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Regulation of Metallothionein Gene Expression in Mammalian Cells by Gold Compounds

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SUMMARY

Metallothioneins are a class of low molecular weight, cysteine-rich proteins. Metallothioneins bind heavy metals and are thought to play a role in metal metabolism. Auranofin, an antiarthritic gold compound, is a potent inducer of metallothionein in Chinese hamster ovary cells. The induction of metallothionein by auranofin was mediated by active transcription of the gene and new mRNA was accumulated within 30 min after the exposure of Chinese hamster ovary cells to the drug. The extent of metallothionein induction was related to the concentration of the compound and was affected by the nature of the ligand attached to the gold molecule. A subline of these Chinese hamster ovary cells was established by growing them in the presence of normally cytotoxic concentrations of auranofin. In this auranofin-resistant cell line, the metallothionein genes were actively transcribed in the presence of auranofin, suggesting a relationship between cytotoxic action of auranofin and metallothionein gene transcription. Regulation of metallothionein gene transcription may play an important role in the molecular mechanism(s) of action of auranofin and resistance to it.

Auranofin (AF), 1-thio-β-D-glucopyranosato triethylphosphine gold 2,3,4,6-tetraacetate (see Fig. 1), is an orally absorbed gold compound active in the treatment of rheumatoid arthritis (1–4). Current evidence suggests that an important therapeutic target of the drug is the macrophage, which plays a critical role in the etiology of rheumatoid arthritis (5). It has been shown that AF alters suppressor macrophage function by retarding the production of IL-2 (5). More recent studies in our laboratory indicate that there exists a dynamic equilibrium of the drug between the cell membrane, cytoplasm, and the nucleus (6). A model describing the influx and efflux of the drug in tissue culture cells which involves shuttling of the gold molecule between various sulfhydryl-containing cellular moieties has been proposed (6). Furthermore, it is very likely that the active moiety of AF is the gold molecule which interacts with sulfhydryl-containing proteins. Gold may modulate activities of certain proteins by replacing a metal cofactor, by blocking the active site of the protein, or by causing conformational changes in enzymes. For example, it has been reported that gold compounds preferentially inhibit DNA polymerase α, a sulfhydryl-containing enzyme, and not DNA polymerase β (7).

AF has also been shown to have antitumor activity in mice inoculated with the lymphocytic leukemia, P388 (8, 9), but limited activity in other tumor models (9). A number of other gold compounds have been shown to have more significant antitumor activities *in vitro* and *in vivo* (10). Mechanisms by which gold compounds induce the cytotoxic effect have not been defined.

One site at which gold-containing drugs may interact is with the sulfhydryl-rich, small molecular weight proteins, i.e., MTs (11). MTs are evolutionarily conserved proteins, distributed throughout mammals, plants, and yeast (11–13). MTs are thought to play a role in heavy metal detoxification and zinc homeostasis (11). Mammalian cells studied thus far contain two forms of MT protein, designated as MT-I and MT-II. In humans, MTs are encoded by a complex gene family (14). Heavy metals such as Cd, Zn, Cu, Ag, Co, Hg, and Bi elevate the MT level by increasing the transcription of the MT genes (15). In addition to heavy metals, glucocorticoids and interferons also appear to regulate MT gene expression (15–18). DNA sequences responsible for the cadmium-mediated increase in transcription have been identified in the mouse MT-I and human MT-IIA genes (17, 19). Recent studies indicate that DNA elements required for cadium-induced transcription are distinct from those responsible for glucocorticoid-mediated gene activation of human MT-IIA gene (17).

A number of reports document the interactions of gold molecules with MTs (20–24). Studies performed by Schmitz *et al.* (20) demonstrated that sodium gold thiomalate was able to replace Zn or Cd that was associated with rat liver MT *in vitro*. Moreover, when rats were administered gold or cadmium, the gold moiety coeluted with the MT fraction isolated from liver and kidney (20). Additionally, it has been shown that the repeated administration of gold chloride (10 mg/kg, seven doses, every other day) to rats lead to an increase in a 12-kDa, cysteine-rich protein (22). Since gold compounds appear to

ABBREVIATIONS: AF, auroanofin; MT, metallothionein; CHO, Chinese hamster ovary; CDDP, *cis*-diaminedichloroplatinum; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetate.

interact with MTs and the data suggest that they may induce an MT-like protein, we have examined whether the gold regulates MT gene transcription. We have studied the effects of various gold complexes on CHO cells in tissue culture. Our studies demonstrate that gold is a potent inducer of MT gene transcription. The extent of MT induction was related to the concentration of the compond and affected by the nature of the ligand(s) attached to the gold molecule. Moreover, we have shown that AF is cytotoxic to CHO cells and that resistance to the drug is mediated by increased transcription of the MT gene.

