Cell-Free Synthesis of Metallothionein Directed by Rat Liver Polyadenylated Messenger Ribonucleic Acid

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Total RNA was isolated from rat liver polyribosomes and fractionated by oligo(dT)–cellulose chromatography to obtain polyadenylated mRNA. The mRNA was translated in a wheat-germ cell-free protein-synthesizing system containing [³H]glycine, [³H]lysine and [³H]serine. Most of the newly synthesized ³H-labelled polypeptides were removed from the cell-free products by precipitation at pH 4.0. ³H-labelled thionein chains, which were soluble at pH 4.0, were purified by activated-thiol–Sepharose 4B chromatography or by gel-filtration chromatography. Polyribosomal thionein mRNA was found to increase by at least 3-fold after parenteral administration of 32 μ mol of Zn²⁺. The increase was maximal 5 h after Zn²⁺ administration and by 20 h thereafter the ratio of thionein mRNA to total mRNA approached that found in controls. Actinomycin D administration *in vivo* blocked the Zn²⁺-induced increase in polyribosomal thionein mRNA content. These data strongly suggest that metallothionein is an inducible protein. The mechanism of regulation appears to involve changes in the synthesis *de novo* of thionein mRNA and hence the pool of thionein mRNA available for translation.

Metallothionein has been demonstrated to have an important role in zinc metabolism (Bremner & Davies, 1975; Richards & Cousins, 1975). Synthesis of this hepatic Zn²⁺-binding protein appears to be induced after short-term increases in Zn²⁺ status (Richards & Cousins, 1976*a,b*). Experiments with isolated rat liver parenchymal cells have shown that Zn²⁺ accumulation is markedly and specifically enhanced by glucocorticoids (Failla & Cousins, 1978*a,b*). The ligand to which newly accumulated Zn²⁺ is bound is metallothionein, indicating that this metalloprotein is an important link in intracellular zinc metabolism.

We have demonstrated that the rate of synthesis of thionein, the metal-free form of metallothionein, increases to a maximum by 5–6 h after parenteral Zn²⁺ administration and subsequently decreases to a basal value by 15 h (Squibb *et al.*, 1977). Zn²⁺ binding is maximal by 18 h after the metal is administered, indicating that the accumulation of functional thionein chains lags behind an increase in the rate of synthesis. The incorporation of both [³⁵S]cystine and ⁶⁵Zn into metallothionein is blocked by either actinomycin D or cordycepin. These latter data strongly suggest that the regulation of metallo-

thionein biosynthesis involves changes in the transcription of thionein mRNA.

Liver ferritin synthesis is stimulated by iron (Drysdale & Munro, 1966). The iron-induced increase in ferritin synthesis occurs concomitantly with a 2-fold increase in polyribosomal ferritin mRNA (Zahringer *et al.*, 1975). Similarly, Squibb & Cousins (1977) demonstrated that liver polyribosomes from Zn²⁺-treated rats directed the synthesis of more thionein-like polypeptide chains than did control liver polyribosomes. However, unlike metallothionein, ferritin synthesis is not inhibited by either actinomycin D or cordycepin. On the basis of these data it is likely that the regulation of ferritin synthesis is centred at translation, as suggested by Zahringer *et al.* (1976), whereas metallothionein synthesis involves primarily changes in transcription of thionein mRNA (Squibb *et al.*, 1977).

In an effort to define the mechanism of regulation of metallothionein synthesis by Zn²⁺ further, the metallothionein mRNA content of polyribosomes was assayed in a wheat-germ protein-synthesizing system. The results of these experiments, which we report here, indicate that Zn²⁺ produces a measurable, actinomycin D-inhibitable, increase in polyribosomal metallothionein mRNA. The time course of this response coincides with the Zn²⁺-induced stimulation of metallothionein synthesis *in vivo*.

