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REGULATION OF INDUCED THIONEIN GENE EXPRESSION IN CULTURED MAMMALIAN CELLS: EFFECTS OF PROTEIN SYNTHESIS INHIBITION ON TRANSLATABLE THIONEIN mRNA LEVELS IN REGULATORY VARIANTS OF THE CHO CELL

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SUMMARY

The effects of protein synthesis inhibitors, cycloheximide and puromycin, on functional thionein mRNA levels have been examined to further delineate the regulation of thionein gene expression in Cd^{2+}-resistant CHO sublines proficient in Cd^{2+}- or Zn^{2+}-stimulated synthesis of metallothioneins. Inhibition of protein synthesis at or shortly before the time of metal-mediated induction only slightly reduces the usual increase in thionein mRNA. Protein synthesis inhibition alone does not significantly increase thionein mRNA levels. However, addition of cycloheximide 4 h after Zn^{2+} induction increases thionein mRNA activity measured 8 h after induction by at least 40%, suggesting that protein synthesis is required for normal turnover of thionein mRNA.

INTRODUCTION

The synthesis of thionein, a small protein rich in cysteine (1), is induced in animal cells *in vivo* or in culture by Cd^{2+} or Zn^{2+}. Although cultured Chinese hamster cells (line CHO) are relatively deficient in this response, Cd^{2+}-resistant variants of this cell line have been derived (2) that respond better than CHO. The ease with which CHO and its variants may be grown in suspension and exposed to and removed from inducers and inhibitors makes them a useful system to study the phenomenon of induced thionein gene expression. Previous studies addressed the relationship of thionein synthesis rates to cytoplasmic translatable mRNA levels in the variant $Cd^{r}2C10$ during induction, deinduction, and superinduction (3). In all instances, mRNA levels measured by cell-free translation were found to correspond closely to cellular thionein synthesis rates. Those studies suggested that thionein synthesis in the $Cd^{r}2C10$ variant is controlled primarily at the level of transcription of rapidly turning-over thionein mRNA ($mRNA_{th}$). While specific probes of thionein structural gene sequences do not yet exist for delineating the transcriptional and post-transcriptional regulation of thionein gene expression, we have proceeded

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to determine the role of protein synthesis both in transcriptional control (i.e., the Cd^{2+}- or Zn^{2+}-stimulated appearance of functional $mRNA_{th}$) and in the post-transcriptional control (i.e., following induction of translatable $mRNA_{th}$). In the absence of better-defined probes, the use of protein synthesis inhibitors provides an indirect approach to this problem. Specifically, if a rapidly turning-over inducer-receptor protein is required for transcriptional activation of specific mRNA as suggested by studies of the glucocorticoid regulated induction of tryptophan oxygenase mRNA (4) then inhibition of protein synthesis prior to or at the time of addition of inducing metal could block the increase in translatable $mRNA_{th}$ levels. Conversely, if a rapidly turning-over protein is involved in repression of specific, inducible genes as indicated by studies of the hormonally-regulated expression of tyrosine aminotransferase mRNA (5), protein synthesis inhibition alone should cause an increase in functional $mRNA_{th}$. Further, if post-transcriptional regulation of specific mRNA levels requires protein synthesis as suggested by the cycloheximide-mediated superinduction of glucorticoid-induced tyrosine aminotransferase and tryptophan oxygenase mRNA levels (6), then inhibition of protein synthesis after induction of thionein mRNA is established may lead to a superinduction of $mRNA_{th}$ levels. Results will be presented to show the following: a) Inhibition of protein synthesis does not prevent a Zn^{2+}- or Cd^{2+}-induced increase in thionein mRNA; b) inhibition of protein synthesis 4 to 7 h after addition of inducer increases the level of thionein mRNA measured 8 h after induction; and c) cycloheximide by itself does not induce the appearance of cytoplasmic translatable thionein mRNA.