Materials and Methods

Chemicals. Analytical grade chemicals were used throughout this study. Cadmium chloride was obtained from Aldrich Chemical Co. Gold compounds used in the present studies were synthesized at Smith Kline and French Laboratories (1, 10). The chemical structures of the gold compounds employed are shown in Fig. 1: AF, chloro (triethylphosphine) gold (AF without the substituted glucose), chloro (cyclohexylemine) gold (a nonphosphine gold), β-D-thioglucose tetraacetate (substituted glucose moiety), and *cis*-diaminedichloroplatinum (CDDP). CDDP was obtained from Sigma Chemical Co. Stock colutions (1–10 mM) of the gold compounds were prepared in 95% ethanol or dimethyl sulfoxide. Radioactive nucleotides were purchased from New England Nuclear. Diethylpyrocarbonate was purchased from Sigma Chemical Co.

Cell culture. CHO cells Cd^r 20F4 (25), subsequently referred to as CHO, are resistant to 20 μM $CdCl_2$ in continuous culture conditions. These cells were subcultured in the absence of cadmium for several months. Induction of MT gene transcription by gold compounds was performed on cells maintained in the absence of cadmium. Gold- and cadmium-resistant CHO cell lines were maintained on monolayer cultures with Ham's F-10 medium, supplemented with 15% newborn calf serum (Gibco). Cadmium-resistant CHO cells were kindly provided by Dr. E. C. Hildebrand of Los Alamos National Laboratory (26).

AF-resistant cells (Au^r) were obtained by the following method. Cell cultures in T-75 flasks (grown in the absence of cadmium) were treated with 5 μM AF for 5 hr. This treatment results in approximately 50% cell death. Dead cells were removed by changing the media and the surviving cells were cultured for 2 weeks in 5 μM AF. The growth media were changed every second day and fresh AF was added. Under these conditions the doubling time for Au^r cells was similar to that of parent cell line (18 hr), grown in the absence of AF. The AF concentration was increased to 10 μM and surviving cells were adapted to AF by continuous subculturing for 8 weeks. Single clones were selected as previously described (26). Cells which were resistant to 10 μM AF were designed Au^{r10}. Aliquots of logarithmically growing cultures were frozen in Ham's F12 media and 10% glycerol and in the absence of AF at −70°.

Cell survival assay. Asynchronous populations of cells were harvested by trypsinization. The cells were then washed with media and counted. The cells were diluted to a density of 1×10^5/ml, and 0.5 ml of cells were incubated at 37° with at least five different concentrations of each drug to obtain the IC_{50}. After incubation with the compound the cells were diluted to a density of 1 cell/μl and 5 ml of cells (5000 cells) were plated on 60 × 15-mm tissue culture plates. Plates were incubated for 4–6 days at 37° in a CO_2 (5%) incubator. Thereafter, the media were removed, the plates were washed with 0.15 M NaCl, fixed, and stained with 1% crystal violet, and colonies were counted by a New Brunswick Automatic Colony Counter (New Brunswick, NJ). Means and standard deviations of triplicate samples were determined for each drug concentration. Viability was measured by the ability of cells to form colonies of the survival fraction (number of colonies in the drug-treated plates per number of colonies in control) versus the drug concentration.

Analysis of MT. Monolayers of CHO or CHO Au^r cells were subcultured to semilogarithmic stages in the presence or absence of test compounds and incubated with 30–100 μCi of [^{35}S]cysteine (NEG 002T, New England Nuclear) for 3 hr. Cells were used during logarithmic growth. After the incubation, cells were washed with PBS and harvested. Approximately 1×10^6 cells were labeled with [^{35}S]cysteine per data point. Cells were pelleted in 1.5-ml Eppendorf Microfuge tubes and lysed in 200 μl of 20 mM Tris-HCl, pH 8, 10 mM β-mercaptoethanol, 0.4% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride (lysis buffer). The lysate was centrifuged for 5 min in a microcentrifuge (Beckman Microfuge) to pellet the debris, supernatant was recovered for further analysis.

Equal amounts of protein (100 μg) were aliquoted from each sample supernatant. Supernatants were saturated with cadmium by addition of 1 mM cadmium chloride. Each sample was subjected to 18% polyacrylamide gel electrophoresis in the absence of urea or sodium dodecyl sulfate (12). The gel was fixed in acetic acid and treated with Autofluor (National Diagnostics), dried, and autoradiographed.

