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Induction of Metallothionein-I mRNA in Cultured Cells by Heavy Metals and Iodoacetate: Evidence for Gratuitous Inducers

DIANE M. DURNAM AND RICHARD D. PALMITER*

Howard Hughes Medical Institute Laboratory, Department of Biochemistry, University of Washington, Seattle, Washington 98195

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A mouse hepatocyte cell line selected for growth in 80 μM $CdSO_4$ (Cd^r_{80} cells) was used to test the role of metallothioneins in heavy metal detoxification. The cadmium-resistant (Cd^r_{80}) cells have double minute chromosomes carrying amplified copies of the metallothionein-I gene and accumulate ca. 20-fold more metallothionein-I mRNA than unselected cadmium-sensitive (Cd^s) cells after optimal Cd stimulation. As a consequence, the amount of Cd which inhibits DNA synthesis by 50% is ca. 7.5-fold higher in Cd^r_{80} cells than in Cd^s cells. Cd^s and Cd^r_{80} cells were compared in terms of their resistance to other heavy metals. The results indicate that although Zn, Cu, Hg, Ag, Co, Ni, and Bi induce metallothionein-I mRNA accumulation in both Cd^r_{80} and Cd^s cells, the Cd^r_{80} cells show increased resistance to only a subset of these metals (Zn, Cu, Hg, and Bi). This suggests that not all metals which induce metallothionein mRNA are detoxified by metallothionein and argues against autoregulation of metallothionein genes. Metallothionein-I mRNA is also induced by iodoacetate, suggesting that the regulatory molecule has sensitive sulfhydryl groups.

The influx of heavy metals into the environment as industrial waste products warrants concern for the effects of metals on living systems. Clinically, it has been possible to correlate heavy metal toxicity with a number of disease symptoms, including growth retardation, decreased reproductive capacity, teratogenesis, carcinogenesis, and early mortality (22, 24). Such clinical symptoms can often be explained by the effect of the toxic metal on one or more specific organs; Cd, for example, exerts its effects primarily on the kidney, where it accumulates and eventually causes renal dysfunction (11, 12, 24). The molecular mechanisms by which heavy metals exert their toxicity are not well understood. It is currently thought, however, that metals are toxic because of their affinity for essential ligands such as sulfhydryl, amino, and phosphate groups (24). Through such interactions, toxic metals can inactivate essential proteins by displacing metal cofactors, blocking active sites, or causing allosteric changes (24). Many metals bind to membrane components, altering both membrane permeability and enzyme activity (24). In addition, the ability of metals, such as Cd and Hg, to induce gross chromosomal aberrations may account for the mutagenic, carcinogenic, or teratogenic effects of these metals (11).

One possible mechanism for detoxifying some heavy metals involves sequestration of the metal ions by small, sulfhydryl-rich proteins called metallothioneins (MT). MTs are highly conserved proteins which have been identified in all invertebrate and vertebrate species examined (18). Many species have two homologous MTs designated MT-I and MT-II. Although Zn-thionein is the predominant form of MT in vivo, smaller amounts of Cd-, Cu-, and Hg-thionein have also been identified (18, 20). The high sulfhydryl content of MT (20 of its 61 amino acids are cysteinyl residues) allows each protein molecule to bind many heavy metal ions, e.g., seven Cd ions (26), thus helping to protect the cell from the toxic effects of the metal.

Support for the role of MTs in metal detoxification comes from the observation that the levels of renal, hepatic, and intestinal MTs increase in response to exposure to heavy metals such as Zn, Cd, Hg, or Cu (4, 18, 28, 33). More recently, a wide variety of cells in culture have been shown to accumulate MT in response to heavy metal administration (19, 29). These metal-induced increases in MT levels result from an increased rate of MT gene transcription, MT mRNA accumulation, and MT synthesis (2, 6). The data suggest that the concentration of MT in a cell is precisely regulated by cellular metal concentrations.

The hypothesis that MTs detoxify Cd receives additional support from the analysis of cultured cells that have been selected to grow in high, normally toxic levels of Cd. These cells synthesize much more MT than unselected cells (1, 2, 17, 23, 30), with MT synthesis accounting for as much as 70% of total cysteine incorporation (2). In most of the cadmium-resistant cells examined so far, the increase in MT synthesis reflects an amplification of both the amount of MT mRNA present in the cell and the number of MT genes (1, 2, 13, 23).

Previous studies have shown that in addition to Cd, Zn, Cu, and Hg, a wide spectrum of metals, including Ag, Ni, and Co, can induce MT accumulation (9, 27, 33, 34). In most cases, however, it has not been possible to demonstrate that these metal ion inducers actually bind to the newly synthesized MT. Thus, the role of MT in detoxifying these metals has remained questionable. In the present study, we took a genetic approach to examining the range of metals detoxified by MT. We initially describe the selection of a hepatoma cell line which grows in 80 μM $CdSO_4$ (Cd^r_{80} cells), amplifies the MT-I gene, and can be induced to synthesize ~20-fold more MT-I mRNA and protein than the parental Hepa cell line (unselected cadmium-sensitive cells [Cd^s cells]). We then asked whether the increased MT synthetic capability of the Cd^r_{80} cells increases their resistance to a variety of other metal ions that induce MT synthesis. The results bear on the question of the mechanism of MT gene regulation.

MATERIALS AND METHODS

Cell culture. Mouse hepatoma cell line Hepa 1A (5) was kindly provided by G. J. Darlington. Cells were grown in monolayer culture in Nutrient Mixture F12 (Ham) with 10%

* Corresponding author.

fetal calf serum, unless otherwise indicated. Zn-free F12 medium was specially prepared by GIBCO Laboratories; atomic absorption analysis showed <0.1 μM Zn compared with 3 μM for normal F12 medium. Selection of Cd-resistant Hepa 1A cells was initiated by the addition of 10 μM $CdSO_4$ to the culture medium. Thereafter, the $CdSO_4$ concentration was increased by 10-μM increments whenever the surviving cells grew well. This process was repeated until cells capable of growing in 80 μM Cd were selected.

