Evidence for differences in the post-transcriptional regulation of rat metallothionein isoforms

M. Helena VASCONCELOS*, Shuk-Ching TAM†, John H. BEATTIE* and John E. HESKETH*‡

*Division of Biochemical Sciences, Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, and †Department of Chemistry, University of Aberdeen, Aberdeen AB9 2UE, Scotland, U.K.

The expression of metallothionein (MT)-1 and -2 mRNAs in rat liver following administration of Cd or Cu was investigated using specific oligonucleotides. The specificity was confirmed using a competitive prehybridization assay. Cd injection caused a biphasic induction of both isoform mRNAs, whereas Cu induced a sustained, monophasic response. Analysis of polyribosomal RNA showed that, after both Cd and Cu treatments, the recruitment of MT-1 mRNA into polyribosomes paralleled the increase in transcription, but the increase of polyribosomal MT-2 mRNA was less than that of total MT-2 mRNA. This indicates that not all the MT-2 mRNA induced was translated, suggesting that there is translational control of MT-2 mRNA expression, but not of MT-1 mRNA. This hypothesis was supported by the observation that, after Cu treatment, the induction of MT-1 protein was induced to the same extent as MT-1 mRNA, whereas the total MT protein (MT-1 + MT-2) was increased far less (7-fold) than MT-2 mRNA (30-fold).

INTRODUCTION

Metallothionein (MT) is a metal-binding, cysteine-rich protein which has a number of highly conserved isoforms [1]. The number of isoforms varies between species and tissues, but there are only two MT proteins preferentially expressed in rat liver, namely MT-1 and MT-2 [2]. It has been proposed that these proteins play a major role in the control of intracellular metal concentrations and detoxification [3,4,5], but there is increasing evidence that they possibly have antioxidant functions in the cell [6,7].

MT genes possess both metal- and glucocorticoid-responsive promoters [8,9] which are responsible for the induction of MT genes by metals and glucocorticoids. However, in some circumstances there is evidence for post-transcriptional control of MT gene expression. Inhibition of protein synthesis stabilizes MT mRNA [10], and the MT mRNA half-life depends specifically on the inducing metal [11], thereby suggesting that there is regulation of MT mRNA turnover. In rat liver, MT synthesis during development has been reported not to parallel changes in mRNA levels [12,13], and dexamethasone treatment increased both isoform mRNAs, but only MT-2 protein [14], both observations indicating some control of MT mRNA translation.

Such evidence for translational or post-transcriptional control is indirect, and some of the observations could be explained by differences in protein stability; in addition, in some cases the individual MT isoforms or their mRNAs were not examined. The aim of the present work was to study directly the proportion of both MT-1 and MT-2 mRNAs which are translated during MT induction in rat liver after Cd or Cu injection. To determine the levels of MT-1 and MT-2 mRNAs in total and polyribosomal RNA, hybridization techniques and specific probes for the two rat isoform mRNAs were used. The coding region sequences of the MT isoforms are highly conserved, such that, in the case of the sheep isoforms, probes to the untranslated sequences are required to distinguish between isoform mRNAs [15]. In the present experiments, oligonucleotides specific to sequences in the 5′ untranslated regions of rat MT-1 and MT-2 mRNAs were designed to allow specific analysis of the two rat MT isoform mRNAs. The specificity of the probes was validated by developing a competitive prehybridization assay.

MATERIALS AND METHODS

Animals and treatments

Male rats of the Rowett Hooded Lister strain were used throughout. The animals were maintained in accordance with the Animals (Scientific Procedures) Act 1986. The animals were group-housed, in a 12 h light/12 h dark cycle, were given a standard pelleted diet *ad libitum* and had free access to drinking water. All injection volumes were 1 ml/kg body weight, and all the injection concentrations were verified by atomic absorption spectrophotometry (AAS).

(a) Study 1: specificity of probes

Seven rats were injected subcutaneously (s.c.) with different sterile solutions: 0.9% NaCl, 10, 30, 100 or 300 μmol/kg of $ZnCl_2$ in 0.9% NaCl or 10 μmol/kg $CdCl_2$,2.5H_2O in 0.9% NaCl and 10 μmol/kg dexamethasone disodium phosphate in 0.9% NaCl. At 6 h after injection the animals were killed by intraperitoneal (i.p.) injection of an overdose of sodium pentobarbitone (Euthesate; Willow Francis Veterinary, Crawley, Sussex, U.K.). Livers were perfused through the portal vein with 50 ml of ice-cold perfusion solution (1 mM EGTA/0.154 M

Abbreviations used: MT, metallothionein; AAS, atomic absorption spectrophotometry; TNF-α, tumour necrosis factor-α; s.c., subcutaneously; i.p., intraperitoneal(ly); DEPC, diethyl pyrocarbonate; BCH, blood-cell homogenate.

‡ To whom correspondence should be addressed.

KCl, pH 7.0), removed, immediately frozen in liquid nitrogen and subsequently stored at −80 °C.

