Induction of metallothionein mRNA in rat liver and kidney after copper chloride injection

Samantha A. WAKE and Julian F. B. MERCER*

Birth Defects Research Institute, Royal Children's Hospital, Flemington Road, Parkville, Vic. 3084, Australia

(Received 21 November 1984/29 January 1985; accepted 12 February 1985)

The kinetics of the increase of metallothionein mRNA in rat liver and kidney after CuCl_2 injection was determined by cell-free translation and dot-blot hybridization of total RNA isolated at various times after the injection. Both assay procedures gave essentially the same result: a 16-fold increase in hepatic metallothionein mRNA was observed 7 h after CuCl_2 injection, with a decline to basal values by 15 h. The response in the kidney was less dramatic, with a 6-fold increase in metallothionein mRNA 5 h after injection, and basal values were attained by 12 h. The rise in Cu^{2+} concentration in both organs was closely correlated with the increase in metallothionein mRNA; hepatic Cu^{2+} was increased 5.9-fold by 5 h after injection and renal Cu^{2+} was increased 4.3-fold 5 h after injection. The Zn^{2+} concentration in the liver had not risen significantly within 5 h of Cu^{2+} injection. Renal Zn^{2+} concentrations did not alter appreciably in the Cu^{2+} -treated animals. These results support the conclusion that Cu^{2+} is acting as a primary inducer of metallothionein mRNA in the rat.

The synthesis of the low- M_r cysteine-rich metal-binding proteins, metallothioneins, has been shown to be inducible by administration of heavy metals such as cadmium and zinc (Kagi & Nordberg, 1979). This induction is mediated by an increase in metallothionein mRNA (Andersen & Weser, 1978; Shapiro *et al.*, 1978) resulting from an increase in transcriptional rates of metallothionein genes (Durnam & Palmiter, 1981). The ability of copper salts, however, to act as inducing agents of metallothionein biosynthesis has been the subject of much controversy. Studies at the protein level have often been conflicting, owing to the intractable nature of copper-thionein. It has been suggested that injection of copper salts into rats induced an hepatic protein termed copper chelatin (Winge *et al.*, 1975) rather than metallothionein, although this was refuted by Bremner & Young (1976) and has since been retracted by Winge *et al.* (1981). Studies using a cell-free translation system (Mercer *et al.*, 1981) have shown that CuCl_2 injection into rats resulted in an increase in hepatic translatable metallothionein mRNA. Durnam & Palmiter (1981), using a mouse metallothionein-1 cDNA probe, have also shown that a CuSO_4

injection gave good induction of metallothionein-1 mRNA in mouse liver.

Although these results suggest that Cu^{2+} is a primary inducer of metallothionein biosynthesis, the evidence is not unequivocal. For example, in rat liver, Bremner & Davies (1976) showed that Zn^{2+} concentrations rose after Cu^{2+} injection, and this raised the possibility that the metallothionein-mRNA induction could have been a result of this secondary influx of Zn^{2+} . Other discordant results are the very poor induction of renal metallothionein-1 mRNA by CuSO_4 injection into mice (Durnam & Palmiter, 1981), and the lack of significant induction of metallothionein mRNA by Cu^{2+} in cultured human fibroblasts in comparison with that with Zn^{2+} and Cd^{2+} (Richards *et al.*, 1984). Other studies have also shown that in rats given a copper-supplemented diet, only negligible amounts of Cu^{2+} were present on metallothionein, whereas substantial amounts of copper-metalllothionein were found in the livers of rats injected with copper salts (Mehra & Bremner, 1984). These results suggest that the route of administration of Cu^{2+} is important in determining its ability to induce metallothionein synthesis. In the present paper we report the kinetics of induction of metallothionein mRNA in rat liver and kidney after CuCl_2 injection and show that amounts of metallothionein mRNA are clearly correlated with

Abbreviations used: SDS, sodium dodecyl sulphate; SSC, 0.15M-NaCl/0.015M-sodium citrate.