Inducing reagents. Inducing reagents were as follows: $3(CdSO_4) \cdot 8H_2O$, $CoCl_2 \cdot 6H_2O$, and $NiCl_2 \cdot 6H_2O$ were obtained from Mallinckrodt; $ZnSO_4 \cdot 7H_2O$ and $HgCl_2$ were obtained from Allied Chemical and Dye Corp.; $CuSO_4 \cdot 5H_2O$ was obtained from Matheson, Coleman and Bell; $AgNO_3$ was obtained from Merck & Co.; and $Bi(NH_4)$ citrate from K & K Laboratories, Inc. Iodoacetate, iodoacetamide, and *N*-ethylmaleimide were purchased from Sigma Chemical Co. All solutions were prepared in glass-distilled, deionized water, except for $Bi(NH_4)$ citrate which was prepared in 1.5 M NH_4OH. Atomic absorption analysis revealed no contamination by Cd in any of the solutions except the $ZnSO_4$ solution which contained 0.0008% Cd.

Metal toxicity measurements. Cd^s or Cd^r_{80} cells withdrawn from Cd for 10 to 14 days were incubated with various concentrations of each metal for 14 h. During the last hour, [^{3}H]thymidine (20 Ci/mmol; New England Nuclear Corp.) was added at 3 μCi/ml. Cells were then washed three times in physiological saline and harvested in 1× SET buffer (SET buffer is 1% sodium dodecyl sulfate, 5 mM EDTA, and 10 mM Tris-HCl [pH 7.5]) plus 50 μg of proteinase K per ml. After incubation at 45°C for 1 h, samples were extracted with phenol-chloroform and concentrated by ethanol precipitation. Portions of the resulting total nucleic acid solutions were used to determine acid-precipitable radioactivity and the concentration of nucleic acid in each sample. Total nucleic acid concentrations were determined spectrophotometrically, assuming that 1 mg/ml has an absorbance of 20 at 260 nm. Results of the analyses are expressed as counts per minute per microgram of total nucleic acid.

MT-I mRNA measurements. Portions of the same total nucleic acid samples used to determine metal toxicity were hybridized to MT-I cDNA as described previously (7). The number of MT-I mRNA molecules per cell was calculated with values of 12.6 pg of DNA per Cd^s cell and 19.5 pg of DNA per Cd^r_{80} cell.

Polyacrylamide gel electrophoresis. Cd^s cells, Cd^s cells grown in 8 μM $CdSO_4$ (Cd^s_8) for 1 week, and Cd^r_{80} cells grown in 80 μM $CdSO_4$ were incubated with 5 μCi of [^{35}S]cysteine (~600 Ci/mmol; Amersham Corp.) per ml for 14 h, washed three times with physiological saline, and subsequently harvested in 25 mM Tris-HCl (pH 7.5)–5 mM $MgCl_2$–1 mM dithiothreitol–0.2% Triton X-100. Portions (~80,000 cpm of acid-precipitable radioactivity) were incubated with 20 mM iodoacetate in 150 mM Tris-HCl (pH 8.6) at 37°C for 20 min in the dark before being subjected to electrophoresis on a 10 to 20% polyacrylamide-sodium dodecyl sulfate gel. The gel was analyzed by fluorography (3).

High-performance liquid chromatography. [^{35}S]cysteine-labeled proteins were isolated and chromatographed on Sephadex G-75 as described previously (2). The MT-containing peak was then analyzed by high-performance liquid chromatography on an Altex TSK SW3000 column (0.75 by 60 cm) as described previously (31). Fractions of the eluant were analyzed for [^{35}S]cysteine by scintillation counting and for Cd by atomic absorption.

Analysis of MT-I gene amplification. Serial dilutions of total nucleic acid were made in 2 M NaCl–0.2 N NaOH–100 μg of herring sperm DNA per ml. Dilutions were boiled for 2 min, and then 5 μl was spotted onto nitrocellulose, baked at 80°C for 2 h, and hybridized to the MT-I-specific sequences of plasmid $m_1pEH_{0.4}$ (8) labeled with ^{32}P by nick translation. Posthybridization washes were carried out in 2× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate [pH 7.0]) at 68°C followed by 0.5× SET buffer at 45°C. Filters were analyzed by autoradiography and by cutting out individual spots and counting them in toluene-Omnifluor scintillation fluid.

RESULTS

Selection and characterization of cadmium-resistant Hepa cells. We initiated our study by selecting a population of Hepa cells resistant to 80 μM $CdSO_4$ (Cd^r_{80}) in the hopes that, like other Cd-resistant cell lines (1, 2, 13, 23), these cells would amplify their MT genes and thus increase their MT synthetic capability. Figure 1 shows that Cd^r_{80} cells differ from unselected (Cd^s) Hepa 1A cells not only in their resistance to Cd but also in terms of their ability to accumu-

