Partial Purification, Characterization and Translation *in vitro* of Rat Liver Metallothionein Messenger Ribonucleic Acid

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Poly(A)⁺ (polyadenylated) mRNA coding for metallothioneins was purified 13-fold from rat liver polyribosomes and was identified by its ability to direct the biosynthesis of these proteins in a wheat-germ cell-free system. The carboxymethylated products of the protein-synthesizing system *in vitro* were analysed with sodium dodecyl sulphate/20% polyacrylamide-gel electrophoresis. The labelled compounds [³H]serine and [³⁵S]cysteine were incorporated at high specific radioactivity into proteins that co-migrated with authentic metallothioneins. No [³H]leucine incorporation was found, in agreement with the amino acid composition of the metallothioneins. Metallothionein mRNA had a sedimentation coefficient of 9S and carried a maximum of four ribosomes. At 5h after a subcutaneous injection of ZnCl₂ or CdCl₂ (10 μmol/kg body wt.), the amount of this mRNA increased approx. 2- and 4-fold respectively, on the basis of translation *in vitro*. The increase in metallothionein mRNA (defined by translation in the wheat-germ system) was transient and, after CdCl₂ treatment, fell back to control values by 17h. Metallothioneins constituted a maximum of 0.8% of the total protein products synthesized in the wheat-germ system by total mRNA isolated from rat liver after CdCl₂ treatment.

Metallothioneins are low-molecular-weight cysteine-rich proteins which were first isolated from horse kidney by Margoshes & Vallee (1957). They were initially characterized by Kägi & Vallee (1960, 1961) as proteins binding Zn²⁺ and Cd²⁺, and containing traces of copper and other ions. Since then, metallothioneins have been found in a number of species, including mouse (Nordberg *et al.*, 1971), rat (Shaikh & Lucis, 1971), rabbit (Piscator, 1964) and human (Pulido *et al.*, 1966). Sequence analysis of horse kidney (Kojima *et al.*, 1976), human liver (Kissling & Kägi, 1977) and mouse liver (Huang & Yoshida, 1977) metallothioneins shows these proteins to contain 61 amino acid residues, of which 20 are cysteine. Remarkable sequence homology is found among these proteins, suggesting a conservation of function during the course of evolution as strong as that in the cytochrome *c* molecule. Two or more electrophoretically distinguishable forms of metallothionein are usually found. This fact, and the occasional presence of microheterogeneity seen in the sequence analysis (Kojima *et al.*, 1976; Bühler & Kägi, 1974), suggests the presence of multiple alleles

for the metallothioneins. The multiple forms of these proteins do not appear to be functionally different.

The amino acid compositions of rat liver metallothioneins 1 and 2 (Andersen *et al.*, 1978) differ only slightly. In addition, the amino acids arginine, histidine, tyrosine, leucine, phenylalanine and tryptophan are absent. No disulphide bonds exist in the metallothioneins, whose cysteine residues participate in the binding of the metal ions Zn²⁺ and Cd²⁺. Metallothioneins isolated from human (Rydén & Deutsch, 1978) or bovine (Hartman & Weser, 1977) foetus contain copper and zinc ions. This foetal (Cu + Zn)-thionein appears to be localized in the mitochondrial subcellular fraction, perhaps in heavy lysosomes (Porter, 1974). It is not found in adults to any great extent, except in the case of patients having Wilson's disease (Evans *et al.*, 1973) or in copper-loaded rats (Winge *et al.*, 1975; Riordan & Gower, 1975; Bremner & Young, 1976). This suggests that the synthesis of foetal copper-thioneins in the liver may be under developmental control.

The rate of biosynthesis of metallothioneins increases in response to exposure to heavy metals (Shaikh & Lucis, 1970; Squibb & Cousins, 1974; Chen *et al.*, 1975; Shaikh & Smith, 1975; Andersen *et al.*, 1978). The nature of this biosynthetic response has been attributed to either translational control of

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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protein biosynthesis (Webb, 1972) or transcriptional control of mRNA biosynthesis (Richards & Cousins, 1975; Squibb *et al.*, 1977) on the basis of studies in rats treated with cycloheximide, actinomycin D or cordycepin. A homologous cell-free system for the translation of metallothioneins from rat liver polyribosomes has been reported (Squibb & Cousins, 1977), and the cell-free synthesis of these proteins has been demonstrated in the wheat-germ system (Shapiro *et al.*, 1978). We have also used a heterologous system, with wheat germ, for the translation *in vitro* of metallothionein mRNA from rat liver. We report here the results of a preliminary characterization of the poly(A)⁺ (polyadenylated) mRNA for metallothionein and the effects of the heavy-metal ions Cd²⁺ and Zn²⁺ on changes in the cytoplasmic concentration of this mRNA.

Materials and Methods

Experiments

Deionized quartz-distilled water was used in all experiments. Solutions were autoclaved, or treated with 0.1% diethyl pyrocarbonate and autoclaved, and stored at 4°C; all glassware was sterilized; polyallomer ultracentrifuge tubes and all tubing and connections in the gradient fractionator were rinsed with 0.1% diethyl pyrocarbonate and sterile water; reagents or enzyme solutions not autoclavable were prepared with sterile water and stored at -20°C; manipulations of polyribosomes and mRNA were done at 0-4°C; incubations were carried out in sterile 0.5 ml or 1.5 ml polypropylene centrifuge tubes (Eppendorf) or in 4 ml polypropylene test tubes in a thermostatically controlled water bath at 25-45°C; polyribosomes and mRNA solutions were stored in liquid N₂. The $A_{260}^{1\text{mg/ml}}$ for poly(A)⁺ mRNA was taken as 25.