(b) Study 2: a time-course study for Cd

A total of 25 rats (mean body weight 280 g) were injected s.c. with a sterile solution of 8.9 μmol/kg $CdCl_2$,2.5H_2O in 0.9 % NaCl. Ten rats were injected with a sterile solution of 0.9 % NaCl, and five rats received no injection. Groups of five animals injected with Cd were killed at 4, 6, 12, 24 and 48 h after injection, groups of two saline-injected controls were killed at the same times, and all the non-injected controls at 0 h.

At the specified times the animals were anaesthetized with sodium pentobarbitone and then injected with 0.2 ml of a solution of 20 mg/ml heparin (Sigma, Poole, Dorset, U.K.) in saline, administered into the posterior vena cava. Allowing several seconds for the heparin to circulate, the animals were then killed by exsanguination, and the liver was removed from the animals and perfused with 20 ml of sterile, ice-cold 0.154 M KCl, pH 7.0. Each liver was frozen immediately in liquid nitrogen and then stored at −80 °C.

(c) Study 3: a time-course study for Cu

A total of 30 rats (mean body weight 230 g) were injected s.c. with 8.7 μmol/kg $CuCl_2$,2H_2O in 0.9 % NaCl. A total of 12 rats (controls) were injected with 0.9 % NaCl. Five animals injected with Cu and two injected with saline were killed as described in (b) at each of the following times: 4, 6, 12, 24, 48 and 72 h. Five animals without any injection were killed at 0 h.

Oligonucleotide design and synthesis

Sequences of up to 30 bases were systematically examined by computer as candidates for oligonucleotide probes using criteria including sequence similarity between MT-1 and MT-2 primary transcripts of less than 60 % and a high melting temperature (T_m). The potential formation of dimers and secondary structures were determined using a PC-based computer program, Oligo (Sequence Analysis Workbench, University of Alabama, Birmingham, AL, U.S.A.), and possible cross-hybridization with other rat mRNAs was evaluated using a standard computerized algorithm technique (Fasta) and the EMBL sequence database. Sequences were selected on the basis of minimal cross-hybridization and dimer formation and corresponded to equivalent parts of the 5′ untranslated region, namely (a) a 28-mer for rat MT-1:

5′-GGTCCATTCCGAGATCTGGTGAATCTGG-3′

and (b) a 30-mer for rat MT-2:

5′-ATGGCGAATGGAGGCGGCAGTTGGAGATCA-3′

Oligonucleotides were synthesized by the standard cyanoethyl phosphoramidite method in an automated DNA synthesizer (PS250; Cruachem, Glasgow, Scotland, U.K.) and purified using oligonucleotide purification cartridges (COP; Cruachem).

Separation of total and polyribosomal RNA

All glassware and muslin filters were treated with DEPC (diethyl pyrocarbonate) and autoclaved. The ultracentrifuge tubes were rinsed, first with denaturing solution [4M guanidinium thiocyanate/25 mM sodium citrate (pH 7.0)/0.5 % *N*-dodecylsarcosine] and then with DEPC-treated water. All solutions were treated with DEPC, autoclaved, and kept ice-cold. All operations were carried out at 4 °C. Approx. 1 g samples of liver were transferred immediately after weighing to a measuring cylinder containing 10 ml of Buffer A [50 mM Tris (pH 7.6)/25 mM KCl/5 mM $MgCl_2$/5 mM 2-mercaptoethanol/0.25 M sucrose]. Liver volumes were noted and the volumes adjusted to 3.5 times the liver volumes. Samples were minced with scissors in a beaker and homogenized with 12 strokes of a Teflon/glass homogenizer. After the addition of 1 mM ribonucleoside vanadyl complexes (Sigma), samples were centrifuged at 2000 ***g*** in a Chilspin 2 centrifuge for 10 min to sediment nuclei and unbroken cells and the resulting supernatants centrifuged at 11000 ***g*** ($r_{av.}$ 6 cm; MR 1822 Jouan centrifuge) for 12 min to sediment mitochondria. The second supernatants were then filtered through two layers of muslin and 1 ml aliquots of the filtrate were taken for extraction of total RNA. Sodium deoxycholate (Sigma) was added to the remaining supernatant to a final concentration of 0.5 % and, after 5 min on ice, samples were layered over 15 ml of Buffer B [50 mM Tris (pH 7.6)/500 mM KCl/5 mM $MgCl_2$/5 mM 2-mercaptoethanol/40 % (w/v) sucrose/200 mM ribonucleoside vanadyl] and polysomes pelleted by centrifugation for 17 h at 25000 ***g*** ($r_{av.}$ 73.5 mm) in a Ti55.2 rotor using a Beckman L8-80 ultracentrifuge [16,17]. After centrifugation, pellets were processed for polysomal RNA extraction.

RNA extraction and hybridization

All RNA extractions were carried out by the acid/guanidinium/phenol/chloroform method of Chomczynski and Sacchi [18], and the preparations assessed by the A_{260}/A_{280} absorbance ratio. RNAs were then separated by electrophoresis through a denaturing 2.2 M formaldehyde/1.2 % agarose gel [19] and transferred by capillary blotting to a nylon membrane (Du Pont, Stevenage, Herts., U.K.). For slot-blotting, aliquots of RNA containing 2 μg were diluted to 20 μl with DEPC-treated water and denatured with 60 μl of 6.15 M formaldehyde and 10 × SSC (0.15 M sodium citrate/1.5 M NaCl), heated to 65 °C for 15 min and then loaded into a Minifold II 96-well slot-blot apparatus. The wells were then washed twice with 200 μl of 10 × SSC. For both Northern and slot blots, RNA was fixed by exposure to UV light and the membranes stored dry until required.

