Volume 7 Number 1 1979 Nucleic Acids Research

Thionein gene expression in Cd^{++}-variants of the CHO cell: correlation of thionein synthesis rates with translatable mRNA levels during induction, deinduction, and superinduction

M.D.Enger, L.B.Rall and C.E.Hildebrand

Cellular and Molecular Biology Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, NM 87545, USA

Received 27 March 1979

ABSTRACT

The relationship of thionein synthesis rates to translatable cytoplasmic thionein mRNA levels was investigated for the first time in a cultured cell system. Thionein synthesis was induced in Cd^r, a cadmium-resistant variant of CHO, by exposure to 2 μM $CdCl_2$. Following a short (1.5 hr) lag, thionein synthesis increases to a rate that is at least 30 times the uninduced rate 7-8 hr after addition of Cd^{++}. This increase is blocked by the coincident addition of actinomycin D. Cytoplasmic thionein mRNA levels, measured by translation in a modified wheat germ system, increase rapidly following induction to values approximately 25 times uninduced levels within 6-8 hr. The increases in thionein mRNA precede proportionate increases in thionein synthesis by 0.5-1.0 hr. Continued exposure to Cd^{++} results in a decreased thionein synthesis rate after 8 hr. By 30 hr, the rate is one-half that seen 6-8 hr after induction. Removal of Cd^{++} after 8 hr results in a rapid decrease in thionein synthesis ($t_{1/2}$ ~ 4 hr). Both decreases are inhibited by the addition of actinomycin. In all instances--induction, deinduction, and actinomycin-mediated "superinduction"--translatable thionein mRNA levels and thionein synthesis rates increase, decrease, or are maintained coordinately. The results suggest that thionein synthesis in Cd^r is controlled primarily by the level of translatable cytoplasmic thionein mRNA.

INTRODUCTION

Thionein is a small metal-binding protein of unusual composition. Cysteine represents 20 of the 61 amino acids found in human, mouse, and equine thionein, and there are no aromatic residues (1,2). Several lines of evidence suggest that this protein functions in the homeostasis of Zn^{++} and in the detoxification of Cd^{++} (1). When isolated from animal tissues, thionein is found complexed with both cations (as metallothionein) in varying ratios, but with an essentially constant ratio (3:1) of cysteine residues to total bound divalent metal (1).

Metallothionein was first found in equine kidney (3). Since then it has been shown to be ubiquitous, occurring at different levels in tissues of

© Information Retrieval Limited 1 Falconberg Court London W1V 5FG England

Downloaded from https://academic.oup.com/nar/article/7/1/271/2359743 by guest on 25 April 2024

higher animals as well as in lower forms of life, including shell fish, yeast, and fungi (1). There is some variation in amino acid composition throughout its range of occurrence; however, the essential features that distinguish the protein and identify it as a metallothionein are maintained (i.e., cysteine richness, low molecular weight, deficiency of aromatic amino acids, and binding of Group I and Group II soft acids).

Most studies of metallothionein have emphasized its role in metal homeostasis and detoxification. However, the induced synthesis of this protein provides a unique system for studying mechanisms involved in gene expression. The small size and high cysteine content of metallothionein facilitate measurement of its synthesis. Metallothionein synthesis can be induced in cultured cells or _in vivo_ (1,4-9), in higher or lower animals (1), in fibroblasts as well as epithelial cells (4-7,10,11), using physiological (Zn^{++}) or nonphysiological (Cd^{++}, Hg^{++}) inducers (1). Further, with cultured cell systems, stable variants possessing increased inducibility (i.e., greater resistance to Cd^{++}) are obtained readily (6,12) and can be applied to identify the mechanisms regulating thionein induction.

Previous studies on the regulation of thionein induction were performed primarily in animals. These studies showed that thionein synthesis _in vivo_ is sensitive to actinomycin D during the early stages, suggesting that induction occurs at the level of increased mRNA synthesis (4,9,13). Moreover, Zn^{++}-mediated induction _in vivo_ results in a maximal response 5-6 hr after administration (9), and its synthesis then decreases unless cycloheximide or actinomycin D (AMD) is present (14), suggesting that thionein mRNA turns over rapidly unless RNA or protein synthesis is inhibited.

In comparison with animal systems, cultured cells offer the advantages of more precise control over the administration and removal of the inducing agent, absence of secondary hormonal effects, more exact determination of cell number and viability, and the ability to generate stable genetic variants. In our Laboratory, the effects of Cd^{++} on the growth and viability of cultured Chinese hamster cells (CHO), as well as the uptake of Cd^{++} and partitioning in CHO, have been studied in detail (5). A stable variant (Cd^{r}) has been derived which is ~ 100-fold more resistant to the cytotoxic effects of Cd^{++} and which responds to Cd^{++} exposure with increased thionein synthesis, while cellular uptake of the metal is the same (12). Thionein induction kinetics and the extent of increase in metallothionein during Cd^{++} exposure in Cd^{r} are comparable to the response of liver cells exposed to Cd^{++} _in vivo_ or in culture (7,15).

Downloaded from https://academic.oup.com/nar/article/7/1/271/2359743 by guest on 25 April 2024

In this paper, the regulation of cellular thionein synthesis is examined during the primary induction (i.e., continuous exposure to an inducing agent; in this case, Cd^{++}) and deinduction (i.e., the period following removal of the inducing agent after attainment of maximal thionein synthesis) by comparing relative thionein synthesis rates and translatable mRNA levels. These studies allow a comparison of induced thionein synthesis rates and translatable mRNA levels with those obtained in vivo. They provide also, for the first time, data on such rates and levels during deinduction and AMD-mediated superinduction. We find that Cd^r cells respond to $CdCl_2$ exposure by increasing their rate of metallothionein synthesis at least 30-fold within 6-8 hr. This synthesis is sensitive to high (5 μg/ml) or low (0.05 μg/ml) levels of actinomycin D during the first hour of induction and occurs in the absence of comparable increases in general RNA or protein synthesis. In the continued presence of Cd^{++}, the rate of thionein synthesis decreases between 8 and 30 hr to about 50% of the peak value. Removal of Cd^{++} at the time of maximal thionein synthesis results in a rapid decrease in thionein synthesis rate. The decreases in rate of thionein synthesis observed during both continued induction and deinduction are largely inhibited by high levels of actinomycin D, mimicing the phenomenon of superinduction (i.e., increased specific protein synthesis in actinomycin-treated cells or tissue relative to untreated controls) reported in other inducible systems. Thionein mRNA levels, quantitated by cell-free translation, compare closely with relative thionein synthesis rates during induction, deinduction, and superinduction.