Animals

Female Sprague-Dawley rats (Suddeutsche Versuchstierfarm G.m.b.H., Voss KG, Germany) weighing 180-250g were used. The animals were kept in plastic cages with stainless-steel lids and supplied with Altromin Standard Diet (Alma Futter, F. Botzenhardt KG, Kempten, Germany) *ad libitum*. Animals were deprived of food for 18h, killed by decapitation and exsanguinated. The livers were excised, washed in sterile medium B (5mM-MgCl₂/25mM-NaCl/50mM-Tris/HCl, pH7.6) and placed into medium B containing 0.25M-sucrose and 500µg of heparin/ml at 0°C (see under 'Preparation of rat liver mRNA and polyribosomes').

Chemicals

Tris base, Hepes, amino acids and yeast tRNA were from Sigma Chemical Co., St. Louis, MO,

U.S.A.; Sephadex G-25 was from Pharmacia Fine Chemicals, Uppsala, Sweden; oligo(dT)-cellulose was from Collaborative Research, Waltham, MA, U.S.A.; nucleotides, phosphocreatine (disodium salt) and creatine kinase were from Boehringer Mannheim Biochemicals, Mannheim, Germany; spermine and dithioerythritol were from Serva Feinbiochemica, Heidelberg, Germany; all other chemicals were of reagent grade. Phenol was distilled under N₂ and stored at 4°C; iodoacetic acid was recrystallized from light petroleum (b.p. 30-60°C).

Equipment

Screw-cap polypropylene tubes (30 and 200ml) and 15ml corex tubes were used for preparative centrifugation in a Beckman model J-21B centrifuge with JA-14 and JA-20 rotors; 14 ml and 38.5 ml polyallomer tubes were used for all ultracentrifuge studies in a Beckman model L2-65B ultracentrifuge with Ti-60 ($r_{\text{max.}} = 9.0\text{cm}$), SW-27 ($r_{\text{max.}} = 16.12\text{cm}$) and SW-40 ($r_{\text{max.}} = 15.87\text{cm}$) rotors; gradients were fractionated with an Isco gradient fractionator; 1 cm diameter Visking dialysis tubing was boiled 24h before it was used to retard the passage of low-molecular-weight polypeptides.

Radioisotope use and radioactivity counting

[³H]Leucine (60Ci/mmol) and [¹⁴C]serine (168Ci/mol), from Amersham Buchler, Braunschweig, Germany, and [³H]serine (8.9Ci/mmol), from New England Nuclear, Boston, MA, U.S.A., were used as supplied; [³⁵S]cystine (35.7Ci/mmol), from New England Nuclear, was reduced with 18mM-dithioerythritol in 73mM-Hepes, pH7.6, at 37°C for 1h before use. Radioactivity counting was done in Aquasol (New England Nuclear) for liquid samples and in toluene scintillant [5g of 2,5-diphenyloxazole + 0.1g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene in 1 litre of toluene] for filter-paper discs; Corrections were made for background and, where double-label studies were done, for channel overlaps (0.02% for ³H overlap on the ³⁵S channel; 49% for ³⁵S overlap on the ³H channel). Liquid-scintillation counting was done in a Packard model 2450.

Isolation and carboxymethylation of metallothioneins

Metallothioneins 1 and 2 were isolated from rat liver as previously reported (Andersen *et al.*, 1978). To each of two protein solutions, containing 3.05 and 2.29µmol of thiol groups (161 and 109nmol of protein) in metallothioneins 1 and 2 respectively, was added 55µl of 1M-HCl in a total volume of 0.5ml. Each solution was chromatographed separately on Sephadex G-25 in 0.1M-HCl, the protein peak in the excluded volume purged with N₂, titrated to pH7.6 with 1M-Tris base, made 6M in

guanidine hydrochloride and made to react with 25 μ mol of iodoacetic acid under continuous bubbling with N_2 at 25°C in the dark. Carboxymethyl-metallothioneins were desalted by dialysis against water and freeze-dried.

Preparation of rat liver polyribosomes

Polyribosomes were isolated by a modification of the method of Noll *et al.* (1963). Rat livers were weighed and minced into two parts (w/v) of medium B + 0.25 M-sucrose + 500 μ g of heparin/ml, homogenized with five strokes of a glass/Teflon homogenizer and centrifuged for 10 min at 16000 rev./min (30000 g_{max}) in a JA-20 rotor. The postmitochondrial supernatant was made 1% (w/v) in Triton X-100 and sodium deoxycholate respectively. Approx. 15 ml/tube was layered above 8 ml of 2.0 M-sucrose + 13 ml of 0.5 M-sucrose (in medium B + 50 μ g of heparin/ml), and centrifuged at 50000 rev./min (302000 g_{max}) for 5 h in the Ti-60 rotor. The sucrose layers were aspirated away, and the pellets washed briefly in medium B and resuspended in medium B or in water. The yield was more than 600 A_{260} units/25 g of liver.

Polyribosomes to be fractionated were redissolved in medium B to $A_{260} = 190$, and 95 A_{260} units (0.5 ml) were layered over a linear gradient made from 18 ml of 0.5 M-sucrose + 18 ml of 1.5 M-sucrose in 0.1 M-NaCl/5 mM-MgCl₂/20 mM-Tris/HCl (pH 7.5) + 50 μ g of heparin/ml, and centrifuged at 25000 rev./min (121000 g_{max}) in the SW-27 rotor for 5 h. Fractions (1.4 ml) were collected and immediately frozen in liquid N_2 .

