Induction of rat liver metallothionein mRNA and its distribution between free and membrane-bound polyribosomes

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Total, membrane-bound and free polyribosomes were purified from livers of Zn^{2+} -treated and control rats. Polyadenylated RNA was separated from the polyribosomal RNA extracts by oligo(dT)–cellulose chromatography and translated in a wheat-germ cell-free translation system. Newly synthesized ^{35}S -labelled metallothionein was isolated from the other [^{35}S]methionine-labelled translation products by activated-thiol–Sephacrose 4B chromatography. The purity of the ^{35}S -labelled metallothionein product was substantiated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Zinc administration resulted in an elevation of metallothionein mRNA activity to 11% of the total polyribosomal mRNA activity. The vast majority of biologically active metallothionein mRNA was localized in the free polyribosomal pool, at least 94% and 97% in control and zinc-treated rats respectively. The increase in the percentage of polyribosomal mRNA coding for metallothionein after zinc administration was 3-fold, whether measured directly in total polyribosomal mRNA or as a combination derived from membrane-bound and free polyribosomal mRNA. These data indicate that the induction of metallothionein mRNA by zinc involves only free polyribosomes and suggest that the function of metallothionein is limited to intracellular processes.

Metallothionein appears to have a short-term storage or processing function for zinc (Cousins, 1979). Studies with intact rats have shown that the amount of cytoplasmic zinc bound to metallothionein can be increased by elevating the zinc content of the diet (Richards & Cousins, 1976*a,b*) or by parenteral zinc administration (Bremner & Davies, 1975; Richards & Cousins, 1975*a,b*; Squibb *et al.*, 1977), both of which increase the zinc content of the liver. Subsequent return of liver zinc concentration to within the normal range after these treatments is associated with degradation of this protein (Feldman & Cousins, 1976; Bremner *et al.*, 1978; Andersen *et al.*, 1978). Starvation, exposure to environmental stresses, partial hepatectomy and acute infections, all of which induce redistribution of tissue zinc pools, also increase the amount of liver zinc-metallothionein (Bremner & Davies, 1975; Richards & Cousins, 1976*b*; Oh *et al.*, 1978; Sobocinski *et al.*, 1978; Ohtake *et al.*, 1978). Moreover, glucocorticoid-induced zinc accumulation in rat liver parenchymal cells in primary

monolayer culture is almost entirely accounted for as zinc-metallothionein in the cytosol fraction of lysed cells (Failla & Cousins, 1978).

The transient nature of liver metallothionein suggests the possibility that its rate of synthesis could be altered by various stimuli. Initial experiments with [^{35}S]cystine indicated that only small amounts of metallothionein are synthesized, unless zinc metabolism is sufficiently perturbed, e.g. by parenteral administration of zinc. The rate of metallothionein synthesis *in vivo* increases to a maximum 5–6 h after parenteral zinc administration and returns to a basal value by 15 h thereafter (Squibb *et al.*, 1977). The induction is blocked by either actinomycin D or cordycepin. The ability of polyribosomal mRNA to direct metallothionein synthesis *in vitro* is directly proportional to the zinc-stimulated increase in the rate of synthesis and is inhibited by prior administration of actinomycin D (Shapiro *et al.*, 1978).

A similar, but less pronounced, increase in the rate of liver metallothionein synthesis was observed in adrenalectomized rats after administration of the synthetic glucocorticoid dexamethasone (Etzet *et al.*, 1979). The hormone-stimulated increase in the rate

Abbreviation used: poly(A)⁺ RNA, poly(A)-containing RNA.

of synthesis is also associated with elevated polyribosomal metallothionein mRNA activity. Collectively these data support earlier suggestions (Richards & Cousins, 1975*a,b*; Squibb & Cousins, 1977) that the induction of metallothionein requires synthesis *de novo* of its mRNA.

The inducible nature of metallothionein, especially under conditions in which metallothionein-bound zinc may be an intermediate in the eventual redistribution of the metal, such as in regenerating liver, during starvation or stress, or during marked changes in dietary zinc content, suggests that it has an intracellular function related to zinc metabolism (Cousins, 1979). However, an extracellular role of metallothionein has been proposed as well (G. F. Nordberg, 1972; M. Nordberg, 1978), in which the protein might appear in the plasma. Our interest in developing a model to examine the contribution of metallothionein to zinc metabolism in hepatocytes and to the control of zinc-requiring and perhaps other cellular processes prompted us to investigate further the regulatory aspects of metallothionein biosynthesis. In the present study we examined the effect of zinc administration on the distribution of metallothionein mRNA between membrane-bound and free rat liver polyribosomes. The results of these experiments indicate that metallothionein is synthesized almost entirely on free polyribosomes. This finding, coupled with our earlier observation, substantiated here, that metallothionein synthesized *in vitro* is the same size as that purified from rat liver (Shapiro *et al.*, 1978) is consistent with a model limiting metallothionein function to intracellular processes (Blobel & Dobberstein, 1975).