Radioactively labelled oligonucleotide probes were prepared using 100 ng of oligodeoxynucleotide, 1.85 MBq of [γ-^{32}P]ATP (Amersham International) and 1 μl of T4 kinase (New England Biolabs, Hitchin, Herts, U.K.). The reaction was carried out at 37 °C for 30 min, stopped by addition of 7.5 μl of 0.05 M EDTA and the volume adjusted to 40 μl by adding 7.5 μl of TE buffer (10 mM Tris/1 mM EDTA, pH 7.5)/0.1 M NaCl. Purification of the labelled oligonucleotides was carried out using Chroma Spin-10 columns (Cambridge Biosciences, Cambridge, U.K.).

Membranes were prehybridized for at least 6 h at 42 °C in 10 % dextran sulphate/0.2 % BSA/0.2 % polyvinylpyrrolidone/0.2 % Ficoll/50 mM Tris/HCl (pH 7.5)/0.1 % $Na_4P_2O_7$/1 % SDS/50 % deionized formamide/1.5 M NaCl/0.1 mg/ml salmon sperm DNA. The labelled probe was then added and hybridization carried out at 42 °C for 16 h. The membranes were washed to remove non-specifically bound DNA: for rat MT-1, 18 S rRNA and poly(T) oligonucleotide probes, the membranes were washed in 6 × SSC for 10 min, at room temperature, twice, followed by 6 × SSC/0.1 % SDS at 42 °C for 20 min; for rat MT-2 an additional final wash in 2 × SSC, 0.1 % SDS at 42 °C for 20 min was carried out. Specific hybridization was detected and quantified directly in a Canberra Packard Instant Imager; where

necessary, autoradiography was carried out using pre-flashed Hyperfilm-MP at −80 °C. After acquisition of the data, bound probe was removed by heating the membrane in 0.1 % SDS at 90–95 °C for 5–10 min and the membranes rehybridized. The amount of RNA loaded per lane (Northern blots) or per slot (slot-blots) was assessed by hybridization with a specific oligonucleotide probe to the 18 S rRNA [20] or a poly(T) oligonucleotide respectively.

RIA for rat MT-I protein

Liver homogenates (20 %, w/v) were prepared in gelatin buffer [50 mM Tris (pH 8.0)/0.1 % gelatin/0.1 % sodium azide] by sonication for 2 min at an amplitude of 16 μ using a Soniprep 150 (MSE) sonicator. Samples were centrifuged at 10000g (r_{av}. 6 cm; MR 1822 Jouan centrifuge) for 20 min and the supernatant fluids frozen at −80 °C until analysed; these were diluted in gelatin buffer and the MT-1 concentration was determined by RIA [21].

Silver saturation assay for total MT protein

Liver homogenates (20 %, w/v) were prepared in double-distilled water, then sonicated, centrifuged and stored as described for the RIA. Aliquots of each sample or blank were analysed by a Ag saturation micro method adapted from that described by Scheuhammer and Cherian [22].

Aliquots (0.1 ml) of each liver sample or blanks were vortex-mixed in 2.0 ml microcentrifuge tubes (Sigma) with 0.14 ml of glycine buffer (1 M glycine, pH 8.5). To each tube, 0.1 ml of freshly prepared Ag solution (20 mg/l of Ag as $AgNO_3$; Sigma) was added, the tubes vortex-mixed and then incubated at room temperature for 5 min. A 20 μl aliquot of blood-cell haemolysate (BCH) was then added to remove excess Ag. This BCH was prepared from saline-washed rat red blood cells by repeated freezing and thawing in double-distilled water. Tubes were vortex-mixed, closed with lid-locks and heated for exactly 1.5 min at 100 °C, immediately cooled on ice and centrifuged at 13000 ***g*** (r_{av}. 6 cm; MR 1822 Jouan centrifuge). The step of BCH addition, heating and centrifugation were repeated twice more without removing the protein pellet. The supernatant fluids were centrifuged again at high speed in a MSE Micro Centaur centrifuge for 5 min and the Ag concentration in the final supernatant fluids was analysed by AAS (Pye–Unicam SP9 atomic absorption spectrometer) using a micropipetting technique [20] and Ag standards (BDH, Poole, Dorset, U.K.) prepared in glycine buffer.

Statistical analysis

Results are expressed as means ± S.E.M. Statistical analysis of the data was carried out using a non-parametric Mann–Whitney U-test, except in the case of the competitive assay, where data were analysed using a one-way analysis of variance.

RESULTS

Specificity of probes

Only single bands were observed on Northern blots of rat liver mRNA using the specific oligonucleotide probes (Figure 1). In each case, the bands corresponded to mRNAs of approximate size 500 bases, and their abundance increased dramatically following the administration of metals or dexamethasone, confirming the ability of the oligonucleotides to detect the MT mRNAs.