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Experimental

Animals

Male rats (Sprague-Dawley strain; Charles River; Wilmington, MA, U.S.A.) weighing 100–200 g were maintained on a commercial natural diet (50 p.p.m. of zinc) and tap water, both provided *ad libitum*. The animals were deprived of food overnight before each experiment.

Reagents and materials

All chemicals were reagent grade. All water was distilled and then deionized (Ultrapure Resin; Barnstead Co., Framingham, MA, U.S.A.) before use. L-[³⁵S]Cystine (135 mCi/mmol), [2-³H]glycine (3.4 Ci/mmol), L-[4,5(n)-³H]lysine hydrochloride (25 Ci/mmol) and L-[3-³H]serine (8.9 Ci/mmol) were obtained from Amersham-Searle Corp. (Downers Grove, IL, U.S.A.). Glycine, lysine and serine were combined in equal amounts to produce an aqueous solution with a total ³H content of 1 mCi/ml. Wheat germ was a gift from General Mills (Vallejo, CA, U.S.A.). All chromatography steps utilized products from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.).

Induction of metallothionein mRNA

In the experiments to investigate time-related changes in liver polyribosomal metallothionein mRNA content, rats were injected intraperitoneally with 2 mg of Zn²⁺ (as ZnSO₄) at 0, 2½, 5, 8 and 20 h before killing. Control rats, at each time point, were given injections of 0.9% NaCl (w/v) instead of Zn²⁺. The animals were killed by decapitation (two rats per time point) and their livers were quickly excised for preparation of polyribosomal RNA. In some experiments actinomycin D (0.8 mg/kg in 50% propylene glycol) was administered, intraperitoneally, 4 h before Zn²⁺.

Precipitation of polyribosomes and extraction of RNA

Livers were immediately minced and a 10% homogenate was prepared in 25 mM-Tris/HCl buffer, pH 7.5, containing 25 mM-NaCl, 5 mM-MgCl₂, 1 g of heparin/litre and 20 ml of Triton X-100/litre. A postmitochondrial supernatant was prepared (27000g, *r*_{av}. 8.0 cm, 5 min) for preparation of the polyribosomes by Mg²⁺ precipitation. The RNA was extracted by a sodium dodecyl sulphate/phenol/chloroform procedure. These methods have been described in detail (Palmiter, 1974). The final RNA precipitate was not washed. The yield of RNA was typically about 100 A₂₆₀ units/g of liver (1 A₂₆₀ unit = 42 µg of RNA). Polyribosomal RNA was stored in water (250–350 A₂₆₀ units/ml) in the vapour phase of liquid N₂ until further purified.

Purification of mRNA

Poly(A)⁺ (polyadenylated) mRNA was purified from the total polyribosomal RNA by oligo(dT)-cellulose affinity chromatography (type T-2; Collaborative Research, Watham, MA, U.S.A.) as described by Green *et al.* (1976), except that the second elution buffer was omitted. The RNA (up to 1800 A₂₆₀ units) was brought to a final concentration of 30–40 A₂₆₀ units/ml in starting buffer (0.5 M-KCl/0.01 M-Tris/HCl, pH 7.4) and applied to a column (0.9 cm × 6 cm) of oligo(dT)-cellulose (1.5 g) equilibrated with that buffer. The column was then eluted (20–25 ml/h) with starting buffer (6 ml fractions) until the A₂₆₀ of the eluate reached a constant baseline. The buffer was changed to 0.01 M-Tris/HCl, pH 7.4, and poly(A)⁺ mRNA was eluted from the column in 3 ml fractions. Poly(A)⁺ mRNA was precipitated at –20°C overnight by the addition of 0.1 vol. of 20% (w/v) potassium acetate, pH 5.5, and 2 vol. of 95% ethanol. The mRNA was collected by centrifugation at 11000g (*r*_{av}. 8.0 cm) for 10 min, dissolved in sterile water and stored in the vapour phase of liquid N₂.