Experimental

Materials

All chemicals were reagent grade. Water was glass-distilled and then deionized (Ultrapure Resin; Barnstead Co., Framingham, MA, U.S.A.) before use. All solutions and glassware used in the preparative procedures were autoclaved, except solutions used in the extraction of RNA, which were treated with diethyl pyrocarbonate as described by Palmiter (1974), and glassware that came in contact with purified RNA and mRNA, which was washed in chromic acid/H₂SO₄ and heated at 180°C for 2 h. Ribonuclease-free sucrose (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used throughout. L-[³⁵S]Methionine (547 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). Bovine serum albumin, cytochrome *c* and insulin were purchased from Sigma Chemical Co.

Wheat germ was a gift from General Mills Corp. (Vallejo, CA, U.S.A.). Oligo(dT)-cellulose (type T₂) was purchased from Collaborative Research

(Waltham, MA, U.S.A.). Other chromatography products were obtained from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.).

Animals

Male rats (CD strain; Charles River, Wilmington, MA, U.S.A.) weighing 150–300 g were fed on a commercial diet (Ralston Purina Co., St. Louis, MO, U.S.A.; containing 50 p.p.m. of Zn) and tap water *ad libitum* until death, whereas rats used in preparation of total polyribosomes were starved overnight before death to decrease liver glycogen. Zinc was administered in a manner previously shown to induce polyribosomal metallothionein mRNA maximally (Squibb *et al.*, 1977; Shapiro *et al.*, 1978). A dose of 2 mg of Zn²⁺ (as ZnSO₄) in 0.25 ml of 0.9% NaCl (w/v) per 150 g body wt. was given by intraperitoneal injection. Control rats received a comparable volume of 0.9% NaCl. All animals were killed 5 h after injection.

Preparation of total polyribosomes

The rats were killed by decapitation and the livers were immediately excised and minced. A 10% (w/v) homogenate was prepared in 25 mM-Tris/HCl buffer, pH 7.5, containing 25 mM-NaCl, 5 mM-MgCl₂, 0.1% (w/v) heparin and 20 ml of Triton X-100/litre by using a glass/Teflon Potter-Elvehjem homogenizer. Polyribosomes were extracted by the procedure of Palmiter (1974), as described previously (Shapiro *et al.*, 1978).

Preparation of membrane-bound and free polyribosomes

Membrane-bound and free polyribosomes were isolated in a similar manner to that described by Ramsey & Steele (1976). Rats were anaesthetized with diethyl ether, and the livers perfused via the hepatic portal vein with 200 ml of ice-cold 250 mM-sucrose/5 mM-MgCl₂, followed by 100 ml of cold homogenization buffer {250 mM-sucrose, 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (pH 7.4), 75 mM-KCl, 5 mM-MgCl₂, 3 mM-glutathione (reduced)}. The livers were immediately excised and placed in ice-cold homogenization buffer. When all perfusions were completed, the livers were cut into small pieces and homogenized in 3 vol. of ice-cold homogenization buffer with ten strokes of the homogenizer. The homogenates were centrifuged at 740 g (*r*_{av}, 11.3 cm; 4°C) for 2 min, and then at 131 000 g for 12 min. Subsequent procedures were performed in a 4°C room. The supernatants, from which free polyribosomes were prepared, were removed to within 0.5 cm of the soft pellets, and stored on ice. The small amount of supernatant that remained was decanted and its volume was measured for correction of free polyribosomal yield. The pellets were

resuspended in cell sap (adjusted to 250 mM-KCl; see below). These suspensions were pooled, $\frac{1}{3}$ vol. of 10% (w/w) Triton X-100 was added and the contents were mixed. The mixtures were centrifuged at 1470 g (r_{av} 8.0 cm) for 5 min (4°C) and the pellets were discarded. Then $\frac{1}{3}$ vol. of 13% (w/w) sodium deoxycholate was added to each supernatant to complete solubilization of the membrane-bound polyribosomes.

The appropriate supernatants were then subjected to discontinuous-sucrose-gradient centrifugation for purification of the free and membrane-bound polyribosomes. Gradient buffers were made by dilution of a stock gradient solution [2.3 M-sucrose, 10 mM-Hepes (pH 7.4), 250 mM-KCl, 5 mM-MgCl₂, 3 mM-glutathione] with sufficient cell sap (250 mM-KCl) to give the required sucrose concentrations. Polyribosomal supernatant (5 ml) was applied to a 5 ml gradient consisting of 3 ml of 1.38 M-sucrose overlaying 2 ml of 2.0 M-sucrose, both in gradient buffer. The gradients were centrifuged for 20 h at 174 000 g (r_{av} 5.9 cm; 4°C). The supernatant solutions were removed and the polyribosomal pellets were resuspended in the buffer. When necessary, polyribosomal suspensions were stored in liquid N₂ for subsequent analysis.

