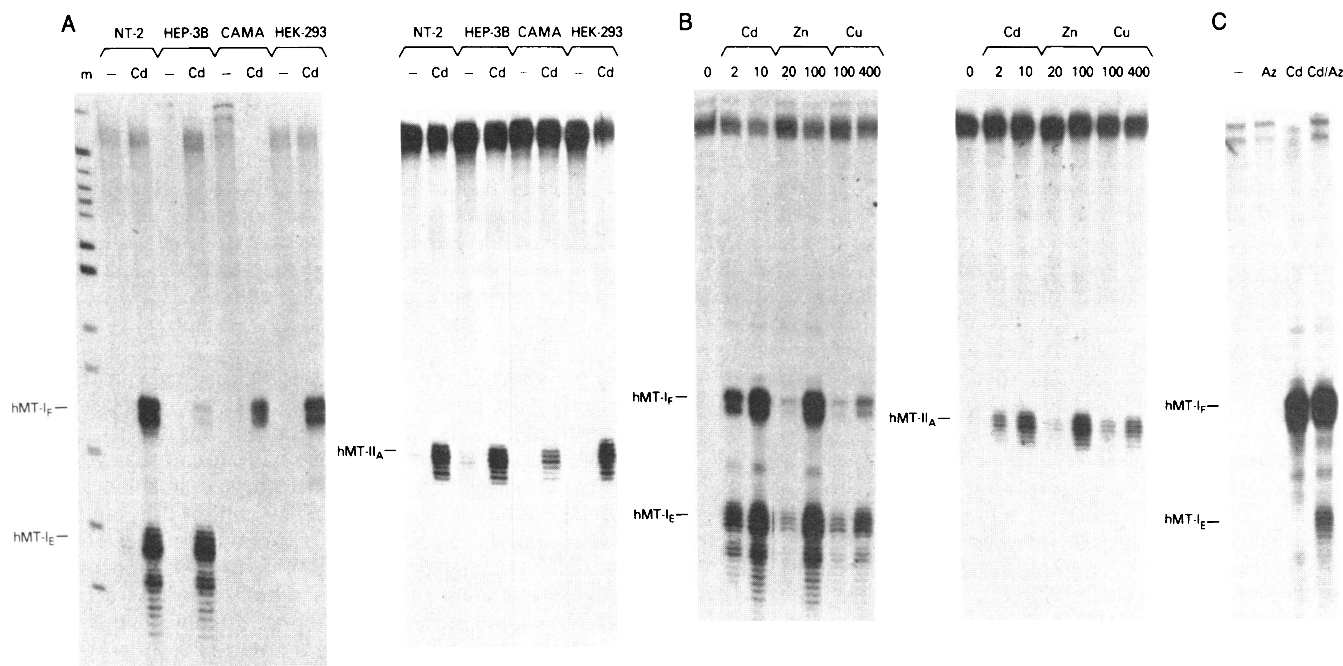

FIG. 1. Nuclease S1 mapping of MT transcripts. In each lane, 10 μg of total RNA was hybridized with the gene-specific probes. MT-I probes were mixed to permit analysis of the same RNA sample. The assignment of the bands to hMT-I_E and hMT-I_F transcripts was determined previously by individually assaying each probe. (*A*) Results from four different cell lines either uninduced (lanes –) or treated with 10 μM cadmium (lanes Cd) for 8 hr. Lane m contains the nucleotide size markers, which were generated by 5′-end-labeling *Hpa* II-digested pBR322. (*B*) Coordinate induction of *MT1E, MT1F*, and *MT2A* in NT-2 cells. In this experiment, cells were treated with the indicated metal concentrations (μM) for 8 hr, and RNA was extracted and subjected to nuclease S1 mapping. (*C*) Effect of 5-acacytidine treatment upon *MT1* expression in HeLa cells. Cells were treated for 16 hr with 16 μM 5-azacytidine. The drug was then removed, and the cells were either untreated (lane Az) or induced with 10 μM $CdCl_2$ (lane Az/Cd). In lane Cd, cells were induced with 10 μM $CdCl_2$ but received no 5-azacytidine.

protected bands is presumably due to the presence of duplicate transcription initiation sites for the *MT1E* and *MT1F* genes (15) and the use of a uniformly labeled probe; the results were not substantially altered by changing the nuclease S1 concentration.