MATERIALS AND METHODS

Cell Culture.--Cd^r, a stable, cloned, Cd^{++}-resistant variant of CHO, was cultured in suspension in Ham's F-10 medium, supplemented with 15% newborn calf serum and antibiotics. Cultures were negative when tested for PPLO (16). Cd^r cultures grew exponentially between 70,000 and 500,000 cells/ml at generation times of 16-19 hr. Experiments were performed while the cells were in exponential growth. Cells were not concentrated prior to exposure to inducer or to isotopically labeled compounds.

Measurement of Thionein Synthesis Rate.--The relative rate of thionein synthesis was determined by measuring the kinetics of [^{35}S]cysteine (New England Nuclear Corporation, 260 Ci/mmol) incorporation into metallothionein prior to and at various times during Cd^{++} exposure. Cultures exposed to $CdCl_2$ were labeled with 0.350 μCi/ml [^{35}S]cysteine for 30 min prior to the times indicated in the figures. Pulse labeling was stopped by pouring the

Downloaded from https://academic.oup.com/nar/article/7/1/271/2359743 by guest on 25 April 2024

cultures (100 ml) over frozen 0.25 M sucrose cubes and stirring the suspension for 30 sec. Cells were harvested, washed, and lysed as described below except that 0.1 mM DTT was included in the lysis buffer and deoxycholate was omitted. After centrifugation of the cell lysate at 500 x g for 5 min, the supernatant cytoplasm was transferred to polystyrene tubes and stored at -20°C. The amount of [^{35}S]cysteine incorporated into metallothionein was determined by separating the low molecular weight metallothioneins from the other, larger, cytoplasmic components using molecular sieve chromatography. The use of Sephadex G-75 or G-50 to effect this separation is a standard procedure (4-15).

Sephadex G-75 columns (1.2 x 85 cm) were equilibrated with 0.05 M Tris (pH 8.4) at 20°C and eluted with the same buffer at 20°C. The elution profile of ^{35}S activity from these columns showed three distinct peaks: the first peak contained material excluded from the column (70,000 daltons), the second peak was identified with metallothionein, and the peak eluting last contained unincorporated [^{35}S]cysteine. The identity of the metallothionein peak was confirmed in separate experiments showing that (a) its chromatographic behavior on Sephadex G-75 is indistinguishable from that reported for well-characterized metallothionein from other sources (4,6,11,14,15); (b) it preferentially binds Cd^{++} in cells exposed to $^{109}CdCl_2$; (c) its synthesis is dependent upon exposure of cells to Cd^{++} or Zn^{++}; and (d) it displays relative cysteine richness and leucine deficiency (unpublished data). The relative rate of metallothionein synthesis is expressed as the ratio of ^{35}S-metallothionein to ^{35}S-labeled nonmetallothionein proteins. Measurements of the specific activity of nonmetallothionein proteins (those eluting in the excluded peak on Sephadex G-75 columns) were found to be approximately constant during the Cd^{++} exposure intervals described in this report. Therefore, this procedure for calculating the relative thionein (the apoprotein of metallothionein) synthesis rate is valid and also avoids requirements for maintaining constant cell concentrations from one labeling period to the next. Similar calculations have been made in other inducible systems (17).

Isolation of Cytoplasmic Poly A RNA.--Cells were harvested by centrifugation, and those exposed to inducer for less than 8 hr were first chilled by pouring over frozen 0.25 M sucrose. The cell pellets were washed once with cold 0.25 M sucrose, suspended in $K_{10}T_{10}M_{1.5}$ (10 mM KCl, 10 mM Tris, pH 7.4 at 25°C, 1.5 mM $MgCl_2$) at about 2 x 10^7/ml, frozen in Dry Ice-ethanol bath, and stored at -70°C. After thawing, the cells were lysed by the addi-

Downloaded from https://academic.oup.com/nar/article/7/1/271/2359743 by guest on 25 April 2024

tion of 1/10 volume 10% NP-40, followed by vigorous mixing (10 sec). After 15 min at ice bath temperature, the lysate was again mixed, 1/10 volume 5% sodium deoxycholate was added and mixed, and the lysate held another 15-20 min in ice prior to removal of nuclei by centrifugation. The supernatant cytoplasm (usually 20 ml) was placed in an A211 IEC centrifuge tube, underlaid with 1.5 ml 1 M sucrose in $K_{10}T_{10}M_{1.5}$, and polysomes and ribosomes were pelleted by spinning 3 hr at 140,000 g average, 4°C. The pellet was rinsed 5 times with distilled water, suspended in 10 ml $Na_{100}T_{10}E_{10}S_{0.5}$ (100 mM NaCl, 10 mM Tris, 10 mM EDTA, 0.5% SDS), and extracted once with chloroform-phenol (1:1) and twice with chloroform-isoamyl alcohol (24:1) prior to ethanol precipitation. The ethanol precipitate was dissolved in $Na_{120}T_{10}E_{1}S_{0.05}$ buffer and applied to a 4-g column of oligo(dT)cellulose (T3 grade, Collaborative Research). The column was washed with the same buffer. Poly A^+ RNA was then eluted with 0.05% SDS. The eluate was heated for 2 min at 60°C prior to adjusting the buffer concentration and rechromatography. The second 0.05% SDS eluate was extracted with chloroform-phenol and chloroform-isoamyl alcohol prior to ethanol precipitation to remove SDS. The poly A^+ RNA was reprecipitated from $Na_{100}T_{10}E_{1}$ prior to use.

