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Regulation of Cd^{2+}/Zn^{2+}-Stimulated Metallothionein Synthesis during Induction, Deinduction, and Superinduction†

C. E. Hildebrand* and M. D. Enger

ABSTRACT: Metallothioneins (MTs) are low-molecular-weight, thiol-rich, metal-binding proteins which are synthesized in animal tissues or in cultured cells in response to Cd^{2+} or Zn^{2+} exposure. We have examined regulation of MT synthesis in a Cd^{2+}-resistant (Cd^r) Chinese hamster cell which is proficient in induction of MT synthesis following exposure to either Cd^{2+} or Zn^{2+}. The MTs synthesized by the Cd^r cell were characterized by Sephadex G-75 column chromatography, nondenaturing polyacrylamide gel electrophoresis, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The Cd^r cell MTs are cysteine rich and leucine deficient, bind Zn^{2+} and Cd^{2+} with high affinity, and are synthesized only during exposure of the cells to Cd^{2+} or to excess Zn^{2+}. Both of the major isoMTs reported to be synthesized in cells in vivo or in culture are induced coordinately in Cd^r cells during Cd^{2+} or Zn^{2+} exposure. Following addition of Cd^{2+} (2 μM) or Zn^{2+} (100 μM) to the growth medium, MT synthesis in Cd^r cells increases above the low basal level within 2 h, reaches a maximal synthesis rate (at least 30-fold > basal level) by 7 h, and thereafter declines slowly (by 24 h) to a constant rate of approximately 40–60% of the maximal rate. Induction of MT synthesis is inhibited by 5 μg/mL actinomycin D (AM), indicating that RNA synthesis is required for induction. MT synthesis also is dependent upon the continued exposure to inducing metal since withdrawal of inducer at the time of maximal MT synthesis results in a deinduction of MT synthesis with a half-time of 2–3 h. Studies of AM effects at both high (2 μg/mL) and low (0.05 μg/mL) levels on induction and deinduction kinetics revealed the phenomenon of "superinduction" of MT synthesis at the high AM level but not at the low level. Analyses of induction and deinduction kinetics, together with the results of RNA synthesis inhibitor studies, suggest that induction of MT synthesis is regulated primarily at the level of transcription of thionein mRNA. Further, examination of primary and secondary induction (i.e., secondary exposure to inducer following deinduction from a primary exposure) events indicates that translational control over MT synthesis is not a major regulatory factor in this cellular system. Finally, the existence of the AM-mediated superinduction of MT synthesis, a phenomenon originally observed in steroid-mediated induction of specific hepatic enzymes, suggests that similar regulatory mechanisms may operate in these quite different inducible systems.

Metallothioneins are low-molecular-weight, thiol-rich, metal-binding proteins (Margoshes & Vallee, 1957; Kägi & Vallee, 1960, 1961). They are ubiquitous among eukaryotes and have been reported in microorganisms [reviewed by Kojima & Kägi (1978)]. These proteins, or thioneins, are synthesized de novo by cells both in vivo (Piscator, 1964; Webb, 1972; Richards & Cousins, 1975a; Winge et al., 1975) and in culture (Lucis et al., 1970; Webb & Daniel, 1975; Hildebrand et al., 1979, and references therein) as a consequence of exposure to Zn^{2+}, Cd^{2+}, and, in some cases, Hg^{2+} or Cu^{2+} (Kojima & Kägi, 1978). Although a well-defined physiological function of metallothionein has not been established, much of the previous work on metallothionein has been performed in the context of trace-element metabolism [e.g., in homeostatic regulation of essential trace metals (viz., Zn^{2+})] (Cousins, 1979) and detoxification of nonessential heavy metals (viz., Cd^{2+} or Hg^{2+}) (Kojima & Kägi, 1978; Cherian & Goyer, 1978). Only a few studies have focused on the mechanisms involved in the regulation of induction of thionein synthesis (Richards & Cousins, 1975b; Squibb et al., 1977; Day et al., 1978; Panemangalore & Brady, 1978). Several reports suggest that the primary induction of thionein synthesis (i.e., the response occurring upon initial exposure to the inducing metal) requires transcription of thionein-specific mRNA (Richards & Cousins, 1975b; Squibb & Cousins, 1977; Squibb et al., 1977; Anderson & Weser, 1978; Hidalgo et al., 1978; Panemangalore & Brady, 1978; Enger et al., 1979b). However, some studies suggest that control of induction of thionein synthesis also is exerted at the level of translation of thionein mRNA (Squibb et al., 1977; Anderson & Weser, 1978); Panemangalore & Brady, 1978).

In the present study, we have utilized a Cd^{2+}-resistant variant of the cultured Chinese hamster cell (line CHO), which has been shown to be especially proficient in the Cd^{2+}- or Zn^{2+}-mediated induction of metallothionein (MT)[1] synthesis (Hildebrand et al., 1979), to examine the regulation of metal-mediated MT synthesis. Further, the phenomenon of ac-

† From the Genetics Group, Life Sciences Division, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87545. *Received August 31, 1979.* This work was performed under the auspices of the U.S. Department of Energy.

[1] Abbreviations used: MT, metallothionein; AM, actinomycin D; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; TEMED, *N,N,N',N'*-tetramethylethylenediamine; cpm, counts per minute; Cx, cycloheximide; Puro, puromycin.

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tinomycin D mediated "superinduction" of inducible enzyme systems (Garren et al., 1964; Tomkins et al., 1972) was examined in this inducible system. Finally, experiments were performed to study the mechanisms involved in the regulation of MT synthesis during a cycle of primary metal-mediated induction, deinduction (following withdrawal of metal), and secondary induction. These experiments were performed to determine whether the primary response is controlled transcriptionally, while the secondary response is regulated at the level of translation as well as at the level of transcription, as suggested by studies in vivo (Squibb et al., 1977; Anderson & Weser, 1978; Panemangalore & Brady, 1978).