Isolation of mRNA and from CHO cells. The effect of gold compounds on the regulation of MT mRNA transcription was analyzed by Northern plot analysis as described previously (12). Total RNA was analyzed from cells challenged with various concentrations of compounds. For the preparation of total RNA, cells (5×10^6) grown in T-75 flasks were challenged with various gold compounds for appropriate periods of time. Cyclohexylimide was added to the cultures (50 μg/ml) 5 min before harvesting. The cell layer was washed with 5 ml of PBS and suspended in 5 ml of PBS. Cells were pelleted, washed, and lysed in 0.50 ml of 10 mM Tris-HCl, pH 8, 1 mM EDTA, 1X vanadium

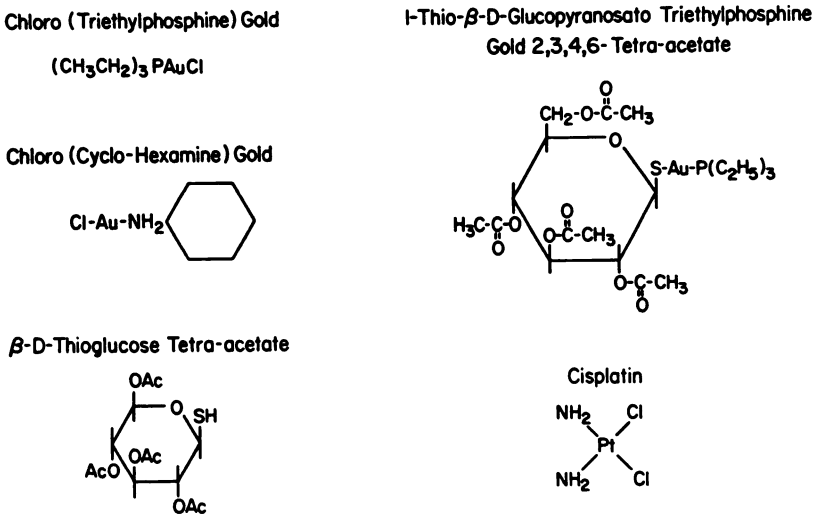

Fig. 1. Chemical structures of the gold compounds employed.

ribonucleoside complex (Bethesda Research Laboratories), and 0.4% Nonidet P-40 for 10 min at 4°. The lysate was centrifuged in a microcentrifuge for 3 min and the RNA-containing supernatant was extracted three times with equal volumes of phenol (Tris-saturated). The RNA was washed two times with 70% ethanol. RNA was denatured for 5 min at 65° in 50 % formamide, 6% formaldehyde in MOPS buffer (20 mM morpholinepropane sulfonic acid, pH 8.0, 5 mM sodium acetate, and 1 mM EDTA). Samples were electrophoresed in 1.5% agarose-formaldehyde gel as described previously (12, 27). After electrophoresis the RNA was transferred to nylon membranes according to the manufacturer's instruction (Pall, Glen Cove, NY). Filters were hybridized with 2×10^6 cpm/ml of ^{32}P-labeled-nick-translated cDNA from CHO MT-I and MT-II (28) and autoradiographed.

Results

To allow better monitoring of MT synthesis, the CHO cell line employed in this study had been previously adapted to grow in 20 μM $CdCl_2$ (25). This cell line has amplified its MT-I and MT-II genes 6-fold as compared to the parent cell line (25). Before challenging the CHO cells with AF or other compounds, the cells were allowed to grow in the absence of $CdCl_2$ for 5 months. Fig. 2 describes the kinetics of MT mRNA production in CHO cells after being challenged with AF at 3 μM for various periods of time. Total cellular RNA from treated or untreated cells was electrophoresed on 1.5% formaldehyde-agarose gels and analyzed as described in Materials and Methods. In the absence of AF, no MT mRNA was detectable under our experimental conditions (Fig. 2, lane *1*). AF induced a time-dependent increase in MT mRNA. The synthesis of MT mRNA was first detected after 30 min of treatment (fig. 2, lane *3*) and decreased with time (Fig. 2, lanes *4–6*). The amount of mRNA induced by 3 μM AF in 6 hr was approximately equal to the RNA induced by 20 μM $CdCl_2$ in 15 hr (Fig. 2, lane *7*).

To determine the sensitivity of these cells to AF and $CdCl_2$, colony survival assays were performed. Parent CHO cells which were derived from a cadmium-resistant clone maintained their cadium resistance (IC_{50}, 140 μM $CdCl_2$). The IC_{50} value for AF in the same cell populations was 7 μM (see Table 1). The apparent difference in IC_{50} values of $CdCl_2$ between this and the previous work relates to the different protocol used to determine the IC_{50} values.