Cell-Free Translation.--Wheat germ embryos were prepared and extracted as reported (18,19) with the exception that chloride salts were substituted for acetate salts (18). The final reaction mixture contained in a total volume of 20 µl: 3.0 mM $MgCl_2$, 70 mM KCl, 0.1 mg/ml creatine phosphokinase (Boehringer Mannheim), 8 mM creatine phosphate (K^+ salt, Calbiochem), 1 mM ATP (Na^+ salt, P. L. Biochemicals), 0.16 mM GTP (Na^+ salt, P. L. Biochemicals), a mixture of 19 amino acids varying in concentration from 7-133 µM (20), 0.5 µCi [^{35}S]cysteine (~ 79 nM), and 10 µl of the wheat germ extract. The labeled amino acid was prepared by the reduction (21) of [^{35}S]cysteine (New England Nuclear) with 10 mM dithiothreitol (DTT, P. L. Biochemicals). The above components were combined on ice, 16 µl was transferred to 25°C for 10 min, and then 4 µl of RNA or sterile water (endogenous activity) was added for an additional 125 min. The final translation product was analyzed in one of three ways:

(1) Total trichloroacetic acid (TCA) precipitable counts.--At specified times, 5-µl aliquots of the reaction mixture were spotted on Whatmann 3MM filter discs pretreated with 50 µl of 10% TCA containing 0.1 M cysteine. The filters were batch-washed in cold 5% TCA and then heated at 90°C for 30 min to hydrolyze aminoacylated transfer RNA. They were washed twice in cold 5% TCA, twice in cold ethanol, and finally in diethyl ether. Following

Downloaded from https://academic.oup.com/nar/article/7/1/271/2359743 by guest on 25 April 2024

air-drying, the filters were counted in 10 ml of a toluene-PPO-POPOP scintillation fluid.

(2) SDS polyacrylamide gel electrophoresis.--To terminate a 20-µl translation assay, 10 µl of 4x SDS buffer [200 mM Tris-Cl (pH 8.9), 8% SDS, 50% glycerol] was added and the samples transferred to ice. After all the reactions were terminated, the samples were warmed to 37°C to dissolve the SDS, 10 µl of 100 mM DTT was added, and the mixture was boiled for 5 min. The dissociated proteins were then carboxymethylated [50°C, 15 min (22)] by adding 7 µl of 1 M iodoacetate (Sigma) freshly dissolved in water and adjusted to approximately pH 8. SDS polyacrylamide gel electrophoresis was carried out as reported (23) using cylindrical (0.5 x 10 cm) 15% gels. Generally, 20-µl samples were applied and they, as well as the running buffer, were prewarmed to 37°C to avoid protein aggregation (see Fig. 5). Following electrophoresis (1.5 mA/gel), the gels were sliced into 1.8-mm sections, dissolved (60°C, 16-24 hr) in 1 ml of 30% hydrogen peroxide, and counted in 10 ml of Aquasol (New England Nuclear).

(3) Native slab gel electrophoresis.--Native slab gels (7.5%, 10 cm in length) were poured essentially according to Davis (24), but with several modifications. Ammonium persulfate and TEMED were added separately (23), the lower gel buffer was adjusted to pH 9.5 as opposed to pH 9, both the stacking (4.5%) and running (7.5%) gels contained the same acrylamide/bis ratio of 37.5, and finally each gel contained Triton X-100 at a concentration of 0.05%. In all cases, the gels were pre-electrophoresed for ~ 3.5 hr at 10 mA per slab. Translation samples (20 µl) were diluted with 30 µl of a solution containing 34% glycerol, 0.16% β-mercaptoethanol, and 0.32% Triton X-100. Twenty µl of this mixture was layered onto the stacking gel and, after the dye (bromophenol blue) band reached the end of the gel, the slab was cut into 3 x 10-mm sections that were prepared for liquid scintillation counting as described above.

To calibrate both gel systems, metallothionein was isolated from exponentially growing Cd^r cells that had been labeled continuously with [^{35}S]-cysteine (0.1 µCi/ml) or $^{109}Cd^{++}$ (0.01 µCi/ml of suspension culture) during a 24-hr exposure to 2 µM Cd^{++}. The cells were harvested by centrifugation, washed once in 0.25 M sucrose, and resuspended in lysis buffer containing 10 mM Tris-Cl (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM DTT, and 1% NP40 (Shell Oil Company). The suspension culture was agitated by vortexing for 30 sec, allowed to stand at 4°C for 15 min, and then revortexed. The cell lysate was centrifuged (500 x g, 5 min) and chromatographed (4,11,13,25) on

Downloaded from https://academic.oup.com/nar/article/7/1/271/2359743 by guest on 25 April 2024

Sephadex G-75 (1.2 x 85-cm column, 10 mM Tris-Cl, pH 8.4, 20°C) to separate metallothionein from larger proteins and unincorporated label. Metallothionein-containing fractions were identified, pooled, and rechromatographed. The twice eluted material was collected, lyophilized, and analyzed by non-denaturing polyacrylamide gel electrophoresis. Greater than 95% of the ^{35}S-labeled proteins co-migrated in approximately equal proportions with the two $^{109}Cd^{++}$-containing metallothioneins (data not shown).

RESULTS

Accurate Quantitation of Levels of Translatable Thionein-Specific mRNA.--The wheat germ system has been used by a number of investigators to measure levels of specific messages (18,19,26). We show here that it may be adapted to measure translatable thionein mRNA levels. Poly A-containing RNA was prepared from the cytoplasm of Cd^r cells exposed to high, but subtoxic, levels of cadmium for 8 hr. Under these conditions, the rate of cellular metallothionein synthesis is maximal (12,27). Cysteine was selected as a label, since metallothioneins contain about 30 mol % (1). As shown in Fig. 1, the radioactive amino acid is rapidly incorporated into TCA-

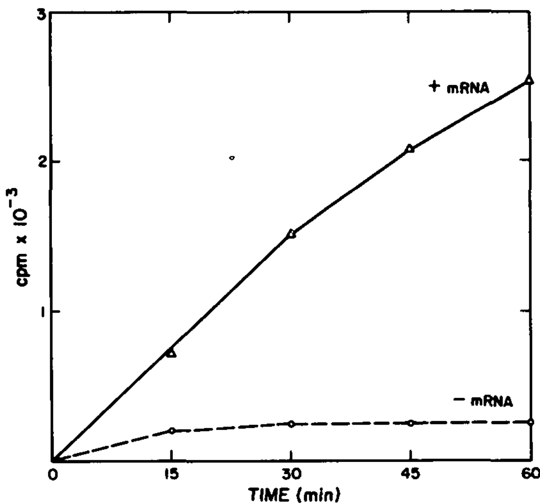

Fig. 1. Reaction kinetics of [^{35}S]cysteine incorporation into TCA-precipitable protein. Each point represents the TCA-precipitable material in a 5-µl aliquot of a 50-µl wheat germ assay containing 2 mM Mg^{++}, 75 mM K^+, and 37 µg/ml RNA. The poly A-containing RNA was isolated from the cytoplasm of Cd^r cells exposed to 1 µM Cd^{++} for 8 hr.