The *MT2A* gene was highly expressed in response to cadmium in all cell types examined. In most cells the induction ratio was 10- to 30-fold, although in some lines it was lower because of increased basal expression levels. For example, fibroblasts generally had a 5- to 7-fold greater basal level than other cell types. The uninduced primary liver cells also contained a high basal level of MT-II transcripts, perhaps due to induction by stress of the patient prior to surgery.

The two MT-I isoform genes exhibited a much different pattern of activity. In most cell types examined they were expressed in a reciprocal fashion. For example, hepatocarcinoma cells expressed high levels of hMT-I_E RNA in response to cadmium but only very low levels of MT-I_F RNA. In contrast, under identical inducing conditions, mammary carcinoma and embryonic kidney cells contained high levels of hMT-I_F but no detectable hMT-I_E RNA. The only excep-

Table 1. MT transcript levels in various cell types

Human cell line	Cell type	Ref.	hMT-I_E –	hMT-I_E Cd	hMT-I_F –	hMT-I_F Cd	hMT-II_A –	hMT-II_A Cd
			Normalized transcript levels					
NT-2	Teratocarcinoma	22	1	23	1	25	1	25
Hep G2	Hepatoblastoma	23	1	15	1	18	1	20
Liver	—	—	5	15	1	3	10	20
Hep 3B	Hepatocarcinoma	23	1	20	1	3	1	18
SW1116	Colon carcinoma	24	1	10	1	3	1	23
T24	Bladder carcinoma	25	10	20	ND		11	18
GM 323	Fibroblast	26	1	5	ND		7	20
AdGM 323	Transformed fibroblast	26	1	5	ND		5	15
WI-38	Fibroblast	27	1	5	ND		7	25
Hs 578	Mammary carcinosarcoma	28	10	20	ND		10	20
HOS	Osteosarcoma	29	1	20	ND		1	27
HEK 293	Embryonic kidney	30	ND		1	18	1	21
HeLa	Cervical carcinoma	31	ND		1	17	1	15
H9	Lymphoma	32	ND		1	17	1	15
Cama-1	Mammary carcinoma	33	ND		1	10	1	18

The levels of hMT-I_E, hMT-I_F, and hMT-II_A transcripts were determined in uninduced (columns –) or cadmium-induced (columns Cd) cells as shown in Fig. 1. All results were normalized to give a value of 1 for uninduced NT-2 teratocarcinoma cells. ND, none detected.

tions noted to this reciprocal expression rule were in teratocarcinoma cells (NT-2 and three other lines not shown) and a hepatoblastoma line (Hep G2). In these cells the *MT1E* and *MT1F* genes were expressed at nearly equivalent levels in response to cadmium.

The inducibility of hMT mRNA by various concentrations of cadmium, zinc, and copper was tested in NT-2 cells, a line in which all three isoform genes are active. As shown in Fig. 1*B*, cadmium and zinc induced all of the genes to equivalent levels. Copper also coordinately induced *MT1E, MT1F*, and *MT2A*, although it failed to give the full induction achieved with cadmium or zinc; high copper concentrations gave decreased MT mRNA levels presumably due to toxic effects. These results show that all three genes are intrinsically capable of coordinate induction in response to these metals in the NT-2 teratocarcinoma cells.

Because DNA methylation has been correlated with *MT* gene expression (34), we tested the effect of the hypomethylating agent 5-azacytidine on *MT1* expression in HeLa cells, a cervical carcinoma cell line that express MT-I_F but not MT-I_E transcripts in response to cadmium. As shown in Fig. 1*C*, treatment with 5-azacytidine for 18 hr followed by 8 hr of cadmium exposure led to detectable transcription of *MT1E*, whereas either agent alone had no effect. Similar results were obtained in HEK 293 embryonic kidney cells (data not shown).