* To whom reprint requests should be addressed.

an influx of Cu^{2+} , demonstrating that Cu^{2+} can indeed act as a primary inducer of metallothionein mRNA in both organs when Cu^{2+} is administered by injection. This induction does not involve adrenal hormones, an acute-phase response or metallothionein-gene amplification.

Material and methods

Chemicals

Guanidine hydrochloride (ultra-pure; Bethesda Research Laboratories) was used for all RNA isolations. L-[4,5- ^3H]Leucine (131 Ci/mmol), L-[^{35}S]cysteine (1100 Ci/mmol) and deoxycytidine 5'-[α - ^{32}P]triphosphate (approx. 3000 Ci/mmol) were supplied by Amersham International, Amersham, Bucks., U.K. Nitrocellulose (0.4 μm pore size) was obtained from Schleicher and Schuell, Dassel, West Germany. All other chemicals were of analytical grade.

Animals

Adult male Sprague-Dawley rats (200 g) were used in all experiments. These rats were maintained on a dry cube diet (Clarke King and Co.) containing 10 μg of Cu^{2+} /g dry wt. and 90 μg of Zn^{2+} /g dry wt. Water was provided *ad libitum*. All rats were starved overnight before total-RNA isolation.

Trace-metal analysis

Samples of tissue (approx. 0.1 g) were removed with scissors and forceps that had been soaked in 2% EDTA, then rinsed with distilled water to remove traces of Cu^{2+} and Zn^{2+} . Tissue samples were stored at -20°C . Cu^{2+} and Zn^{2+} contents were analysed by flame atomic-absorption spectrometry, with a Perkin-Elmer model 5000 atomic-absorption spectrophotometer.

Metal injections

Rats were injected intraperitoneally with 1.5 mg of Cu^{2+} (as CuCl_2)/kg body wt in 0.9% NaCl. Control rats were either untreated or injected with an equivalent volume of 0.9% NaCl. The numbers of animals used in each experiment are indicated in the Figure legends.

Total-RNA isolation

Total RNA was prepared by a modification of the method used by Liu *et al.* (1979). Briefly, tissue was homogenized (Sorvall Omni-Mixer) at 4°C in 7.5 M-guanidine hydrochloride/50 mM-sodium citrate/0.1% Sarkosyl/100 mM-2-mercaptoethanol, pH 7.0 (10 ml/g of tissue). After centrifugation for 10 min at 4°C and 10000 g ($r_{\text{av.}} = 10.9\text{ cm}$), the supernatant (6 ml) was layered over a 5.7 M-CsCl

cushion (1 ml). Tubes were centrifuged for 18 h at 4°C at 185000 g ($r_{\text{av.}} = 11.3\text{ cm}$). The resulting pellet was redissolved in the same guanidine hydrochloride solution as above, then precipitated by the addition of acetic acid to 50 mM and 0.5 vol. of 100% ethanol (2 h, -20°C). The resulting pellet was then redissolved in water and precipitated overnight at -20°C by addition of sodium acetate, pH 5.0, to 100 mM and 2 vol. of 100% ethanol. The total RNA was pelleted by centrifugation (10000 g for 10 min at 4°C ; $r_{\text{av.}} = 10.9\text{ cm}$), washed twice with 70% ethanol, dried under vacuum, then dissolved in water and stored at -70°C . The yield was determined by assuming that 1 A_{260} unit was equivalent to 40 μg of total RNA.

Cell-free protein synthesis

Wheat germ was obtained from Adelaide Mills (Adelaide, S.A., Australia) and an S-30 fraction was prepared by the method of Marcu & Dudock (1974), except that the buffer used throughout was 20 mM-Hepes/3 mM-magnesium acetate/50 mM-potassium acetate/1 mM-dithiothreitol, pH 7.6. The S-30 fraction was stored in batches in liquid N_2 .