Downloaded from https://academic.oup.com/nar/article/7/1/271/2359743 by guest on 25 April 2024

precipitable material. After 60 min, the messenger-stimulated activity is an order of magnitude greater than the endogenous activity and, in separate experiments, incorporation plateaus at 120 min. Therefore, unless otherwise specified, translation reactions were carried out for 125 min. This time-course, as well as the salt optima of the reaction (3 mM Mg^{++}, 70 mM K^{+}), and the RNA dose-response curve (maximal activity between 40 and 80 μg/ml) are characteristic of poly A-containing fractions from other sources and are within the ranges reported for translation of thionein mRNA from rat liver (28,29).

The [^{35}S]cysteine-containing products from the cell-free assay were analyzed in two different electrophoretic systems, each calibrated using partially purified metallothionein from Cd^{++}-stimulated Cd^{r} cells. As shown in Fig. 2A, under the dissociating conditions of SDS electrophoresis that completely removes bound metal (data not shown), Cd^{r} thionein migrates as a single peak with a relative mobility of 0.88. The monodispersity of this pattern depends upon blocking the highly reactive thiol groups. In the absence of carboxymethylation, aggregates form and ^{35}S-labeled thionein is distributed across the gel. It should be noted that, in our experience, it was unnecessary to fractionate (28) or dialyze (29) the cell-free product prior to polyacrylamide gel electrophoresis.

To compare the cell-free translation products with the proteins synthesized by the cells in culture, maximally induced Cd^{r} cells were pulse-labeled with [^{35}S]cysteine, and total cytoplasmic proteins were resolved by SDS electrophoresis. As shown in Fig. 2B, approximately 20% of the label co-migrates with carboxymethylated thionein. This percentage agrees with the percentage of [^{35}S]cysteine incorporated into metallothionein as determined by Sephadex G-75 column chromatography of the same cytoplasmic samples as analyzed in Fig. 2B.

In contrast to cellular thionein synthesis, poly A-containing RNA from the same maximally induced Cd^{r} cells directs the synthesis of predominantly (~ 70% of the incorporated label) one class of protein equivalent in mobility to carboxymethylated thionein (Fig. 2C). Three further experiments suggest that this peak is thionein. First, the peak is leucine-poor but cysteine-rich, a characteristic of all isolated thioneins (1). In separate experiments, the ratio of [^{35}S]cysteine to [^{14}C]leucine incorporated into material in the thionein peak (e.g., Fig. 2C) is 5.8, while that ratio in the nonthionein region of the gel (e.g., relative mobility 0.2 to 0.6) is 0.91. Second, the peak is not present using poly A-containing RNA from uninduced

Downloaded from https://academic.oup.com/nar/article/7/1/271/2359743 by guest on 25 April 2024

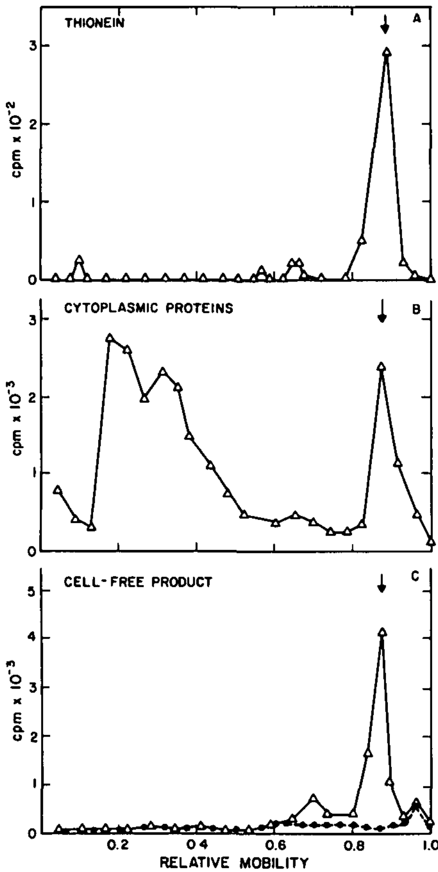

Fig. 2. SDS polyacrylamide gel electrophoresis of carboxymethylated proteins. (A) Thionein standard. Thirty µl of partially purified metallothionein was lyophilized and dissolved in 35 µl of a standard endogenous wheat germ reaction, minus [^{35}S]cysteine and including the SDS dissociation buffer. The reaction was boiled for 4 min and treated (50°C, 15 min) with 6 µl of 1 M iodoacetate. Ten µl of this solution was resolved electrophoretically, as described in the Materials and Methods section. (B) Cellular proteins. Cd^r cells were maximally induced (2 µM Cd^{++}, 8 hr) and then pulse-labeled (30 min) with [^{35}S]cysteine (0.1 µCi/ml of suspension culture). Cytoplasm was prepared from 7 x 10^7 cells, lyophilized, and then dissolved in 120 µl of SDS sample buffer. The sample was reduced, carboxymethylated, and 20 µl resolved as described in the Materials and Methods section. (C) Cell-free product. Poly A-containing RNA was isolated from the cytoplasm of uninduced Cd^r cells (-●-) or Cd^r cells exposed to 2 µM Cd^{++} for 8 hr (-Δ-) and translated in the optimized wheat germ system at a final concentration of 74 µg/ml. The product was reduced, carboxymethylated, and resolved as described in the Materials and Methods section. The arrows in panels (A), (B), and (C) indicate the relative mobility of authentic thionein.