Extraction of polyribosomal RNA

Polyribosomal pellets were suspended in 20 mM-Hepes (pH 7.5) buffer and RNA was extracted at room temperature by using the sodium dodecyl sulphate/phenol/chloroform procedure of Palmiter (1974). Resuspended polyribosomes (20–100 A_{260} units of RNA/ml) were mixed with an equal volume of 0.1 M-sodium acetate (pH 5.0) and $\frac{1}{3}$ vol. of 10% (w/v) sodium dodecyl sulphate. Phenol (saturated with 0.1 M-sodium acetate) was then added in equal volume and the mixture was shaken several times over a period of 5 min. Chloroform was added in equal volume to the phenol, and the shaking procedure was repeated. The contents were then centrifuged for 1 min at 15 000 g (r_{av} 8.0; 23°C). The aqueous phase and interphase were re-extracted twice with twice the volume of chloroform used previously. RNA was precipitated from the final aqueous phase at –20°C overnight in 2 vol. of 95% ethanol. The precipitate was collected by centrifugation for 10 min at 27 000 g (r_{av} 8.0 cm; 4°C). The pellet was rinsed twice with 2 ml of 3.0 M-sodium acetate (pH 6.0), dissolved in water at a concentration greater than 60 A_{260} units/ml and stored in liquid N₂.

Purification of poly(A)⁺ mRNA

Poly(A)⁺ mRNA was purified from polyribosomal RNA preparations by oligo(dT)–cellulose affinity chromatography (Aviv & Leder, 1972) essentially as described previously (Shapiro *et al.*,

1978). However, owing to the differences in the amount of RNA extracted from membrane-bound and free polyribosomes, adjustments were made in the chromatographic procedure so that the concentration of poly(A)⁺ mRNA in the final elution peak was sufficient for recovery [greater than 8 μ g of mRNA/ml, assuming 41.7 μ g of RNA/ A_{260} unit (Shapiro *et al.*, 1978), in the buffer/ethanol mixture]. The recovered poly(A)⁺ mRNA was stored in liquid N₂.

Preparation of rat liver cell sap

Livers from several rats were pooled, minced, and homogenized in 1 vol. of the same homogenization buffer used to prepare free and membrane-bound polyribosomes. The homogenate was centrifuged at 17 000 g for 10 min (r_{av} 8.0 cm; 4°C) and the resulting supernatant was centrifuged at 226 000 g (r_{av} 5.9 cm; 4°C) for 163 min. The clear upper portion of the supernatant (cell sap) was removed and adjusted to 250 mM-KCl by addition of 2 M-KCl in homogenization buffer.

Sedimentation analysis of membrane-bound and free polyribosomes on sucrose gradients

For each sedimentation profile, 10 A_{260} units of polyribosomes in a total volume of 100 μ l of buffer [10 mM-Hepes (pH 7.4)/75 mM-KCl/5 mM-MgCl₂], were layered on a 12 ml linear 10–40% (w/v) sucrose gradient in the same buffer adjusted to 0.5 mM-EDTA. The gradients were centrifuged for 2 h at 115 000 g (r_{av} 11.3 cm; 4°C). The gradients were removed in ascending fashion with 2 M-sucrose. Distribution of the polyribosomes in the gradient was assayed by measuring A_{260} .

Translation of mRNA

A wheat-germ extract was prepared by the procedure of Efstratiadis & Kafatos (1976) as previously modified (Shapiro *et al.*, 1978). mRNA was translated in a total reaction volume of 25 or 50 μ l containing 2 mM-ATP, 0.2 mM-GTP, 2 mM-dithiothreitol, 8 mM-phosphocreatine, 0.8 mM-spermidine, 100 mM-KCl, 1.5 mM-magnesium acetate, 20 mM-Hepes (pH 7.0), 50 μ M in all amino acids except methionine, 5–8 μ Ci of [³⁵S]methionine/50 μ l, an appropriate amount of mRNA and wheat-germ extract (15 μ l/50 μ l of incubation mixture).

The translation mixtures were allowed to react for 1 h at 25°C. Then 5 μ l samples were spotted on cellulose triacetate filters (Metricel, 0.45 μ m pore size; Gelman, Ann Arbor, MI, U.S.A.) for measurement of total amino acid incorporation (Mans & Novelli, 1961; Daniel *et al.*, 1977). This was standardized for recovery of metallothionein and corrected for geometry of liquid-scintillation counting on filter discs.

The remainder was stored at -20°C for further analysis.