Materials and Methods

Cell Culture. A Cd^{2+}-resistant variant subline (Cd^r2C10, designated Cd^r hereafter) of the Chinese hamster (line CHO) cell was isolated and characterized for its resistance to Cd^{2+} exposure (up to 2.0 μM in the medium), its proficiency to induce MT synthesis, and its phenotypic stability during prolonged growth in the absence of Cd^{2+} (Hildebrand et al., 1979). Details of the isolation and characterization of the variant are provided elsewhere (Hildebrand et al., 1979). Zinc concentrations in growth medium and in stock solutions were determined by atomic absorption analysis. Dilutions of the stock solutions were made into sterile, deionized, glass-distilled water and were stored frozen at −20 °C in polyethylene tubes to minimize adsorptive loss. All experiments reported here were performed by exposing Cd^r cells to metal ions in suspension culture. High (5.0 or 2.0 μg/mL) or low (0.05 μg/mL) levels of AM were used to inhibit total RNA synthesis or nucleolar RNA synthesis (Perry & Kelley, 1970; Enger & Hanners, 1978), respectively. Cycloheximide (8.4 μg/mL, the Upjohn Co.) or puromycin (200 μg/mL, Calbiochem-Behring Corp.) was used to inhibit protein synthesis.

Radioisotopic Labeling and Cell Fractionation. For demonstration of the existence of low-molecular-weight metal-binding proteins in this cultured cell system, Cd^r cells were labeled with $^{109}Cd^{2+}$ by including tracer levels of $^{109}CdCl_2$ in the growth medium during exposure to Cd^{2+}. Alternatively, for detection of the presence of the metallothioneins on the basis of their cysteine content, cells (typically 100 mL of culture at >200 000 cells/mL) were labeled with 0.350 μCi/mL [^{35}S]Cys (New England Nuclear Corp., >260 Ci/mmol) for 30 min. Cell harvesting and fractionation procedures are described elsewhere (Hildebrand et al., 1979).

Measurement of Metallothionein. Measurement of metallothionein was carried out in one of three ways.

(1) *Sephadex G-75 Column Chromatography.* These procedures have been reported previously (Hildebrand et al., 1979).

(2) *Sodium Dodecyl Sulfate (NaDodSO$_4$)–Polyacrylamide Gel Electrophoresis of Carboxymethylated Thionein.* Samples of ^{35}S-labeled total cytoplasm or of [^{35}S]MT, partially purified by Sephadex G-75 column chromatography, were prepared for electrophoresis and analyzed on 15% NaDodSO$_4$–polyacrylamide gels as reported previously (Enger et al., 1979a).

(3) *Polyacrylamide Gel Electrophoresis of Native Metallothionein.* Native polyacrylamide gel electrophoresis was used to measure the levels of the isometallothioneins, which have been reported in other systems (Nordberg et al., 1972; Winge et al., 1975; Rudd & Herschman, 1979). Procedures for sample preparation and electrophoresis have been described elsewhere (Enger et al., 1979a).

Measurement of Metallothionein Synthesis Rate. Sephadex G-75 column chromatography of cytoplasm from [^{35}S]Cys pulse-labeled cells (shown in Figure 1B) separated the ^{35}S]MT from larger ^{35}S-labeled cytoplasmic components and from unincorporated [^{35}S]Cys. The relative rate of MT synthesis was determined as 100 times the ratio of ^{35}S cpm in the MT peak to the ^{35}S cpm in nonMT proteins eluting before MT. This method for determining the relative rate of synthesis of inducible proteins has been used previously (Tomkins et al., 1972; Steinberg et al., 1975a), and further evidence for the validity of this method in the present application is provided in the Results. Total protein was determined by the method of Lowry et al. (1951).

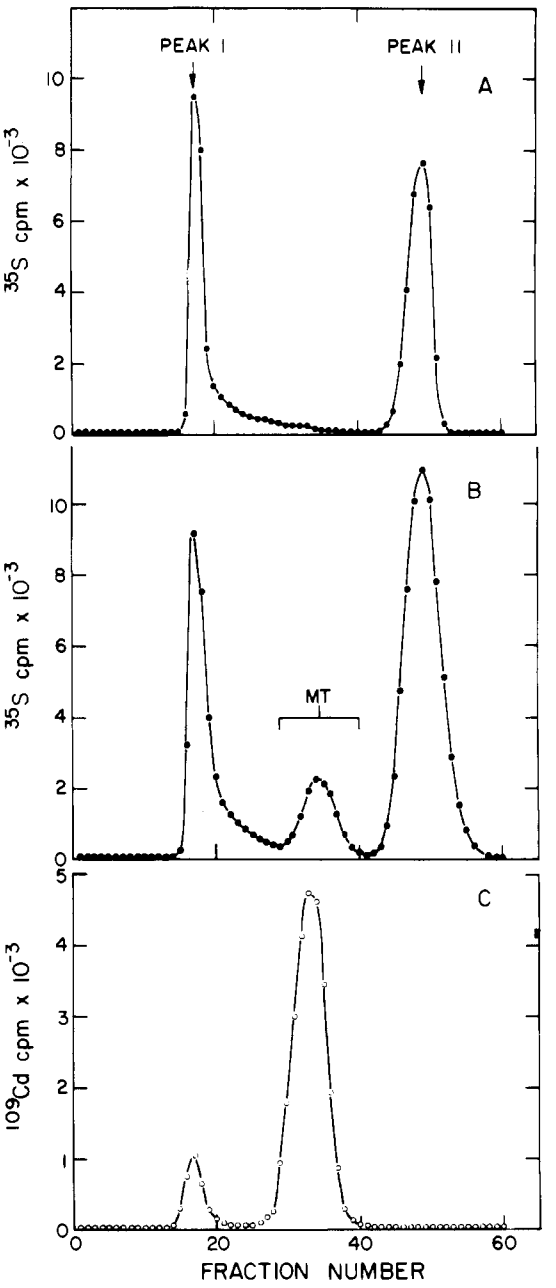

FIGURE 1: Sephadex G-75 column chromatography of cytoplasm from Cd^r cells that were (A) pulse labeled with [^{35}S]Cys in the absence of inducing concentrations of metal ions, (B) pulse labeled with [^{35}S]Cys during exposure to a maximally inducing concentration of Zn^{2+} (100 μM, 8 h), and (C) exposed to a maximally inducing level of $CdCl_2$ with tracer levels of ^{109}Cd for 8 h. Recovery of labeled material from the column was °95% [^{109}Cd].