For the cell-free translations, each 50 μl reaction mixture contained ATP (1.0 mM), GTP (20 μM), phosphocreatine (8 mM), creatine kinase (40 $\mu\text{g}/\text{ml}$), potassium acetate (110 mM), magnesium acetate (2 mM), dithiothreitol (2.2 mM), 19 unlabelled amino acids (each 25 μM), spermine (4 μM), Hepes, pH 7.6 (24 mM), and 3 μCi of [^3H]leucine or 7.5 μCi of [^{35}S]cysteine. Total RNA concentration ranged from 100 to 160 $\mu\text{g}/\text{ml}$. Each assay contained 10 μl of wheat-germ S-30. Incubation was for 90 min at 26°C . Incorporation of radioactivity into polypeptides was measured as described by Marcu & Dudock (1974). The translation products were carboxamidomethylated as described by Andersen & Weser (1978), with iodoacetamide in place of iodoacetic acid.

RNA dot-blot hybridizations

Total RNA (0.5 or 1.0 μg) was dotted on to nitrocellulose, presoaked in $10 \times \text{SSC}$, in volumes of 5 μl containing 2.5 M-formaldehyde and $6 \times \text{SSC}$, by the method of White & Bancroft (1982). The amount of total RNA used was within the linear range for quantification purposes (results not shown). The filter was air-dried, baked for 2 h at 80°C under vacuum, then prehybridized for 16 h at room temperature in a hybridization mixture which contained 50% formamide, $5 \times \text{SSCE}$ (0.75 M-NaCl/0.075 M-sodium citrate/25 mM-EDTA, pH 8.0), $1 \times$ Denhardt's solution (2 mg each of polyvinylpyrrolidone, Ficoll and bovine serum albumin) and 250 μg of denatured herring sperm DNA/ml. Filters were then hybridized for 24 h at 42°C .

in a fresh solution of the hybridization mixture which also contained 100 μ g each of poly(U) and poly(C)/ml as well as a portion (10 ng/ml) of the 400-base-pair *Eco*RI–*Hind*III fragment of mouse metallothionein-1 cDNA, which included the entire coding sequence (Durnam *et al.*, 1980) labelled to a specific radioactivity of 10^8 c.p.m./ μ g of DNA by nick translation with [α - 32 P]dCTP. After hybridization, the filter was washed in $2 \times$ SSC/0.1% SDS for 30 min at 20°C, then in $0.5 \times$ SSC/0.1% SDS for 1 h at 55°C. After autoradiography to detect the dots, they were excised, and radioactivity was measured by dissolving the nitrocellulose in methylCellosolve and counting with a toluene-based scintillation fluid in a Packard liquid-scintillation counter. Counts were corrected relative to an internal standard.

Electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out essentially as described by Laemmli (1970): 20% acrylamide/0.16% bisacrylamide/0.1% SDS gels were used for the identification of metallothionein synthesized *in vitro*. Slab gels were 20 cm long and 0.7 mm thick. Samples were dissolved in 2% SDS/5% 2-mercaptoethanol/62.5 mM-Tris/HCl (pH 6.8)/10% glycerol/0.005% Bromophenol Blue by heating for 5 min at 95–100°C. Electrophoresis was carried out at 150 V for 6 h. After electrophoresis, gels were fixed and stained overnight in a solution of 0.25% Coomassie Brilliant Blue in methanol/acetic acid/water (9:2:9, by vol.). Gels were destained for 1 h in 10% ethanol/10% acetic acid and then prepared for fluorography (Bonner & Laskey, 1974).

Results

Change in translatable hepatic metallothionein mRNA after CuCl₂ and ZnSO₄ injections

RNA was isolated from rat livers and kidneys at various times after injection of CuCl₂ or saline as a control and translated in a cell-free protein-synthesis system prepared from wheat germ. Translation products were labelled with [35 S]cysteine or [3 H]leucine and carboxamidomethylated to facilitate clear identification of metallothioneins (Mercer *et al.*, 1981). Fig. 1 shows a fluorogram of the translation products of hepatic RNA which had been separated on an SDS/20%-polyacrylamide gel. The metallothionein is clearly seen as a peptide of apparent M_r 14000, heavily labelled with [35 S]cysteine (e.g. lanes 3–8), but which was not labelled with [3 H]leucine (lane 12), as we had previously observed (Mercer *et al.*, 1981). Visual inspection of the gel reveals that the maximal amount of metallothionein mRNA occurred between 5 and 8 h after the CuCl₂ injection, and