METHODS

Preparation and growth of the Cd^{2+}-resistant variant $Cd^{r}2C10$ have been described (2). Quantitation of cytoplasmic thionein mRNA has been described in detail (3). It involves cell-free translation of poly A^{+} RNA in a modified wheat germ system, followed by reduction and carboxymethylation of product and resolution of [^{35}S]cysteine-labeled thionein from other cytoplasmic proteins by SDS-gel electrophoresis. These studies involve translation of poly A^{+} RNA, since translatable thionein mRNA is represented only in the poly A^{+} fraction of total cytoplasmic RNA. This was demonstrated by translating equivalent amounts of 7 to 11S poly A^{+} and poly A^{-} cytoplasmic RNA resolved by sucrose gradient zone sedimentation. (The 7 to 11S region contained most of the thionein mRNA activity as determined in separate experiments using cytoplasmic RNA.) Greater than 96% of total thionein mRNA activity was found in the 7 to 11S poly A^{+} fraction.

Cycloheximide (Sigma) was added to a concentration of 10 µg/ml. This concentration was found to inhibit incorporation of [^{3}H]lysine into TCA-precipitable, Pronase-soluble material by 96% after both 45 min and 3.75 h. Puromycin (Calbiochem) was used at 50 µg/ml and actinomyin D (Sigma) at 2 µg/ml.

RESULTS AND DISCUSSION

Inhibition of Protein Synthesis prior to Induction does not Prevent the Increase in Translatable Thionein mRNA--The kinetics of Zn^{2+}-induced cytoplasmic

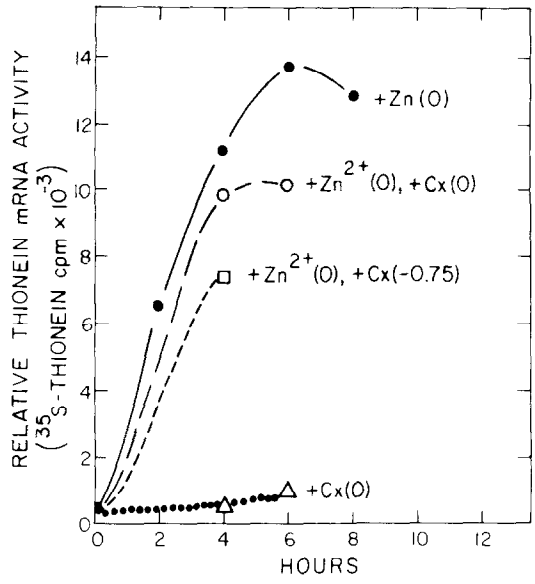


FIG. 1. Relative thionein mRNA activities in poly A^+ RNAs derived from Cd^r2C10 cells exposed to 100 μM Zn^{2+} (-●-), to 10 μg/ml cycloheximide (-Δ-), to Zn^{2+} and cycloheximide simultaneously (-O-), and to cycloheximide 45 min prior to Zn^{2+} (-□-). The numbers in parentheses on this figure indicate the time in hours of addition of Zn^{2+} or cycloheximide (Cx). It should be noted that in several experiments, the level of translatable thionein mRNA observed after a 6 h exposure to Cx alone (-Δ-) is not significantly different from an untreated control.