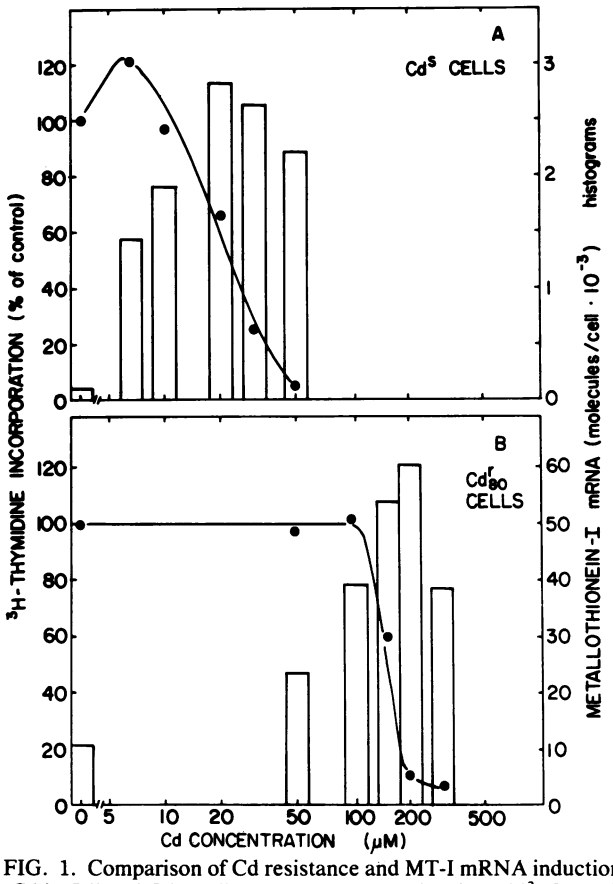

FIG. 1. Comparison of Cd resistance and MT-I mRNA induction by Cd in Cd^s and Cd^r_{80} cells. (A) MT-I mRNA levels and [^{3}H]thymidine incorporation in Cd^s cells after a 14-h incubation with 0 to 50 μM $CdSO_4$. Cells were labeled for the last hour with [^{3}H]thymidine. Total nucleic acids were prepared and used for both assays. (B) Same as (A), except that Cd^r_{80} cells withdrawn from Cd for 10 days were analyzed, and the Cd concentrations studied ranged from 0 to 300 μM. Cells were grown and tested in F12 medium containing 10% fetal calf serum. In both panels, MT-I mRNA levels are expressed as molecules per cell and are indicated by histograms; [^{3}H]thymidine incorporation, expressed as the percentage of control (no Cd), is shown (●).

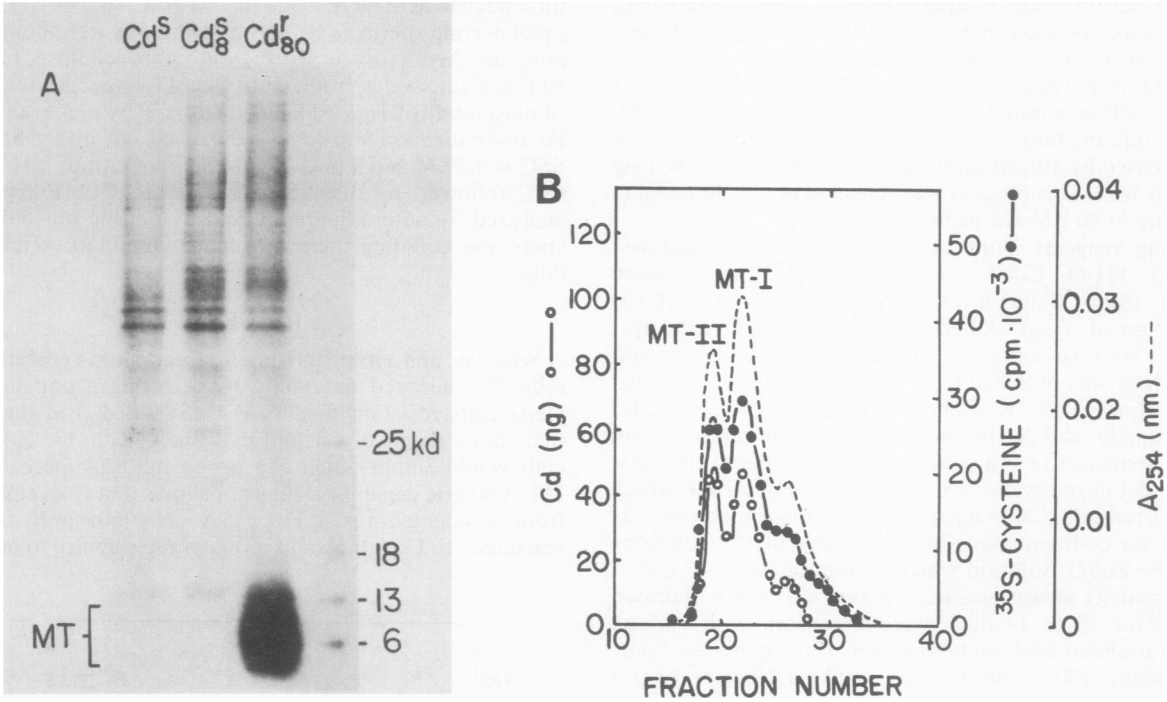

FIG. 2. Analysis of MT protein levels in induced and uninduced Cd^s cells and in Cd^r_{80} cells. (A) Fluorogram of polyacrylamide gel of [^{35}S]cysteine-labeled proteins. Cd^s cells, Cd^s cells grown in 8 μM $CdSO_4$ for 1 week (Cd^s_8), and Cd^r_{80} cells growing in 80 μM $CdSO_4$ were labeled for 14 h with [^{35}S]cysteine. Total ^{35}S-labeled proteins were carboxymethylated, and ~80,000 acid-precipitable cpm were loaded into each well of a 10 to 20% polyacrylamide-sodium dodecyl sulfate gel. After electrophoresis, the gel was analyzed by fluorography. The sizes of protein markers are indicated; the arrow indicates the position of mouse MT. (B) High-performance liquid chromatographic analysis of partially purified MT from Cd^r_{80} cells. Cd^r_{80} MT sample was prepared as described in the text. The dashed line shows absorbance at 254 nm. [^{35}S]cysteine (●) and Cd (○) levels are also shown.