Fig. 1. Translation products of hepatic RNA after CuCl₂ injection

RNA was isolated from livers of rats at various times after CuCl₂ (1.5 mg of Cu²⁺/kg; one animal for each time point) or saline injections and translated in a wheat-germ cell-free protein-synthesis system; products were labelled with [35 S]cysteine unless otherwise indicated. After carboxamidomethylation the products were resolved by SDS/20%-polyacrylamide-gel electrophoresis and the products were detected by fluorography. Lane 1, products made in absence of added RNA, [35 S]cysteine-labelled; lane 2, products made in absence of added RNA, [3 H]leucine-labelled; lane 3, uninjected animal; lane 4, 1 h after Cu²⁺ injection; lane 5, 2.5 h after Cu²⁺ injection; lane 6, 5 h after Cu²⁺ injection; lane 7, 8 h after Cu²⁺ injection; lane 8, 12 h after Cu²⁺ injection; lane 9, 15 h after Cu²⁺ injection; lane 10, 20 h after Cu²⁺ injection; lane 11, 5 h after saline injection; lane 12, 5 h after Cu²⁺ injection, [3 H]leucine-labelled. The positions of the M_r standards (Amersham) used are shown: myosin, 200000; phosphorylase *b*, 92500; bovine serum albumin, 69000; ovalbumin, 46000; carbonic anhydrase, 30000; lysozyme, 14300.

this was determined by densitometry to be about 10-fold higher than in the zero-time animal (results not shown). This increase in metallothionein mRNA was closely correlated with hepatic Cu²⁺ concentrations (see below). The saline-injected control (at 5 h) (Fig. 1, lane 11) showed only a slight increase in metallothionein mRNA compared with the zero-time sample (lane 3). Very few translation products were formed in the absence of added RNA with either [35 S]cysteine or [3 H]leucine (lanes 1 and 2).

Close inspection of the fluorogram revealed that a number of unidentified translation products were also increased after CuCl₂ injections, e.g. several can be seen in the 30000–69000- M_r range in lanes 5–10; all of these translation products were also increased after injection of ZnSO₄ (results not shown).

Changes in translatable renal mRNA after CuCl₂ injections

Fig. 2 shows the carboxamidomethylated translation products of renal RNA isolated at various

Fig. 2. Translation products of renal RNA after CuCl_2 injection

RNA was isolated from the kidneys of rats at various times after copper chloride (1.5 mg of Cu^{2+} /kg; one animal for each time point) injection, translated and analysed as described in Fig. 1. Lane 1, uninjected animal; lane 2, 1 h after Cu^{2+} injection; lane 3, 2.5 h after Cu^{2+} injection; lane 4, 5 h after Cu^{2+} injection; lane 5, 8 h after Cu^{2+} injection; lane 6, 15 h after Cu^{2+} injection; lane 7, 20 h after Cu^{2+} injection; lane 8, 5 h after saline injection; lane 9, 5 h after Cu^{2+} injection, [^{35}S]leucine-labelled; lane 10, no added RNA, [^{35}S]cysteine-labelled; lane 11, no added RNA, [^3H]leucine-labelled. M_r markers are as shown in Fig. 1.

times after CuCl_2 injections. In contrast with CuCl_2 injection into the mouse at this dose (1.5 mg of Cu^{2+} /kg; Durnam & Palmiter, 1981), we observed a considerable increase in metallothionein mRNA in the rat kidney. As in the liver, metallothionein is the predominant cysteine-labelled translation product in the maximally induced animals. Quantification by densitometry (results not shown) showed that the maximal amount of metallothionein mRNA was attained at 5 h (lane 4) and was about 4–5-fold greater than in the uninduced animals; by 15 h (lane 6) metallothionein mRNA had returned to basal values. The increase in metallothionein mRNA was closely correlated with the influx of copper into the kidney (see below). Most of the other translation products are unaltered after the injection; however, one, of approx. M_r 40 000, increased in both Cu^{2+} -injected (Fig. 2) and Zn^{2+} -injected animals (results not shown).