To address the question of whether the resistance to AF was associated with an increase in MT production, the cadmium-adapted CHO cells were treated with increasing concentrations of AF as described in Materials and Methods. The CHO Au^r cells thus derived were grown in 7.5 μM and 10 μM AF, drug concentrations which are toxic to the parent cadmium-adapted cells. Interestingly, the IC_{50} for $CdCl_2$ in $Au^{r7.5}$ cells increased to 302 μM from 140 μM in the parent cells as shown in Table 1. The level of resistance to AF was demonstrated by colony survival assays described previously. The IC_{50} for AF in $Au^{r7.5}$ cells was 17.5 μM (see Table 1). Fig. 3 shows the analysis of MT mRNA from $Au^{r7.5}$ cells. As shown in lane 2, Au^r cells contained increased amounts of MT mRNA as compared to the parent cells (Fig. 3, lane *1*) which did not contain any detectable RNA hybridizable to CHO MT-I and MT-II. Removal of AF for 4–8 hr had virtually no effect on quantities of MT mRNA (Fig. 3, lanes *2* and *3*). However, no MT mRNA was detected at 24 or 33 hr after the removal of AF (Fig. 3, lanes *5* and 6). The band at the top of the gel is due to nonspecific interactions at MT mRNA with 28 S ribosomal RNA. Ribosomal RNA is the major component of total cellular

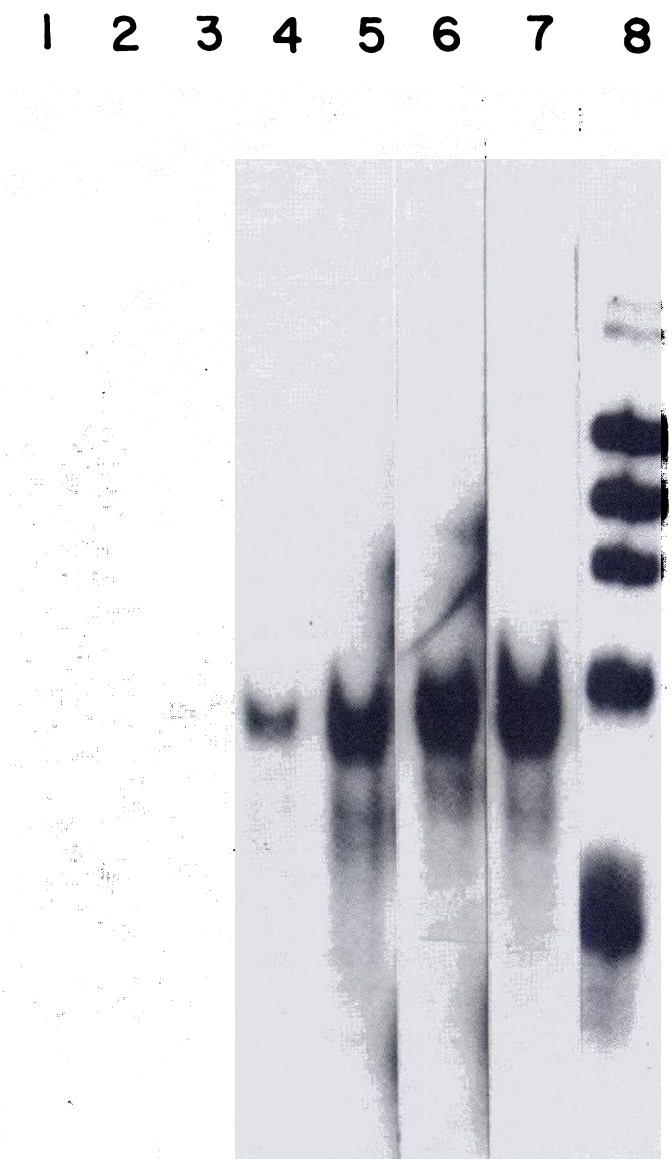

Fig. 2. Kinetics of MT mRNA induction by AF. CHO cells were treated with 3 μM AF for various periods of time. Total RNA was prepared and 10 μg of RNA from each sample were analyzed by electrophoresis on formaldehyde-agarose gels. The RNA was transferred to nylon membranes (Pall) and hybridized with [^{32}P]dCTP nick-translated MT-I and MT-II cDNA probe. After hybridization autoradiographs were developed. *Lane 1*, control, no drug treatment; *lanes 2–6*, AF treatment for 10 min, 30 min, 1.5 hr, 3 hr, and 6 hr, respectively; *lane 7*, cells treated with 20 μM $CdCl_2$ for 15 hr; *lane 8*, ^{32}P-labeled molecular weight standards, Hind III digested of λ DNA and Hea III-digested ϕ ×174 DNA.

RNA. These results provide evidence that resistance to AF is associated with the increased expression of MT gene, and once the drug is removed, the mRNA of MT is turned over between 12 and 24 hr. More recent results indicate that the half-life of the MT mRNA was identical (12 hr) whether induced by AF or $CdCl_2$ (data not shown). These results suggest that the increased signal in the MT mRNA band is not due to a decrease in breakdown of the mRNA but very likely due to new transcription of the gene.