cells (Fig. 2C) that synthesize little metallothionein in cell culture (12, 27). Finally, the cell-free translation product from induced cells separates into two peaks on native gels, as does the partially purified cellular product (Fig. 3). These two forms of metallothionein are characteristic of metallothioneins from other sources and reflect charge differences due to variations in amino acid composition (25). In this case, the gels were pre-electrophoresed and β-mercaptoethanol was included in the sample to avoid oxidative crosslinking of metallothionein. This treatment does not dissociate bound metal, since $^{109}Cd^{++}$ tracks with [^{35}S]cysteine during electrophoresis of partially purified cellular metallothionein (data not shown). The fact that the cell-free products co-migrate with the authentic metallo-

Downloaded from https://academic.oup.com/nar/article/7/1/271/2359743 by guest on 25 April 2024

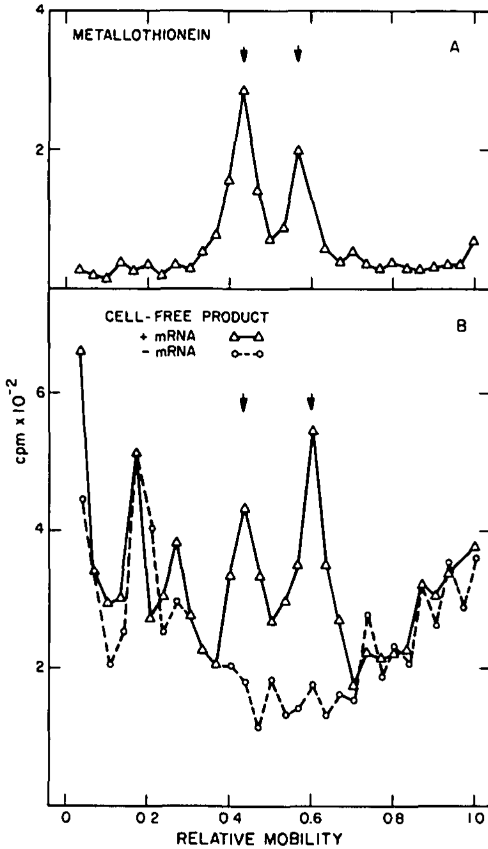

Fig. 3. Resolution of authentic metallothionein (A) and cell-free products (B) on native slab gels. The wheat germ translation products (endogenous and RNA-stimulated) are those containing [^{35}S]cysteine as described in Fig. 5. Samples (20 μl) were prepared for electrophoresis, as described in the Materials and Methods section. Fifteen μl of partially purified metallothionein was mixed with 9 μl of 25% glycerol, 0.25% β-mercaptoethanol, 0.5% Triton X-100, and 20 μl of this solution was used to calibrate the gel system.

Downloaded from https://academic.oup.com/nar/article/7/1/271/2359743 by guest on 25 April 2024

thioneins (Figs. 3A and 3B) is evidence that the cell-free products are in the form of metallothionein (most likely by binding available Zn^{++}), since metal-deficient metallothionein does not migrate in this gel system (unpublished results).

Thus, by several criteria, thionein is effectively synthesized in a wheat germ assay that has been optimized on the basis of cysteine incorporation. Under conditions of either dissociating or native gel electrophoresis, a majority of the label co-migrates with either cellular carboxymethylthionein or metallothionein(s), respectively. [In the case of the native gel pattern (Fig. 3), this is especially evident if the endogenous counts are subtracted from the stimulated profile.] As anticipated, the cell-free product contains little or no leucine and is absent when translating RNA from cells that synthesize only trace amounts of metallothionein (Fig. 2C).

However, the proportion of [^{35}S]cysteine associated with thionein is unexpectedly high. That is, if total cytoplasmic poly A-containing RNA reflects the proteins synthesized in a short labeling period and if the initiation rates are uniform in the wheat germ extract, then the cellular and cell-free profiles should be similar. As shown in Figs. 2B and 2C, this is not true: 80% of the incorporated cysteine appears in proteins other than thionein if cells synthesizing thionein at a maximal rate are pulse-labeled. In the cell-free reaction, the reverse is observed. Thus, the wheat germ extract is highly efficient in translation of thionein mRNA.

In addition to the efficient translation of thionein mRNA in the wheat germ extract, the results shown in Fig. 2C indicate that synthesis of one other major [^{35}S]cysteine-labeled translation product (relative mobility 0.70) is directed specifically by cytoplasmic poly A^+ RNA from Cd^{++}-induced Cd^r cells. This observation suggests the existence of other Cd^{++}-mediated induction specific mRNAs in the Cd^r cell. Further studies are in progress to define the nature of this cell-free translation product and to determine whether synthesis of this protein is induced in Cd^r cells by exposure to Cd^{++}.

In our hands, the reticulocyte lysate system (20) also efficiently and preferentially translates thionein mRNA. However, a low percentage of free [^{35}S]cysteine oxidatively crosslinks to hemoglobin during the course of the reduction and carboxymethylation procedures. Due to the large mass of hemoglobin relative to other proteins in this system and because carboxymethylated hemoglobin subunits migrate close to modified thionein, it is difficult to assess the level of [^{35}S]cysteine incorporated into this protein (data not shown).

Can the wheat germ system, then, be used to quantitate the levels of thionein-specific mRNA? As reported previously (26,29), the stimulation of protein synthesis is a complex function of RNA levels. We have verified those findings using poly A^+ RNA from Cd^{++}-induced cells. The thionein synthesis response is linear with RNA concentration only up to 20 μg/ml, peaks at 74 μg/ml, and thereafter declines gradually as the RNA level is increased. However, if the total RNA concentration is maintained at a constant value (74 μg/ml) by combining poly A-containing RNA from cadmium-stimulated cells with that isolated from nontreated cells (taking advantage of the fact that RNA from nontreated cells produces very little thionein synthesis, i.e., only ~ 2-fold greater than endogenous incorporation in SDS gels), then the total counts associated with the thionein peak (SDS gels) are

Downloaded from https://academic.oup.com/nar/article/7/1/271/2359743 by guest on 25 April 2024

proportional to the fraction of RNA contributed by the metal-challenged cells (Fig. 4). This procedure for obtaining linear RNA dose-thionein synthesis response over a broad range of RNA concentrations circumvents the difficulties encountered in the nonlinear responses reported recently (29). Hence, although the absolute level of thionein messenger in a given preparation is not known, it can be expressed accurately on a relative basis (Fig. 4). Thus, in the following experiments, all measurements of translatable thionein mRNA were made using poly A^+ RNA at 74 μg/ml in the cell-free assay.