Results

Characterization of Chinese Hamster Metallothioneins. The cysteine richness of MTs and their high affinity for trace metals (viz., Cd^{2+} and Zn^{2+}) facilitate the labeling of these

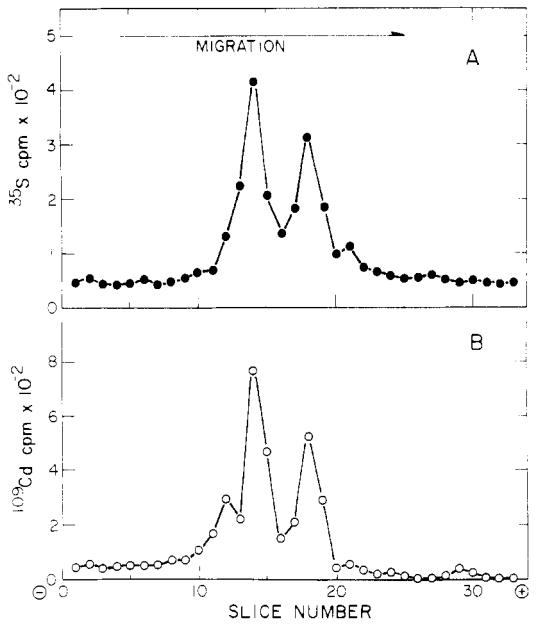

FIGURE 2: Nondenaturing polyacrylamide gel electrophoresis of metallothionein(s) from the MT peak of the Sephadex G-75 column chromatograph. Recovery of both ^{35}S and ^{109}Cd label from the gels was >90%.

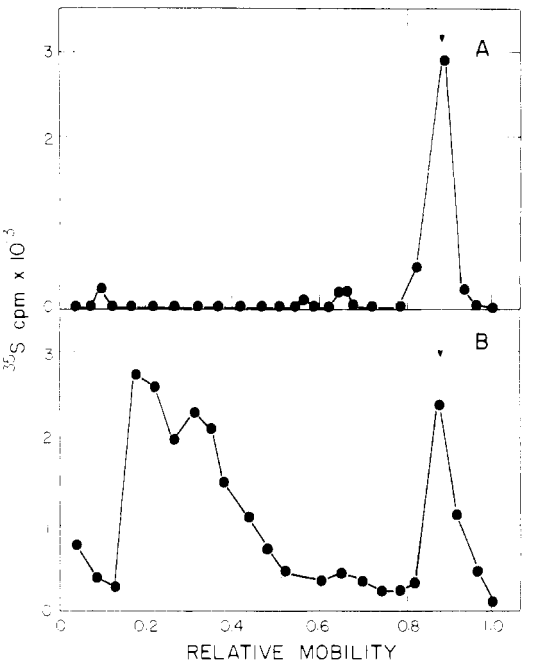

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of (A) carboxymethylated [^{35}S]MT from the Sephadex G-75 column chromatograph and (B) carboxymethylated ^{35}S-labeled cytoplasm from maximally induced (2.0 μM Cd^{2+}, 8 h) Cd^r cells.

proteins with radioisotopes of cysteine, Cd^{2+} or Zn^{2+}. Because of the small size (6000–7000 daltons) of MTs synthesized in response to Cd^{2+} or Zn^{2+}, gel filtration chromatography is a standard procedure for separating MTs from other cellular components and from unincorporated radioactive precursors (Hildebrand et al., 1979, and references therein). For a demonstration of the effectiveness of this separation using gel filtration chromatography, Cd^r cells were exposed to maximally inducing concentrations of Cd^{2+} or Zn^{2+} (as determined by studies detailed below) and labeled with either [^{35}S]Cys or [^{109}Cd]$^{2+}$. Sephadex G-75 chromatography of cytoplasm from cells exposed to 100 μM Zn^{2+} added to the growth medium and pulse-labeled for 30 min at 7.5 h after beginning exposure (Figure 1B) showed the ^{35}S label eluting in three peaks. Peak I material eluted with an excluded marker (data not shown) and corresponded to cytoplasmic particles and large proteins. Peak II material contained unincorporated [^{35}S]Cys. The MTs appeared at an elution volume intermediate between peak I and peak II. The elution volume for this MT ($V_e/V_0 \simeq$ 1.8–2.0). Figure 1A shows that the ^{35}S peak identified tentatively as MT was absent in [^{35}S]Cys pulse-labeled cells which had not been exposed to added Cd^{2+} or Zn^{2+}. For the determination of whether the material in the presumptive MT peak also had an affinity for binding trace metals, cells were exposed to a maximally inducing level (2.0 μM) of Cd^{2+} in the growth medium with tracer amounts of [^{109}Cd]$^{2+}$ for 8 h, and the cytoplasm was analyzed by Sephadex G-75 chromatography (Figure 1C). This result shows that the material identified as MT (Figure 2B) sequestered a large fraction of the cytoplasmic Cd^{2+} (which turns out to be >90% of the total cellular Cd^{2+}). Hence, these results are consistent with previous reports in that a cysteine-rich, low-molecular-weight, metal-binding protein was synthesized in response to either Cd^{2+} or Zn^{2+} exposure.

The presence of at least two distinct isometallothioneins in the Sephadex G-75 MT peak has been reported in other species by several investigators (Shaikh & Lucis, 1971; Nordberg et al., 1972). For the determination of whether Cd^r cells synthesized both (or at least the two major) forms of MT in response to Cd^{2+} exposure, the MT peak (labeled with either [^{35}S]Cys or [^{109}Cd]$^{2+}$) was isolated by Sephadex G-75 chromatography and was subjected to nondenaturing polyacrylamide gel electrophoresis. The results of this experiment (Figure 2) showed that both [^{35}S]MT (Figure 2A) and [^{109}Cd]MT (Figure 2B) separate into two major components. Thus, both of the major forms of MT were synthesized coordinately in the Cd^r cell in response to Cd^{2+} (or Zn^{2+}, data not shown) exposure.