To determine whether the gold-regulated expression of MT mRNA was translated into MT protein, Au^{r10} cells were labeled with [^{35}S]cysteine and total cellular proteins were analyzed by

TABLE 1
Properties of cell lines employed in present study

Cell line	Phenotype	Parent	IC_{50} for AF	IC_{50} for $CdCl_2$
			μM	μM
CHO	Resistant to 20 μM $CdCl_2$	Refs. 24, 28	7	140
$CHOAu^{r7.5}$	Resistant to 7.5 μM AF	CHO	17.5	302
$CHOAu^{r10}$	Resistant to 10 μM AF	CHO	22.5	not determined

polyacrylamide gel electrophoresis. The results described in Fig. 4 demonstrate that the CHO Au^{r10} cell line synthesized both isoforms of MT (Fig. 4, lane *3*).

To determine what portion of AF may be responsible for MT gene regulation, the effects of AF and analogues on MT gene expression were determined. Fig. 5A describes the results from the analysis of an experiment in which cells were treated with three different concentrations of chloro(triethylphosphine) gold (AF molecule without tetraacetyl glucose moiety) (Fig. 5A, lanes *1–3*). There was a clear induction of MT gene transcription upon treatment of the cells with this compound. However, no MT gene transcription was observed upon treatment of the cells with a metal-free ligand of AF (β-D-thioglucose tetraacetate) (Fig. 5A, lanes *4–6*). Lanes *4–6* (Fig. 5B) also demonstrate the induction of the MT gene by chloro(cycloeylemine)gold(I), a gold compound without triethylphosphine. These results further confirm that the active MT-inducing agent of AF is gold. In contrast, 1, 5, and 20 μM CDDP (24) did not induce MT gene transcription in these cells under identical experimental conditions (Fig. 5A, lanes *7–9*).

Discussion

Although several studies have demonstrated that MT binds gold when presented in several compounds (20, 21, 23) and it has been reported that the administration of gold chloride to rats resulted in the induction of a 12-kDa protein in the kidney, the regulation of MT gene expression has not been documented (22). To enhance our ability to observe the induction of MT genes, we studied CHO cells in which the MT gene has been amplified 6-fold by continuous exposure to $CdCl_2$ (25). Our studies demonstrate that AF, gold triethyl phosphine chloride, and cyclohexylemine gold chloride induced transcription of MT genes and this resulted in the production of a sulfhydryl-rich, 6-kDa protein with characteristics equivalent to both isoforms of MT (11).

The induction of MT gene transcription by AF is rapid. A significant amount of MT mRNA was detected within 30 min following exposure of 3 μM AF. Furthermore, the induction increased with increasing time of exposure to AF for up to 6 hr. The kinetics of induction of MT mRNA by AF in these cells are comparable to those of $CdCl_2$ (Fig. 2).[1] Thus, it appears that the mechanisms of MT gene activation for AF may be analogous to that of Cd. The detailed mechanisms of MT gene expression have not been elucidated in mammalian systems. However, in yeast, mutations in the chromosomal copper MT gene have been used to show that the MT autoregulates its gene transcription (29). The observation that gold compounds are potent inducers of MT gene transcription suggests that gold or cadmium MT may interact with the regulatory elements in an analogous manner to promote transcription of the gene.

Deinduction of the MT gene transcription after removal of AF was also observed in Au^r cells. No differences in MT mRNA were detected at 3 or 6 hr after the removal of AF. However, 24 or 33 hr after removal of AF, minimum amounts of MT mRNA were observed. Inasmuch as only one cell division could have occurred in 24 hr, the loss of MT mRNA cannot be explained by a failure to maintain the amplified MT gene in the progeny.