Translation of mRNA

Translation of purified poly(A)⁺ mRNA was performed in a wheat-germ cell-free system by a modification of the procedure outlined by Efstratiadis & Kafatos (1976). Preparation of the wheat-germ extract varied from that described in two ways. Extraction was carried out by grinding 11 g of wheat germ with 11 g of sterile glass beads. Only the first one-third of the turbid fractions in the Sephadex G-25 eluate were pooled, to avoid amino acid contamination.

Translation of mRNA was performed in a total reaction volume of 50 µl. The translation mix contained 2 mM-ATP, 0.2 mM-GTP, 2 mM-dithiothreitol, 8 mM-phosphocreatine, 0.8 mM-spermidine, 64 mM-KCl, 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (pH 7.0), 5 µCi of a mixture of [³H]serine, [³H]glycine and [³H]lysine, 50 µM each of 17 non-radioactive amino acids, an appropriate amount of mRNA [usually 0.09 A₂₆₀ unit (3.75 µg)] and 15 µl of wheat-germ extract containing residual creatine phosphokinase from the preincubation, and sufficient KCl and magnesium acetate to make the translation mix 1.5 mM in magnesium acetate and 100 mM in KCl.

The translation mixture was allowed to react for 1 h at 25°C and was immediately placed at –20°C until the cell-free products were characterized. If trichloroacetic acid precipitation was to be performed, translation was stopped by placing the reaction tubes on ice and adding at least 20 vol. of cold 10% (w/v) trichloroacetic acid. Then, after 12 min in a 90°C water bath to uncharge precipitated tRNA

species, the contents of the reaction tubes were cooled, filtered through cellulose triacetate filters (Metricel, 0.45 μ m pore size; Gelman, Ann Arbor, MI, U.S.A.), and incorporation of ^3H into protein was measured by liquid-scintillation counting.

Characterization of cell-free products

A large percentage (approx. 69%) of the translated *in vitro* products were precipitated by the addition of 1 ml of 0.1M-sodium acetate/0.3M-NaCl/1mM-EDTA, pH4 (buffer A), and removed from the soluble products by centrifugation at 2000g (r_{av} . 10cm) for 5min. Purified rat liver zinc-thionein (16 μ g), prepared as described previously (Feldman & Cousins, 1976), was added to the supernatant as carrier. The mixture was then applied to a Sephadex G-25 gel-filtration column (1.6 cm $\times$ 30cm) equilibrated with buffer A. The fractions at the void volume of the eluate were pooled and assayed for metallothionein in two ways.

The first method was covalent chromatography on activated-thiol-Sepharose 4B, previously used by Squibb & Cousins (1977). Before loading the column (0.9cm $\times$ 6cm), the gel was washed with buffer A until the A_{280} of the wash was less than 0.05 A unit. The sample was then applied and the column eluted with 36ml of buffer A. Fractions (2ml) were collected at a flow rate of 20ml/h for this and subsequent elution steps. The column was re-equilibrated at pH8 by washing with 36ml of 0.1M-Tris/HCl/0.3M-NaCl/1mM-EDTA, pH8.0 (buffer B). Metallothionein was then eluted by washing the column with 36ml of 50mM-cysteine in buffer B. The fractions collected from each step elution were dissolved in 15ml of Scintiverse (Fisher Scientific Co., Pittsburgh, PA, U.S.A.) and the ^3H content was assayed by liquid-scintillation counting. The columns were standardized with ^{35}S -labelled hepatic metallothionein (Feldman & Cousins, 1976) prepared from rats injected with Zn^{2+} and [^{35}S]cystine.

The second approach was gel-filtration chromatography on a standardized Sephadex G-75 column (2.6cm $\times$ 60.0cm) equilibrated with 0.01M-Tris/acetate/0.1% azide, pH8.6. ^3H -labelled polypeptides were eluted with the same buffer at 25ml/h (5ml fractions) and the ^3H was measured as above.