Cellular Thionein Synthesis and Relative Thionein mRNA Levels during Induction and Deinduction.--Exposure of Cd^r cultures to 1 or 2 μM $CdCl_2$ results in a rapid increase in thionein synthesis that is evident within 1.5 hr and is maximal 7-8 hr after induction at a rate that is about 30 times the uninduced rate (Fig. 5A). Thereafter, despite continuing exposure to Cd^{++}, the synthesis rate decreases and is 30-50% of the maximal rate by 24 hr after the addition of inducer. The intracellular concentration of Cd^{++} increases throughout this period and plateaus after 24 hr at 500-1000 times the extracellular concentration (unpublished results). During the same period, the cellular content of Cd-thionein increases approximately 170-fold

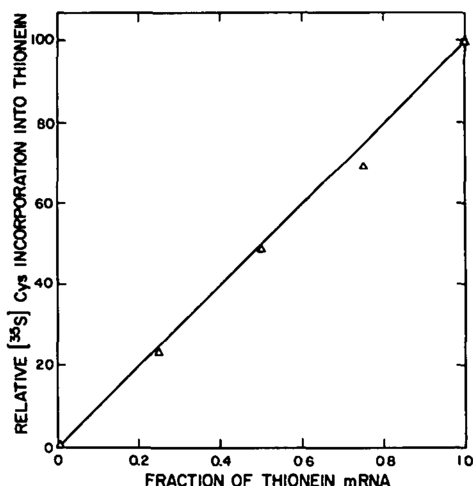

Fig. 4. Quantitation of relative levels of thionein mRNA. Poly A-containing RNA was isolated from maximally induced and uninduced Cd^r cells. The final RNA concentration in the translation reaction was held constant at 74 μg/ml, while the fraction of RNA from induced cells was varied from 0 to 1.0. The cell-free products were resolved by SDS electrophoresis, and the total counts associated with the thionein peak were corrected for endogenous activity.

Downloaded from https://academic.oup.com/nar/article/7/1/271/2359743 by guest on 25 April 2024

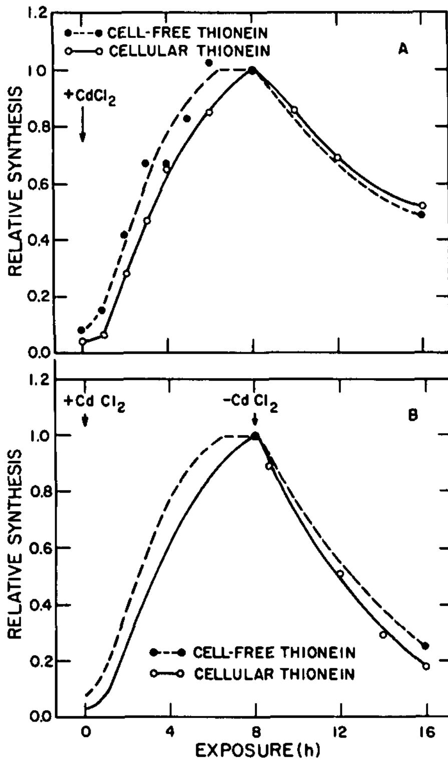

Fig. 5. (A) Induction of metallothionein synthesis in cultured CHO cells measured in a cellular and in a cell-free system. Cd^r cells were exposed to 2 µM $CdCl_2$ and, at the indicated times, the rate of metallothionein synthesis was determined by pulse-labeling (30 min) with [^{35}S]-cysteine. Under similar conditions, poly A-containing RNA was isolated from the metal-treated cells and translated in the wheat germ system.
(B) Deinduction of metallothionein synthesis in cultured CHO cells. Cd^r cells were exposed to 2 µM $CdCl_2$ for 8 hr, and then growth was continued in a metal-free medium. At the indicated times, the rate of cellular metallothionein synthesis and the level of thionein mRNA were determined, as described in the Materials and Methods section. In both panels (A) and (B), values of relative thionein synthesis are expressed relative to the 8-hr point. In panel (B), the 0- to 8-hr cell-free and cellular thionein induction kinetics are reproduced as the dashed and solid curves, respectively.

from the low basal level (unpublished results). Quantitation of thionein poly A^+ mRNA levels by translation in an optimized wheat germ system shows that thionein mRNA levels increase following induction in such a fashion that the maximal (8 hr) value appears to be attained 1 hr sooner than the corresponding thionein synthesis rate (Fig. 5A). The magnitude of the increase in thionein-specific poly A^+ mRNA relative to the uninduced basal rate (~ 25-fold, as found by averaging four independent measurements) is similar to the increase in thionein synthesis rate (~ 30-fold), although in both cases the low uninduced levels are difficult to measure.

Deinduction was achieved by removing the cells from the $CdCl_2$-containing medium and suspending them in fresh Cd^{++}-free medium. A decrease in thionein synthesis rates occurs ~ 1 hr after the removal of Cd^{++}, and the rate drops rapidly thereafter, with a half-life of about 4 hr (Fig. 5B). At the same time, the relative level of translatable thionein mRNA decreases in concert with the relative rate of cellular metallothionein synthesis (Fig. 5B).

Downloaded from https://academic.oup.com/nar/article/7/1/271/2359743 by guest on 25 April 2024

Actinomycin D Effects on Thionein Synthesis and Thionein mRNA Levels.-- Actinomycin D (5 μg/ml) added within the first 1 hr after induction almost completely inhibits the response assayed 7 hr postinduction (Table I). This inhibition also may be effected by low (0.05 μg/ml) levels of actinomycin that do not block general mRNA synthesis (30), but the inhibition is less marked.