Chromatography on oligo(dT)-cellulose

The method of Kabat (1975) was used at room temperature. Polyribosomes suspended in medium B were diluted with three to four parts of 0.5 M-NaCl/0.1 M-Tris/HCl (pH 7.4) + 0.5% sodium dodecyl sulphate (added to buffer shortly before use) to give 50 A_{260} units/ml, and were applied to 1 g of oligo(dT)-cellulose in a 1 cm-diameter column. The column was washed with 20 ml of the above buffer, then 20 ml of this buffer without sodium dodecyl sulphate, and eluted with either 0.01 M-Tris/HCl, pH 7.4, or water. The poly(A)⁺ mRNA was precipitated overnight at -20°C in 0.1 M-NaCl + 2 vol. of ethanol. The yield was 2-6% of total applied A_{260} units. Sucrose-gradient ultracentrifugation showed this mRNA to be contaminated with rRNA, as discussed below. Poly(A)⁺ mRNA free of rRNA was obtained by redissolving the mRNA in a small volume of water, heating to 70°C for 3 min, quickly cooling in ice/water, and repeating the oligo(dT)-cellulose chromatography.

Translation of mRNA in a wheat-germ cell-free system

Raw wheat germ, obtained from the Park Mill, Mannheim, Germany, was extracted as described by Marcu & Dudock (1974). The 30000 g_{max} supernatant (S-30) was preincubated in the presence of 1 mM-ATP, 200 μ M-GTP, 2 mM-dithioerythritol, 7.5 mM-phosphocreatine, 40 μ g of creatine kinase/ml, 3.5 mM-MgCl₂, 2 mM-CaCl₂, 100 mM-KCl and 20 mM-Hepes, pH 7.6, for 20 min at 30°C. The mixture was then chromatographed on Sephadex G-25 (coarse grade) in 20 mM-Hepes, pH 7.6, containing 5 mM-magnesium acetate, 120 mM-KCl and 1 mM-dithioerythritol, centrifuged at 16000 rev./min (30000 g_{max}) for 15 min in the JA-20 rotor and the supernatant frozen in liquid N_2 in 100 μ l portions in 0.5 ml Eppendorf centrifuge tubes. The entire preparation required 60 min. The yield was 2-3 ml of extract (110-190 A_{260} units) from 5 g of wheat germ.

The cell-free biosynthesis was carried out as described by Roberts & Paterson (1973) in a system consisting of 1 part of 'master mix', 1 part of wheat-germ supernatant S-30 (preincubated), MgCl₂, KCl, labelled amino acids (amounts given in the legends to the Figures), mRNA and water to a total of 5 parts by volume. The 'master mix' consisted of: 200 μ M of each amino acid (omitting labelled amino acids), 75 mM-Hepes, pH 7.6, 7.5 mM-ATP, 2.5 mM-GTP, 75 mM-phosphocreatine, 10 mM-dithioerythritol, 0.4 mM-cysteine (omitted from [³⁵S]-cysteine experiments), 0.25 mM-spermine and 0.5 mg of creatine kinase/ml. Incubation was carried out in a total volume of 25-100 μ l for 2 h at 25°C.

Carboxymethylation of translation products produced in vitro

This was adapted from the method of Schechter & Burstein (1976). The incubation mixture described above was made 4 M in the amino acid(s), which were labelled, and a 5 μ l portion was removed for trichloroacetic acid precipitation. Radioactivity was determined by liquid-scintillation counting (see Roberts & Paterson, 1973). The remainder of the incubation mix was made 0.5 M in Tris base and incubated for 30 min at 36°C to dissociate ribosome-bound nascent polypeptides. After neutralization with 0.5 M-acetic acid, the mixture was made 1 M in Tris/HCl, pH 8.2, 6 M in guanidine hydrochloride and 14 mM in dithioerythritol and reduced for 1 h at 36°C. Iodoacetic acid (1 M, neutralized with NaOH) was added to a final concentration of 0.12 M and carboxymethylation was allowed to proceed for 30 min at room temperature in the dark. The reaction was stopped by adding dithioerythritol to a final concentration of 63 mM, and the preparation was dialysed overnight against 5 mM-Tris/HCl, pH 7.5, at 4°C. The material remaining in the dialysis

bag was stored at -20°C . Samples for sodium dodecyl sulphate/20% polyacrylamide-gel electrophoresis were evaporated to dryness and redissolved in 25–50 μl of sample buffer (see under 'Electrophoresis').

Sucrose-gradient ultracentrifugation of RNA

RNA (250 μg), isolated by oligo(dT)-cellulose chromatography of sodium dodecyl sulphate-dissociated polyribosomes, was dissolved in 75 μl of water, heated to 70°C for 3 min, quickly cooled to 0°C , applied to a 13 ml linear gradient of 5–30% sucrose in 0.1 M-LiCl/1 mM-EDTA/10 mM-Tris/HCl (pH 7.5) and centrifuged for 14 h at 39 000 rev./min (277 000 g_{max}) in the SW-40 rotor; 30 fractions (0.4 ml each) were collected. After addition of 25 μg of yeast tRNA per tube, the RNA was precipitated with 1 ml of ethanol at -20°C , washed in 70, 90 and 100% (v/v) ethanol, and the pellets were redissolved in 25 μl of water and stored in liquid N_2 . Sedimentation coefficients were calculated relative to yeast tRNA (4 S) and rat liver rRNA (18 S), which were found in fractions 8 and 22 respectively.