Quantitation of Metallothionein Synthesis Rate. Since Sephadex G-75 column chromatography resolves [^{35}S]MT or [^{109}Cd]MT from other cytoplasmic [^{35}S]Cys- or [^{109}Cd]-containing species, it should be possible to measure the rate of MT synthesis relative to nonMT synthesis by integrating the area under the MT peak (Figure 1B) and by comparing it to the area under the nonMT regions of the chromatographic profile (e.g., Figure 1A, peak 1). However, it is possible that during the cell fractionation procedures a portion of the cysteine-rich MT may become oxidatively cross-linked to larger cytoplasmic proteins, thereby causing an underestimation of MT based solely on the MT peak. A procedure was developed to analyze total cytoplasmic material by $NaDodSO_4$–polyacrylamide gel electrophoresis, thereby eliminating artifacts arising from nonspecific aggregation (i.e., oxidative cross-linking) of MT with nonMT proteins. This was achieved by carboxymethylation of total cytoplasm with iodoacetate following protein denaturation, metal removal, and disulfide reduction. The results from $NaDodSO_4$–polyacrylamide gel electrophoresis of carboxymethylthionein prepared from the Sephadex G-75 [^{35}S]MT peak and of total cytoplasm from maximally induced (2.0 μM Cd^{2+}) Cd^r cells are illustrated in Figure 3, A and B, respectively. In Figure 3, [^{35}S]MT was mixed with unlabeled cytoplasm (an amount equivalent to that used in Figure 3B) prior to preparation for electrophoresis. In this gel system, carboxymethylthionein migrated as a single, high-mobility (0.88) protein (Figure 2). The absence of detectable ^{35}S label migrating with the larger unlabeled cytoplasmic proteins indicated that no significant oxidative cross-linking occurred during sample preparation for $NaDodSO_4$–polyacrylamide gel electrophoresis. Finally, the

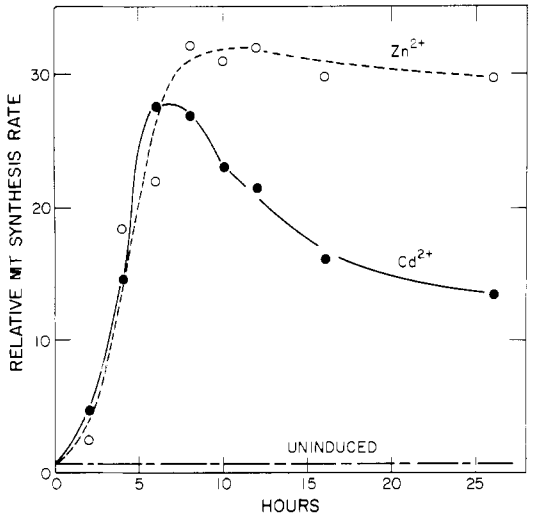

FIGURE 4: Kinetics of induction of MT synthesis in Cd^r cells exposed to 2.0 μM $CdCl_2$ (●) or to 100 μM $ZnCl_2$ (○).

Table I: Inhibitors of Protein and RNA Synthesis: Effects on MT Synthesis[a]

drugs	μg/mL	time of drug addition (h post-induction)	rel MT synthesis rate[b] (7-h post-induction)
none	–	–	100
Cx	8.43	6	0 (10)[c]
Puro	200	6	0 (11)[c]
AM	0.05	0	3
AM	2.0	0	<1

[a] Cd^r cells were exposed to 1.0 μM $CdCl_2$ beginning at 0 h. [b] The relative synthesis rates were determined as described under Materials and Methods for [^{35}S]Cys pulse labeling from 6.5 to 7.0 h. [c] Relative synthesis rates in cultures treated with protein synthesis inhibitors were determined as ^{35}S cpm incorporated into total protein per mg of total protein per 30 min and taken as a percent of the untreated control. No MT synthesis was detected in either the Cx or Puro treatments. The numbers in parentheses indicate the percent of nonMT synthesis relative to the untreated control.

percent of ^{35}S label migrating as carboxymethylthionein was determined from Figure 3B and compared with the percent of ^{35}S label incorporated into MT determined by Sephadex G-75 chromatography of a portion of the same cytoplasm. These percentages were 17% and 18% for the $NaDodSO_4$–polyacrylamide gel electrophoresis analysis and Sephadex G-75 analysis, respectively.

Optimization of Cd^{2+} and Zn^{2+} Exposures for Induction of MT Synthesis. Measurements of cell growth kinetics (data not shown) indicated that Cd^r cells could tolerate Cd^{2+} exposure levels up to 2.0 μM in the growth medium without any growth inhibition (doubling time 16–17 h). Similar measurements for Zn^{2+} exposure gave a maximal tolerable level of 100 μM Zn^{2+} added to the growth medium. It should be noted that the culture medium without serum contains 0.1 μM $ZnCl_2$ and that the serum contributes an additional $\sim$2 μM Zn^{2+} as determined by atomic absorption analysis (data not shown), in agreement with similar measurements reported by Failla & Cousins (1978).

Maximal induction of MT synthesis was observed at the maximal subtoxic level for both Cd^{2+} and Zn^{2+} (2.0 and $\sim$100 μM, respectively) (unpublished results). Induction of MT synthesis could be observed at Cd^{2+} and Zn^{2+} levels down to 0.2 and $\sim$10 μM, respectively. The kinetics of Cd^{2+}- and Zn^{2+}-mediated induction of MT synthesis are compared in Figure 4. In both instances a rapid increase in the rate of MT synthesis was obtained, and the induction kinetics are similar up to the times of maximal MT synthesis rate (6–8 h) but, thereafter, differ significantly (Figure 4). This interesting contrast in Cd^{2+}- and Zn^{2+}-mediated induction kinetics can be explained, in part, on the basis of recent studies of the dependence of MT turnover rates on the metal composition both in vivo (Chen et al., 1975; Feldman & Cousins, 1976, 1978; Shaikh & Smith, 1976; Cain & Holt, 1979) and in vitro (Feldman et al., 1978). In several studies (Chen et al., 1975; Shaikh & Smith, 1976; Feldman & Cousins, 1978; Cain & Holt, 1979), the half-life reported for the turnover of Cd-thionein (i.e., MT synthesized during Cd^{2+} exposure) was 2.8–5 days while in the case of Zn^{2+} exposure the half-life of Zn-thionein was 20 h (Feldman & Cousins, 1976). Further, in vitro studies of the half-lives of Cd^{2+}- and Zn^{2+}-thioneins showed that, in the presence of trypsin or lysosomal extracts, Zn-thionein was degraded approximately twofold faster than Cd-thionein (Feldman et al., 1978). When those findings are extrapolated into the present study, it might be expected that during continuous metal uptake by Cd^r cells, as observed for both Cd^{2+} (Hildebrand et al., 1979) and Zn^{2+} (Hidebrand et al., 1979), a faster MT turnover rate (expected in the case of Zn^{2+} exposure would require an increased synthesis rate (as observed for Zn^{2+} exposure in Figure 4) in order for cells to accommodate incoming metal ions. Althugh the results shown in Figure 4 are consistent with this prediction, further studies of thionein turnover rates during Cd^{2+} and Zn^{2+} exposure in the Cd^r cell will be required to quantitatively test the validity of this explanation for the differential Cd^{2+}- and Zn^{2+}-mediated MT induction kinetics.