Competitive prehybridization studies with the two oligonucleotides showed that they are specific to different MT isoform mRNAs. Hybridization competition studies were developed to confirm the specificity of each probe for mRNA on slot-blots using the following prehybridization conditions: (a) the standard prehybridization mixture; (b) the standard mixture supplemented with 100 ng of non-labelled oligonucleotide probe of different sequence to that subsequently used in the hybridization (the other isoform oligonucleotide); (c) the standard mixture supplemented with 100 ng of non-labelled oligonucleotide probe with the same sequence as that subsequently used in the hybridization. It was anticipated that if the oligonucleotides were specific for each isoform, the signal obtained in (a) and (b) would be the same, but the signal obtained in (c) would be lower, because the labelled and non-labelled oligonucleotide would compete for binding to the target mRNA.

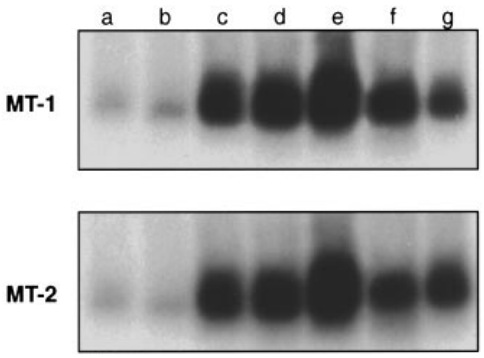

Figure 1 Northern blot showing the specificity of the MT-1 and the MT-2 oligonucleotide probes

Total mRNA from seven rat livers was probed by Northern hybridization for MT-1 (top) and reprobed for MT-2 (bottom). Animals were killed 6 h after s.c. injection with 0.9% NaCl (lane a), with 10, 30, 100 or 300 μmol of $ZnCl_2$/kg in 0.9% NaCl (lanes b, c, d and e respectively), with 10 μmol of $CdCl_2$,2.5H_2O/kg in 0.9% NaCl (lane f) and lane g with 10 μmol of dexamethasone disodium phosphate/kg in 0.9% NaCl (lane g).

As shown in Table 1, prehybridization in the presence of unlabelled MT-2 oligonucleotide had virtually no effect on the hybridization signal obtained with the MT-1 oligonucleotide. In contrast, when the prehybridization mixture contained unlabelled MT-1 oligonucleotide, the hybridization was reduced by 56.5 % ($P < 0.05$). Similarly, analysis of MT-2 mRNA showed a negligible decrease in hybridization of the labelled oligonucleotide when the prehybridization mixture contained unlabelled MT-1 but a 42 % reduction ($P < 0.05$) when prehybridization was carried out in the presence of unlabelled MT-2 oligonucleotide. The results show that the presence of MT-1 oligonucleotide did not interfere with hybridization of the labelled MT-2 oligonucleotide and vice versa, indicating that each oligonucleotide is specific for a different MT isoform mRNA.

Cd post-transcriptional studies

Induction of MT by Cd administration caused a rapid increase in both MT-1 and MT-2 mRNAs (Figure 2). The induction of MT-1 was considerably greater than that of MT-2 (10-fold as opposed to 3-fold), and the induction of both isoform mRNAs was biphasic, with an initial rapid, transient response peaking at 4 h for MT-1 and 6 h for MT-2, and a second response, which was evident by 24 h. At all the time points analysed the increase in MT-1 mRNA expression in polyribosomes was not different from that of total MT-1 mRNA expression (Figure 2a), and the ratio of polyribosomal MT-1 mRNA to total MT-1 mRNA did

Table 1 Competitive prehybridization assay

Quantification of bound oligonucleotide under different hybridization conditions. The amount of ^{32}P-labelled oligonucleotide bound after hybridization with labelled MT-1 (left) and MT-2 (right) oligonucleotides is shown under different prehybridization conditions: normal prehybridization (first row), normal supplemented with the non-labelled oligonucleotide for MT-1 (second row) and normal supplemented with the non-labelled oligonucleotide for MT-2 (third row). Results are from slot-blots of 2 μg of total RNA. Results are mean values ± S.E.M. from three slots.

	MT-1 probe		MT-2 probe	
Prehybridization conditions	(counts ± S.E.M.)	(% of control)	(counts ± S.E.M.)	(% of control)
Normal (control)	1758 ± 287	100	2410 ± 245	100
Added MT-1	765 ± 52	43.5	2223 ± 312	92
Added MT-2	1683 ± 162	96	1405 ± 203	58