MT-I mRNA in response to Cd. For this and subsequent studies with Cd^r_{80} cells, the Cd^r_{80} cells were withdrawn from Cd for 10 to 14 days before the readministration of the Cd concentrations indicated. Such withdrawal allows the intracellular concentration of Cd to decrease while having no significant effect on either the number of MT-I genes or the ability of the cells to grow in 80 μM $CdSO_4$ (data not shown). The data in Fig. 1 show that the dose of Cd that decreases [^{3}H]thymidine incorporation by 50% relative to incorporation in the absence of metal (ED_{50}) is 7.5-fold higher for Cd^r_{80} cells than for Cd^s cells, having increased from ~20 μM in Cd^s cells to ~155 μM in restimulated Cd^r_{80} cells. There is a similar increase in the dose of Cd that optimally induces MT-I mRNA in the two cell types. In addition, the maximal level of MT-I mRNA induced by Cd in Cd^r_{80} cells is ~20-fold greater than the level induced in Cd^s cells.

This increase in MT-I mRNA levels led to a comparable increase in the rate of MT synthesis. Fluorography on a polyacrylamide gel of [^{35}S]cysteine-labeled proteins from Cd^s cells, Cd^s growing in 8 μM Cd (Cd^s_8), and Cd^r_{80} cells growing in 80 μM $CdSO_4$ was carried out (Fig. 2A). These Cd concentrations were selected because they induced MT-I mRNA levels to ~50% of the maximum, yet they showed no toxic effects, at least during a 14-h incubation period (Fig. 1). Note that Cd^r_{80} cells synthesize much more MT than either Cd-induced or -untreated Cd^s cells. Furthermore, when [^{35}S]cysteine-labeled MT is analyzed by gel filtration (G-75 Sephadex) and the amount of Cd associated with MT is measured by atomic absorption, it is clear that (i) Cd^r_{80} cells have 50-fold more Cd associated with MT per cell than Cd^s

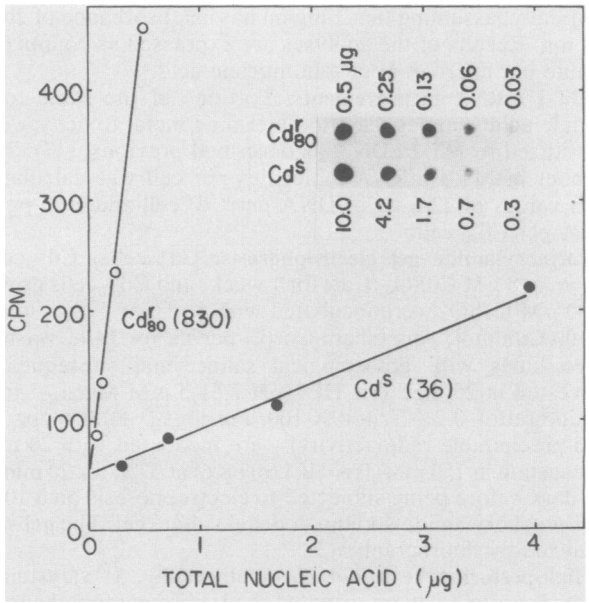

FIG. 3. Analysis of MT-I gene number. (A) Total nucleic acids from Cd^s and Cd^r_{80} cells were serially diluted, denatured, and spotted onto nitrocellulose. After hybridization to nick-translated plasmid $m_1pEH_{0.4}$ sequences, the filter was analyzed by autoradiography (inset) before the individual spots were cut out and analyzed by scintillation counting. The data are expressed as the number of counts per minute hybridized as a function of the amount of nucleic acid spotted. The slope of each line is indicated in parentheses.