Specificity of Metal-Mediated Stimulation of MT Synthesis. The rate of incorporation of [^{35}S]Cys into both MT and nonMT proteins was linear for the pulse-labeling periods examined (up to 1 h) and extrapolated to zero for zero pulse length, indicating the absence of significant effects of cellular amino acid pools. Measurements of the amount of [^{35}S]Cys incorporated during a 30-min pulse into cytoplasmic nonMT proteins showed that the relative rate of nonMT protein synthesis (determined as ^{35}S cpm incorporated/mg of nonMT protein during a 30-min [^{35}S]Cys pulse label) remained approximately constant during a 48-h exposure to 1.0 μM Cd^{2+}. This observation provided additional support for the validtiy of the measurement of relative MT synthesis rates. Further, the method for calculating the relative MT synthesis rate circumvents the problems associated with (1) changing cell concentrations from one pulse period to the next and (2) differences in specific activities of [^{35}S]Cys (due to ^{35}S decay) from one experiment to the next.

Control of Metal-Mediated Induction of MT Synthesis. Experiments were performed initially with inhibitors of protein synthesis [cycloheximide (Cx) and puromycin (Puro)] to verify further that the incorporation of [^{35}S]Cys into cytoplasmic material analyzed by Sephadex G-75 chromatography was due to de novo protein synthesis and not to some other nonsynthetic mechanism (e.g., oxidative cross-linking of [^{35}S]Cys to thiol-rich proteins). The results in Table I show that both Cx and Puro blocked $\sim$90% of total protein synthesis and inhibited MT synthesis below detectable levels.

Several lines of evidence obtained from studies of Cd^{2+}- or Zn^{2+}-mediated stimulation of MT synthesis in vivo suggest that part of the increased MT synthesis can be attributed to de novo synthesis of mRNA coding for thionein (the apoprotein of MT) (Richards & Cousins, 1975a,b; Squibb & Cousins, 1977; Anderson & Weser, 1978; Shapiro et al., 1978;

Enger et al., 1979b). For an assessment of the extent to which RNA synthesis is required for stimulation of MT synthesis in the Cd^r cell, cell cultures were induced with 2.0 μM Cd^{2+}, and AM was added immediately so that the final concentration in culture was either 5.0 μg/mL, which inhibits total RNA synthesis, or 0.05 μg/mL, which preferentially inhibits nucleolar RNA synthesis (but, in most reported cases, allows continued synthesis of mRNA) (Perry & Kelley, 1970; Enger & Hanners, 1978). The results in Table I show that the high level (5.0 μg/mL) of AM inhibited stimulation of MT synthesis when assayed at the time of expected maximal MT synthesis, indicating that transcription of thionein mRNA was required for increased MT synthesis. However, unexpectedly, the low level of AM also strongly inhibited MT synthesis, suggesting that, relative to other mRNAs, transcription of thionein mRNA was unusually sensitive to AM. This observation will be discussed in more detail below.

Additional experiments with AM provided evidence for AM-mediated superinduction of metal-induced MT synthesis analogous to effects of AM reported in other inducible systems (Tomkins et al., 1969, 1972; Steinberg et al., 1975a; Chatterjee et al., 1979). When 2 μg/mL AM is added to maximally induced Cd^r cells (2μM Cd^{2+} for 8 h; Figure 5A), the MT synthesis rate is increased significantly relative to that in the AM-free control cells. [Separate experiments showed that AM at 2.0 μg/mL was as effective as at 5.0 μg/mL for inhibiting RNA synthesis (Perry & Kelley, 1970; M. D. Enger, unpublished results).] These results indicated that the decreasing rate of MT synthesis following maximal induction in the presence of inducer (control curve, Figure 5A) occurred via a process requiring RNA synthesis. In contrast to the superinduction of MT synthesis by 2 μg/mL AM, the low level of AM (0.05 μg/mL) did not superinduce MT synthesis (Figure 5B). In fact, the rate of MT synthesis decreased slightly faster than that in the untreated control. The possible interpretations of these findings will be discussed below.

Another question that arose was whether the presence of inducing metal altered the AM effects, especially the differential effects of high and low levels of AM. To answer this question, maximally induced Cd^r cells (2 μM Cd^{2+} for 8 h) were removed from Cd^{2+}-containing medium, and growth was continued in Cd^{2+}-free medium in three separate cultures: one without AM, a second with 2.0 μg/mL AM, and the third with AM at 0.05 μg/mL. The results in Figure 5B indicate clearly that (1) in the absence of both inducer and AM, the rate of MT synthesis (control curve) decreased rapidly with a half-time of 2–4 h; (2) AM at the high level maintained MT synthesis at a nearly maximal rate for up to 8 h after removal of inducer; and (3) a low concentration of AM accelerated the decay of MT synthesis rate compared with the untreated control. Thus, the absence of inducer did not alter the conclusions regarding the regulation of MT synthesis during treatment with high or low levels of AM. The preceding results indicate that both induction of MT synthesis and regulation of MT synthesis rate during continued exposure to inducer or during deinduction are controlled ultimately at the level of transcription.