Results

Induction of metallothionein mRNA

Poly(A) $^+$ mRNA was obtained from liver polyribosomes in amounts between 1.6 and 3.3% of the total ribosomal RNA content. The mRNA from all preparations was active in the wheat-germ cell-free system in directing the incorporation of ^3H -labelled amino acids into protein. The incorporation was linear up to an mRNA concentration of approx. 6.25 μ g/translation tube (Fig. 1). A concentration of

Fig. 1. Influence of poly(A) $^+$ mRNA concentration on protein synthesis in the wheat-germ cell-free system

Protein synthesis *in vitro* was carried out, as described in the Experimental section, by using liver polyribosomal poly(A) $^+$ mRNA. Protein synthesis was measured as the incorporation of [^3H]glycine, [^3H]lysine and [^3H]serine into proteins precipitable in 10% trichloroacetic acid. Means of two determinations are shown.

mRNA near the centre of the linear region (3.75 μ g) was used for all translations. Therefore an increase or decrease in the amount of metallothionein synthesized reflected an increase or decrease in metallothionein mRNA relative to the total mRNA. Since the poly(A) $^+$ mRNA content of the polyribosomes was not decreased after Zn^{2+} administration, increases in the metallothionein mRNA fraction indicate increases in the actual amount present in the polyribosomes.

Synthesis of metallothionein *in vitro* was assayed by activated-thiol-Sepharose 4B covalent chromatography. The thiol groups of metallothionein form disulphide linkages with the support at pH4, but can be replaced by simple thiols at pH8 and eluted from the column. The elution profiles of samples of purified ^{35}S -labelled rat liver metallothionein (both A and B forms) from the columns are shown in Fig. 2. Almost all of the metallothionein initially adheres to the column. Recovery of ^{35}S exceeds 90%, and approx. two-thirds of this was not eluted until it was replaced during the 50mM-cysteine elution step.

Samples of cell-free product whose synthesis was directed by poly(A) $^+$ mRNA isolated from liver polyribosomes of control and Zn^{2+} -injected rats

Fig. 2. Covalent chromatography of purified [^{35}S]cysteine-labelled rat liver metallothioneins on activated-thiol-Sepharose 4B

A, B and C, Elution of the columns with 36 ml of buffer A, 36 ml of buffer B and 36 ml of 50 mM-cysteine in buffer B respectively (see the Experimental section). (a) Metallothionein, A form; (b) metallothionein, B form.

were partially purified by pH4 precipitation and Sephadex G-25 gel-filtration chromatography as described in the Experimental section and applied to activated-thiol-Sepharose columns. These chromatographic data are shown in Fig. 3. The synthesis of protein containing groups chemically similar to those in metallothionein, i.e. ^3H eluted at the 50 mM-cysteine step, increased more than 3-fold in response to Zn^{2+} administration. Similar thiol-elution patterns have been demonstrated for both liver cytosol after Zn^{2+} treatment and cell-free products directed by polyribosomes from Zn^{2+} -treated rats (Squibb & Cousins, 1977).

Additional cell-free product was similarly partially purified by pH4 precipitation and Sephadex G-25 gel-filtration chromatography and then was applied to a standardized Sephadex G-75 column (Fig. 4).

Fig. 3. Influence of Zn^{2+} administration on induction of liver mRNA coding for protein distinguishable as metallothionein by thiol covalent chromatography

Rats were injected with 2 mg of Zn^{2+} or 0.9% NaCl (control) 5 h before killing. Equal amounts of purified polyribosomal poly(A) $^{+}$ mRNA, obtained from Zn^{2+} -injected ($\bullet$) or control rats ($\circ$), were translated in a wheat-germ protein-synthesizing system. The cell-free products were partially purified as described in the Experimental section. The ^3H -labelled metallothionein polypeptides were identified by covalent chromatography on activated-thiol-Sepharose 4B. A, B and C are as in the legend to Fig. 2.