Activated-thiol-Sepharose 4B covalent-chromatographic assay of metallothionein synthesized in vitro

^{35}S -labelled metallothionein was isolated by activated-thiol-Sepharose 4B covalent chromatography (Squibb & Cousins, 1977; Shapiro *et al.*, 1978). Samples of cell-free product were adjusted to pH 4.0 by the addition of 1 ml of buffer A [0.1 M-sodium acetate/HCl (pH 4.0), 0.3 M-NaCl, 1 mM-EDTA] and acid-insoluble components were removed by centrifugation at 2000 g (r_{av} , 10 cm) for 5 min at room temperature. After addition of 25 μg of purified rat liver zinc-metlothionein (Feldman & Cousins, 1976) as carrier, free [^{35}S]methionine was removed from the supernatant by passage through a Sephadex G-25 column (1.5 cm $\times$ 30 cm) eluted with buffer A. Protein eluted in the void volume was applied to a column (0.9 cm $\times$ 10 cm) of activated-thiol-Sepharose 4B equilibrated with buffer A. The column was eluted with buffer A (36 ml) and then re-equilibrated to pH 8.0 with 36 ml of buffer B [0.1 M-Tris/HCl (pH 8.0), 0.3 M-NaCl, 1 mM-EDTA]. Metallothionein was eluted with 36 ml of 50 mM-cysteine in buffer B (buffer C). The fractions (2 ml) were measured for ^{35}S by liquid-scintillation counting (in Scintiverse; Fisher Scientific Co., Pittsburgh, PA, U.S.A.).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of products of translation in vitro

^{35}S -labelled metallothionein, which was purified from cell-free product by thiol chromatography as described above, was dialysed overnight against 1 mM-Tris/HCl (pH 7.4) at 4°C , freeze-dried and then denatured by addition of 8 M-urea (50 μl), 20% (v/v) sodium dodecyl sulphate (2.5 μl) and β -mercaptoethanol (1 μl) and boiling for 2 min before electrophoresis. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed by the procedure of Laemmli (1970) with some modifications. Because of the small size of metallothionein, a 15%-polyacrylamide (2.5% cross-linked) separating gel was employed, overlaid by a 3%-polyacrylamide (2.5% cross-linked) stacking gel. The gels were formed in a 12 cm $\times$ 12 cm slab-gel apparatus. Electrophoresis was carried out at 20 mA (constant current) for approx. 3 h, without cooling. The gels were stained with 0.25% Coomassie Brilliant Blue in methanol/water/acetic acid (227:227:46, by vol.). Destaining was carried out in methanol/water/acetic acid (45:48:7, by vol.). Gels containing radioactively labelled protein were cut into 1 mm slices. Each slice was placed in a liquid-scintillation vial, 100 μl of 30% (v/v) H_2O_2 was added, and the gels were solubilized at 60°C

overnight. The ^{35}S content was assayed by liquid-scintillation counting.

Results

Translation of total polyribosomal mRNA in vitro and induction of metallothionein mRNA

Poly(A)⁺ mRNA was isolated from total rat liver polyribosomal RNA by oligo(dT)-cellulose chromatography and translated in the wheat-germ cell-free system. As shown in Fig. 1, mRNA from livers of both control rats and those given zinc 5 h before death exhibited similar activity with respect to directing the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable protein (see the Experimental section). ^{35}S incorporation was linear up to a concentration of about 75 μg of mRNA/ml of translation mixture (3.75 $\mu\text{g}/50\mu\text{l}$).

The effect of zinc administration on the metallothionein-mRNA activity of total liver polyribosomal mRNA is shown in Fig. 2. Metallothionein synthesized *in vitro* was isolated from the other [^{35}S]methionine-labelled pH 4-soluble products by activated-thiol-Sepharose 4B covalent chromatography. Most of the ^{35}S from ^{35}S -labelled purified rat liver metallothionein (both 1 and 2 forms) was eluted by the 50 mM-cysteine elution step in this procedure.

Radioactivity incorporated *in vitro* that was eluted by the pH 8 buffer was not identifiable as

Fig. 1. Stimulation of protein synthesis *in vitro* by total polyribosomal mRNA from rat liver

Total protein synthesis in the wheat-germ system was measured by incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material. Each point represents incorporation in 10 μl of a total reaction mixture of 50 μl at the indicated concentration of mRNA. Incorporation values are corrected for endogenous wheat-germ mRNA activity. mRNA was from zinc-treated rats (●) or control rats (○).