Glucocorticoid Induction. Glucocorticoids in addition to heavy metals are inducers of *MT* gene transcription (8, 9). Fig. 2 shows that dexamethasone, a potent synthetic glucocorticoid, strongly induced *MT2A* and *MT1E* in WI-38 fibroblasts but had no effect on *MT1F*. Because *MT1F* also showed no detectable response to metal in this line, we repeated the experiment in HeLa cells in which *MT1F* is inducible by cadmium. Although *MT2A* was strongly induced, *MT1F* again failed to show a dexamethasone-dependent response (data not shown).

Effect of c-Ha-*ras*. Two cell lines, T24 bladder carcinoma and HS578 mammary carcinosarcoma, had consistently 10-fold higher basal levels of both hMT-I_E and hMT-II_A RNA than other cell lines. Because both lines have been shown to contain an activated c-Ha-*ras* oncogene (35–37), we were curious to determine if this might be affecting the basal levels of *MT* gene expression. For this purpose, we compared the levels of mouse MT-I transcripts in isogenic 3T3 cell lines transformed with various *ras* oncogenes. Fig. 3 shows that the endogenous mouse gene for mMT-I had a 3- to 5-fold higher basal expression level in 3T3 cells transformed with the T24-activated c-Ha-*ras* oncogene than in nontransformed control cells. 3T3 cells transformed with the virally derived Harvey oncogene (v-Ha-*ras*) also contained elevated basal levels of MT-I. However, cells transformed with Kirsten murine sarcoma virus (v-Ki-*ras*) contained MT-I basal levels similar to the nontransformed 3T3 cells.

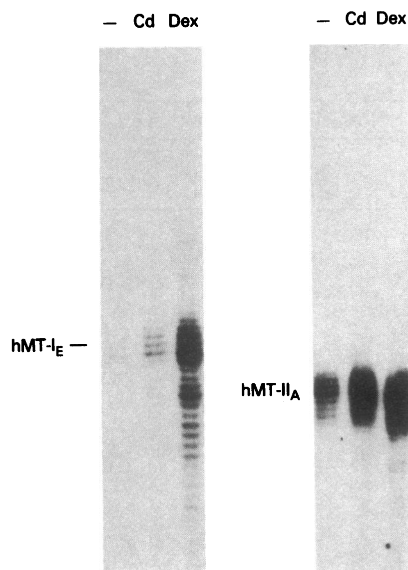

FIG. 2. Glucocorticoid induction of WI-38 fibroblast cells. Ten micrograms of total RNA was analyzed from cells uninduced (lanes –) or induced with either 10 μM $CdCl_2$ (lanes Cd) or 100 nM dexamethasone (lanes Dex) for 8 hr.

DISCUSSION

MTs are generally considered "housekeeping" proteins because they are present in a variety of tissues and cell types. The current results suggest that in humans the situation is more complex. In particular, we have found that while the single *MT2A* subtype gene is expressed in all cell types examined, two *MT1* subtype genes exhibit differential patterns of activity. Typically the expression of these genes is reciprocal, with high levels of hMT-I_E transcript corresponding to low or undetectable levels of hMT-I_F or vice versa. The ability of cadmium, zinc, and copper to coordinately induce all three genes in NT-2 teratocarcinoma cells shows that this is not simply due to differences in metal sensitivity. Instead, the reciprocal expression observed in other cell types may reflect cell- or tissue-specific modifications of *MT1* gene activity.