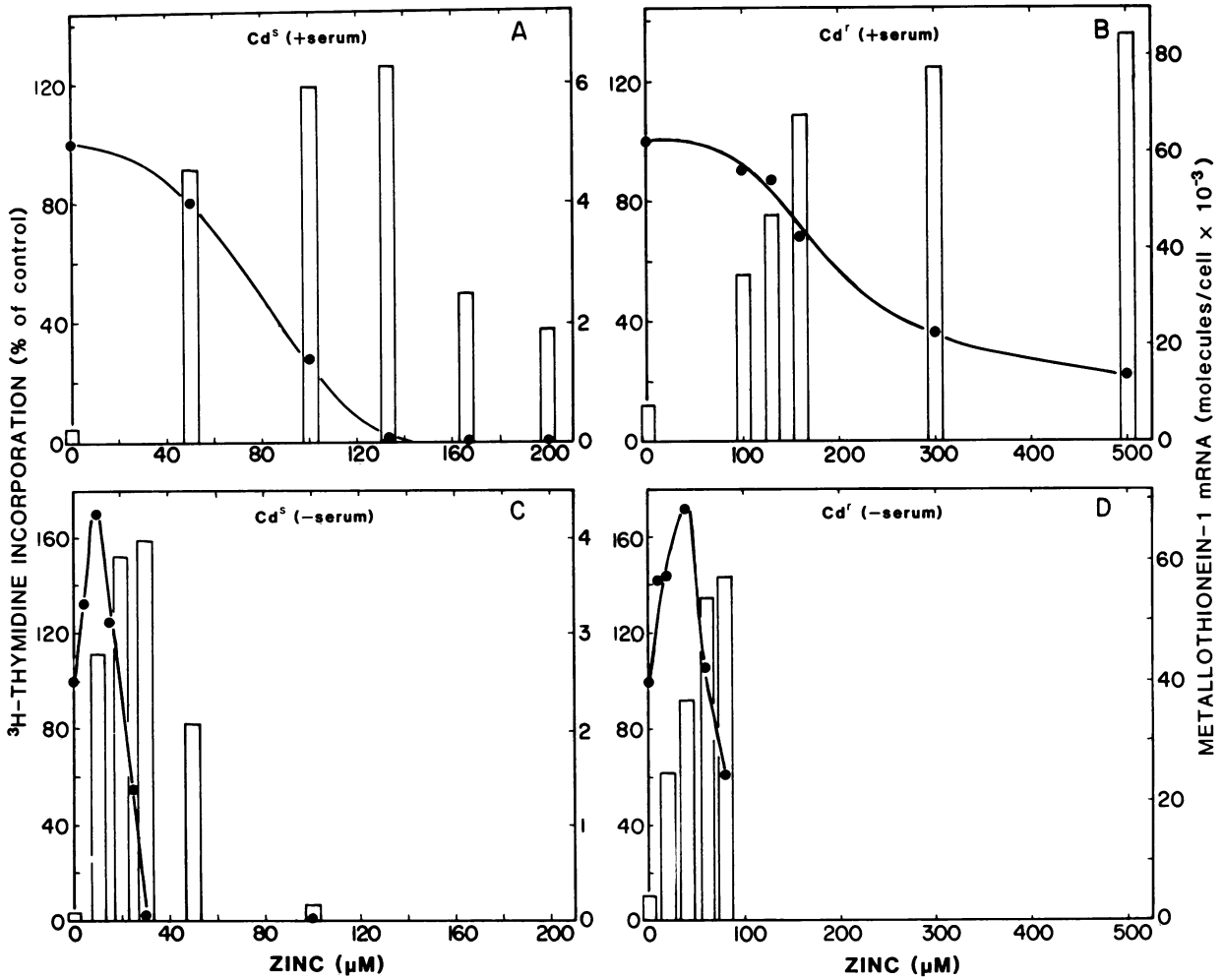

FIG. 4. Comparison of Zn resistance and MT-I mRNA induction in Cd^s and Cd$^r_{80}$ cells. (A) and (B) show the effect of Zn when the experiment is carried out with normal medium. (C) and (D) show the results of carrying out the experiment with Zn-free, serum-free medium. (A) and (C) MT-I mRNA levels and [^{3}H]thymidine incorporation in Cd^s cells after a 14-h incubation with Zn concentrations ranging from 0 to 200 μM. [^{3}H]thymidine was added to the culture medium during the last hour of the incubation. Total nucleic acids were isolated and used for both assays. (B) and (D) Same as (A) and (C) except Cd$^r_{80}$ cells were analyzed with Zn concentrations ranging from 0 to 500 μM. Cd$^r_{80}$ cells were withdrawn from Cd for 10 days before restimulation with Zn. In all panels, MT-I mRNA levels are expressed as molecules per cell and are shown by histograms; [^{3}H]thymidine incorporation, expressed as the percentage of control (no metal), is shown (●).

cells and (ii) although Cd^s cells have ~45% of their intracellular Cd bound to MT, Cd$^r_{80}$ cells have >90% bound to MT (data not shown). Further analysis of the MT peak from Cd$^r_{80}$ cells by high-performance liquid chromatography (31) resolved three peaks of radioactivity and absorbance at 254 nm (Fig. 2B). The two most prominent species correspond to purified mouse MT-I and MT-II; the identity of the third species is unknown but could be a degradation product of MT (32). Atomic absorption analysis indicates the presence of Cd in all three peaks. High-performance liquid chromatographic analysis of the MT peak from Cd$^s_8$ cells revealed a similar but much lower [^{35}S]cysteine profile; levels of absorbance at 254 nm and Cd levels were below detection.

We next determined whether the elevated MT-I mRNA levels and increased MT synthesis in Cd$^r_{80}$ cells reflected an amplification of the MT-I gene. Serial dilutions of total nucleic acid from Cd^s and Cd$^r_{80}$ cells were spotted and baked onto nitrocellulose, hybridized to a ^{32}P-labeled probe specific for MT-I DNA sequences, and analyzed by autoradiography; then, the individual spots were cut out and quantitated by scintillation counting (Fig. 3). This analysis indicates that Cd$^r_{80}$ DNA contains ~23-fold more MT-I genes per microgram of DNA than does Cd^s DNA. Controls show that MT-I mRNA is not detectable by this assay due to alkaline hydrolysis. Determination of the amount of total DNA per cell revealed that Cd$^r_{80}$ cells have ca. 1.4-fold more DNA per cell than do Cd^s cells (data not shown). Thus, the 23-fold difference we measured by hybridization analysis actually reflects a 35-fold amplification of the number of MT-I genes per cell. Chromosomal analysis of the cells indicates that this increase in DNA content results, in part, from the fact that the cells changed from being subtetraploid to being suboctaploid and accumulated ca. 85 double minute chromosomes during the selection process (1). Purification of double minute chromosomes by differential centrifugation also greatly enriched for MT-I genes (data not shown). Since MT-I and MT-II genes lie within 10 kilobases of each other (Searle et al., manuscript submitted), the data indicate that both genes are coamplified on double minute chromosomes.

Comparison of Zn toxicity in Cd^s and Cd$^r_{80}$ cells. Having characterized the Cd$^r_{80}$ cells with respect to Cd resistance, we then determined whether Cd$^r_{80}$ cells were resistant to

normally toxic levels of Zn as well. Figure 4 shows a comparison of the response of Cd^s and Cd^r_{80} cells to increasing concentrations of Zn. These experiments were carried out in both normal medium (Ham F12 medium plus 10% fetal calf serum) and Zn-free, serum-free medium to ensure that serum components were not interfering with induction and detoxification. The data indicate that in normal medium, the ED_{50} for Zn increased from ~90 μM in Cd^s cells to ~235 μM in Cd^r_{80} cells (Fig. 4A and B). Approximately 90,000 molecules of MT-I mRNA were induced in the Cd-resistant cells with optimal Zn concentrations. This induction is ca. 15-fold higher than the levels induced by Zn in Cd^s cells. Similar results were obtained when Zn-free, serum-free medium was used (Fig. 4C and D), except that both cell types were ~threefold more sensitive to the toxic effects of Zn in the absence of serum. As before, there was a 15-fold increase in the level of MT-I mRNA induced in Cd^r_{80} cells, as compared with that induced in Cd^s cells, although in both cases, the maximal mRNA levels were only ~70% of the levels induced in normal medium. These data suggest that the amplified MT-I genes in Cd^r_{80} cells respond to Zn, as well as to Cd, and that these cells have become more resistant to the toxic effects of Zn. The major effect of serum is the shift in the dose-response curves toward a higher Zn concentration, presumably because serum proteins bind Zn, thereby lowering the concentration of available Zn.