Further experiments were done to determine whether significant translational control of thionein mRNA could be observed in our cultured cell system. Such control mechanisms have been suggested both by studies of regulation of ferritin synthesis (Zähringer et al., 1976) and by investigations of the regulation of MT synthesis in animals exposed to Cd^{2+} or excess Zn^{2+} (Squibb et al., 1977; Anderson & Weser, 1978; Panemangalore & Brady, 1978). Translational control of MT

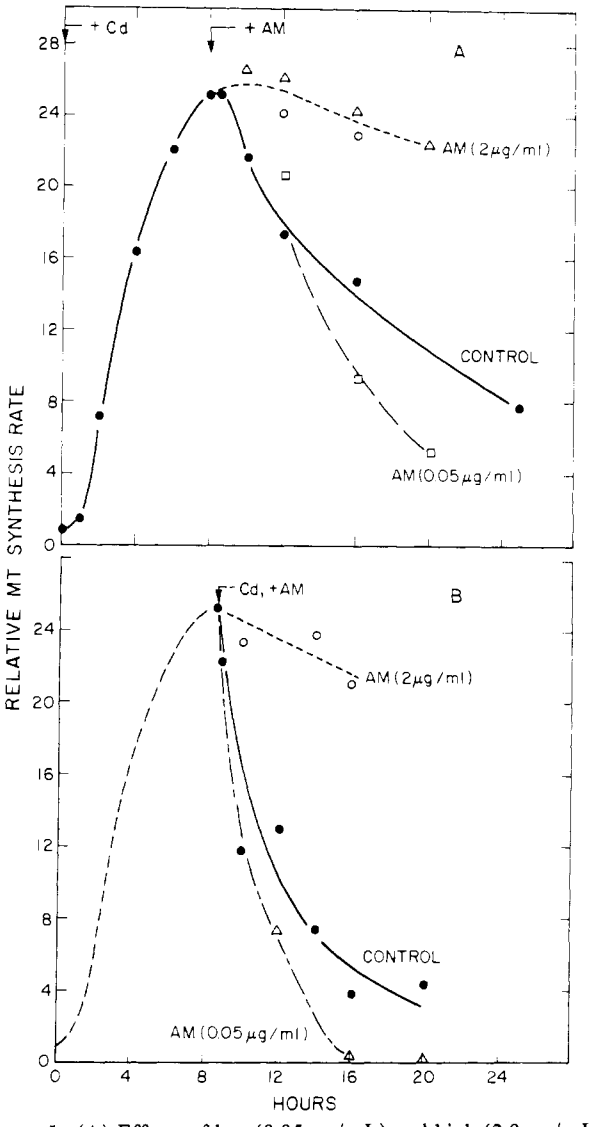

FIGURE 5: (A) Effects of low (0.05 μg/mL) and high (2.0 μg/mL) levels of AM on MT induction kinetics in Cd^r cells exposed to 2.0 μM $CdCl_2$. AM was added at 8 h. (B) Effects of low (0.05 μg/mL) and high (2.0 μg/mL) levels of AM on kinetics of deinduction of MT synthesis. At 8 h, cells were removed from Cd^{2+}-containing medium and resuspended in fresh Cd^{2+}-free medium with or without AM as indicated.

synthesis would imply that thionein mRNA could undergo transitions between translationally active and inactive states depending upon cellular levels of inducing metal. For the determination of whether translational regulation operates in the cultured cell system, Cd^r cells were induced maximally with 1.0 μM Cd^{2+} (Figure 6). This initial exposure to Cd^{2+} was designated the "primary induction". At the time of maximal MT synthesis, cells were withdrawn from Cd^{2+} exposure and deinduction of MT synthesis was continued for 16 h, when the rate of MT synthesis reaches the basal uninduced level. The culture was then split into three equal volumes. All three cultures were induced with 1.0 μM Cd^{2+}. This exposure to Cd^{2+} has been designated the "secondary induction". Two cultures received AM (5.0 μg/mL) either 0.5 h prior to or 0.5 h following the secondary induction. If the thionein mRNA synthesized during the primary induction was changed to a translationally inactive state during deinduction, then the secondary induction should have occurred without the requirement for transcription of new thionein mRNA. However, the fact that AM blocked the secondary induction strongly suggested that transcription of thionein mRNA was required

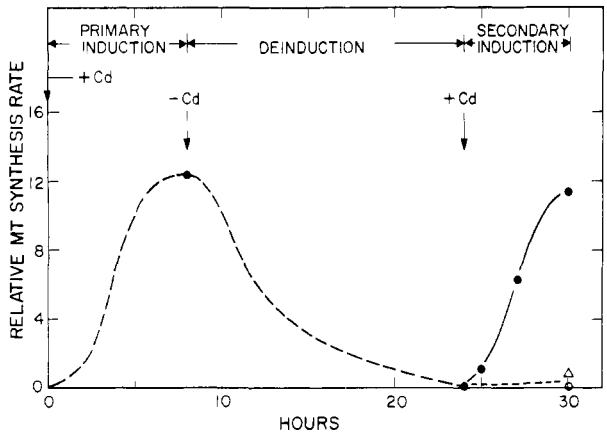

FIGURE 6: Effects of AM (5.0 μg/mL) on secondary induction of MT synthesis. Primary induction of Cd^r cells was attained by an 8-h exposure to 1.0 μM $CdCl_2$. Cells were then removed from Cd^{2+}-containing medium and cultured in Cd^{2+}-free medium for 16 h. Secondary induction was begun by addition of $CdCl_2$ (1.0 μM) at 24 h to each of three deinduced cultures containing no AM (●) or having AM (5.0 μg/mL) added at 23.5 h (△) or 24.5 h (○).

for the secondary, as well as for the primary, induction. This conclusion is not altered by taking into account the dilution of thionein mRNA that would occur due to cell growth and division during the deinduction period.