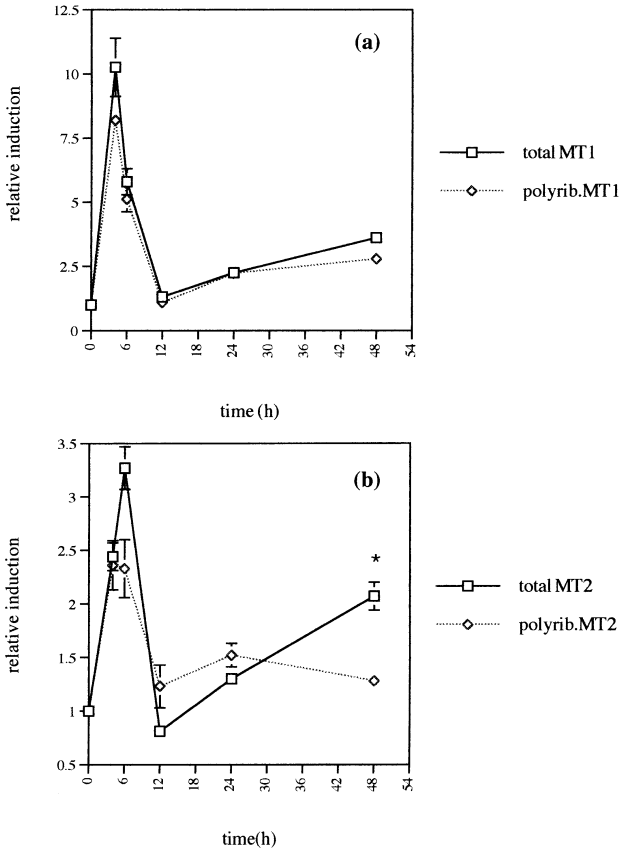

Figure 2 Cd time course study: relative increase in total and polyribosomal (polyrib.) mRNA

Time-course induction of total and polyribosomal mRNA levels for (**a**) MT-1 and (**b**) MT-2 after an injection of 8.9 μmol of Cd/kg. Results are relative to control values ($n = 10$–13), considering these equal to 1. These control values are expressed as the 0 h time point, since no difference was found between zero time and all the other times control animals. Results are the mean for four animals, except for 12 h and 24 h, where $n = 3$ for total MT-1 and total and polyribosomal MT-2 mRNA, and for 48 h, where $n = 5$ for total MT-1, total MT-2 and polyribosomal MT-2 mRNA. Bars represent the S.E.M., and * indicates $P \leqslant 0.05$ (Mann–Whitney U-test) between total and polyribosomal mRNA for the same isoform. Results are from two replicate slot-blots, one of which was probed for MT-1 and the other for MT-2. Both blots were corrected for loadings by subsequently measuring poly(A).

not change significantly during induction (Table 2). In contrast, the increase of MT-2 mRNA in polyribosomes was significantly lower ($P < 0.05$) than in total RNA at 48 h after Cd injection (Figure 2b) and the polysomal/total ratio for MT-2 was also significantly reduced ($P < 0.05$) at 6 and 48 h (Table 2). These observations provide evidence that not all the induced MT-2 mRNA was translated at these times. No difference was found in basal MT mRNA expression and in the polyribosomal/total ratio between saline-injected and uninjected controls.

Cu post-transcriptional studies

The induction of the two MT isoform mRNAs was rapid after Cu injection and sustained for up to 12 h (Figures 3a and 3b), but in this case the MT-2 mRNA was induced to a greater extent (30-fold) than the MT-1 mRNA (less than 10-fold). This very large induction may partly reflect the low MT-2 mRNA expression in the control group; the expression was lower than in controls from the Cd study, and this may be age-related, since the controls were younger in this Cu study. As was the case after Cd injection, the extent of MT-1 mRNA induction in polyribosomal RNA was similar to that for total RNA (Figures 3a and 4a, Table 3), although between 0 and 6 h after Cu treatment the increase in polyribosomal MT-1 mRNA appeared to be greater than that in total RNA; however, this difference was not statistically significant. This probably reflects a situation in which the amount of MT-1 mRNA associated with polyribosomes was apparently increased to a greater extent than the increase in mRNA synthesised from the gene. However, the increase of MT-2 polyribosomal mRNA was significantly smaller than that of MT-2 total mRNA at 4 (Figure 3b and Figure 4b), 12, 24 and 72 h (Figure 3b) and the ratio of MT-2 mRNA in polyribosomal mRNA/total mRNA was reduced at 4, 6, 12 and 24 h compared with controls (Table 3). This suggests that not all the MT-2 mRNA was translated throughout the induction of MT-2 by Cu.

This apparent translational control was further investigated by analysis of MT-1 protein and total MT protein in the same liver samples. As can be seen in Figure 5, there was an increase of MT-1 and total MT protein, which was detectable at 4 h and peaked at 12 h after treatment. The relative increase of MT-1 protein was approx. 5-fold, whereas the increase of MT-1 mRNA, at its peak, was approx. 9-fold (Figure 3a). Although a specific assay for measuring MT-2 protein is not available, the data on total MT protein show clearly that the relative increase in total MT protein was approx. 7-fold, similar to the increase in MT-1 protein. This suggests that the increase in MT-2 protein would be in the same range. The relative increase in MT protein, and, accordingly, in MT-2 protein, more closely parallels the increase in MT-2 in polyribosomal mRNA than the increase in MT-2 in total mRNA and is small compared with the 30-fold increase in

Table 2 Cd time-course study

Abundance of total and polyribosomal mRNA and polyribosomal/total mRNA ratios for MT-1 and MT-2 during a time-course induction by Cd. Results were calculated as counts of MT probe bound per unit of counts of a poly(T) oligonucleotide bound and are expressed in arbitrary units. Means ± S.E.M. for four animals per treatment are shown, except in (a) $n = 10$, (b) $n = 13$ and (c) $n = 11$. * represents P values < 0.05 for differences between control ratios at 0 h and the ratio at that specific time.