After continous exposure of cadmium-adapted CHO cells to

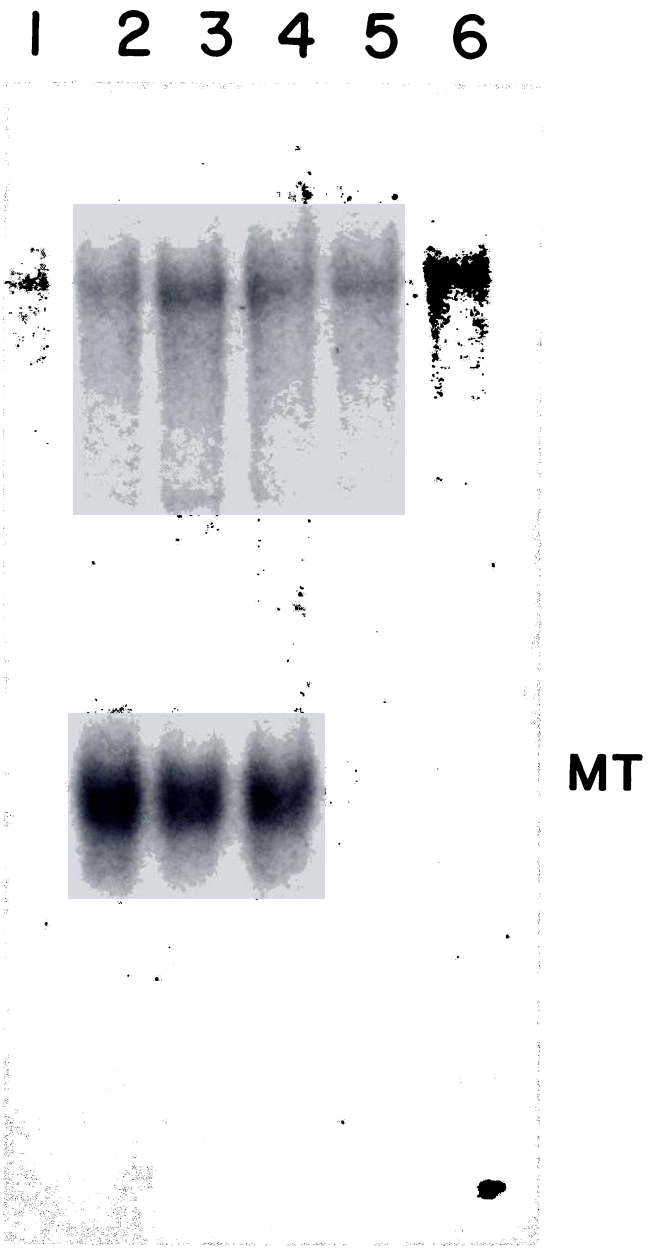

Fig. 3. Analysis of MT mRNA in AF-resistant cell line. CHO cells adapted to 7.5 μM AF were analyzed for steady state MT mRNA production in the presence and absence of AF. Ten μg of total RNA were analyzed on formaldehyde-agarose gel as described in Fig. 2. *Lane 1*, parent CHO cell, no drug treatment; *lane 2*, $Au^{r7.5}$ cell in the presence of 7.5 μM AF; *lanes 3–6*, AF removed from the $Au^{r7.5}$ cell media for 3, 8, 24.5, and 33 hr, respectively.

[1] T. R. Butt, E. J. Sternberg, C. K. Mirabelli, and S. T. Crooke, unpublished results.