Metallothionein mRNA amounts after CuCl_2 injection into adrenalectomized rats

To assess the role of adrenal hormones in the induction of metallothionein mRNA after CuCl_2 administration, rats were injected with CuCl_2 3 days after adrenalectomy and RNA was prepared from the liver and kidneys 5 h after the injection. Analysis of the RNA by translation showed that the degree of induction in both the liver and kidney was unchanged in the adrenalectomized animals

compared with normals (results not shown). In addition, all the other mRNA species that increased in response to the CuCl_2 injection were also increased in the adrenalectomized animals, indicating that adrenal hormones do not play a role in the induction of these mRNA species.

Quantification by dot-blot hybridization of metallothionein-mRNA changes after CuCl_2 injection

To determine metallothionein-mRNA induction kinetics more accurately, we used dot-blot hybridization of RNA bound to nitrocellulose, by the procedure of White & Bancroft (1982). We chose to use a mouse metallothionein-1 probe for these experiments in place of the rat metallothionein-1 cDNA (Mercer & Hudson, 1982), since the latter gave more binding to rRNA, resulting in increased background, whereas the mouse probe did not give any signal with rRNA under the hybridization conditions used (results not shown). Northern-blot analysis has shown that the mouse and rat probes gave equivalent signals (results not shown), as would be expected from their high degree of sequence homology (Durnam *et al.*, 1980; Mercer & Hudson, 1982). We are reasonably confident that the probe was not detecting metallothionein-2 mRNA, since hybridization using the 3'-non-coding region of the mouse probe has shown only a weak (<5%) signal with rat mRNA compared with a coding-region probe; since the 3'-non-coding regions of mouse metallothionein-1 and rat metallothionein-1 are 81% homologous (Mercer & Hudson, 1982) and mouse metallothionein-1 and metallothionein-2 coding regions are about 80% homologous (Searle *et al.*, 1984), this strongly suggests that our probe will not detect rat metallothionein-2. This conclusion is supported by the observation (Searle *et al.*, 1984) that the mouse metallothionein-1 and metallothionein-2 probes cross-hybridize only under conditions of lower stringency than those used in our experiments.

Fig. 3(a) shows the results of our dot-blot determination of hepatic metallothionein-1 mRNA after CuCl_2 injection. These are the results of between 5 and 20 dot-blot determinations with two different RNA preparations for each time point, and additional time points are included to obtain a more accurate estimate of the time of highest mRNA amounts. The results show the same general trend as shown in Fig. 1, with a 16-fold elevation at 7 h after CuCl_2 injection and a fairly rapid decline to basal values by 15 h. Saline-injected animals showed a 2-fold increase in metallothionein-1 mRNA. We found no difference in metallothionein-1 mRNA in starved and non-starved animals.

Fig. 3(b) shows the renal metallothionein-1 mRNA amounts estimated by dot-blots. As shown

Fig. 3. Variation in (a) hepatic and (b) renal metallothionein-1 mRNA after CuCl_2 injection

RNA was isolated from (a) livers or (b) kidneys of rats at various times after CuCl_2 injection, and dotted on to nitrocellulose as described in the Materials and methods section. After hybridization to a ^{32}P -labelled mouse metallothionein-1 cDNA, the extent of hybridization was estimated by scintillation counting of the excised dots. In (a), amounts are expressed as means $\pm$ 2 S.E.M. for between four and twelve determinations relative to the mean of two uninduced animals; two to four animals were used for each point, except for 10h, 12h, 15h and 20h points, which were for one animal. In (b), amounts are expressed relative to an uninduced animal, as means $\pm$ 2 S.E.M. for five determinations; one animal was used for each time point, except for the zero-time, for which two animals were used.

by the translation data, the induction is less dramatic than that observed in the liver (6-fold at 5h), with a decline to basal values by 12h. Saline-injected animals showed a 1.6-fold increase in metallothionein-1 mRNA. The basal amounts of metallothionein-1 mRNA in the kidney were generally about 2-fold higher than those in the liver; however, the amounts at maximal induction were 1.4-fold higher in the liver RNA compared with the kidney, indicating that both the relative and absolute increases in metallothionein mRNA were greater in the liver.