Discussion

The present report provides a detailed characterization of the regulation of cellular synthesis of the thiol-rich, metal-binding proteins (MTs) produced in response to Cd^{2+} or Zn^{2+} in a Cd^{2+}-resistant subline (Cd^r) of the cultured Chinese hamster (CHO) cell. The MTs synthesized in these cells are similar in every characteristic examined to the MTs produced in animals exposed to Cd^{2+} or excess levels of Zn^{2+} or in cultured cells obtained from a variety of sources (Kojima & Kägi, 1978; Rudd & Herschman, 1978; Hildebrand et al., 1979, and references therein). The properties that identify the Chinese hamster (Cd^r) cell MTs with those from other sources are the (1) high cysteine content and leucine deficiency (Enger et al., 1979a) relative to other cellular proteins (Kojima et al., 1976), (2) high affinity of the MTs for Zn^{2+} (unpublished results) or for Cd^{2+} (Figure 1C) (Kagi & Vallee, 1960, 1961), (3) low molecular weight indicated by the chromatographic behavior of the MTs on Sephadex G-75 columns (Figure 1) (Shaikh & Lucis, 1971; Nordberg et al., 1972; Squibb et al., 1977; Rudd & Herschman, 1978) and by the electrophoretic behavior of carboxymethylthionein in Na-DodSO_4 gels (Figure 3), (4) separation of the two isometallothioneins (isoMTs) by nondenaturing PAGE (Figure 2) (Shaikh & Lucis, 1971; Vander Mallie & Garvey, 1978), and (5) immunological identity of these MTs with those isolated from rat liver [see results quoted in Vander Mallie & Garvey (1979) and unpublished results]. The isoMTs in the Cd^r cell system are synthesized coordinately in response to inducing metal ions—in agreement with results reported by several investigators studying Mt synthesis in vivo (Shaikh & Lucis, 1971; Winge et al., 1975) or in other cultured cell systems (Rudd & Herschman, 1978).

Several features of the Cd^r cell system make it especially attractive for studies of Cd^{2+}- or Zn^{2+}-mediated induction of MT. The Cd^{2+}-resistant phenotype was stable for over 6 months of continuous culture (in monolayer) in the absence of the selective agent Cd^{2+} (Hildebrand et al., 1979). The induction of the MT synthesis response in the Cd^r cell following metal exposure was rapid, occurring in 6–8 h, and reached a maximal rate at least 30-fold greater than basal rate. Thus, in contrast to other studies of MT synthesis in vivo or in culture, which show both high uninduced levels of MT synthesis and only a 3- to 5-fold increase in MT synthesis upon induction (Anderson & Weser, 1978; Shapiro et al., 1978); the Cd^r cell has a well-defined induction response. This striking change in synthesis of a specific gene product occurs in the absence of any comparable increase in general cellular protein synthesis and alters RNA synthesis only a few percent during the period of maximal induction (Enger et al., 1979b).

A primary concern is to define the level(s) at which metal-mediated induction of MT synthesis is regulated. For example, does the inducing metal act to increase MT synthesis by increasing transcription of thionein mRNA, or does the inducer act by stabilizing thionein mRNA without altering its rate of production? One approach to this question was based on an analysis of induction and deinduction kinetics of steroid-regulated tyrosine aminotransferase synthesis in cultured hepatoma cells [discussed in detail by Steinberg et al., (1975b); Nickol et al., 1978]. The results of this analysis applied to MT synthesis induction and deinduction kinetics (e.g., Figure 5A,B) were consistent with the conclusion that the half-life of thionein mRNA (2–3 h) was the same during induction and deinduction, suggesting that inducer does not act by stabilization of existing thionein mRNA (results not shown).

Another approach to the question of regulation of induction of MT synthesis utilized the inhibitor of RNA synthesis, actinomycin D (AM). Although a considerable controversy has surrounded quantitative interpretation of the results of some applications of this inhibitor (cf. Chatterjee et al., 1979), the use of AM to inhibit general RNA synthesis or nucleolar RNA synthesis, specifically, has proven fruitful in numerous studies and has been applied extensively in the investigation of inducible systems (Tomkins et al., 1972; Killewich et al., 1975; Steinberg et al., 1975a; Chatterjee et al., 1979). In the present study, it was found (Table I) that induction of MT synthesis is inhibited not only by a high level of AM (2.0–5.0 μg/mL), which blocks all RNA synthesis, but also by a low level of AM (0.05 μg/mL), which preferentially inhibits nucleolar RNA synthesis (Perry & Kelly, 1970). This observation indicates that the induction of MT synthesis is controlled at the level of RNA transcription, as suggested by several other studies of regulation of MT synthesis in rat liver in vivo (Richards & Cousins, 1975a,b) and in cultured rat liver cells (Hidalgo et al., 1978). The result that a low level of AM inhibits induction of MT is consistent with the finding of Thompson et al. (1966), who reported that 0.1 μg/mL AM blocks the steroid-mediated increase in the activity of tyrosine aminotransferase.

The phenomenon of AM-mediated "superinduction" has been observed in several inducible systems as noted above. Superinduction of tyrosine aminotransferase activity (and enzyme synthesis rates) has been observed in cultured hepatoma cells (Tomkins et al., 1969, 1972; Steinberg et al., 1975a) after AM administration (at 0.1–5.0 μg/mL) to cells exposed to steroid hormones. Although previous studies of MT synthesis in the livers of animals exposed to Cd^{2+}, Cu^{2+}, or excess Zn^{2+} showed evidence for AM superinduction of MT levels, the superinduction was conditional upon the inducing agent used (Day et al., 1978; Panemangalore & Brady, 1978). Further, the superinduction of the rate of MT synthesis was not determined in those studies. In this report, we have examined the phenomenon of superinduction of MT synthesis *rate* in some detail primarily to determine at what level(s) of

macromolecular synthesis production of MT is regulated and to compare these with results obtained in other inducible systems. Our results indicated that AM administered at 2.0–5.0 μg/mL to cells maximally induced with Cd^{2+} caused a slight increase in the rate of MT synthesis relative to the AM-free control (Figure 5A). However, a low level of AM (0.05 μg/mL) did not maintain the elevated rate of MT synthesis and appeared to accelerate the decay of MT synthesis rate below that of the AM-free control (Figure 5A). The AM-mediated (2.0–5.0 μg/mL) stabilization of maximal MT synthesis levels also was found even when the inducing metal was removed just prior to administration of AM (Figure 5B). Again, however, the low level of AM did not alter the deinduction kinetics relative to the AM-free control and, as in the case of continued metal exposure, may even have accelerated the deinduction kinetics. These observations fall within the scope of a model (McAuslin, 1963; Tomkins et al., 1969) proposing that synthesis of a rapidly turning over component involved in destabilizing the product mRNA (in this case, thionein mRNA) is inhibited by high levels of AM, leading to increased stability of thionein mRNA and, therefore, to continued high rates of MT synthesis. However, if thionein mRNA synthesis is preferentially inhibited relative to synthesis of other RNA either acting directly or coding for a component involved in the destabilizing mechanism, then an accelerated decrease in thionein mRNA stability, reflected by decreased MT synthesis rates, might result. This prediction is supported by our findings (Figure 5A,B). As mentioned above, the suggestion that the rates of MT synthesis reported in this study reflect relative thionein mRNA levels is based on recent measurements using cell-free translation of poly A^+ RNA from Cd^r cells during induction, deinduction, and superinduction (Enger et al., 1979b).