Electrophoresis

Sodium dodecyl sulphate (0.1%)/polyacrylamide-gel electrophoresis was carried out as described by Laemmli (1970). Cylindrical gels were 5 mm (diam.) $\times$ 100 mm long ('running' gel); the 'stacking' gel was 10 mm long. Slab gels were 1.4 mm thick by 150 mm long and had slots 5 mm (18 samples) or 10 mm (9 samples) wide and spaced 3 mm apart. Samples were dissolved in a buffer containing 0.33% sodium dodecyl sulphate, 17 mM-Tris/HCl, pH 6.8, and 0.0003% Bromophenol Blue. Native carboxymethyl-metallothioneins migrated faster than Bromophenol Blue in running gels containing less than 15% (w/v) acrylamide. A gel composition of 20% acrylamide and 0.54% bisacrylamide was finally selected. The gels were run for 5–6 h (cylindrical) or 8–9 h (slab) at 10 V/cm after an initial 30 min at 3.3 V/cm. Cylindrical gels were cut into 1 mm slices with a gel slicer. Slices were incubated in 1 ml of Protosol (New England Nuclear)/toluene/water (10:9:1, by vol.) in scintillation vials for 18 h at 37°C and radioactivity was measured after adding 10 ml of toluene scintillant (see under 'Radioisotope use and radioactivity counting'). Slab gels containing ^3H -labelled compounds were soaked for several hours in dimethyl sulfoxide, then overnight in dimethyl sulfoxide/2,5-diphenyloxazole (4:1, w/w) and finally 4–8 h in several changes of water. Gels were dried in a press under vacuum and fluorograms prepared by exposure to presensitized DuPont Cronex medical X-ray film at -70°C for 1–5 weeks (Laskey & Mills, 1975). Densitometry was performed with an Optronics Photoscan model P-1000 digitalized densitometer by

using a photometer window with a 200 μm raster and averaging the accumulated film density in a strip 2 mm wide through the peak centre (unless otherwise stated).

Results

Isolation and translation *in vitro* of rat liver poly(A)⁺ mRNA

Poly(A)⁺ RNA was isolated from polyribosomes by dissociating these in 0.5% sodium dodecyl sulphate and chromatographing through oligo(dT)-cellulose (Kabat, 1975). Yields of 6.6, 4.7 and 3.7% of applied A_{260} units were obtained from control, Cd^{2+} - and Zn^{2+} -treated animals respectively. Sucrose-gradient ultracentrifugation showed these RNA species to contain considerable 18 S and 28 S rRNA in addition to 9 S and 11 S mRNA peaks (Fig. 1; similar profiles were obtained with the poly(A)⁺ RNA prepared either from a phenol extraction of the postmitochondrial supernatant or from total rat liver nucleic acids). These mRNA preparations gave, in the wheat-germ system, a maximum 30-fold stimulation of [^3H]leucine incorporation and a maximum 18-fold stimulation of [^3H]serine incorporation into trichloroacetic acid-insoluble products, with 8–10 μg of RNA/50 μl of incubation mixture.

A double-label experiment with [^3H]leucine + [^{35}S]cysteine was carried out to identify the products of biosynthesis *in vitro* from a wheat-germ system directed by control mRNA and mRNA species

Fig. 1. Sucrose-gradient ultracentrifugation of poly(A)⁺ RNA from Cd^{2+} -treated rats

Poly(A)⁺ RNA (250 μg in 75 μl) isolated from rats 5 h after CdCl_2 treatment (10 $\mu\text{mol/kg}$ body wt.) was fractionated by sucrose-gradient ultracentrifugation as described in the Materials and Methods section. Sedimentation values were calculated relative to yeast tRNA (4 S) and rat liver rRNA (18 S). The 28 S rRNA sedimented to the bottom of the tube in this experiment. An analysis of the translation products directed by mRNA species from tubes 7–19 is shown in Fig. 6.

Fig. 2. Electrophoresis of [^3H]leucine + [^{35}S]cysteine double-labelled translation products *in vitro* directed by rat liver poly(A) $^+$ mRNA species from CdCl_2 - and ZnCl_2 -treated and control animals

Each sample was incubated for 2 h in a final volume of 100 μl containing 3.5 mM- Mg^{2+} , 65 mM- K^+ , 3.8 A_{260} units of wheat-germ S-30, 8.35 μCi of [^{35}S]cysteine (reduced), 10 μCi of [^3H]leucine, 28.4 μg of poly(A) $^+$ mRNA from CdCl_2 -treated animals (a + d), 16.3 μg of poly(A) $^+$ mRNA from ZnCl_2 -treated animals (b + e), and 36.1 μg of poly(A) $^+$ mRNA from controls (c + f). Samples were carboxymethylated and electrophoresed as described in the Materials and Methods section. [^3H]Leucine incorporation is shown in (a), (b) and (c), and the corresponding [^{35}S]cysteine profiles are shown in (d), (e) and (f). Further details are given in the text. Protein molecular-weight standards used were: a, bovine serum albumin (mol.wt. 65400); b, ovalbumin (46000); c, chymotrypsinogen (25700); d, ribonuclease (13700); e, cytochrome c (12400); f and g, carboxymethyl-metlothioneins 1 (7100, 19 thiol groups) and 2 (7300, 21 thiol groups) respectively.

isolated by sodium dodecyl sulphate-dissociated polyribosomes from Cd^{2+} - and Zn^{2+} -treated animals. Alkylation of these products with iodoacetic acid in 6M-guanidine hydrochloride was carried out as described by Schechter & Burstein (1976) (see the Materials and Methods section), and the carboxymethylated labelled proteins were separated by electrophoresis in 20% polyacrylamide gels, 0.1% in sodium dodecyl sulphate (Laemmli, 1970). This high acrylamide concentration was chosen to facilitate the identification of carboxymethyl-metlothioneins 1 and 2, which, as the native proteins, have mobilities relative to Bromophenol Blue in this gel system of

0.72 and 0.74 respectively. Products ranging in molecular weight from several thousand up to 65000–70000 were found in gel slices from polyacrylamide-gel electrophoresis when monitoring the radioactivity from [^3H]leucine (Figs. 2a, 2b and 2c) and from [^{35}S]cysteine (Figs. 2d, 2e and 2f). A product that migrated in sodium dodecyl sulphate/20% polyacrylamide gels identically with the metlothioneins was found (Figs. 2d, 2e and 2f; centred on slices 53–55; Bromophenol Blue at slice 74) that contained [^{35}S]cysteine at high radioactivity relative to that in other proteins incorporating ^{35}S . This product did not incorporate [^3H]leucine (Figs. 2a,