Effect of other metals on Cd^s and Cd^r_{80} cells. To address the question of the role of MT in detoxifying other heavy metals, we first analyzed a variety of heavy metals for their ability to induce MT-I mRNA in Cd^s and Cd^r_{80} cells. Table 1 shows that Ag, Hg, Cu, Ni, Co, and Bi also induce MT-I mRNA in both cell types. The effective concentration range for induction by the metals varies over 100-fold. Ag, for example, is similar to Cd in that it induces optimally in Cd^s cells at a concentration of 20 to 50 μM; comparable inductions by Ni and Bi require concentrations of about 1 mM. Analysis of the amount of MT-I mRNA induced by the metals in Cd^s and Cd^r_{80} cells shows that, like Cd and Zn, these metals induce 5- to 25-fold more MT-I mRNA in Cd^r_{80} cells than in Cd^s cells. Unlike Cd and Zn, however, the optimal concentration range for induction by Ag, Hg, Co, Bi, and Ni is identical for the

TABLE 1. Comparison of the effects of a variety of metals on Cd^s and Cd^r_{80} Hepa cells[a]

Metal	ED_{50} (μM)		Increased metal resistance[b]	Maximal MT-I mRNA accumulation (molecules per cell)[c]			
	Cd^s	Cd^r_{80}		Cd^s		Cd^r_{80}	
Cd	20	155	+++	2,770	(20)	30,850	(200)
Zn	80	220	+++	6,640	(130)	84,350	(500)
Cu	225	500	++	1,880	(200)	8,580	(500)
Ag	50	30	−	4,050	(40)	13,500	(20)
Hg	15	20	+	2,970	(50)	41,150	(50)
Co	200	180	−	940	(300)	16,650	(300)
Ni	310	340	−	2,580	(1,000)	50,700	(1,000)
Bi	770	1,120	+	1,560	(1,000)	38,950	(1,000)
Se	30	30	−				
CH_3Hg	6	6	−				

[a] Cd^s or Cd^r_{80} cells withdrawn from Cd for 10 days were grown in F12 medium plus 10% fetal calf serum and then challenged with five different concentrations of the indicated metals for 14 h.

[b] +++, ≥ threefold; ++, twofoled; +, < twofold; −, no change.

[c] The basal level of MT-I mRNA in the absence of added metals was 150 molecules per cell for Cd^s cells and 8,890 molecules per cell for Cd^r_{80} cells; these values were subtracted. The metal concentrations (in micromolar), which give maximal induction of MT-I mRNA, are shown in parentheses.