The generally reciprocal nature of *MT1* gene activity suggests the possibility of a developmentally controlled switch. The only cell lines that effectively express both *MT1E* and *MT1F* are NT-2 teratocarcinoma and hepatoblastoma cells. NT-2 represents a pluripotent cell that can be experimentally induced to differentiate into many cell types (22). The developmental state of the hepatoblastoma line is less clear, although the pathology of the original tumor suggests that it may represent an early blast stage in hepatocellular development (23). The embryonic germ layer origins of the other cell types, which express the *MT1* genes in a reciprocal fashion, are diagrammed in Fig. 4. All cells derived from the intermediate and lateral mesoderm or from ectoderm contain

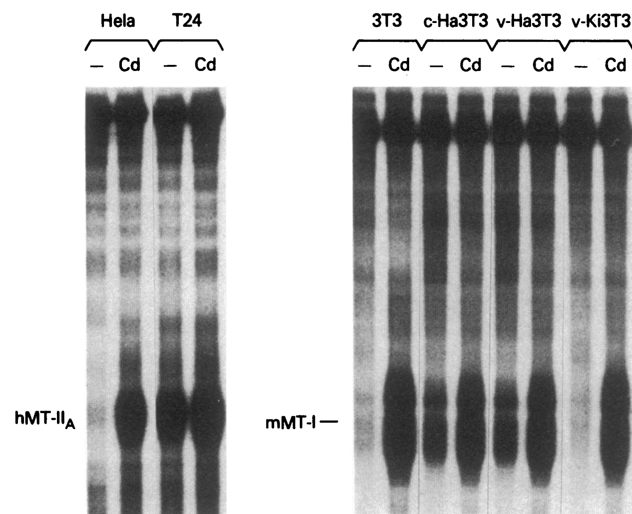

FIG. 3. Ha-*ras* affects the basal level of MT expression. Ten micrograms of total RNA isolated from either uninduced (lanes –) or cadmium-induced (lanes Cd) cells was hybridized with single-stranded gene-specific probes and analyzed by nuclease S1 mapping. Cells from which the various RNAs were isolated are indicated at the top of the lane. c-Ha3T3, v-Ha3T3, and v-Ki3T3 refer to 3T3 cells that were transformed with the corresponding cellular (c) or viral (v) *ras* oncogene.