2b and 2c; arrows labelled f and g indicate metallothioneins 1 and 2 respectively). The amino acid compositions of rat liver metallothioneins 1 and 2 (Andersen *et al.*, 1978) show cysteine to comprise one-third of the amino acids in these proteins, and show leucine to be entirely absent. On this basis we identify as metallothioneins the [^{35}S]cysteine-labelled products of biosynthesis *in vitro* having mobilities in the range 0.72–0.74. Attempts to isolate native metallothioneins from a wheat-germ system by addition of CdCl_2 or ZnCl_2 to the incubation mixture followed by gel filtration of a 40000 g_{max} . (30min) supernatant failed. The formation of a precipitate between the metal ion and dithioerythritol prevented recovery of the translation products in a soluble form.

Total [^{35}S]cysteine radioactivity incorporated into metallothioneins was compared between control (Fig. 2f; 3312 c.p.m.), Zn^{2+} -dosed (Fig. 2e; 5746 c.p.m.) and Cd^{2+} -dosed (Fig. 2d; 12252 c.p.m.) cases. ^{35}S c.p.m. for Cd^{2+} and Zn^{2+} dosage were normalized to the controls from the data in Fig. 2 by the formula:

$$[^{35}\text{S}] = ([^{35}\text{S}]_{\text{Cd,Zn}} \times [^3\text{H}]_{\text{control}} / [^3\text{H}]_{\text{Cd,Zn}})$$

A 1.7-fold stimulation of metallothionein biosynthesis with mRNA from Zn^{2+} -treated animals, and a 3.7-fold stimulation with mRNA from Cd^{2+} -treated animals, were found in comparison with controls. Poly(A) $^+$ RNA was also isolated from the livers of control and Cd^{2+} -treated animals by extraction of total nucleic acids with phenol/chloroform/2-methylbutan-2-ol followed by poly(U)-Sephadex affinity chromatography. Incorporation of [^3H]serine into the products of cell-free biosynthesis directed by this mRNA showed a 3.0-fold stimulation of metallothionein biosynthesis directed with the mRNA from Cd^{2+} -treated animals as compared with controls (results not shown).

The wheat-germ system operated optimally with rat liver mRNA at a pH of 7.2 (pH of the stock solution of Hepes buffer was 7.6). Spermine was added to a concentration of 50 μM to ensure the completion of translation products (Tse & Taylor, 1977). The Mg^{2+} - and K^+ -dependence of the yield of trichloroacetic acid-precipitable translation products and of the metallothioneins was determined with [^3H]serine. Values of 4–6 mM- Mg^{2+} and 65–85 mM- K^+ were found to give optimum stimulation of total protein biosynthesis and slightly narrower limits of 4–5 mM- Mg^{2+} and 75–85 mM- K^+ were found for the metallothioneins. At a Mg^{2+} concentration of 2 mM or lower, biosynthesis in the wheat-germ system was inhibited. Incorporation of labelled amino acids proceeded linearly with time in this system for the first 45–60 min and had stopped completely by 2 h (results not shown). The poly(A) $^+$ RNA concentration giving optimum results was over 100 $\mu\text{g}/\text{ml}$. This

rather high value reflects the large amount of rRNA found in these preparations. After sucrose-gradient ultracentrifugation to eliminate the rRNA species,

Fig. 3. Time-dependence of metallothionein biosynthesis and mRNA formation in rat liver after a single injection of CdCl_2

Rats were injected subcutaneously with 10 μmol of CdCl_2/kg body wt. in experiments both *in vivo* and *in vitro*. In the study *in vivo* (taken from Andersen *et al.*, 1978) an injection of [^3H]cysteine was given intraperitoneally 1 h before determining total radioactivity incorporated into metallothioneins (expressed as c.p.m./ μCi of injected isotope in a single animal). The metallothioneins were isolated in this experiment by Sephadex G-75 chromatography and the label was shown to be incorporated into these proteins by polyacrylamide-gel disc electrophoresis. In the study *in vitro*, poly(A) $^+$ mRNA was isolated from polyribosomes (as described in the Materials and Methods section) at the times indicated (two animals per isolation). Translation of each messenger was done in the wheat-germ system for 2 h in a final volume of 50 μl containing 4 mM- Mg^{2+} , 75 mM- K^+ , 1.14 A_{260} units of wheat-germ S-30 and 10 μCi of [^3H]serine. Poly(A) $^+$ mRNA addition to each incubation was: control, 9 μg ; 1 h, 2 μg ; 2 h, 5 μg ; 5 h, 5 μg ; 17 h, 2 μg . Samples were carboxymethylated and electrophoresed, and a fluorogram was prepared as described in the Materials and Methods section; 75000 c.p.m. of ^3H from each of the final dialysis residues was applied to each gel slot. The radioactivity incorporated into metallothioneins was determined by densitometry of the fluorogram (see under 'Electrophoresis'). ●, Biosynthesis *in vivo*; ■, biosynthesis *in vitro* of metallothioneins.

comparable stimulation was found at a 5–10-fold lower total RNA concentration.