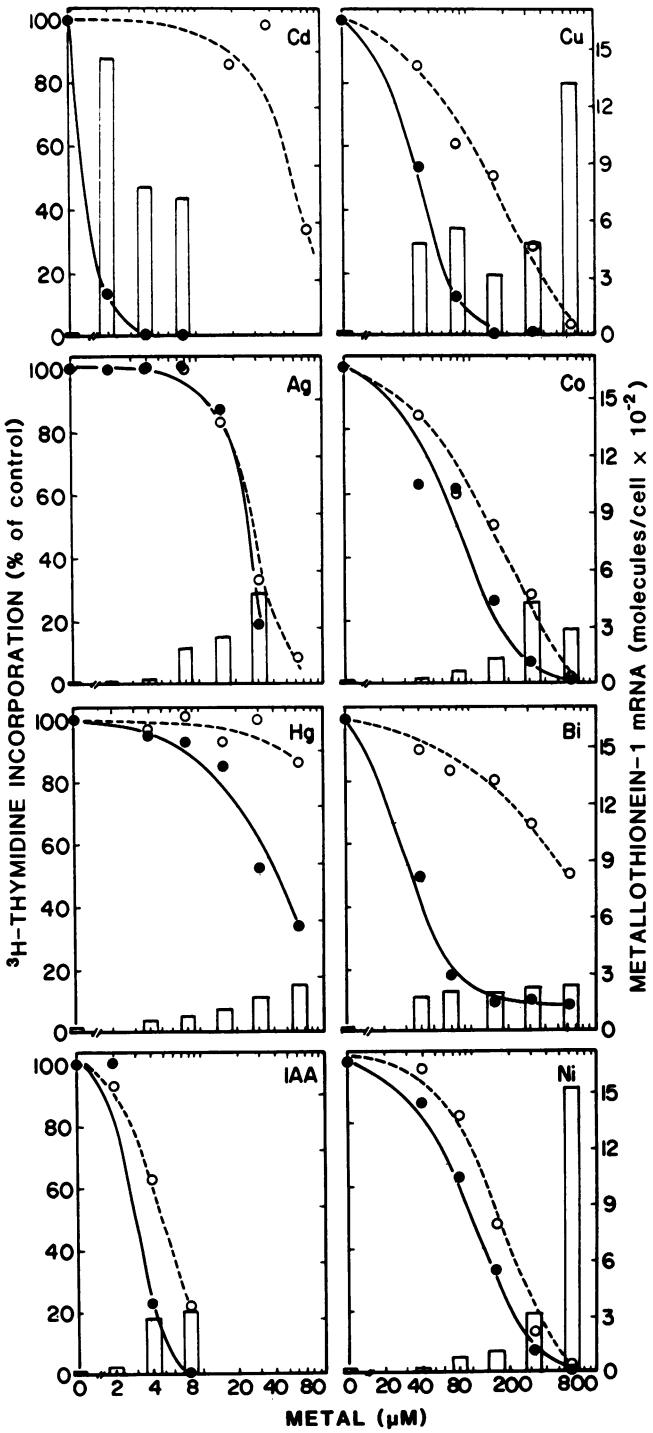

FIG. 5. Induction of MT-I mRNA in Cd^s and Cd^r_{80} cells by a variety of metals in Zn-free, serum-free media. Cd^r_{80} cells were grown in F12 medium containing 10% fetal calf serum and 150 μM $ZnSO_4$ for 7 days; then, Cd^r_{80} and Cd^s cells were plated in normal medium with 10% serum. The next day the cells were washed with saline, the medium was changed to Zn-free, serum-free F12, and the metals or iodoacetate (IAA) were added as indicated. After 14 h, total nucleic acids were isolated and analyzed by hybridization to determine the number of MT-I mRNA molecules per cell in the Cd^s cells (histograms). [^{3}H]thymidine was added during the last hour. In all panels, cell types are Cd^s (●) and Cd^r_{80} (○).

two cell types. Cu shows only a slight (~twofold) induction of MT-I mRNA in Cd^r_{80} cells, perhaps because optimal Cu concentrations for induction were too high to be maintained in solution in the culture medium. Se, CH_3Hg, Fe, and *cis*-diamminedichloroplatinum(II) (cisplatin) were also analyzed but showed no induction. Pb showed a slight induction of MT-I mRNA in Cd^s cells, but only at very high (2 mM) concentrations; the induction of MT-I mRNA in Cd^r_{80} cells was not analyzed.

The effect of these metals on DNA synthesis in Cd^s and Cd^r_{80} cells was measured in the same experiment (Table 1). Of the metals analyzed, only Cd, Zn, Cu, Hg, and Bi show an increased ED_{50} in Cd^r_{80} cells relative to Cd^s cells. ED_{50} of Se and CH_3Hg are also included in Table 1 as controls; these compounds do not induce MT-I mRNA, and the ED_{50} are the same.

We also compared the effects of the metals on uninduced Cd^s cells and on Cd^r_{80} cells that were grown in the presence of 150 μM $ZnSO_4$ for 10 days. In this experiment, the results do not depend on the induction of MT by the metal itself, but rather on whether the metal can be detoxified by the large amounts of Zn-MT already present in the Cd^r_{80} cells. These data (Fig. 5) demonstrate that, as in the previous experiments, Cd, Hg, Cu, and Bi show elevated ED_{50} for the Cd^r_{80} cells, as compared with the Cd^s cells, suggesting that MT can detoxify these metals. In contrast, Ag, Ni, and Co showed slight or no increases in ED_{50}. We conclude that although MT-I mRNA can be induced by eight different metals, MT only detoxifies a subset of the metals studied.

We conducted the latter experiments in Zn-free, serum-free medium because we were concerned that the various metals might induce MT-I mRNA indirectly, e.g., by displacing inducing agents from serum components. Zn was a likely possibility since Ham F12 medium has an unusually high (3 μM) concentration of Zn. Figure 5 shows that all of the metals which induced MT-I mRNA in complete medium also did so in Zn-free, serum-free medium. MT-I mRNA measurements were made only on Cd^s samples because the Cd^r_{80} cells were fully stimulated by Zn in this experiment. With some metals (Ag, Hg, Co, Bi) the maximum induction was not as high, whereas Cd, Cu, and Ni induced MT-I mRNA equally well in either medium. We conclude that all of the metals tested can induce MT-I mRNA by acting directly on the cells, although normal serum components may contribute to the induction in some cases. By comparing the ED_{50} of the Cd^s cells in normal medium (Table 1) and in zinc-free, serum-free medium (Fig. 5), it is apparent that all metals except Hg are less toxic in the presence of serum; the ED_{50} for Ag, Co, and Ni are ~twofold higher, whereas for Cd, Cu, Bi, and Zn (Fig. 4), the ED_{50} are 10- to 20-fold higher in the presence of serum. These differences probably reflect the affinity of the different metals for serum proteins.

Induction of MT-I mRNA by iodoacetate. Because it seems unlikely that metal ions interact specifically with the MT-I gene, we assume that the metals bind initially to a cellular protein that regulates MT-I gene transcription. Because most of the metals that induce MT-I mRNA accumulation also bind to sulfhydryl groups, we addressed the question of whether other, nonmetallic, sulfhydryl-binding reagents could also induce MT-I mRNA accumulation. Figure 6 shows that iodoacetate is an effective inducer of MT-I mRNA, inducing as well as Cd after a 6-h incubation period. Longer incubations (10 and 17 h) with iodoacetate showed a rapid loss of MT-I mRNA, most likely due to its extreme toxicity. In contrast, iodoacetamide and *N*-ethylmaleimide were ineffective inducers of MT-I mRNA, presumably because they do not enter the cells. Iodoacetate also induces MT-I mRNA in Zn-free, serum-free medium (Fig. 5), suggesting a direct action on the cells. In addition, Cd^r_{80} cells are slightly more resistant to iodoacetate toxicity than Cd^s cells (Fig. 5), as might be expected, considering that iodoacetate can displace metals from MT.