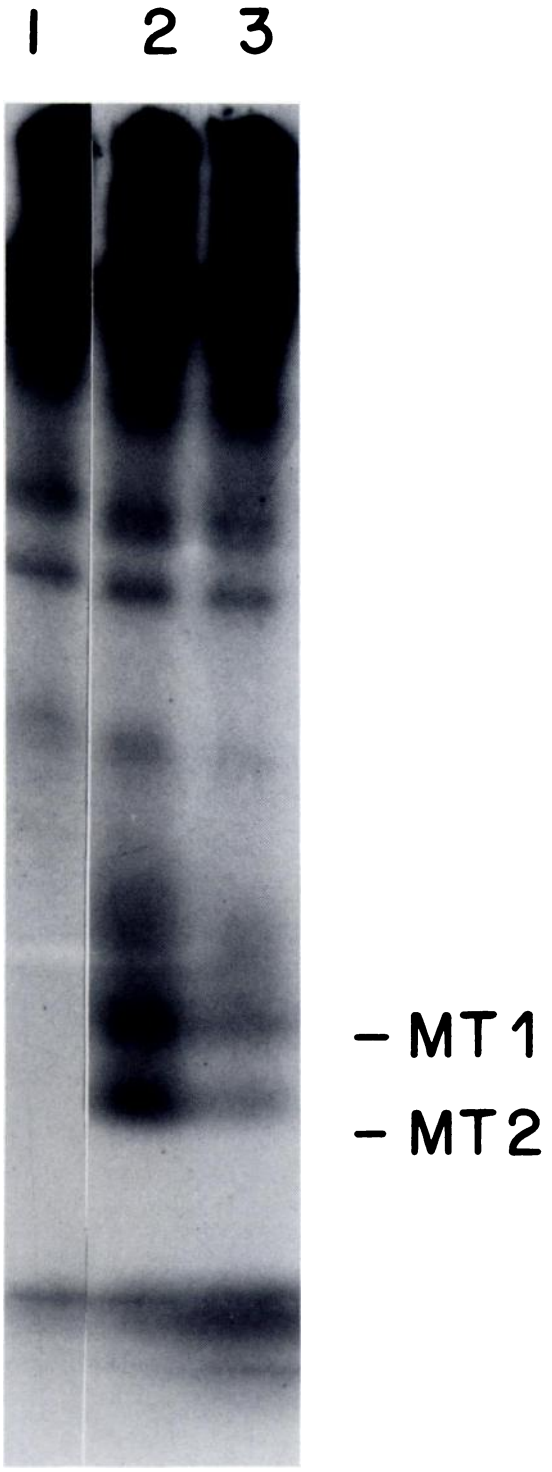

Fig. 4. Analysis of MT protein from CHO and CHO Au^{r10} cells by polyacrylamide gel electrophoresis. Cells were grown in the presence of AF or $CdCl_2$. [^{35}S]Cysteine was added at 4 μCi/ml and incubated for 3 hr, and cell lysates were prepared as described in Materials and Methods. Proteins (100 μg) from each sample were analyzed on the gel. *Lane 1*, CHO cell, control; *lane 2*, CHO cells treated with 20 μM $CdCl_2$ for 15 hr; *lane 3*, CHO Au^{r10} grown in the presence of 10 μM AF.

AF, the $Au^{r7.5}$-resistant clones exhibited in IC_{50} of 17.5 μM for AF, an increase from 7 μM in the parent cell line. The increased resistance can be explained on the basis of increased MT by AF. Although we have not precisely measured the levels of MT mRNA, no significant difference in the mRNA-synthesizing capacity of CHO $Au^{r7.5}$ or CHO cells was observed as shown in Figs. 2 and 3. We have also compared the copy number of the MT gene in CHO $Au^{r7.5}$ and CHO cells by restriction enzyme analysis of total DNA and Southern blots as documented by Crawford *et al.* (25). No difference in the copy number of the MT gene was observed among the two populations of cells.[1] Several lines of evidence suggest that induction of MT was at least, in part, responsible for the observed resistance. All of the cell clones which were resistant to AF demonstrated elevated levels of MT mRNA. All of the clones that displayed induced MT synthesis were resistant to AF. Moreover, the induction of MT mRNA by AF was rapid and the removal of AF was associated with deinduction of the MT gene (Fig. 4). Nevertheless, proteins other than MT may be responsible for resistance to AF and they are the subject of a study in progress.[2]

An important issue in these studies, as well as any other studies on MT gene expression, is whether the inducer regulates MT gene expression by direct interaction with the regulatory factor or by altering the uptake of zinc or by replacement of protein-bound cellular zinc with gold molecules. A definitive answer to these questions will only be obtained after a cell-free system is established in which regulation of gene expression can be monitored upon addition of inducer and the contents of free and protein-bound metal(s) can be precisely determined. Previous studies have shown that protein-bound zinc and not copper is more likely to be exchanged by gold molecules (21). These studies further indicated that treatment of gold compounds to rats did not alter the levels of zinc or cytosolic high molecular weight proteins (21). In addition, we note that the level of MT mRNA induced by 3 μM AF is approximately equal to the level of MT mRNA induced by 20 μM $CdCl_2$. The above studies would suggest that gold may be the direct inducer of the gene transcription; however, we cannot exclude the possibility that induction of MT by AF may be a secondary event resulting from the alteration of intracellular zinc.

Although the mechanism of action of AF and other gold compounds is not defined, recent studies suggest that effects of AF on macrophages may be important (5). Furthermore, studies in our laboratory have shown that the gold in AF is taken up by cells via a sulfhydryl exchange process and that principal sites of interaction in the cell are sulfhydryl-containing proteins, particularly those in the plasma membrane (6). The present studies suggest that an important mechanism by which some cells may protect themselves from the effects of gold-containing compounds may be the synthesis of MT which effectively tritates out the gold, both in the cytosol and via ligand exchange processes out of the plasma membrane. This may be important in defining which cells in the immune system are sensitive to AF and the types of toxicities observed. Therefore, modulation of the MT levels in cells may modulate their response to gold compounds and, thus, affect the therapeutic response to gold compounds.

Additionally, recent studies in our laboratory have shown that gold compounds are cytotoxic and that many have significant *in vivo* antitumor activities against a number of tumor types (10). Moreover, treatment of CHO cells with CDDP did not induce MT gene transcription. Our observations suggest that the induction of MT synthesis may be an important mechanism of resistance to gold compounds and that cell lines

[2] T. R. Butt, E. J. Sternberg, C. K. Mirabelli, and S. T. Crooke, manuscript in preparation.