Kinetics of metallothionein biosynthesis after CdCl₂ treatment

A single subcutaneous injection of CdCl₂ causes a transient stimulation of metallothionein biosynthesis in rat liver (Chen *et al.*, 1975; Andersen *et al.*, 1978). Total polyribosomal poly(A)⁺ mRNA isolated from animals treated 5 h previously with CdCl₂ (10 µmol/kg body wt.; injected subcutaneously) directed the biosynthesis *in vitro* of metallothioneins in 3.7-fold excess over controls (Fig. 2). The time-dependence of this effect is shown in Fig. 3, in which the incorporation of [³H]serine into metallothioneins was determined. For comparison, a study *in vivo* of the time-dependence of [³H]cysteine incorporation into rat liver metallothioneins, with a 1 h labelling time, is also shown [from Andersen *et al.* (1978)]. The maximum stimulation *in vitro* was 2.8-fold, in comparison with a stimulation *in vivo* of 15-fold, at 5 h. Both the stimulation *in vitro* and that *in vivo* of metallothionein biosynthesis returned to control values by 17 h.

Translation of poly(A)⁺ mRNA from polyribosomal fractions

Rat liver polyribosomes from Cd²⁺-treated animals were fractionated by sucrose-gradient ultracentrifugation (Fig. 4). Clearly defined, slightly overlapping, peaks were obtained for two, three, four and five ribosomes/polyribosome. Material from each peak was pooled and the poly(A)⁺ mRNA was purified and translated *in vitro*. The translation products from each fraction were analysed by electrophoresis in sodium dodecyl sulphate/20% polyacrylamide slab gels followed by fluorography (Fig. 5: (a) [³H]leucine incorporation; (b) [³H]serine incorporation; the absence of incorporation in slot 1 demonstrates the low endogenous activity of the wheat-germ system). Total poly(A)⁺ mRNA from control, Cd²⁺- and Zn²⁺-treated animals was also translated in the wheat-germ system, and these translation products can be seen in slots 2–4 respectively in Fig. 5. The metallothioneins are identified in Fig. 5(b) as the fastest moving spot in the fluorogram (band d) and by the conspicuous absence of radioactivity from the corresponding position in Fig. 5(a) (where labelling was with

Fig. 4. Sucrose-gradient ultracentrifugation of polyribosomes from Cd²⁺-treated rats and identification of metallothionein polyribosomes

Polyribosomes were fractionated by sucrose-gradient ultracentrifugation as described in the Materials and Methods section. Fractions containing *n* = 2, 3, 4 and 5 ribosomes/polyribosome were pooled from tubes 11–12, 13–14, 15–17 and 17 respectively, and poly(A)⁺ mRNA species isolated by oligo(dT)–cellulose chromatography in 0.5% sodium dodecyl sulphate as described in the Materials and Methods section. Recovery of poly(A)⁺ mRNA in this step was 0.5–1.0 A₂₆₀ unit/fraction. After ethanol precipitation the mRNA was redissolved in 25 µl of water, and 5 µl portions were translated in the wheat-germ system (see Fig. 5). Translation conditions were identical with those in Fig. 5. [³H]Serine radioactivity incorporated into metallothioneins was determined by sodium dodecyl sulphate/20% polyacrylamide-gel electrophoresis of the carboxymethylated translation products followed by fluorography and densitometry. Total radioactivity in bands c (upper bar) and d (lower bar) from Fig. 5(b) is shown superimposed on the polyribosome profile over the respective pooled fractions. Digital densitometry in this experiment was done with a 50 µm raster and scanned over a 5.5 mm strip through each peak.

Fig. 5. Electrophoresis of [^3H]leucine- and [^3H]serine-labelled translation products produced *in vitro* by poly(A) $^+$ mRNA species from rat liver polyribosomes and $n = 2$ –5 polyribosome fractions

Each sample was incubated for 2 h in a final volume of 50 μl containing 3.5 mM- Mg^{2+} , 65 mM- K^+ , 1.4 A_{260} units of wheat-germ S-30 and 10 μCi of [^3H]leucine (a) or [^3H]serine (b). Poly(A) $^+$ mRNA additions to each incubation were: slot 1, none; slot 2, 22.6 μg from controls; slot 3, 7.1 μg from CdCl_2 -treated animals; slot 4, 8.2 μg from ZnCl_2 -treated animals; slots 5, 6, 7 and 8 ($n = 2, 3, 4$ and 5 ribosomes/polyribosome respectively), 8–16 μg from CdCl_2 -treated animals. Samples were carboxymethylated and electrophoresed as described in the Materials and Methods section. [^3H]Leucine applied to each slot was: slot 1, 100 000 d.p.m.; slots 2–4, 400 000 d.p.m.; slot 5, 132 000 d.p.m.; slot 6, 220 000 d.p.m.; slot 7, 400 000 d.p.m.; slot 8, 393 000 d.p.m. [^3H]Serine applied to each slot was: slot 1, 100 000 d.p.m.; slots 2–4, 200 000 d.p.m.; slot 5, 75 900 d.p.m.; slot 6, 122 000 d.p.m.; slot 7, 200 000 d.p.m.; slot 8, 149 000 d.p.m. The stimulation of incorporation was a maximum of 30-fold over endogenous (slot 1) with [^3H]leucine and a maximum of 12-fold with [^3H]serine. Further details are given in the text and in Fig. 4.

[^3H]leucine, absent from metallothioneins). An increase in metallothionein mRNA concentrations after Cd^{2+} or Zn^{2+} treatment is seen in the [^3H]serine fluorogram (Fig. 5b, slots 3 and 4). Integration of the peak areas gave control/ Zn/Cd proportions of 1:1.6:3.1. Two other pronounced changes are evident from Fig. 5. A band having a mobility of 0.26 (apparent mol.wt. 22 000) has appeared after metal treatment, which was absent from or very weak in controls and which shows up in both the [^3H]leucine and [^3H]serine fluorograms (Fig. 5; slots 3 and 4; band b). The slowest migrating band of radioactivity, at the top of the gel, appears decreased in intensity after metal treatment (Fig. 5; slots 2–4; band a). This band corresponds to a polypeptide of mol.wt. 65 000–70 000. Bovine serum albumin migrates just ahead of this band in the 20% polyacrylamide-gel system (Fig. 2a).