The question arises as to whether actinomycin D superinduction is observed in the thionein system. This phenomenon is characterized by the actinomycin D-mediated (2-5 μg/ml) elevation of the rate of synthesis of a specific product relative to an untreated control when the drug is applied near the time of maximum synthesis (31). To determine whether superinduction occurs in the thionein system, actinomycin D (2 μg/ml) was administered to a maximally induced culture (2 μM Cd^{++}, 8 hr) and, at 16 hr postinduction, the relative rate of metallothionein synthesis and the relative thionein mRNA level were measured. The results of this experiment (Table II) show that actinomycin blocks the coordinate decrease in both values observed during the 8-hr period following maximal induction in either the continued presence or absence of Cd^{++}. This suggests that the decreasing rate of metallothionein synthesis (and mRNA level) following maximal induction is under the control of a process requiring RNA synthesis.

Table I. Actinomycin D (AMD) Inhibition of Induction of Metallothionein Synthesis

Time of AMD Addition (hr postinduction)	AMD (μg/ml)	Rate of Metallothionein Synthesis Assayed 7 hr Postinduction*
--	--	100
0	5.0	< 1
0.5	5.0	< 1
1.0	5.0	6
0	0.05	3

*All cultures were induced with 1 μM Cd^{++} at zero hr, and AMD was added at the indicated times to the desired final concentrations. The rate of metallothionein synthesis in each culture is taken as the amount of [^{35}S]-cysteine incorporated per μg protein per 30 min, and the value is compared to the induced control (given a value of 100). It should be noted that the high level of AMD also partially inhibits net cellular protein synthesis, as reported in tyrosine aminotransferase induction (17).

Downloaded from https://academic.oup.com/nar/article/7/1/271/2359743 by guest on 25 April 2024

Table II. Effects of Actinomycin on Thionein mRNA Levels during Continued Induction and Deinduction*

Cd^{++} Induction Conditions	Relative Metallothionein Synthesis Rate	Relative Thionein mRNA Level
8 hr continuous	1.0	1.0
16 hr continuous	0.52	0.49
16 hr continuous plus AMD last 8 hr	0.94	1.06
8 hr continuous, 8 hr deinduced	0.18	0.25
8 hr continuous, 8 hr deinduced with AMD	0.86	0.99

*Cd^r cells were exposed to 2 μM Cd^{++} and 2 μg/ml actinomycin D as indicated. In each case, the rate of cellular metallothionein synthesis and the level of thionein mRNA were determined as described in the Materials and Methods section. The values are expressed relative to the 8-hr point. For both "8 hr continuous, 8 hr deinduced" and "8 hr continuous, 8 hr deinduced with AMD," cultured Cd^r cells were exposed to Cd^{++} for 8 hr, followed by removal of cells from Cd^{++}-containing medium by centrifugation and resuspension and continued culture in Cd^{++}-free medium either without or with AMD.

DISCUSSION

In this investigation, several features of metallothionein synthesis are examined for the first time in a cultured cell system. The kinetics of cellular metallothionein synthesis and cytoplasmic translatable thionein mRNA accumulation have been measured during both induction and deinduction. Actinomycin D was used to define further the level of regulation of metallothionein synthesis and to explore the phenomenon of superinduction.

Relative to other induced syntheses in eucaryotes, the response of Cd^r cells to Cd^{++} is rapid and is comparable to hormone-mediated effects such as hydrocortisone-induced tyrosine aminotransferase (TAT) synthesis in liver cells (32). During induction, there is a parallel increase in the metallothionein synthesis rate and in translatable cytoplasmic thionein mRNA levels. This suggests that control does not occur at the level of translation, although such a possibility cannot be rigorously excluded until actual thionein mRNA levels are determined directly. These data also do not exclude the possibility that induction is a consequence of more efficient processing or stabilization of a constitutive mRNA or its precursor. However, the sensitivity of the induction process to low levels of actinomycin D and the rapid decrease in cytoplasmic mRNA content upon deinduction are more readily explained by transcriptional regulation of a short-lived mRNA.

Downloaded from https://academic.oup.com/nar/article/7/1/271/2359743 by guest on 25 April 2024

Both high and low levels of actinomycin D inhibit metallothionein induction when added simultaneously with Cd^{++}. High concentrations of the drug block the decrease in metallothionein synthesis observed during either continued induction or deinduction. In both cases, there is an accompanying retention of functional mRNA, which suggests that the mRNA may be stabilized by the action of actinomycin D as opposed to being more efficiently translated (33). This system, therefore, may be used to test the hypothesis of Tomkins et al. (34) that a regulatory gene codes for an earlier repressor protein that specifically binds the mRNA in question and promotes its degradation.

Thionein synthesis rates (and the levels of translatable thionein mRNA) change rapidly in response to increased or decreased Cd^{++} exposure. This tight control is probably essential to cell viability, since continued production of thionein in the absence of Cd^{++} uptake may result in the sequestration of Zn^{++} to the extent that cellular functions catalyzed by Zn^{++} enzymes are impaired.

The specificity of the response is an important question, which can be answered only in part at this time. There is no general increase in protein synthesis and only a slight (~ 10%) elevation in total mRNA production 8 hr postinduction (4,35), a time when metallothionein synthesis is 30 times the uninduced value. However, the active uptake of Cd^{++} to levels 500-1000 times extracellular values suggests that an active process is involved which might be expected to require the synthesis of specific uptake proteins. Such related inducible systems, if detected, would add another dimension to the system in the context of studies of gene expression. The advantages of the thionein system in studying such phenomena lie in the ubiquity of the response among eucaryotes and the ability to isolate variants with stable alterations.