Fig. 2. Activated-thiol-Sepharose 4B covalent chromatography of [^{35}S]methionine-labelled cell-free translation products directed by total polyribosomal mRNA

Translation products that were soluble at pH 4 and larger than 5000 daltons were loaded on the thiol column and eluted stepwise with 36 ml sequential additions of buffers A, B and C (braces A, B and C respectively; see the Experimental section). Metallothionein synthesized *in vitro* was eluted with buffer C (50 mM-cysteine). Cell-free products directed by liver mRNA from control rats (O) and from zinc-treated rats (●).

metallothionein, whereas all the radioactivity eluted by the cysteine buffer behaved chromatographically as metallothionein on gel-filtration chromatography (Shapiro *et al.*, 1978). When equal amounts of polyribosomal mRNA were translated and the pH 4-soluble products analysed, clearly more metallothionein-mRNA activity was found in the polyribosomal mRNA from zinc-treated rats than in controls. On a percentage basis, zinc treatment elevated metallothionein-mRNA activity from 4.1 to 11.2% of the total translational activity *in vitro*.

In order to confirm the purity of metallothionein isolated by covalent chromatography, the protein(s) eluted with 50 mM-cysteine were further characterized by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 3). Virtually all of the ^{35}S eluted from the covalent column migrated with the rat liver metallothionein-2 standard. This corroborates the suitability of the activated-thiol chromatography procedure for the quantification of metallothionein synthesized *in vitro*. Endogenous wheat-germ mRNA does not code for metallothionein in an amount detectable by this procedure. Metallothionein synthesized *in vitro* appears to be the same size as the native protein, and the results suggest that the majority of the metallothionein mRNA translated codes for metallothionein-2.

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of [^{35}S]methionine-labelled metallothionein

Cell-free translation mixture (45 μl) was subjected to activated-thiol-Sepharose chromatography (Fig. 2; see the Experimental section). Fractions eluted with 50 mM-cysteine were dialysed against 1 mM-Tris/HCl (pH 7.4) and freeze-dried. The dried material was electrophoresed on sodium dodecyl sulphate/15% polyacrylamide gels for 3 h at 20 mA. The gels were sliced into 1 mm slices, dissolved and ^{35}S content was measured. Migration of purified rat liver metallothionein-1 (1) and rat liver metallothionein-2 (2) are shown above. (Migration was in the direction of the anode.) Insert: Migration of purified metallothioneins-1 (MT1) and -2 (MT2) compared with marker proteins of known molecular weights: A, bovine serum albumin (68000); B, cytochrome c (12400); C, insulin (6000).

Yield of membrane-bound and free polyribosomes

The yield of polyribosomes was determined by measuring the A_{260} of RNA extracted from each polyribosomal preparation. Total polyribosomal RNA was obtained in the range of 2.5–3.5 mg of RNA/g of perfused liver. As shown in Table 1, approx. 77–81% and 19–23% of the polyribosomal RNA were recovered from membrane-bound and free polyribosomes respectively. This distribution is in agreement with that obtained by Ramsey & Steele (1976) and Yap *et al.* (1978). The percentage of mRNA in the total RNA extract from membrane-bound polyribosomes was similar in both zinc-treated and control groups. However, the percentage of mRNA extractable from free polyribosomes of zinc-treated rats was greater than that from controls (1.5 and 0.9% respectively). Thus the total yield of mRNA was largely of membrane-

Table 1. Yield of membrane-bound and free polyribosomal RNA and mRNA from zinc-treated and control livers. Rats were injected with 2.0mg of Zn^{2+} or 0.9% NaCl and were killed 5 h later. Free and membrane-bound polyribosomes were separated from liver homogenates by sucrose-gradient centrifugation. The polyribosomal RNA was fractionated by oligo(dT)-cellulose chromatography. Values are based on analysis of pooled livers from two rats in each treatment group.

Treatment	Liver wt. (g)	Yield of poly-ribosomal RNA (A_{260} units/g of liver)	Distribution of recovered polyribosomal RNA		Yield of recovered polyribosomal mRNA	
			A_{260} units bound (% bound)	A_{260} units free (% free)	A_{260} units from bound (% of bound RNA)	A_{260} units from free (% of free RNA)
Zinc-injected rats	11.2	67	574 (77)	173 (23)	2.4 (0.4)	2.7 (1.5)
Control rats	12.0	81	786 (81)	186 (19)	4.2 (0.5)	1.7 (0.9)

Fig. 4. Sedimentation-velocity gradient centrifugation of membrane-bound (a) and free (b) liver polyribosomes. Membrane-bound or free polyribosomes ($10A_{260}$ units) from zinc-treated rats (—) and control rats (---) were suspended in buffer and centrifuged through 10–40% (w/v) sucrose gradients.

bound origin in control rat liver, but was nearly evenly divided between membrane-bound and free polyribosomes in liver from the zinc-treated rats.

Sedimentation analysis of polyribosomes on linear 10–40% sucrose gradients (Fig. 4) established that polyribosomes derived from all sources were well aggregated and had similar size distributions. Differences in the yield of polyribosomal mRNA obtained from the two groups of rats therefore do not appear to be the result of differences in integrity of the polyribosomes.