	Time…	0 h	4 h	6 h	12 h	24 h	48 h
MT-1	mRNA abundance in 20 μg of total RNA	0.61 ± 0.04(a)	6.26 ± 0.70	3.53 ± 0.31	0.8 ± 0.09	1.37 ± 0.03	2.21 ± 0.18
	mRNA abundance in 20 μg of polyribosomal RNA	0.77 ± 0.05(b)	6.31 ± 0.27	3.94 ± 0.38	0.83 ± 0.07	1.96 ± 0.16	2.14 ± 0.24
	Polyribosomal mRNA/total mRNA	1.34 ± 0.13(a)	1.05 ± 0.14	1.13 ± 0.12	1.10 ± 0.2	1.44 ± 0.15	0.97 ± 0.02
MT-2	mRNA abundance in 20 μg of total RNA	0.87 ± 0.04(c)	2.12 ± 0.11	2.84 ± 0.17	0.71 ± 0.02	1.13 ± 0.03	1.81 ± 0.11
	mRNA abundance in 20 μg of polyribosomal RNA	0.97 ± 0.06(b)	2.29 ± 0.22	2.26 ± 0.26	1.19 ± 0.2	1.47 ± 0.11	1.24 ± 0.07
	Polyribosomal mRNA/total mRNA	1.15 ± 0.09(a)	1.09 ± 0.15	0.79 ± 0.09 *	1.67 ± 0.22	1.31 ± 0.13	0.7 ± 0.05 *

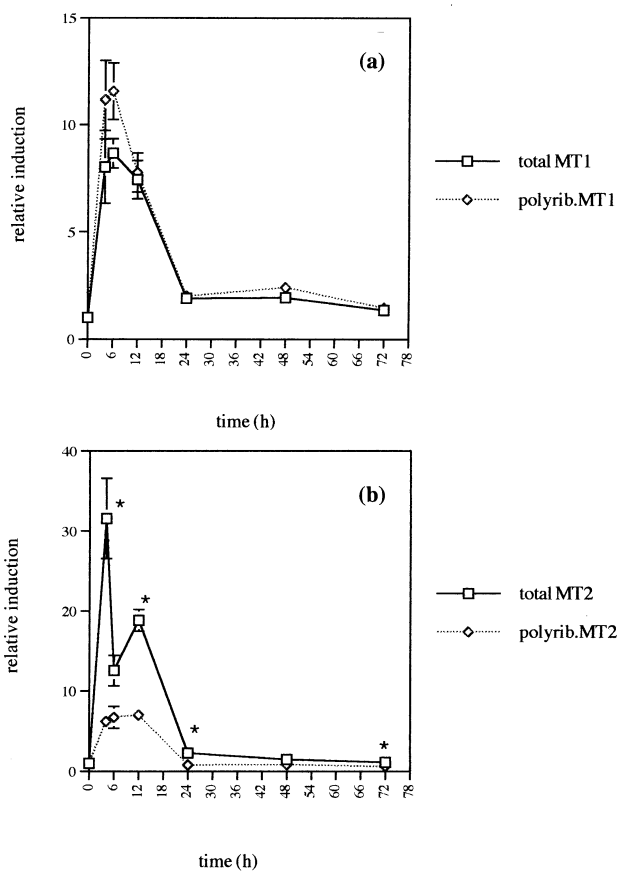

Figure 3 Cu time course study: relative increase in total and polyribosomal (polyrib.) mRNA

Time-course induction of total and polyribosomal mRNA levels for (**a**) MT-1 and (**b**) MT-2 after an injection of 8.7 μmol of Cu/kg. Results are relative to control values, considering these equal to 1. $n = 5$ for all time points, except 4 h ($n = 4$). See the legend to Figure 2 for an explanation of statistics and correction for loadings.

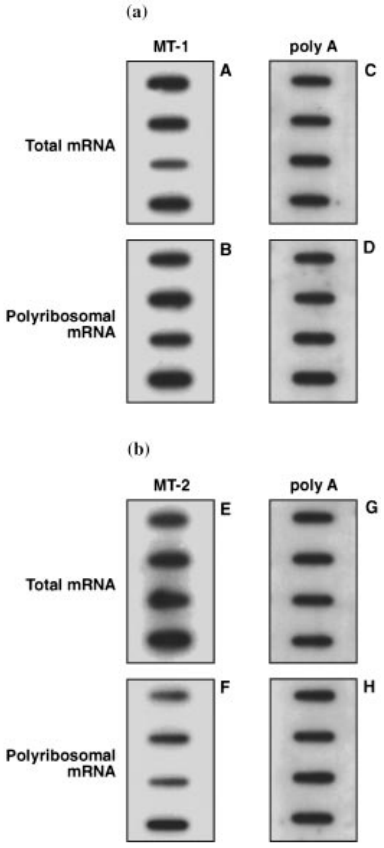

Figure 4 MT-1 and MT-2 total and polyribosomal mRNA levels 4 h after Cu treatment

Slot blots of rat liver total and polyribosomal mRNA (2 μg RNA per slot), probed (**a**) for MT-1 (panels A and B) and re-probed for poly(A) (panels C and D) or probed (**b**) for MT-2 (panels E and F) and re-probed for poly(A) (panels G and H). Animals were treated with 8.7 μmol of Cu/kg and killed 4 h later. Results shown are from four replicate animals.