DISCUSSION

In this study, we applied a genetic approach to test the ability of MT to detoxify various heavy metals. First, we selected a cell line capable of growing in levels of Cd that would normally be toxic. The resistant cell line made about 20-fold more MT mRNA and protein than unselected cells. Analysis of the DNA from these cells revealed that the MT genes were amplified; however, unlike previous examples (2, 23), the amplified MT genes were located on double minute chromosomes. Then, we compared the toxicity of other metals on the sensitive parental line and the derived resistant line. In one set of experiments, the resistant cells were grown in the absence of Cd for 10 days to deplete them of Cd-MT and then challenged with various metals. In this experimental regimen, increased resistance presumably would depend upon the metal inducing MT gene transcription and then being sequestered by the resultant MT proteins. The results indicate that the Cd-resistant cells were also more resistant to Zn, Hg, Cu, and Bi, but they were equally sensitive to Ag, Ni, and Co, even though all of these metals induced MT-I mRNA. In another set of experiments, the Cd-resistant cells were preloaded with Zn-MT to elimi-

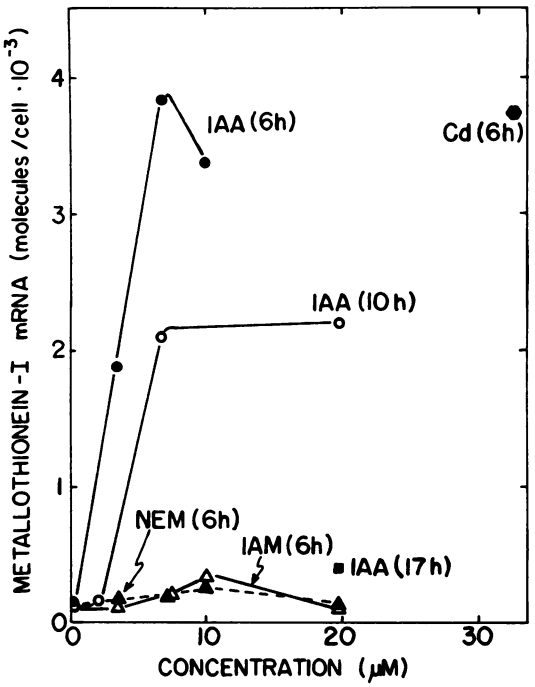

FIG. 6. Induction of MT-I mRNA by alkylating agents. Iodoacetate (IAA), iodoacetamide (IAM), and *N*-ethylmaleimide (NEM) were incubated with Cd^s cells at the concentration and incubation periods indicated. MT-I mRNA levels are expressed as molecules per cell and were determined by hybridization of total nucleic acid samples to MT-I cDNA. Inductions by iodoacetate for 6 h (●), 10 h (○), and 17 h (■) are shown. Six-hour inductions by iodoacetamide (△) and *N*-ethylmaleimide (▲) are indicated. The amount of MT-I mRNA induced in 6 h by 30 μM Cd (●) is shown for comparison.

nate the requirement for mRNA induction, and then their resistance was compared with that of untreated sensitive cells. This regimen also showed increased resistance only to Zn, Hg, Cu, and Bi. The combined results suggest that only a subset of metals are detoxified by MT. Hence, we would predict that of the metals tested, only Cd, Zn, Cu, Hg, and Bi could be used to select for MT gene amplification. The other metals (Ag, Co, and Ni) can be considered "gratuitous inducers" because they induce MT-I mRNA accumulation, but they apparently do not bind to MT under physiological conditions. It seems likely that the MT that is synthesized in response to these gratuitous inducers binds other trace metals, such as Zn and Cu, more avidly.

Our results agree with those of Gick et al. (14) by showing that Cd-resistant cells are also resistant to Zn, but other investigators have not obtained this result (10, 16). In one recent study (10), the effects of Zn, Hg, Cu, Co, and Ni on Cd-resistant CHO cells were tested, but these cells were only resistant to Cd and Hg. These differences may arise from the use of different cell types with different levels of MT gene amplification, but they are more likely due to different experimental designs and assays. We chose to use a cell line derived from liver, an organ which not only synthesizes high levels of MT, but also is one of the primary organs responsible for metal detoxification. In addition, because the half-life of MT is a function of metal content and because many of the assays of MT protein levels are indirect, we chose to measure MT mRNA accumulation rather than protein levels.

In this report, we focused on those metals which induce MT-I mRNA accumulation, although several others were tested (Se, Fe, Pb, and CH_3Hg) and found to be ineffective. Previous studies, based on partial purification of MT from animals injected with various metals, indicated that Cd, Zn, Cu, Hg, and Ag were effective inducers (9, 27, 33). However, inconclusive results were obtained with Co, Ni, Bi, and Pb. Many of the discrepancies in the literature undoubtedly result from the fact that the studies were performed in vivo, where the concentration and distribution of metal ions can vary, depending upon experimental design, and where stress (25) and trauma (15) can also induce MT. In addition, in most of these studies, the induced protein was not positively identified as MT. Analysis of MT mRNA induction in cell culture obviates all of these problems. When comparing the concentration of metals required for MT-I mRNA induction in Zn-free, serum-free medium, there was a 200-fold difference in potency between the extremes. Under these experimental conditions, the effectiveness (measured as the concentration required for one-half the maximal induction) of the metals can be ranked as Cd > Zn > Ag > Hg > Bi > (Co, Cu) > Ni. Different potencies might reflect differences in the affinity of these metals for postulated regulatory molecules involved in MT gene transcription. However, a critical parameter is the "free" concentration of the various metals inside the cell, a value that is difficult to establish. It is noteworthy that in all cases, the concentration range that allows maximal MT-I mRNA induction is also the range in which toxic effects of the metals are apparent.

We included iodoacetate in our studies as a nonmetallic inducer of MT. At physiological pH, iodoacetate interacts primarily with sulfhydryl groups. It has been shown to induce hepatic MT in mice (unpublished data) and rats (21), but it remained unclear whether this was a direct effect of iodoacetate or a response to stress of iodoacetate injection. It is apparent from these studies that iodoacetate can induce MT-I mRNA in Hepa cells. Furthermore, Zn-MT provides some protection against this alkylating agent.

Although the mechanism of MT gene regulation by heavy metals is not yet clear, the existence of gratuitous metal inducers argues against a simple model of autoregulation of MT genes by MT. Thus, we imagine a regulatory molecule with at least two domains: one which binds metals and one which binds to DNA sequences located near the MT gene promoter. The regulatory protein could be either a positive or negative regulator of MT gene transcription, but it presumably has one or more sensitive cysteinyl residues since iodoacetate is an effective inducer.

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