Translation of free and membrane-bound polyribosomal mRNA in vitro

A comparison of translational activity of membrane-bound and free polyribosomal mRNA in the

Fig. 5. Stimulation of protein synthesis in vitro by membrane-bound and free polyribosomal mRNA from rat liver.

Total protein synthesis directed by membrane-bound or free polyribosomal mRNA in the wheat-germ system was measured as described in the legend to Fig. 1. Each point represents incorporation in 5 μ l of a total reaction mixture of 25 μ l at the indicated concentration of mRNA. Incorporation values are corrected for endogenous wheat-germ mRNA activity. mRNA was derived from free polyribosomes from zinc-treated rats (●) or control rats (○), or from membrane-bound polyribosomes from zinc-treated rats (▲) or control rats (△).

wheat-germ cell-free system (Fig. 5) was made by measurement of incorporation of [35 S]methionine into trichloroacetic acid-precipitable protein. Incorporation was linear up to a concentration of 75 μ g of mRNA/ml of translation mixture (1.88 μ g/25 μ l) for mRNA from control and zinc-treated rats, regardless of the source. Membrane-bound and free mRNA from control rats continued to direct linear

incorporation of [³⁵S]methionine up to 133.4 μg of mRNA/ml (3.34 μg/25 μl), the maximum concentration tested, whereas both membrane-bound and free polyribosomal mRNA from zinc-treated rats appeared to saturate the system at this concentration.

Free polyribosomal mRNA from zinc-treated and control rats exhibited greater translational activity than did membrane-bound mRNA, as has been previously reported (Shafritz, 1974; Zahringer *et al.*, 1977). Although activity of the recovered membrane-bound polyribosomal mRNA was essentially unchanged by zinc administration, the activity of free polyribosomal mRNA was increased substantially by parenteral zinc in the range of mRNA concentration where ³⁵S incorporation was uniformly linear. The relationship of zinc treatment and source of mRNA to its translational activity *in vitro* was similar to its relationship to the amount isolated by oligo(dT)–cellulose chromatography (Table 1). The enhanced activity may be related to the inclusion in the mRNA pools of a small, abundant, newly synthesized messenger, i.e. metallothionein mRNA. This possibility is discussed below.

Distribution of metallothionein mRNA between free and membrane-bound polyribosomes

Chromatographic profiles on activated-thiol–Sephacose of pH 4-soluble [³⁵S]methionine-labelled proteins of greater than 5000 daltons that were synthesized *in vitro* from membrane-bound and free polyribosomal liver mRNA are shown in Fig. 6. A greater quantity of ³⁵S-labelled metallothionein was present among the cell-free products directed by mRNA from zinc-treated animals than in control-mRNA directed translations. Zinc administration

increased metallothionein synthesis *in vitro* from 5.7 to 15.5% of the total synthesis of ³⁵S-labelled proteins directed by equal amounts of polyribosomal mRNA (data combined for free and membrane-bound) in the cell-free system (Table 2). Correction for either relative yield of membrane-bound and free polyribosomes (assuming that metallothionein mRNA was recovered with the same efficiency as other species of mRNA with which it was co-extracted) or relative yield of mRNA (assuming

Fig. 6. Activated-thiol–Sephacose 4B covalent chromatography of [³⁵S]methionine-labelled cell-free translation products directed by membrane-bound and free polyribosomal mRNA

Thiol–Sephacose chromatography was performed as described in the legend to Fig. 2. Free (●) or membrane-bound (○) polyribosomal-mRNA-directed cell-free product with mRNA from (a) zinc-treated or (b) control rats.

Table 2. Induction of metallothionein mRNA and its distribution between membrane-bound and free polyribosomes. Rats were injected with 2.0 mg of Zn²⁺ or 0.9% NaCl and were killed 5 h later. Free and membrane-bound polyribosomes were separated from liver homogenates by sucrose-gradient centrifugation. Poly(A)⁺ mRNA was isolated from polyribosomal RNA by oligo(dT)–cellulose chromatography. The poly(A)⁺ mRNA was translated in a wheat-germ cell-free system. Metallothionein was isolated by activated-thiol covalent chromatography. Incorporation of [³⁵S]methionine was corrected for incorporation directed by endogenous wheat-germ mRNA. Exogenous-mRNA-directed incorporation *in vitro* of [³⁵S]methionine into total protein and metallothionein were measured by trichloroacetic acid-precipitable radioactivity (c.p.m.) and activated-thiol chromatography respectively (see the Experimental section). All translation mixtures contained 1.88 μg of mRNA in a total volume of 25 μl. Determinations of % metallothionein mRNA activity are based on comparison of equal amounts of mRNA.