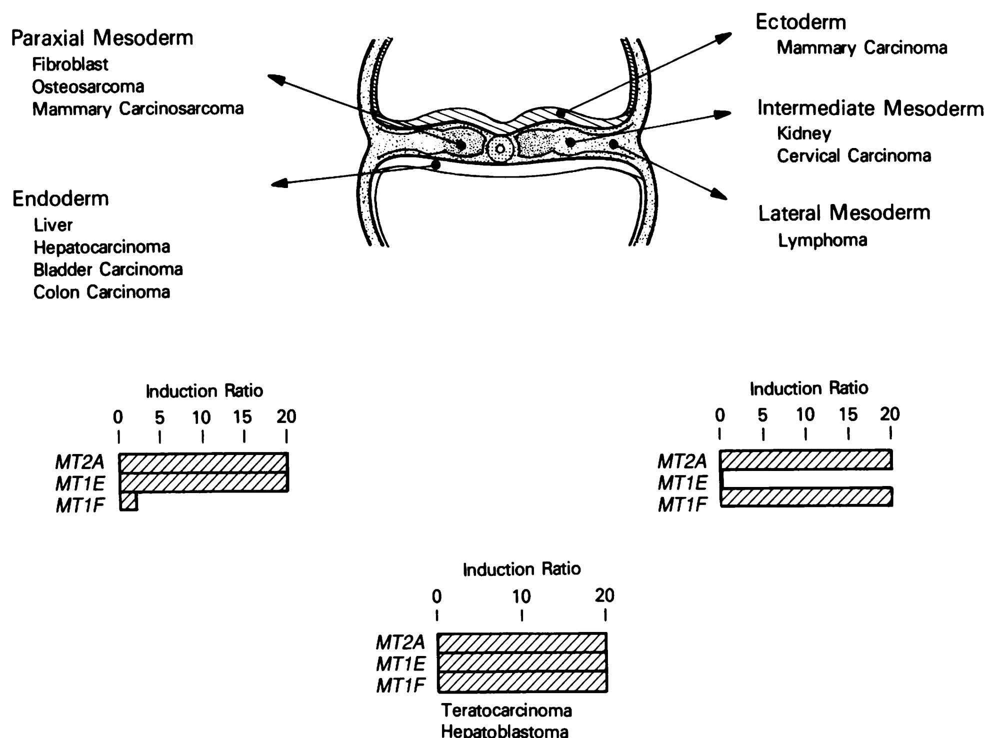

FIG. 4. The embryonic germ layer origin of the various cell lines and tissues. The drawing represents a schematic representation of a transverse section of the embryonic disc. In each panel, the histogram shows the average induction ratio of *MT1E*, *MT1F*, and *MT2A*. The bottom panel presents the induction ratios for the two anomalous cell lines, NT-2 and Hep G2.

high levels of hMT-I_F transcripts but no detectable hMT-I_E transcripts. In contrast, most cell types derived from the endoderm or the paraxial mesoderm express high levels of hMT-I_E but low or undetectable amounts of hMT-I_F. These correlations suggest a model in which the *MT1E* and *MT1F* genes are both expressed in early development. At some point, possibly in the trilaminar embryo, cells would become committed to suppress one of these two *MT1* subtypes, but the actual reduction of activity would not occur until later in development.

What could be the mechanism of such a developmental switch? One possibility is that different cell types synthesize different *trans*-acting, diffusible *MT* gene regulatory factors that specifically interact with *MT1E* or *MT1F* regulatory sequences. A second possibility, which we currently prefer, is that the *MT1* genes are altered by a *cis*-acting, epigenetic event such as DNA methylation. In support of this we have demonstrated that the hypomethylating agent 5-azacytidine partially restores the activity of a dormant *MT1E* gene. It should be possible to distinguish between these possibilities by transfection experiments using the cloned *MT1* genes.

The three *MT* genes examined in this study respond equally well to three different heavy metals. This is similar to the mouse genes for mMT-I and mMT-II (38) but is different from the human *MT1A* gene, which has been reported to respond preferentially to cadmium (14). However, we did note a differential effect of glucocorticoids, with *MT1E* and *MT2A* but not *MT1F* being responsive. While the molecular basis for this difference is unknown, it could indicate either that *MT1F* is intrinsically unresponsive to glucocorticoids or that it has been modified to an unresponsive form in the cell lines examined. A comparison of the nucleotide sequences of *MT1E* and *MT1F* with the consensus sequence for the glucocorticoid control element (7) failed to reveal any significant homologies.

A surprising finding was that bladder and mammary carcinosarcoma cells harboring the activated c-Ha-*ras* oncogene express elevated basal levels of both hMT-II_A and hMT-I_E mRNA. This is not a general characteristic of carcinoma cell lines since several others that we assayed had normal basal levels. It also is not typical for all members of the *ras* gene family since v-Ki-*ras* transformants had normal basal MT mRNA levels. It should be interesting to determine whether the normal c-Ha-*ras* proto-oncogene regulates *MT*, particularly early in development when both genes are expressed at high levels (39, 40).

What are the implications of these results for the still unknown function of MT? The ubiquitous expression of *MT2A* suggests a general role in cellular physiology, perhaps in the metabolism of zinc, while the generally reciprocal expression of the *MT1* subtypes may indicate a more restricted role. It is particularly noteworthy that hMT-I_E and hMT-I_F are differentially synthesized in liver and kidney, the two major organs of heavy metal storage and detoxification. Recently we found that transformed mouse cells producing high levels of hMT-I_E or hMT-I_F accumulate different levels of bound zinc, copper, and cadmium (unpublished data). This suggests that these subtype proteins, despite their close sequence similarity, are not functionally equivalent and that cells might modulate their metal content by differences in the repertoire of expressed *MT* genes.

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