Variation in hepatic and renal Cu^{2+} and Zn^{2+} concentrations after metal salt injection

In order to relate the influx of Cu^{2+} or Zn^{2+} to metallothionein-mRNA increase, we determined the Cu^{2+} and Zn^{2+} concentrations in both the liver and kidney for the 0-23h period after CuCl_2 injections. As shown in Fig. 4(a), hepatic Cu^{2+} concentrations rose rapidly 2.3-fold by 1h after injection, 4.8-fold by 2.5h, and reached a maximum of 6.3-fold 8h after Cu^{2+} administration, compared with saline-treated or uninjected animals. In contrast, mean hepatic Zn^{2+} concentrations did not alter significantly over the 0-5h time interval, but by 15h had risen by about 30%. Since metallothionein mRNA was increased about 13-fold at 5h (e.g. see Fig. 3a), these results suggest that the induction of metallothionein mRNA is not related to entry of Zn^{2+} into the liver. Cu^{2+} and Zn^{2+} concentrations were still maximally elevated 20h after metal injection (5.8- and 1.3-fold respectively). In fact, Bremner & Davies (1976) have shown that hepatic Cu^{2+} and Zn^{2+} concentrations remain elevated until approx. 3 days after Cu^{2+} injection.

Fig. 4(b) shows that renal Cu^{2+} concentrations increased 5-fold by 5h after CuCl_2 injection and thereafter, declined steadily, but were still elevated 20h after injection. In contrast with the liver, the renal Zn^{2+} concentrations did not alter significantly after the CuCl_2 injection.

CuCl_2 injection does not cause any metallothionein-gene amplification

Hepatic DNA isolated at different times after CuCl_2 injection was digested with various restriction endonucleases (*EcoRI*, *PstI*, *HindIII*), electrophoresed on agarose gels, transferred to nitrocellulose and probed with rat or mouse metallothionein-1 nick-translated cDNA probes. Both probes detected the same restriction fragments, e.g. with *EcoRI* digestion fragments were detected at 14.9, 10.5, 8.1, 5.2 and 2.0 kilobases, a similar pattern to that reported by Andersen *et al.* (1983). The complexity of this pattern may indicate that a metallothionein-gene family is present in the rat.

Fig. 4. (a) Hepatic and (b) renal Cu^{2+} and Zn^{2+} concentrations after CuCl_2 injection

Cu^{2+} and Zn^{2+} concentrations were determined in rats after CuCl_2 injection. In (a) one to four animals were used for each time point, and results are expressed as means $\pm$ 2S.E.M. where applicable. In (b) one animal was used for each time point, except for the zero-time point (two animals), and in this case results are expressed as means $\pm$ 2S.E.M.

Neither the band pattern nor the intensity altered after the CuCl_2 injection, indicating that no amplification or detectable rearrangement of the metallothionein gene had occurred.

Discussion

The amounts of metallothionein mRNA after metal-salt injection were assessed by translation assays and by using dot-blot hybridization with a mouse metallothionein-1 probe. The translation

assay presumably measured both metallothionein-1 and metallothionein-2 mRNA species (assuming equal translation efficiency), although our gel system did not resolve metallothionein-1 and metallothionein-2, so estimates of individual amounts could not be achieved. The metallothionein-1 probe is almost certainly specifically detecting metallothionein-1 mRNA under our hybridization conditions, since it has been observed that mouse metallothionein-1 and metallothionein-2 only cross-hybridize under conditions of low stringency (Searle *et al.*, 1984). Since both methods of measurement gave essentially the same results, this suggests that the kinetics of metallothionein-1 and metallothionein-2 induction do not greatly differ after Cu^{2+} injection, assuming approximately equivalent amounts of metallothionein-1 and metallothionein-2 mRNA.