The translation products directed by poly(A) $^+$ mRNA from $n = 2$ –5 polyribosomes are found in slots 5–8 respectively in Fig. 5. The [^3H]serine fluorogram reveals that the metallothionein mRNA is associated mainly with three or four ribosomes. The integrated intensity of each of these bands (Fig. 5b; slots 5–8; band d) is shown in the upper bar graph in Fig. 4. The presence of considerable

[^3H]leucine radioactivity in proteins of apparent mol.wt. 12 000–14 000 (unlabelled arrows in Figs. 5a and 5b) in slots 6–8 ($n = 3$ –5 polyribosomes) suggests that the poly(A) $^+$ mRNA from these fractions contains both metallothionein and other mRNA species. The persistence of the 12 000–14 000-dalton products in $n = 5$ polyribosomes suggests that these proteins are synthesized on slightly larger messengers than are the metallothioneins. A peak migrating with a mobility of 0.66 and with a peak intensity paralleling the metallothionein radioactivity is seen in Fig. 5(b) (band c). The integrated intensity of band c for each polyribosome fraction is shown in the lower bar graph in Fig. 4. This product of biosynthesis *in vitro* may be the result of incomplete reduction of metallothioneins by dithioerythritol and subsequent dimerization before carboxymethylation, or it may be a precursor to these proteins (see the Discussion section). A 13-fold purification of metallothionein mRNA was achieved by isolation of poly(A) $^+$ mRNA from rat liver tetra-ribosomes.

Sucrose-gradient ultracentrifugation of poly(A) $^+$ RNA from polyribosomes

Polyribosomes were prepared from animals

Fig. 6. Analysis of sucrose-gradient ultracentrifugation of poly(A)⁺ mRNA from rat liver polyribosomes isolated from CdCl₂-treated rats by electrophoresis of the translation products produced *in vitro*

Each sample was incubated for 2 h in a final volume of 25 μ l containing 3.8 mM-Mg²⁺, 75 mM-K⁺, 0.57 A₂₆₀ unit of wheat-germ S-30, 5 μ Ci of [³H]-serine and 5 μ l of poly(A)⁺ mRNA from fractions 19, 18, ... 7 (slots 2–14 respectively) from the sucrose-gradient fractionation of rat liver poly(A)⁺ mRNA from CdCl₂-treated rats (see Fig. 1). Samples were carboxymethylated and equal volumes from each final dialysis residue (material remaining in dialysis bag) electrophoresed as described in the Materials and Methods section. Further details are given in the text and in Fig. 1.

treated with CdCl₂ (10 μ mol/kg body wt., injected subcutaneously; 5 h exposure). Poly(A)⁺ RNA was isolated and subjected to sucrose-density-gradient ultracentrifugation as described in the Materials and Methods section. The A₂₅₄ profile of this run is shown in Fig. 1. RNA fractions were collected and translated in the wheat-germ system with [³H]-serine. A fluorogram prepared from electrophoresis of the carboxymethylated translation products in a sodium dodecyl sulphate/20% polyacrylamide slab gel is shown in Fig. 6. Slot 1 in this fluorogram shows the endogenous activity in the wheat-germ system. Slots 2–14 show the translation products from fractions 19, 18, ... 7 respectively from the sucrose-gradient run. Metallothioneins were translated with peak activity in fraction 13 (slot 8), corresponding to a sedimentation coefficient of 9S in comparison with yeast tRNA and rat liver rRNA.

The distribution of translation products *in vitro* seen in Fig. 6 indicates that the metallothionein mRNA is one of the smallest messengers in rat liver.

Discussion

The identification of metallothioneins among the products of biosynthesis *in vitro*, directed by rat liver poly(A)⁺ mRNA, has been made on the basis of the electrophoretic mobility of their carboxymethylated derivatives in a sodium dodecyl sulphate/20% polyacrylamide-gel system. The very high mobility of carboxymethyl-metallothioneins in this gel system is probably due to the large number of carboxymethyl-cysteine residues in these proteins in comparison with other rat liver proteins. The presence of [³⁵S]cysteine and [³H]serine in, and the absence of [³H]leucine from, this region of the gel is further evidence for the correct identification of this peak, on the basis of the amino acid composition of the metallothioneins. We did not characterize the metallothioneins synthesized *in vitro* by peptide or sequence analysis. It is therefore not possible to say if these products contain a short leader sequence (i.e., premetallothionein), which, because of the anomalous rate of migration of these proteins after carboxymethylation, may co-migrate with native metallothioneins. That this may not be the case is suggested by the presence of *N*-acetylmethionine in all metallothioneins sequenced to date [and in rat liver metallothioneins (Andersen *et al.*, 1978)]. This methionine is believed to be the initiator in the biosynthesis of these proteins (J. Kägi, personal communication).

The amount of metallothionein mRNA in total rat liver poly(A)⁺ mRNA during Cd²⁺ induction can be determined from the recovery of protein-biosynthetic products (Fig. 2). Assuming leucine to be 10% of the amino acids in rat liver proteins (many of which carry leucine-rich prepeptides) and correcting for the relative specific radioactivities of [³H]leucine and [³⁵S]cysteine in the incubation *in vitro*, we find that the metallothioneins constitute about 0.8% of the total protein products synthesized.

At least one other mRNA species appears to increase in concentration in rat liver after Cd²⁺ or Zn²⁺ treatment (Figs. 5a and 5b; band b). This translation product, having an apparent mol.wt. of 22000 (based on its gel mobility), contains both leucine and serine. The time-dependence of appearance of this product is different from the metallothioneins, persisting for at least 17 h after Cd²⁺ treatment (results not shown). It is noteworthy that another mRNA species, coding for a major peak in the 65000–70000-dalton size range, is decreased in concentration after Cd²⁺ or Zn²⁺ treatment (Fig. 5; band a). The size and amount of this protein suggest that it may be preproalbumin, whose mRNA con-

stitutes 11% of total rat liver mRNA (Tse & Taylor, 1977).