Source of mRNA	Incorporation of [³⁵ S]methionine (c.p.m./20 μl of incubation mixture)		Total polyribosomal metallothionein- mRNA activity (%)	Metallothionein mRNA in free polyribosomes (%)
	Total protein	Metallothionein		
Free polyribosomes, zinc-treated rats	211 600	34 800	15.5	96.7
Membrane-bound polyribosomes, zinc-treated rats	21 000	1200		
Free polyribosomes, control rats	96 200	6200	5.7	93.7
Membrane-bound polyribosomes, control rats	18 600	400		

that metallothionein mRNA was always recovered with the same efficiency) does not significantly affect the relative or absolute metallothionein-mRNA activity values obtained. These data corroborate the findings presented above, in which zinc was observed to induce nearly a 3-fold increase in the percentage of metallothionein mRNA extracted from total polyribosomes.

Metallothionein mRNA was detected almost exclusively in free polyribosomes in both the control and zinc-treated rats (Table 2). Metallothionein-mRNA activity of free polyribosomal mRNA obtained from zinc-treated rats was 96.7% of the total metallothionein-mRNA activity of equal quantities of free and membrane-bound polyribosomal mRNA, whereas in controls 93.7% of this activity resided in free polyribosomal mRNA.

Discussion

Previous experiments have shown that metallothionein-mRNA amounts in total liver polyribosomes, as measured by translation *in vitro*, are directly proportional to the increase in the rate of metallothionein synthesis after parenteral administration of zinc (Squibb *et al.*, 1977; Shapiro *et al.*, 1978). In the present study, we measured the distribution of translatable metallothionein mRNA in free and membrane-bound liver polyribosomes to establish the relative contribution of these two polyribosome populations to metallothionein synthesis in livers of normal and zinc-treated rats. In an effort to obtain reliable measurements of metallothionein-mRNA activity, we used the uniformly incorporated amino acid [^{35}S]methionine to label the cell-free products. The mixture for translation *in vitro* contained spermidine (0.8 mM) and a relatively high concentration (100 mM) of K^+ . These conditions have been reported to promote accurate synthesis of both small and large proteins (Hunter *et al.*, 1977; Roewekamp *et al.*, 1976; Schmeckpeper *et al.*, 1974). However, measurements of the abundance of newly transcribed small mRNA species, by using translation systems *in vitro*, might be influenced by certain factors. For example, newly transcribed mRNA has longer average poly(A) sequences than other poly(A) $^+$ mRNA molecules (Chan *et al.*, 1977), which can facilitate their recovery by oligo(dT)-cellulose chromatography (Wilt, 1977).

This effect may have influenced the results reported here. Although both membrane-bound and free polyribosomes were received intact and were well aggregated (Fig. 3), the yield of mRNA from polyribosomal RNA (Table 1) varied consistently with the percentage of metallothionein-mRNA activity measured. It should be noted that zinc administration results in a general increase in RNA

polymerase activity (Weser & Hubner, 1970). Therefore zinc-induced synthesis of other mRNA species may also have contributed to the difference noted in recovery and efficiency of translation of mRNA pools between zinc-treated and control rats. Additionally, wheat-germ cell-free protein-synthesizing systems appear to translate small proteins more efficiently than larger ones (Hunter *et al.*, 1977). This preference may have caused the generally greater translational activity of free polyribosomal mRNA that we observed, since this mRNA pool has been shown to code for smaller proteins than do the membrane-bound polyribosomal mRNA species (Shafritz, 1974). This effect could be pronounced for the mRNA coding for a very small protein such as metallothionein, in spite of the precautions taken. It should be noted, however, that other liver mRNA species have been reported to be increased to as much as 20% of the total liver mRNA population under certain conditions of induction (Deeley *et al.*, 1977), so that the high degree of induction reported here may not be uncommon. The efficiency of the method of measurement of individual proteins synthesized *in vitro*, which has typically involved immunoprecipitation, may also affect the apparent extent of synthesis. Additional effects, such as zinc-related stabilization of metallothionein mRNA relative to other messengers, cannot be ruled out, however.

Protein synthesis in rat liver occurs mainly on polyribosomes that are associated with endoplasmic reticulum (membrane-bound polyribosomes) or on polyribosomes that are either free in the cytoplasm or associated with cytoplasmic fine-structural components (free polyribosomes). Proteins that are secreted, e.g. albumin (Redman, 1969) and transferrin (Morgan & Peters, 1971), are made on membrane-bound polyribosomes, and those that remain within the cytoplasm, e.g. ferritin (Zahringer *et al.*, 1977), are made on free polyribosomes. Proteins associated with cellular membrane or organelles may be synthesized on either free polyribosomes, e.g. catalase (Redman *et al.*, 1972), or membrane-bound polyribosomes, e.g. cytochrome c (Gonzalez-Cadavid & de Cordova, 1974).