The model used to describe the phenomenon of superinduction in the tyrosine aminotransferase studies accomodates the findings observed in the MT induction system. In addition, the model provides testable predictions of several features of the regulation of the MT induction system (e.g., whether protein synthesis is required for destabilization of thionein mRNA). These questions are the subject of current work.

The final point that is addressed by these studies is the possiblity of translational control of MT synthesis in the Cd^r cell system. This possibility was investigated by asking whether RNA synthesis is required for a secondary induction response (i.e., induction resulting from exposure to inducer following deinduction after primary, or initial, exposure to inducer). The finding that synthesis of new thionein mRNA is essential for stimulation of MT synthesis during the secondary induction is evidence that translational control of MT synthesis is not a major regulatory mechanism in this cultured cell system.

In summary, these findings suggest that the MT induction system is regulated primarily at the level of synthesis of thionein mRNA. Both the increased synthesis of MT upon exposure to Cd^{2+} or Zn^{2+} and the deinduction of MT synthesis (half-life 2–3 h) are rapidly responding processes that appear to be regulated by an RNA synthesis-requiring mechanism. MT induction in eukaryotes is one of the few examples of induced gene expression in which the induced gene product has a direct functional relationship to the inducing agent. Continuing studies of the regulation of MT synthesis are utilizing CHO, Cd^r, and several more recently isolated sublines having different responses to metal exposure. Analyses of these variants by using definitive biochemical approaches (e.g., cell-free translation of thionein mRNAs, detection of the mRNAs coding for the different isoMTs, and utilization of both labeled and unlabeled thionein cDNA probes) promise to provide further delineation of the epigenetic and genetic mechanisms involved in regulating this interesting inducible system.

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Mechanism of Translocation: Effect of Cognate Transfer Ribonucleic Acids on the Binding of $AUGU_n$ to 70S Ribosomes†

Karl Holschuh, Jutta Bonin, and Hans Günter Gassen*

ABSTRACT: We try to mimic the unidirectional sliding-type movement of the PP-tRNA·mRNA complex with respect to the ribosome by looking at the effect of different combinations of cognate tRNAs on the stability of the 70S·$AUGU_n$ complex. The association constant for the binary complex 70S·$AUGU_3$ was determined as 6.8×10^5 M^{-1}. Addition of $tRNA_f^{Met}$ resulted in a 67-fold increase in the association constant, which with both cognate tRNAs is revised to $K_{assoc} = 2.2 \times 10^8$ M^{-1}. Increasing the chain length of the oligonucleotide from $AUGU_3$ to $AUGU_{13}$ did not further raise the association constant. The data indicate that the stability of the 70S ribosome·mRNA interaction is governed by the presence of the cognate tRNAs and is topographically restricted to the decoding domains. Since a peptidyl group in the tRNA increases the affinity of $AUGU_3$ for the ribosome by up to 15-fold, we conclude that the affinity of the peptidyl transfer center for the peptidyl moiety pulls the PP-tRNA·mRNA complex from the A (aminoacyl-tRNA) site to the P (peptidyl-tRNA) site. EF-G·GTP or EF-G·GMPPCP 5′-(β,γ-methylene)triphosphate] displace $tRNA_f^{Met}$ from the quaternary complex 70S·$AUGU_n$·$tRNA_f^{Met}$·$tRNA^{Phe}$ (n = 3 and 6) at $Mg^{2+} < 25$ mM. From the amount of EF-G·GTP bound to a 70S ribosome, it follows that the elongation factor replaces the deacylated tRNA in a stoichiometric way. These data indicate that the EF-G·GTP-dependent release of the deacylated tRNA from the P site, followed by removal of EF-G·GDP from the 50S subunit, is sufficient to trigger the translocation of the mRNA·PP-tRNA complex.

The EF-G-[1] and GTP-promoted translocation of the mRNA in ribosome-dependent protein synthesis represents a fascinating mechanistic problem and is still one of the unsolved problems of mechanochemical reactions (Lucas-Lenard & Lipmann, 1971; Haselkorn & Rothman-Denes, 1973; Leder, 1973; Brot, 1977; Weissbach, 1979). During this process, the mRNA is translocated by three nucleotides, i.e., 10 Å per GTP hydrolyzed. Translocation involves the following changes in going from the pre- to the posttranslocational ribosomal state: the release of the deacylated tRNA from the P site (Kuriki & Kaji, 1968; Lucas-Lenard & Haenni, 1969; Ishitsuka et al., 1970; Roufa et al., 1970; Skogerson et al., 1971), the movement of the peptidyl-tRNA to the P site, the site at which the peptidyl moiety is reactive with puromycin, and the translocation of mRNA by one codon bringing a new codon into the A site (Erbe & Leder, 1968; Haenni & Lucas-Lenard, 1968; Pestka, 1968; Erbe et al., 1969).

The movement of the mRNA along the ribosome during translocation can be demonstrated experimentally. It was shown that the mRNA fragment which is protected against ribonuclease by the attached ribosome extends three nucleotides further toward the 3′ end in the mRNA·ribosome com-

† From the Fachgebiet Biochemie, Institut für Organische Chemie und Biochemie, Technische Hochschule Darmstadt, D-6100 Darmstadt, Federal Republic of Germany. *Received March 13, 1980.*

[1] Abbreviations used: EF-G, bacterial elongation factor EF-G; GMPPCP, guanosine 5′-(β,γ-methylene)triphosphate; *N*-AcMet-$tRNA_f^{Met}$, methionyl-tRNA in which the α-amino group is acetylated; ME, β-mercaptoethanol; A site, aminoacyl-tRNA binding site of the ribosome; P site, peptidyl-tRNA binding site.

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