Metallothionein mRNA has a sedimentation coefficient of 9S and carries three or four ribosomes in the polyribosome. The mRNA species coding for the next larger proteins in rat liver carry four, five or more ribosomes (Fig. 5, unlabelled arrows) and sediment at 9S or greater (Fig. 6). Metallothionein mRNA is thus one of the smallest messengers found in rat liver.

Other mRNA species that sediment at 9S include rabbit globin (Chantrenne *et al.*, 1967; Labrie, 1969; Gaskill & Kabat, 1971), human β -globin (Marotta *et al.*, 1976; Chang *et al.*, 1977) and uteroglobin (Atger & Milgrom, 1977). These mRNA species contain approx. 600–650 nucleotides. Metallothioneins whose amino acid sequences have been determined are 61 amino acid residues long (Kojima *et al.*, 1976; Kissling & Kägi, 1977; Huang & Yoshida, 1977). Since this is a substantially smaller number of amino acids than in the globins (141 residues in the α -chain, 146 residues in the β -chain) or in pre-uteroglobin (80–90 residues), it predicts that metallothionein mRNA would have correspondingly larger untranslated regions, about 400 nucleotides. This may in fact be the case. Thus the α - and β -globin mRNA species, with lengths of 650–670 nucleotides, have untranslated sequences containing 150–175 nucleotides. Immunoglobulin κ -chain mRNA species are estimated to have untranslated sequences [excluding poly(A)] totalling about 225 out of a total of 1050 nucleotides (Kuehl, 1977). Only about half of the approx. 600 nucleotides in uteroglobin mRNA are necessary to code for pre-uteroglobin. Alternatively, however, metallothionein mRNA is large enough to accommodate two completely independent cistrons of approx. 180 nucleotides each. The existence of metallothionein tetra-ribosomes (Fig. 4 and Fig. 5b) suggests that at least 300 nucleotides are covered by bound ribosomes [assuming a ribosome size of 25 nm (250 Å) and a nucleotide separation of 0.34 nm (3.4 Å) (Lake, 1976)]. There is no known precedent for a eukaryotic mRNA with two initiation sites. It is not known whether the presence of two sites would be required for synthesis of the two forms of metallothionein found in the liver. Whether the mRNA is monocistronic or polycistronic, the translation product having an electrophoretic mobility of 0.66 (Fig. 5b, band c; shoulder in metallothionein peak in Figs. 2d and e) could result from improper termination with read-through into the untranslated region (or into the second cistron). The absence of proteinases or processing enzymes from the wheat-germ system suggests that this minor product is not a premetallothionein that is processed into metallothionein (Fig. 5b; band d).

Quantification of mRNA activity in a cell-free system is complicated by the non-linear response

frequently encountered with increasing concentrations of nucleic acid, particularly at high concentrations (Rhoads *et al.*, 1973). This results from inhibition of initiation (Mathews & Korner, 1970; Lodish *et al.*, 1971). No correction was made for this effect in the present studies and metallothionein biosynthesis has been expressed relative to total protein biosynthesis (determined by [3 H]-leucine incorporation) rather than per μ g of nucleic acid assayed. Accurate quantification of metallothionein mRNA concentrations in the cytoplasm will require the use of a complementary DNA probe in a hybridization assay.

We cannot explain the nature of the increase in metallothionein mRNA concentration during induction of metallothionein biosynthesis by the experiments reported here. An increased rate of transcription (elevated gene activity), release of mRNA from a precursor RNA molecule, stabilization of the mRNA (immediately after biosynthesis or during subsequent processing) or stabilization of the polyribosome are all possible explanations of this effect. Both transcriptional and post-transcriptional mechanisms have precedence in the literature. Thus Orkin & Swerdlow (1977) have reported that increased synthesis of globin RNA follows treatment of murine erythroleukaemic cells with dimethyl sulphoxide, indicating transcriptional activation of the globin genes on induction of differentiation. On the other hand, Melli *et al.* (1977) have shown that the synthesis of histone mRNA does not appear to be coupled to DNA duplication, indicating that regulation of cytoplasmic histone mRNA concentrations is not at the transcriptional level.

The measurement of metallothionein mRNA concentration in the cell may provide a more detailed understanding of the mechanism of transient appearance of foetal (Cu + Zn)-thioneins in the liver (Rydén & Deutsch, 1978; Hartman & Weser, 1977; Bremner & Young, 1976; Porter, 1974). The biosynthesis of this form of metallothionein may be under developmental control, sharing features in common with the mechanism underlying the induction of metallothionein biosynthesis in adult animals after Cd $^{2+}$ or Zn $^{2+}$ treatment. Copperthionein has also been implicated in Wilson's disease (hepatolenticular degeneration; Evans *et al.*, 1973), an inborn error of metabolism. A comparison of metallothionein mRNA concentration and biosynthesis in normal and diseased human liver tissue may shed light on the possible role that the metallothioneins play in this hereditary disease.

Studies on metallothionein biosynthesis and degradation and on the nature of metal binding to these proteins has implicated them in cellular detoxification of heavy metals [for reviews of this subject, see Nordberg (1972) and Webb (1975)], in the maintenance of Zn $^{2+}$ (Richards & Cousins,

1977) and Cu^{2+} (Rydén & Deutsch, 1978), and in participation in enzymic reactions (Prinz & Weser, 1975). A better understanding of the function of these proteins in the body will come, as well, by studying the way in which cells regulate the biosynthesis of metallothioneins, both in response to the presence of specific heavy-metal ions and during cell development.

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