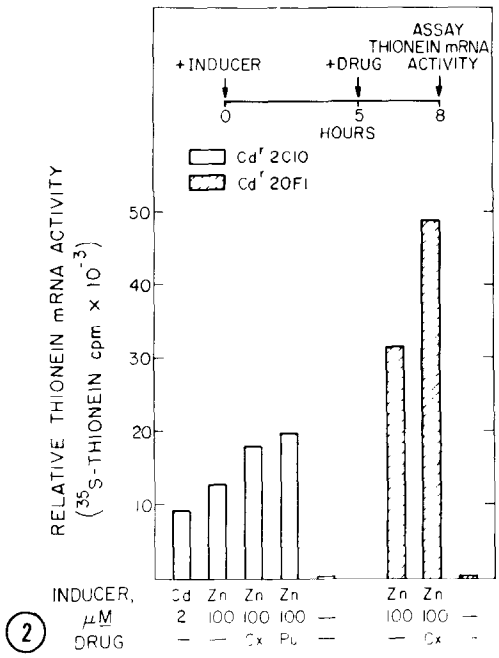
thionein mRNA appearance in Cd^r2C10 (Fig. 1) are similar to those observed previously (3) using Cd^{2+} as inducer when Zn^{2+} and Cd^{2+} are used at the maximum subtoxic levels for this cell (100 and 2 μM, respectively). In both instances, the response occurs relatively rapidly (compared with most induced syntheses in eucaryotes), and the maximum thionein mRNA level obtains 6 to 8 h after the addition of inducer. The maximal value, however, is about 70% greater when Zn^{2+} is used instead of Cd^{2+}. As shown also in Fig. 1, the Zn^{2+}-induced increase in translatable thionein mRNA occurs when protein synthesis is inhibited by the addition of cycloheximide either before or with inducer. When cycloheximide is added with the inducer, the thionein mRNA level is 88% of that in the Zn^{2+}-induced control after 4 h and is 74% of the control level 6 h after the addition of Zn^{2+}. Even when cycloheximide is added 45 min prior to Zn^{2+}, thionein mRNA is 74% of control after 4 h of induction. These data suggest that the Zn^{2+}-induced increase in thionein mRNA is the result of a direct effect (i.e., it is improbable that a gene other than that coding for thionein mRNA is first activated to produce a protein product that, in turn, is involved in activation of the thionein gene). However, these data do not exclude the possibility that stable, cytoplasmic, Cd^{2+}- or Zn^{2+}-receptor proteins may function in the induction of $mRNA_{th}$.

Cycloheximide Alone does not Induce an Increase in Cytoplasmic Thionein mRNA--Cycloheximide alone mediates an increase in specific mRNA activity in at least one instance (5). In the case of cytoplasmic translatable thionein mRNA, however, such a result was not observed when Cd^r2C10 cells were treated for either 4 or 6 h with an amount of cycloheximide that decreases total protein synthesis by 96% (Fig. 1). In contrast, a 4-h treatment with cycloheximide, sufficient to inhibit protein synthesis in rat liver by 95%, resulted in a 6- to 7-fold increase in translatable mRNA for tyrosine aminotransferase (but not in total mRNA or mRNA specific for tryptophan oxygenase). The magnitude of the cycloheximide-mediated increase was reported to be comparable to the inducer-mediated (hydrocortisone) increase. It is evident that such a response to cycloheximide does not occur in the case of thionein mRNA levels in Cd^r2C10. These findings suggest that a short-lived protein is not involved in the repression of metalmediated induction of $mRNA_{th}$.

Inhibition of Protein Synthesis after Zn^{2+} Induction is Established Increases Cytoplasmic Translatable Thionein mRNA--Administration of cycloheximide 4 h after cortisol treatment increased rat liver mRNA activities for tyrosine aminotransferase and tryptophan oxygenase measured 2 h later (6). To determine whether inhibition of protein synthesis could similarly affect thionein mRNA activities following Zn^{2+} induction, Cd^r2C10 cultures were exposed to 100 μM Zn^{2+} for 8 h, and separate culture aliquots were treated with cycloheximide for 4, 3, 2, and 1 h prior to harvest. As shown in Fig. 2, once induction is established, addition of cycloheximide increases thionein mRNA in Cd^r2C10. The effect is maximum when cycloheximide is added 4 h prior to harvest and is lessened as the cycloheximide treatment is shortened, as shown in Fig. 3.

The cycloheximide effect also was measured in a population (Cd^r20F1, derivation and characterization to be described elsewhere) of CHO variants an order of magnitude more resistant to Cd^{2+} than Cd^r2C10 and having over twice the level of thionein mRNA activity 8 h after exposure to 100 μM Zn^{2+}. In this population, administration of cycloheximide 3 h prior to harvest increased the thionein mRNA activity by ∿ 1.56 times. This suggests that the more resistant population has thionein mRNA that is turned over rapidly by a mechanism affected by protein synthesis inhibition in the same fashion as in Cd^r2C10.