Establishing whether Zn^{2+} or Cu^{2+} is the primary inducing agent in the liver is complicated by the increase in Zn^{2+} that occurs in the liver after the Cu^{2+} injection (see Fig. 4). Our results strongly suggest that Cu^{2+} is the primary inducing agent and the increase in Zn^{2+} is a secondary effect, resulting probably from the synthesis of thionein induced by the Cu^{2+} . The evidence for this is as follows. (1) No increase in mean hepatic Zn^{2+} occurred up to 5h after CuCl_2 injection; at this time, Cu^{2+} concentrations in the liver had risen 6-fold and metallothionein mRNA had increased about 12-fold. (2) Most of the increase in Zn^{2+} concentration occurred in the period 7–12h after the Cu^{2+} injection; during this period metallothionein mRNA was decreasing rapidly.

That the CuCl_2 injections resulted in an increase in metallothionein mRNA in the kidney is quite apparent from our results. Metallothionein-1 mRNA increased by a maximum of 6-fold at 5h, which correlated well with the 4.3-fold increase in Cu^{2+} concentration at this time, and no significant alteration in the Zn^{2+} concentration was observed over a 20h period. The relative and absolute metallothionein-1 mRNA increases were less in the kidney than in the liver, even though the Cu^{2+} concentrations increased to a similar extent in both organs, which indicates that in the kidney Cu^{2+} is a weaker inducer of metallothionein. The induction of renal metallothionein mRNA in the rat kidney contrasts with the failure of Durnam & Palmiter (1981) to observe significant induction of metallothionein-1 mRNA in mouse kidneys after injection of an equivalent dose of Cu^{2+} to that used here. This could result from significant differences in Cu^{2+} distribution between tissues in rats and mice after copper salt injection, or could be because the metallothionein gene in mouse kidney is not significantly inducible by Cu^{2+} . Further studies will be needed to clarify this interesting observation.

The translation assays revealed a number of unidentified polypeptides in the liver and kidney which increased in response to CuCl_2 injections. We specifically looked for peptides which may be induced only by Cu^{2+} and not by Zn^{2+} , since this may have enabled identification of inducible proteins involved in copper transport or storage (e.g. copper chelatin). Close comparison of translation products from ZnSO_4 - or CuCl_2 -injected animals showed that, at least in the first 6h, the same polypeptides increased after injections of both metal salts. The nature of these polypeptides is obscure, as is the reason for their increase, although we did find that adrenal hormones are not involved, since equivalent induction was observed in adrenalectomized animals.

Durnam *et al.* (1984) have demonstrated that bacterial endotoxin is a potent inducer of metallothionein-1 mRNA in mouse liver and kidney, and metallothioneins are also induced by injections of turpentine and carbon tetrachloride (Oh *et al.*, 1978). All of these agents induce an acute-phase response in rats, in which large amounts of acute-phase proteins are produced by the liver (Schreiber & Howlett, 1984). Thus it was possible that the injection of CuCl_2 could be inducing an acute-phase response, and the increase in metallothionein mRNA could be part of this rather than being due to the influx of copper. Analysis of the translation products over a 20h period after CuCl_2 injection, however, shows no major production of acute-phase-protein mRNA (in comparison with translation products of turpentine-injected rats; results not shown) or a decrease in albumin synthesis, which is also characteristic of this phenomenon. The minor polypeptides which increased did not appear to correspond in M_r to the acute-phase proteins.

We investigated the possible role of adrenal hormones in the increase of metallothionein mRNA after Cu^{2+} injection into the rats, since corticosteroids are known to be inducers of hepatic (but not renal) metallothionein mRNA synthesis in the mouse (Hager & Palmiter, 1981). Adrenalectomized animals showed the same increase in metallothionein mRNA in both liver and kidney as that observed after CuCl_2 injection into normal rats, demonstrating that a release of adrenal hormones did not significantly contribute to the increase in the hepatic metallothionein mRNA.

Following a report that gene amplification was involved in induction of metallothionein mRNA after cadmium salt injection and in neonatal rat livers (Koropotnik & Duerksen, 1983), we performed Southern blots of DNA isolated from rats at various times after CuCl_2 injection. We found a complex pattern of bands with both the mouse and rat probes, suggesting that a gene family of

metallothioneins may be present in the rat, but none of the bands altered in intensity after CuCl_2 injection. We have also failed to find metallothionein-1-gene amplification in neonatal rat livers (J. F. B. Mercer & A. Grimes, unpublished work), which Koropotnik & Duerksen (1983) also reported, and we consider the possible involvement of metallothionein-gene amplification in the neonatal period or with metal-salt injection to be improbable.