Close similarity is evident in the elution, at the void volume, of high-molecular-weight ^3H -labelled proteins coded for by mRNA isolated from both groups of rats. However, a large increase in synthesis of protein corresponding to the elution volume of purified rat liver metallothionein was observed in the product directed by mRNA from the Zn^{2+} -treated rats.

In order to compare the cell-free products isolated by the two methods of chromatography, ^3H -labelled material eluted in the pH8 equilibration step and the 50 mM-cysteine step from the activated-thiol-Sepharose column were rechromatographed on Sephadex G-75. A single peak, corresponding to purified metallothionein, was obtained from the rechromatography of the ^3H -labelled material from the cysteine elution (Fig. 5). Most of the ^3H in the pH8 elution was retained by the gel-filtration column. It was concluded, on the basis of this chromatographic evidence, that polyribosomal poly(A) $^{+}$ mRNA

Fig. 4. Influence of Zn^{2+} administration on induction of liver mRNA coding for protein distinguishable as metallothionein by gel-filtration chromatography

Rats were injected with 2 mg of Zn^{2+} or 0.9% NaCl (control) 5 h before killing. Equal amounts of purified polyribosomal poly(A)⁺ mRNA, obtained from Zn^{2+} -injected (●) or control rats (○), were translated in a wheat-germ protein-synthesizing system. The cell-free products were partially purified as described in the Experimental section. The 3H -labelled metallothionein polypeptides were identified by gel-filtration chromatography on Sephadex G-75. The column was calibrated with Blue Dextran (V_0), purified rat liver metallothionein (V_m) and tryptophan (V_t).

Fig. 5. Gel-filtration chromatography of cell-free product previously fractionated by activated-thiol-Sepharose covalent chromatography

3H -labelled cell-free product directed by liver polyribosomal poly(A)⁺ mRNA, obtained 5 h after an injection of 2 mg of Zn^{2+} , was partially purified and fractionated by activated thiol-Sepharose covalent chromatography as described in Fig. 3. 3H -labelled products eluted from the activated-thiol-Sepharose 4B column at the pH8 equilibration elution (○) or 50mm-cysteine elution (●) were separately rechromatographed on Sephadex G-75. The gel filtration column was calibrated with Blue Dextran (V_0), purified rat liver metallothionein (V_m) and tryptophan (V_t).

directed the incorporation of [3H]glycine, [3H]lysine and/or [3H]serine into a reactive-thiol-containing polypeptide of approximately the same size as native metallothionein. Moreover, the amount of this mRNA in polyribosomes could be increased by treating rats with Zn^{2+} .

Zn^{2+} -stimulated changes in polyribosomal metallothionein mRNA

In order to determine whether the induction of metallothionein mRNA in the polyribosomal fraction coincided with data for the Zn^{2+} induction of net synthesis of liver metallothionein *in vivo* (Squibb *et al.*, 1977), poly(A)⁺ mRNA was purified from rat liver polyribosomes obtained at 0, 2½, 5, 8 and 20 h after an intraperitoneal injection of Zn^{2+} . Synthesis of thionein chains directed *in vitro* by poly(A)⁺ mRNA obtained from control and Zn^{2+} -treated rats at each time point were compared. The stimulation ($+Zn^{2+}$ minus control) shown in Fig. 6

Fig. 6. Influence of Zn^{2+} administration on polyribosomal metallothionein mRNA

Poly(A)⁺ mRNA was purified from liver polyribosomes 0, 2½, 5, 8 and 20 h after rats were injected with 2 mg of Zn^{2+} , or 0.9% NaCl in the case of controls. The poly(A)⁺ mRNA was translated in the wheat-germ protein-synthesizing system and nascent 3H -labelled metallothionein chains were characterized by activated-thiol-Sepharose covalent chromatography. Enhancement of polyribosomal metallothionein mRNA in Zn^{2+} -treated rats relative to control rats is expressed as the difference, at each time point, in 3H -labelled protein eluted from the activated-thiol-Sepharose 4B columns with 50mm-cysteine.