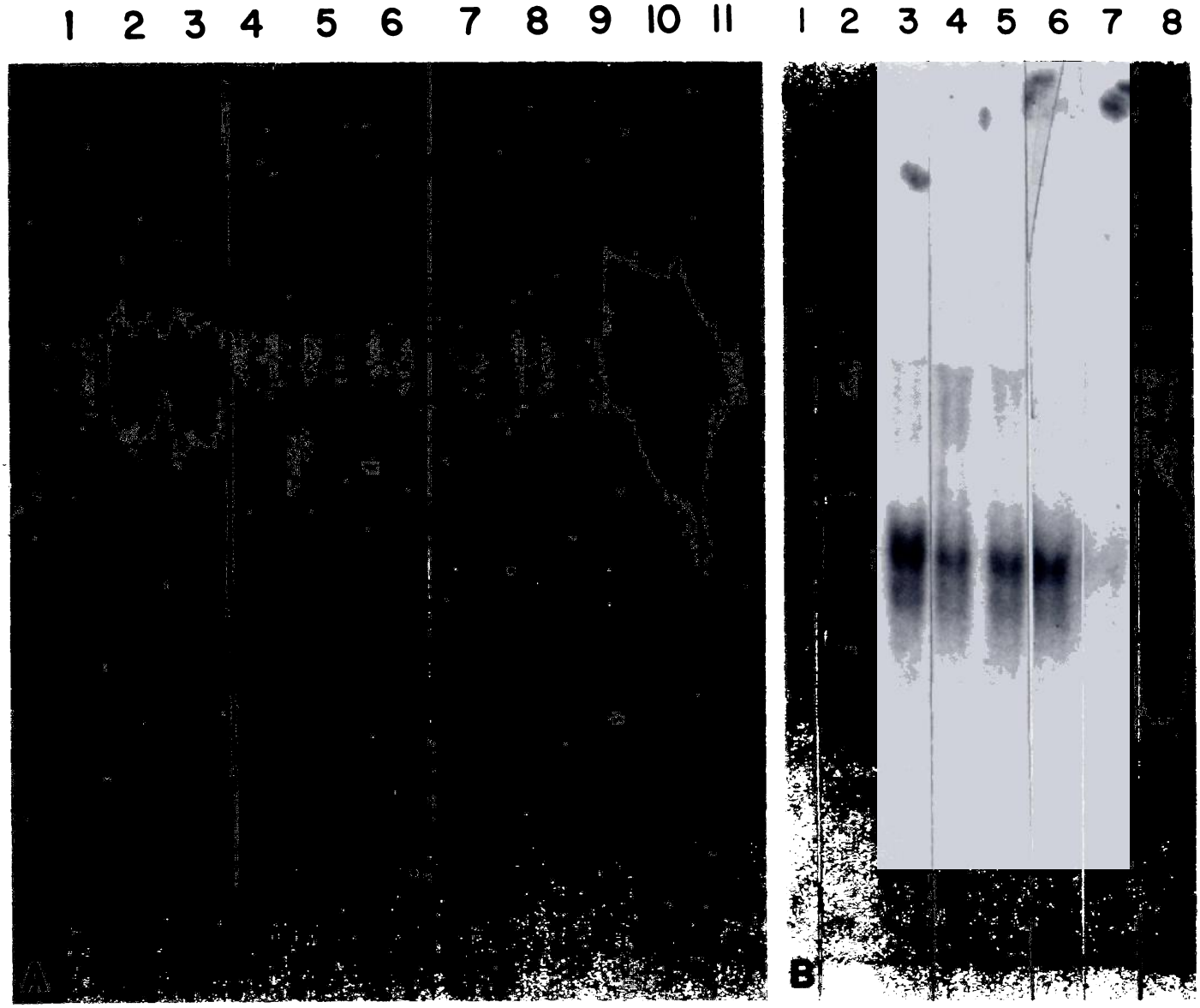

Fig. 5. Regulation of MT mRNA induction by components of AF and CDDP. CHO cells were treated with the following compounds for 12–15 hr; total RNA was prepared after the drug treatment and 10 μg of RNA analyzed by formaldehyde-agarose gel as described for Fig. 2. Autoradiograph A: *lanes 1–3,* cells traeted with 1 μM, 2 μM, and 5 μM chloro(triethylphosphine)gold, respectively, (Au without the substituted glucose); *lanes 4–6,* 1 μM, 2 μM, and 3 μM of thioglucose tetraacetate, respectively (substituted glucose moiety of Au); *lanes 7–9,* 1 μM, 5 μM, and 20 μM of CDDP; *lane 10,* 20 μM $CdCl_2$; and *lane 11,* control, no drug treatment. Autoradiograph B: *lanes 1–3,* cells treated with 1 μM, 2 μM, and 5 μM Au, respectively; *lanes 4–6,* 10 μM, 20 μM, and 50 μM chloro(cyclohexylemine)gold(I), respectively (a nonphosphine gold complex); *lane 7,* control, no drug treatment; lane 8, cells treated with 20 μM $CdCl_2$ for 15 hr.

resistant to CDDP are probably resistant via other mechanisms. These observations also suggest that gold compounds may have a spectrum of clinical anticancer activities different from that of platinum compounds which suggests that, at the cellular level, a combination of gold- and platinum-containing compounds might be additive. However, the value of such a combination in the treatment of human malignancies would be defined by a complex set of mechanisms in addition to the cellular pharmacology of the agents (30).

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