MT-2 total mRNA. This difference in protein and mRNA induction supports the hypothesis that not all the induced MT-2 mRNA was translated after Cu injection. This is further reflected in the observation that the relative increase in MT-1 protein stayed approximately constant after 12 h, but the level of total MT protein decreased to near that of the controls.

DISCUSSION

The specificity of antisense oligonucleotide probes designed for the independent measurement of MT-1 and MT-2 mRNA has been clearly demonstrated using two different techniques. Firstly, we have shown that the probes do not cross-hybridize with other mRNAs, because the intensity of the single band detected on

Table 3 Cu time-course study

Abundance of total and polyribosomal mRNA and polyribosomal/total mRNA ratios for MT-1 and MT-2 during a time-course induction by Cu. Results were calculated as counts of MT probe bound per unit of counts of a poly(T) oligonucleotide bound and are expressed as arbitrary units. Means ± S.E.M. for five animals per treatment are shown, except in (a) $n = 4$. * Represents P values < 0.05 for differences between control ratios at 0 h and the ratio at that specific time.

	Time...	0 h	4 h	6 h	12 h	24 h	48 h	72 h
MT-1	mRNA abundance in 20 μg of total RNA	0.26 ± 0.05	2.09 ± 0.44(a)	2.25 ± 0.18	1.93 ± 0.23	0.49 ± 0.08	0.5 ± 0.1	0.35 ± 0.04
	mRNA abundance in 20 μg of polyribosomal RNA	0.25 ± 0.05	2.79 ± 0.46(a)	2.89 ± 0.33	1.94 ± 0.23	0.5 ± 0.06	0.6 ± 0.09	0.36 ± 0.04
	Polyribosomal mRNA/total mRNA	1.08 ± 0.32	1.48 ± 0.24(a)	1.28 ± 0.07	1.02 ± 0.1	1.05 ± 0.06	1.27 ± 0.11	1.03 ± 0.05
MT-2	mRNA abundance in 20 μg of total RNA	0.08 ± 0.02	2.52 ± 0.4	1.00 ± 0.15	1.51 ± 0.11	0.18 ± 0.02	0.12 ± 0.03	0.09 ± 0.01
	mRNA abundance in 20 μg of polyribosomal RNA	0.19 ± 0.02	1.17 ± 0.17	1.27 ± 0.26	1.33 ± 0.11	0.15 ± 0.02	0.15 ± 0.01	0.11 ± 0.01
	Polyribosomal mRNA/total mRNA	2.33 ± 0.36(a)	0.51 ± 0.12(a)*	1.25 ± 0.09*	0.88 ± 0.05*	0.83 ± 0.12*	2.08 ± 0.89	1.42 ± 0.32

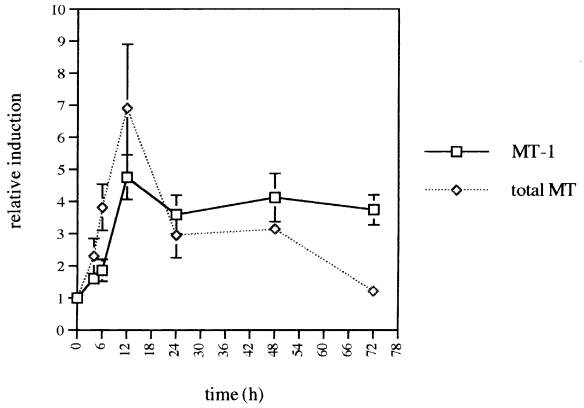

Figure 5 Time course of the relative increase in total MT protein and MT-1 protein following Cu treatment

Results are from Ag saturation assay (total MT protein) and from RIA (MT-1 protein) and are expressed as a relative increase compared with control values, considering these as 1. Results are the mean for five rats at each time point, and bars represent S.E.M.

Northern blots increases dramatically in response to administration of known MT-inducing agents (Figure 1). Secondly, the competitive prehybridization assay shows clearly that each of the oligonucleotides was specific for a particular MT mRNA. This method should be applicable to the assay of any pair of similar mRNAs: to distinguish between mRNAs for closely related proteins, between mRNAs for isoforms or between mRNAs differing in small mutations. The method would allow specificity to be demonstrated when the mRNAs were so similar that they could not be distinguished on the basis of size. Furthermore, the method could be extended to the analysis of DNA and products of DNA amplification; indeed, whilst this work was in progress it was shown that a similar competitive assay could be used to distinguish between amplification products from DNA sequences differing by a single point mutation [23].

Under a wide range of conditions, and in many species, administration of metals results in a rapid and transient induction of MT, usually attributable to increased gene transcription [8]. However, at least in rat liver, there has been no comprehensive study of the comparable effects of different inducers on the relative induction of the MT-1 and MT-2 mRNAs [24]. The present data, using specific oligonucleotides to detect the MT-1 and MT-2 mRNAs, show that both isoform mRNAs are induced by Cu and Cd, but to different extents and with different time courses: Cu induces total MT-2 mRNA more than total MT-1 mRNA and the response is monophasic and prolonged up to 12 h, whereas Cd induces MT-1 mRNA more than MT-2 mRNA and the response of both mRNAs is biphasic, with the first phase being over by 12 h.