demonstrates that shortly after Zn^{2+} administration the fraction of polyribosomal mRNA that codes for metallothionein increased rapidly, reaching a maximum by 5h after the injection. By 20h after the Zn^{2+} injection this mRNA fraction is still greater than controls. It is clear that the time of maximum polyribosomal metallothionein mRNA content coincides with the maximum rate of synthesis of metallothionein observed *in vivo*. These results provide strong evidence for the concept that the increase in liver metallothionein in response to Zn^{2+} loading involves an accelerated rate of synthesis due to an increase in the mRNA content of liver polyribosomes.

Effect of actinomycin D on Zn^{2+} induction of metallothionein mRNA

Sensitivity to actinomycin D was used to determine whether transcription was required for the induction of polyribosomal metallothionein mRNA. Actinomycin D, at a concentration shown to inhibit mRNA synthesis (Prosky *et al.*, 1968) and also the induction of metallothionein by Zn^{2+} (Squibb *et al.*, 1977), was administered before Zn^{2+} treatment. Polyribosomal mRNA from the livers of actinomycin D-treated and control rats was used to direct the

synthesis of metallothionein in the wheat-germ system. Actinomycin D completely blocked the induction of polyribosomal metallothionein mRNA by Zn^{2+} (Fig. 7) at 5h after the injection, i.e. when the induction should be maximal. Thus the increased metallothionein mRNA in the polyribosomal fraction appears to be newly transcribed.

Discussion

The availability of cell-free translation systems, i.e. reticulocyte lysate, Krebs ascites II and wheat-germ extract, provides a reliable method for measuring a pool of translatable mRNA. These techniques are particularly applicable to investigating the mechanism of induction of specific gene products. For example, cortisol has been shown to increase tryptophan oxygenase mRNA (Schutz *et al.*, 1973) and tyrosine aminotransferase mRNA (Roewekamp *et al.*, 1976; Nickol *et al.*, 1976) in liver. It is now known that many other hormones increase the intracellular content of specific mRNA species. For example, intestinal polyribosomes are enriched in calcium-binding-protein mRNA after administration of 1,25-dihydroxycholecalciferol, the hormonally active form of vitamin D (Emtage *et al.*, 1974; Spencer *et al.*, 1976).

In contrast with these examples, ferritin and metallothionein appear to be regulated by the specific metals they bind. There seemed to be major differences, however, in the mechanisms regulating their biosynthesis. Iron administration has been demonstrated to redistribute ferritin mRNA from free cytoplasmic pools to polyribosomes (Zahringer *et al.*, 1975, 1976). Ferritin induction is not inhibited by either actinomycin D or cordycepin, indicating that translational regulation is probable (Zahringer *et al.*, 1976). Munro (1977) has proposed that translational control allows cells to respond rapidly to an intracellular influx of iron, which is highly cytotoxic when unbound.

Although unbound Zn^{2+} is also toxic to cells, the mechanisms of response of metallothionein to changes in intracellular Zn^{2+} are apparently different. A considerable body of evidence indicates that actinomycin D and cordycepin are both able to block the Zn^{2+} -stimulated increase in metallothionein synthesis *in vivo* (Squibb *et al.*, 1977; Richards & Cousins, 1977). This strongly argues in favour of a requirement for transcription of metallothionein mRNA *de novo* after Zn^{2+} administration. That the induction of metallothionein synthesis involves the increased ability of liver polyribosomes to synthesize metallothionein *in vitro* has been shown by Squibb & Cousins (1977). Data presented in the present paper (Fig. 6) demonstrate further that this capacity is due to an enrichment in metallothionein mRNA in the polyribosomes after Zn^{2+} administration.