In conclusion, the close correlation of the hepatic and renal Cu^{2+} concentrations with the increase in metallothionein mRNA strongly supports the conclusion that injection of CuCl_2 results in a primary induction of metallothionein mRNA by Cu^{2+} , with little contribution by adrenal hormones, and does not involve an acute-phase response or metallothionein-gene amplification.

We are grateful to Professor David Danks for his helpful discussions and criticism of the manuscript, and to Neil Francis for his careful determination of the Cu^{2+} and Zn^{2+} concentrations. This work was supported in part by the Royal Children's Hospital Research Foundation and the National Health and Medical Research Council of Australia.

References

- Andersen, R. D. & Weser, U. (1978) *Biochem. J.* **178**, 841–852
- Andersen, R. D., Birren, B. W., Ganz, T., Piletz, J. E. & Herschman, H. R. (1983) *DNA* **2**, 15–22
- Bonner, W. M. & Laskey, R. D. (1974) *Eur. J. Biochem.* **46**, 83–88
- Bremner, I. & Davies, N. T. (1976) *Br. J. Nutr.* **36**, 101–112
- Bremner, I. & Young, B. W. (1976) *Biochem. J.* **157**, 517–520
- Durnam, D. M. & Palmiter, R. D. (1981) *J. Biol. Chem.* **256**, 5712–5716
- Durnam, D. M., Perrin, F., Gannan, F. & Palmiter, R. D. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6511–6515
- Durnam, D. M., Hoffman, J. S., Quaife, C. J., Benditt, E. P., Chen, H. Y., Brinster, R. L. & Palmiter, R. D. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1053–1056
- Hager, L. J. & Palmiter, R. D. (1981) *Nature (London)* **291**, 340–341
- Kagi, J. H. R. & Nordberg, M. (eds.) (1979) *Metallothionein*, Birkhäuser Verlag, Basel
- Koropotnik, J. & Duerksen, J. D. (1983) *J. Cell. Biochem.* **7A**, 107
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Liu, C. P., Slate, O. L., Gravel, R. & Ruddle, F. H. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4503–4506
- Marcu, K. & Dudock, B. (1974) *Nucleic Acids Res.* **1**, 1385–1397
- Mehra, R. K. & Bremner, I. (1984) *Biochem. J.* **219**, 539–546

- Mercer, J. F. B. & Hudson, P. (1982) *Biosci. Rep.* **2**, 761–768
- Mercer, J. F. B., Lazdins, I., Stevenson, T., Camakaris, J. & Danks, D. M. (1981) *Biosci. Rep.* **1**, 793–800
- Oh, S. H., Deagen, J. T., Whanger, P. D. & Weswig, P. H. (1978) *Am. J. Physiol.* **234**, E282–E285
- Richards, R. I., Heguy, A. & Karin, M. (1984) *Cell* **37**, 263–272
- Schreiber, G. & Howlett, G. (1984) in *Plasma Protein Secretion by the Liver* (Glauman, H., Peters, T., Jr. & Redman, C.), pp. 423–449, Academic Press, London and New York
- Searle, P. F., Davison, B. L., Stuart, G. W., Wilkie, T. M., Norstedt, G. & Palmiter, R. D. (1984) *Mol. Cell. Biol.* **4**, 1221–1230
- Shapiro, S. G., Squibb, K. S., Markowitz, L. A. & Cousins, R. J. (1978) *Biochem. J.* **175**, 833–840
- White, B. A. & Bancroft, F. C. (1982) *J. Biol. Chem.* **257**, 8569–8572
- Winge, D. R., Premakumar, R., Wiley, R. D. & Rajagopalan, K. V. (1975) *Arch. Biochem. Biophys.* **170**, 253–266
- Winge, D. R., Geller, B. L. & Garvey, J. (1981) *Arch. Biochem. Biophys.* **208**, 160–166