The response to Cd^{++} stimulation which we obtained in cultured cells may be compared with that seen in livers of rats exposed to Cd^{++} or Zn^{++}. The time-responses for both metallothionein synthesis and increased content of cytoplasmic translatable thionein mRNA are similar in rat liver and in cultured cells (Cd^r). However, the range of response is much greater for thionein mRNA in the cultured cells. In induced rat liver, Shapiro et al. (28) and Andersen and Weser (29) both noted a 2- to 4-fold maximal increase in translatable cytoplasmic thionein mRNA, even though thionein synthesis in the induced liver increased 15-fold (29). This contrasts with the coordinate 25- to 30-fold increases in both cellular thionein synthesis and translatable

Downloaded from https://academic.oup.com/nar/article/7/1/271/2359743 by guest on 25 April 2024

thionein mRNA content seen in induced cultured cells. Further, the basal or uninduced thionein mRNA content is significant in rat liver but was very low in cultured Cd^r cells. Perhaps some of the induced thionein synthesis in rat liver (but not in Cd^r cells) involves increased translation of existing thionein mRNA, as well as an increase in thionein mRNA content. Deinduction and superinduction effects on thionein mRNA content were not performed in the animal experiments and, therefore, cannot be compared with the cultured cell responses.

In summary, the rapidity of induction and deinduction of thionein synthesis rates, the correlation of these rates with translatable cytoplasmic thionein mRNA levels, and the sensitivity of induction to actinomycin suggest that thionein synthesis is closely controlled at the level of transcription of a short-lived mRNA. Proof of this suggestion will be sought using DNA complementary to thionein mRNA to measure actual thionein mRNA synthesis, transport, and turnover rates.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of Helen Barrington, Linda Ferzoco, John Hanners, and Marianne Jones. This work was performed under the auspices of the U. S. Department of Energy and the U. S. Environmental Protection Agency under interagency agreement EPA-IAG-D5-E681.

REFERENCES

1. Kojima, Y. and Kägi, J.H.R. (1978) Trends in Biochem. Sci. 3, 90-93.
2. Huang, I., Yoshida, A., Tsunoo, H. and Nakajima, H. (1977) J. Biol. Chem. 252, 8217-8221.
3. Margoshes, M. and Vallee, B.L. (1957) J. Am. Chem. Soc. 79, 4813-4814.
4. Hidalgo, H.A., Koppa, V. and Bryan, S.E. (1978) Biochem. J. 170, 219-225.
5. Enger, M.D., Hildebrand, C.E., Jones, M. and Barrington, H.L. (1978) Hanford Biology Symposium Proceedings, Battelle Northwest Laboratories, Richland, Washington (October 17-19, 1977), pp. 37-56.
6. Rugstad, H.E. and Norseth, T. (1978) Biochem. Pharmacol. 27, 647-650.
7. Rudd, C.J. and Herschman, H.R. (1978) Toxicol. Appl. Pharmacol. 44, 511-521.
8. Cempel, M. and Webb, M. (1976) Biochem. Pharmacol. 25, 2067-2071.
9. Squibb, K.S., Cousins, R.J. and Feldman, S.L. (1977) Biochem. J. 164, 223-228.
10. Lucis, O.J., Shaikh, Z.A. and Embil, J.A. (1970) Experientia 26, 1109-1110.
11. Rugstad, H.E. and Norseth, T. (1975) Nature 257, 136-137.
12. Hildebrand, C.E., Tobey, R.A., Campbell, E.W., Kissane, R.J. and Enger, M.D. (1978) J. Cell Biol. 79, 385a.

Downloaded from https://academic.oup.com/nar/article/7/1/271/2359743 by guest on 25 April 2024

13. Richards, M.P. and Cousins, R.J. (1975) Biochem. Biophys. Res. Commun. 64, 1215-1223.
14. Day, F.A., Coles, B.J. and Brady, F.O. (1978) Bioinorg. Chem. 8, 93-105.
15. Webb, M. and Daniel, M. (1975) Chem. Biol. Interactions 10, 269-276.
16. House, W. and Wadell, A. (1967) J. Pathol. Bacteriol. 93, 125-132.
17. Tomkins, G.M., Levinson, B., Baxter, J.D. and Dethlefsen, L. (1972) Nature (New Biology) 239, 9-14.
18. Davies, J.W. and Kaesberg, P. (1973) J. Virol. 12, 1434-1441.
19. Astell, C.R. and Ganoza, M.C. (1974) Mol. Biol. Rep. 1, 483-491.
20. Farrell, P.J., Balkow, K., Hunt, T. and Jackson, R.J. (1977) Cell 11, 187-200.
21. Caskey, C.T., Beaudet, A. and Nirenberg, M. (1968) J. Mol. Biol. 37, 99-118.
22. Lane, L.C. (1978) Anal. Biochem. 86, 655-664.
23. Laemmli, U.K. (1970) Nature 227, 680-685.
24. Davis, B.J. (1964) Ann. N. Y. Acad. Sci. 121, 404-427.
25. Winge, D.R., Premakumar, R. and Rajagopalan, K.V. (1975) Arch. Biochem. Biophys. 170, 242-252.
26. Rether, B., Belarbi, A. and Beck, G. (1978) FEBS Lett. 93, 194-199.
27. Hildebrand, C.E. and Enger, M.D. (1978) J. Cell Biol. 79, 340a.
28. Shapiro, S.G., Squibb, K.S., Markowitz, L.A. and Cousins, R.J. (1978) Biochem. J. 175, 833-840.
29. Andersen, R.D. and Weser, U. (1978) Biochem. J. 175, 841-852.
30. Perry, R.P. and Kelley, D.E. (1970) J. Cell. Physiol. 76, 127-140.
31. Thompson, E.B., Granner, D.K. and Tompkins, G.M. (1970) J. Mol. Biol. 54, 159-175.
32. Nickol, J.M., Lee, K. and Kenny, F.T. (1978) J. Biol. Chem. 253, 4009-4015.
33. Killewich, L., Schultz, G. and Feigelson, P. (1975) Proc. Nat. Acad. Sci. 72, 4285-4287.
34. Tomkins, G.M., Gelehrter, T.D., Granner, D., Martin, D., Jr., Samuels, H. and Thompson, E.G. (1969) Science 166, 1474-1480.
35. Enger, M.D., Campbell, E.W., Ratliff, R.L., Tobey, R.A., Hildebrand, C.E. and Kissane, R.J. (1979) J. Toxicol. Environ. Health (in press).

Downloaded from https://academic.oup.com/nar/article/7/1/271/2359743 by guest on 25 April 2024