Fig. 7. Influence of Zn^{2+} and actinomycin D administration on polyribosomal metallothionein mRNA

Actinomycin D was administered (0.8 mg/kg; intraperitoneally) 4h before 2mg of Zn^{2+} was injected. Rats were killed 5h after Zn^{2+} administration, and polyribosomal poly(A)⁺ mRNA was purified and translated in the wheat-germ protein-synthesizing system. Nascent 3H -labelled metallothionein chains were characterized by activated-thiol-Sepharose covalent chromatography. As controls, other rats were injected with 2mg of Zn^{2+} or 0.9% NaCl 5h before the polyribosomes were obtained.

A transcriptional control mechanism for metallothionein induction is also supported by inhibitor studies in this and other systems *in vitro*. We have demonstrated here (Fig. 7) that administration of actinomycin D *in vivo*, at an amount sufficient to inhibit transcription, blocks the increase in metallothionein mRNA in polyribosomes. In isolated rat liver parenchymal cells, actinomycin D also blocks the induction of metallothionein synthesis (Failla & Cousins, 1978b).

The specific action of Zn^{2+} in metallothionein induction is not completely clear. Weser & Bischoff (1970) have reported that Zn^{2+} is quickly accumulated by rat liver nuclei after an intraperitoneal injection of Zn^{2+} . Similarly, Cd^{2+} , which also induces metallothionein, is rapidly associated with the nucleus (Bryan & Hidalgo, 1976). Therefore these metal ions are at the site of transcription sufficiently early to stimulate or mediate the induction of specific mRNA species. Zn^{2+} and Ca^{2+} have also been shown, however, to stimulate the RNA polymerase activity of rat liver nuclei (Weser *et al.*, 1970; Weser & Hübner, 1970), so that general stimulatory effects on mRNA synthesis may also occur.

In the isolated rat liver parenchymal-cell system (Failla & Cousins, 1978a,b) metallothionein synthesis is induced when the intracellular Zn^{2+} concentration is increased by high extracellular Zn^{2+} or by glucocorticoid treatment and physiological extracellular Zn^{2+} concentrations. Glucocorticoids alone are without an effect. This argues for a specific effect of an increase in intracellular Zn^{2+} concentration in stimulating the synthesis of metallothionein mRNA.

The stimulation of metallothionein synthesis is short-lived after a single injection of Zn^{2+} . Intact-animal experiments have shown that, after the maximal rate of metallothionein synthesis is attained, it decreased to initial non-stimulated values (Squibb *et al.*, 1977). This could be interpreted as a decrease in the amount of metallothionein mRNA available, a repression of translation of existing metallothionein mRNA, or an increase in the synthesis of other mRNA species. Total cellular concentrations of this mRNA were not determined in the present experiments, and thus this question remains unanswered. The decrease could also reflect the decrease in nuclear Zn^{2+} after the initial rise noted by Weser & Bischoff (1970), or possibly a decrease in available Zn^{2+} in the cytoplasm occurring as Zn^{2+} is bound to newly synthesized thionein polypeptide chains. A similar decrease in mRNA translation was observed with the 1,25-dihydroxycholecalciferol-induced message for calcium-binding protein (Spencer *et al.*, 1976).

Evidence presented in this paper indicates that metallothionein mRNA is translated in the wheat-germ system as a species of about the same size as observed *in vivo*. A number of small proteins, e.g.

prolactin (Maurer *et al.*, 1976) and parathyroid hormone (Kemper *et al.*, 1974), are translated as units that are considerably larger than the native protein synthesized *in situ*. In contrast, mRNA species for metallothionein (the present study) and Ca^{2+} -binding protein (Spencer *et al.*, 1976) are translated into products that have approximately the same size as products synthesized *in vivo*.

The data presented in this report therefore support the concept that influx of extrahepatic Zn^{2+} induces metallothionein synthesis via the transcription of its mRNA *de novo*. This does not preclude the possibility that translational control of thionein mRNA by Zn^{2+} or other metals may also have a role in regulating the intracellular content of metallothionein.

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