There is evidence to indicate that not all inhibitors of protein synthesis necessarily have the same (stabilizing) effect on specific mRNA levels. Stiles et al. (7) noted that the normally rapid turnover of tyrosine aminotransferase mRNA was inhibited by cycloheximide but not by puromycin or sodium fluoride suggesting that the mRNA stability was provided by ribosome binding. Thus, the effect of puromycin on thionein mRNA levels was determined also. As seen in Fig. 2, puromycin effects an increase in cytoplasmic translatable thionein mRNA



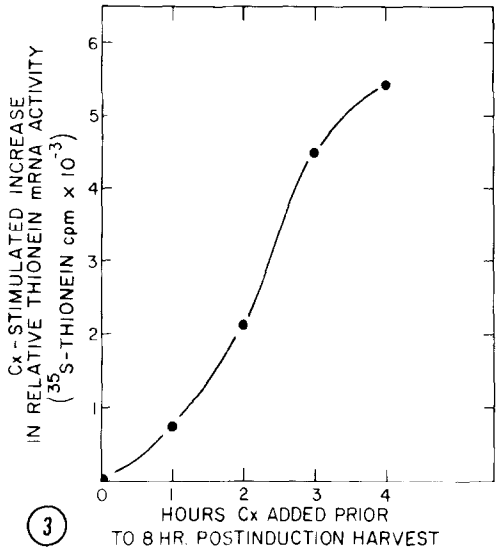


FIG. 2. Relative thionein mRNA activities in poly A^+ RNAs extracted from Cd^r2C10 and Cd^r20F1 cells induced with 2 μM Cd^{2+} or 100 μM Zn^{2+} for 8 h and treated with 10 μg/ml cycloheximide (Cx), or 50 μg/ml puromycin (Pu) 3 h prior to harvest as indicated by the inset at the top of the figure.

FIG. 3. Cycloheximide-stimulated increases in Zn^{2+}-induced thionein mRNA activity as a function of time of cycloheximide addition.

that exceeds that caused by cycloheximide. Hence, these results indicate that superinduction of $mRNA_{th}$ levels by protein synthesis inhibitors is independent of the mode of action of the inhibitor. Specifically, the fact that puromycin-mediated superinduction is observed suggests that this response cannot be ascribed to protection of the mRNA by ribosome binding.

Considerations of the mechanism(s) operative in post-transcriptional control of thionein gene expression, especially in the context of the superinduction phenomenon, should not exclude the possibility that cycloheximide (or puromycin) inhibits synthesis of either a) a labile inhibitor of thionein mRNA synthesis or b) a labile component which destabilizes thionein mRNA. This possibility is suggested by a model for post-transcriptional control of tyrosine aminotransferase gene expression elaborated by Tomkins *et al.* (8). Such a model would predict that cycloheximide treatment would stabilize maximally induced $mRNA_{th}$ levels during the deinduction process following withdrawal of inducing metal. Recent experiments showed that in Cd^r2C10 cells maximally induced with Zn^{2+} inhibition of protein synthesis by cycloheximide at the same time as withdrawal of Zn^{2+} stabilized the $mRNA_{th}$ level for at least 4 h, while in absence of cyclo-

heximide the $mRNA_{th}$ level decreased ~ 50% during the same deinduction interval. While further studies are required to test the validity of the labile repressor hypothesis, the phenomenon of cycloheximide-mediated superinduction of $mRNA_{th}$ levels will very likely be a useful tool for enriching total cytoplasmic poly A^+ RNA for $mRNA_{th}$ sequence, assuming that sequence abundance of $mRNA_{th}$ is correlated with cell-free translational activity. This assumption is supported by recent experiments in our laboratory. Current efforts are directed toward utilizing $mRNA_{th}$-enriched poly A^+ RNA from cycloheximide superinduced Cd^r variants for $mRNA_{th}$ purification and cDNA cloning. The sequence probes obtained by these procedures will provide the key(s) to elucidate further the steps involved in regulation of thionein gene expression.

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