The data obtained in the present study show that virtually all of the liver polyribosomal metallothionein mRNA is located in free polyribosomes of rats after an acute administration of zinc and in untreated rats. On the basis of calculations made by Ramsey & Steele (1976), this polyribosome-preparation procedure should yield membrane-bound polyribosomes that are approx. 3% contaminated with free polyribosomes. If such a correction is applied here, free polyribosomal metallothionein mRNA would account for essentially all (99.9%) of this polyribosomal message in zinc-treated rats, and 97.2% of it in controls. Application of corrections

for polyribosomal yield or mRNA yield in addition to the Ramsey & Steele (1976) contamination corrections does not alter the results for zinc-treated rats, but gives slightly lower results of between 89.0 and 93.3% of the polyribosomal metallothionein mRNA in free polyribosomes in control rats. Since the metallothionein-mRNA activity of membrane-bound polyribosomal mRNA is very low, and nearly unchanged by zinc treatment, these polyribosomes seem to be uninvolved in induction of metallothionein synthesis by zinc. The sharp rise in metallothionein-mRNA content of free polyribosomes after zinc treatment supports other evidence for an intracellular role of this protein.

Although the major body of evidence suggests that metallothionein has an intracellular function (Bremner & Davies, 1975; Oh *et al.*, 1978; Ohtake *et al.*, 1978; Sobocinski *et al.*, 1978; Cousins, 1979), some have proposed that metallothionein could function as a systemic carrier of metals, particularly cadmium (G. F. Nordberg, 1972; M. Nordberg, 1978). The proposal may lack physiological relevance, however, since it is based on limited data from acute and chronic cadmium-toxicity studies. This metal can lead to liver dysfunction, with concomitant loss of liver proteins to the circulation. Radioimmunoassay procedures have shown that metallothionein is not normally found in plasma (VanderMallie & Garvey, 1979).

An intracellular role for metallothionein is also supported by experiments showing that physiological stresses (cold, heat, burns, strenuous exercise) and acute infection with *Salmonella typhimurium* or live *Francisella tularensis* vaccine will induce the protein in liver (Oh *et al.*, 1978; Sobocinski *et al.*, 1978). The underlying physiological basis of some of these effects probably involves mediation by the glucocorticoids, at least in part. We found that administration of dexamethasone to adrenalectomized rats markedly decreased the plasma zinc concentration and simultaneously stimulated the rate of liver metallothionein synthesis (Etzel *et al.*, 1979), which on a temporal basis was nearly identical with that observed after administration of zinc (Squibb *et al.*, 1977). This induction phenomenon was coincident with an increase in the amount of translatable metallothionein mRNA in total liver polyribosomes. Overall, the induction of hepatic metallothionein biosynthesis by administration of zinc or glucocorticoids appears to involve the same mechanisms. Therefore, on the basis of the experiments reported here, the changes in metallothionein mRNA as brought about by physiological stresses or glucocorticoid administration would be expected to involve only free polyribosomes.

Experiments with both isolated liver cells and intact animals have shown that binding of zinc to metallothionein is closely linked to synthesis *de*

novo. Although the function of this protein is probably one of temporary storage, in a manner roughly analogous to that of ferritin, we cannot as yet totally rule out a role in membrane transport of transition metals. However, for such a function, the protein would probably need a hydrophobic region to allow interaction with the plasma membrane, and liver metallothionein does not contain aromatic amino acids (Bremner & Davies, 1975; Bühler & Kagi, 1974). Moreover, this protein does not appear to be synthesized in a precursor form, since the product synthesized *in vitro* is the same size as that found *in situ*. A direct membrane function is thus unlikely.

The findings described in the present paper are in agreement with the distribution between free and membrane-bound polyribosomal populations reported for other liver mRNA species. Yap *et al.* (1978) clearly demonstrated that albumin mRNA is nearly exclusively (98%) associated with membrane-bound polyribosomes in fed rats. Zahringer *et al.* (1976) found that iron administration to rats results in a shift of ferritin mRNA from the postribosomal cytosol into polyribosomes for translation. Although they found a certain amount of ferritin mRNA in the bound-polyribosome fraction, the initial induction of ferritin, an intracellular protein, by iron appeared to be associated with changes in mRNA from free polyribosomes (Zahringer *et al.*, 1977), much as we observed with metallothionein. The fact that the rate of metallothionein synthesis is directly correlated with amount of translatable metallothionein mRNA in polyribosomes (Shapiro *et al.*, 1978), and that both are inhibited by pretreatment with actinomycin D, suggests that the mRNA content of free polyribosomes (as observed in the present study) accounts for virtually all of the metallothionein synthesis observed after zinc treatment. If other cytoplasmic (extra-ribosomal) metallothionein mRNA species are involved in metallothionein synthesis in liver cells, it is doubtful that they contribute to the induction phenomenon as described here and elsewhere.

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