Furthermore, the measurement of mRNAs in polyribosomes provides evidence that there is differential translational control of the two MT isoform mRNAs during induction of MT in rat liver. After injection of either Cu or Cd, the ratio of MT-1 mRNA abundance in polyribosomes to that in total mRNA remained unchanged but the ratio for MT-2 mRNA was reduced, showing that, whilst the increased levels of MT-1 mRNA were paralleled by increased recruitment of the mRNA into polyribosomes, this was not the case for MT-2 mRNA. In contrast with injection with Cd, where MT-2 mRNA recruitment into polyribosomes was reduced only at the peaks of induction, MT-2 mRNA recruitment was reduced during the entire Cu induction process. The data indicate that, after administration of either metal, although induction of MT-1 mRNA was paralleled by its translation, the translation of MT-2 mRNA was increased to a lesser extent than its induction. Thus, in rat liver, in response to both metals, there is a variable capacity to translate the two isoform mRNAs, suggesting some negative control over the translation of MT-2 mRNA. Interestingly, the polyribosomal/total MT-2 ratio was greater than that for MT-1 in the control uninjected group of the Cu experiment, whereas the ratios were similar in the Cd experiment. The reason for this is unclear, but may be related to the fact that younger animals (230 g compared with 280 g) were studied in the second experiment. Therefore the apparent difference in MT-2 mRNA translation may be age-related, a view supported by observations suggesting variation in MT mRNA translation during development [12].

The data on protein levels (Figure 5) provided further evidence for translational control of MT-2 mRNA: although induction of MT-1 protein was of a similar order to that of MT-1 mRNA in total mRNA, total MT protein induction was considerably less than that of total MT-2 mRNA, but similar to that of polyribosomal MT-2 mRNA. Furthermore, the increase in MT-1 protein levels was sustained after 12 h, whereas, by analysis of total MT and MT-1 protein levels, it is possible to conclude that the MT-2 protein levels decreased to lower than control values by the end of the time course (72 h). These data provide further indirect evidence for translational regulation of MT-2. It is unlikely that the fall in total MT protein 24 h after Cu injection is due to increased turnover of MT-2 protein, because the total MT protein half-life is about 17 h in the liver of rats after i.p. injection of 300 μg of Cu^{2+} [25], but MT-1 protein has a shorter half-life than MT-2 [26].

It is established that MT synthesis is regulated at the level of transcription. Furthermore, it has been shown that induction of MT protein by Zn involves an increase in both total and polyribosomal MT mRNA [27], showing that the induced mRNA is at least partially translated. However, there are several observations which suggest that regulation of MT protein synthesis may also occur at the level of translation. For instance, it has been shown that *in vitro* translation of polyribosomal mRNA from Cd-treated rats did not consistently give greater synthesis of MT protein than that from control rats [28]. In addition, studies of rat development have shown that, after birth, total MT-1 and MT-2 mRNA levels remained high, although MT protein levels [12,13], and MT synthesis rates [29], decreased. Furthermore, during the induction of MT by Zn, MT mRNA levels remained higher than control values for up to 36 h, whereas the rate of synthesis of MT was the same as the control by 24 h [30]. Lastly, studies on synergistic induction of MT also suggested a possibility for post-transcriptional regulation, with mRNA levels again not correlating with protein levels [31]. However, the above effects could be accounted for not only by a negative regulation of mRNA translation, but also by changes in MT mRNA stability or protein stability [8]. The present observations support and extend this earlier work by adding more direct evidence that regulation of MT-2 mRNA translation can occur.

The time course of induction of both MT mRNAs was biphasic after injection of Cd, but not of Cu (Figures 2 and 3). This biphasic response, to our knowledge, has not been previously reported, and we investigated the possibility of it being caused by an inflammatory reaction to Cd injection [32] with release of acute-phase cytokines, known to induce MTs as part of an acute-phase response [33]. However, ELISA analysis of tumor necrosis factor-α (TNF-α) concentration in liver and kidney samples after Cd administration provided no evidence for an inflammatory response (results not shown). The cause of the second phase of the MT mRNA increase therefore remains unknown.

The present results suggest that regulation of MT isoform expression by Cu and Cd involves differential translational control, and this contrasts with the regulation of ferritin synthesis by iron, in which the two subunits are differentially regulated at the level of transcription but co-ordinately regulated at the level of translation [34]. The mechanism of translational control of MT mRNA is as yet unknown; whether it involves a protein interacting with the mRNA of each isoform differentially (for a review, see [35]), or if it is dependent on the structure of the mRNAs (for reviews, see [36,37]), is currently being investigated. Differential control of translation of these mRNAs may provide a finer regulation than the transcriptional control, providing a means for a precise synthesis of the required amount of each isoform. This would suggest that the requirements for the two isoforms are different, implying that the two MT isoforms perform different functions in the liver.

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