Environmental Health Perspectives
Vol. 65, pp. 141-147, 1986

Heavy Metal-Induced Gene Expression in Fish and Fish Cell Lines

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Two isoforms of metallothionein (MT) have been isolated from rainbow trout livers following $CdCl_2$ injections. These MTs have been identified by standard procedures and appear to be similar to mammalian MTs. Total RNA from such induced livers was shown to contain high levels of MT-mRNA activity when translated in cell free systems. This activity was demonstrated to be in the 8 to 10S region of a sucrose gradient. The RNA fractions also showed homology to a mouse MT-I cDNA probe. The exposure of rainbow trout hepatoma (RTH) cells to various concentrations of $CdCl_2$ and $ZnCl_2$ induced the expression of MT and MT-mRNA. Exposure of Chinook salmon embryonic (CHSE) cells to these metals, however, did not result in MT synthesis, suggesting that the MT genes have not become committed to transcription. Instead, an unknown low molecular weight (MW = 14 kDa) protein was induced. This metal-inducible protein (MIP) was capable of binding ^{109}Cd and was stable to heating, while the binding of the metal to this protein was not. These characteristics have been reported for a protein induced in rainbow trout liver following environmental exposure to cadmium. We suggest that both MT and MIP may function in detoxification of heavy metals.

Introduction

The prevalence of toxic heavy metals in the environment is of increasing concern to both the environmental and medical communities. Since aquatic animals are often the first life forms to come into contact with these poisons, the detrimental effects, as well as the mechanisms by which these animals cope with such poisons is of great interest. By such studies it may be possible to establish a biological index for heavy metal toxicity in the aquatic environment.

One apparently universal method by which eukaryotes cope with heavy metals is by synthesizing a family of proteins known as metallothioneins (MTs) (*1*). These low molecular weight, thiol-rich proteins are induced at the transcriptional level in response to heavy metals as well as a variety of other agents and environmental stresses (*2–5*).

It is widely believed that MTs function by binding to toxic metals such as Cd and Hg thus detoxifying the system in question (*1*). In addition, MTs function in the homeostasis of essential trace metals such as Cu and Zn (*6,7*). MTs have been quite well characterized in a number of fish such as rainbow trout (*8–10*), salmon (*11*), plaice (*12*), carp (*13*), and eels (*14*), among others.

We report here the induction of MT and MT mRNA in both rainbow trout and a rainbow trout cell line. In addition we demonstrate that MT is by no means the only protein synthesized in response to metals. In fact, a set of genes in fish are capable of being activated following exposure to heavy metals. We describe one of these metal-inducible proteins (MIP) in more detail.

Results

We have previously shown (*8*) that when rainbow trout are subjected to a series of intraperitoneal injections of $CdCl_2$, they respond by producing two isoforms of the low molecular weight metal binding protein metallothionein. These two isoforms, MT-I and MT-II, were purified by standard techniques involving heat denaturation, G-75 column chromatography and ion-exchange chromatography. Figure 1 shows an SDS–polyacrylamide gel demonstrating the MT purification scheme, and Table 1 shows the amino acid analysis of purified MT-I and MT-II. We have also shown (*8*) that RNA isolated from Cd-induced rainbow trout liver contains a high level of MT-mRNA translational activity when compared to controls. When this RNA was fractionated on 5 to 30% sucrose gradient, the bulk of MT-mRNA translational activity was in the 8 to 10S region of the gradient. Such an RNA fractionation is shown in Figure 2. In this case RNA samples from control and Cd induced gradients were immobilized on nitrocellulose and probed with a ^{32}P nick-translated mouse MT-I cDNA probe (a gift of Dr. R. Palmiter). The resulting autoradiograph shown in Figure

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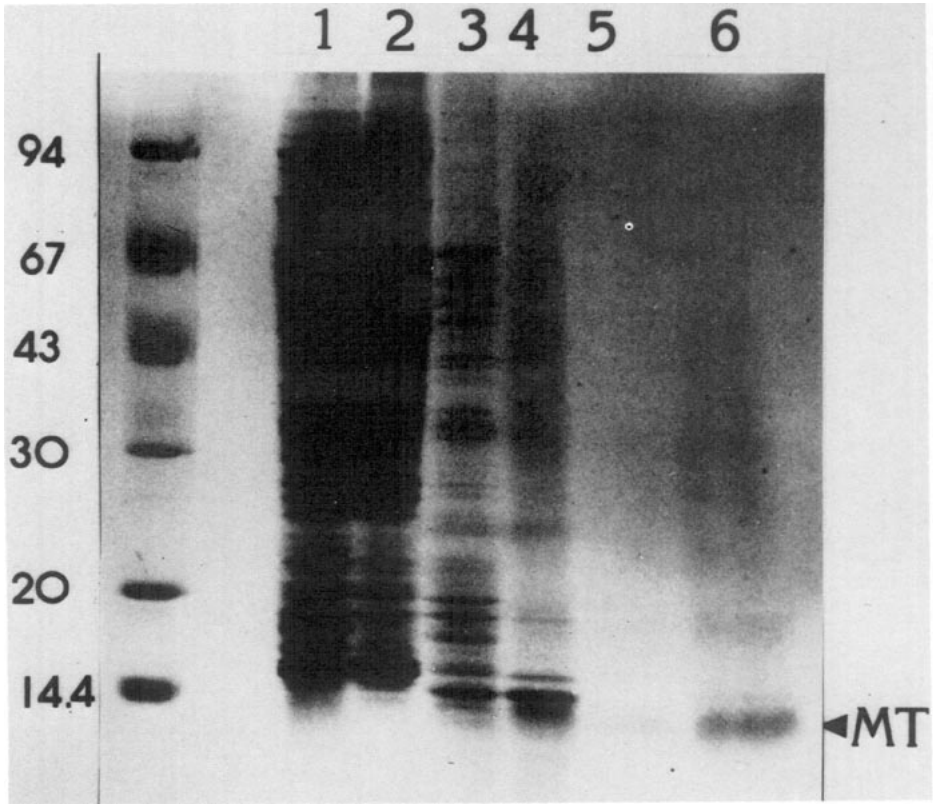

FIGURE 1. Electrophoretogram of MT. Fractions from various purification steps were analyzed by SDS–polyacrylamide gel electrophoresis: (lane 1) total cadmium induced trout liver homogenate; (lane 2) total homogenate supernatant following centrifugation; (lane 3) supernatant following heat treatment and centrifugation; (lane 4) Sephadex G-75 MT fractions; (lane 5), MT-I from DEAE column; (lane 6), MT-II from DEAE column.

2 demonstrates hybridization to RNA fractions previously shown to contain high levels of MT-mRNA translational activity. The corresponding control fractions showed only background hybridization.

The study of the regulation of heavy metal induced gene expression in fish was extended by investigating the

Table 1. Amino acid analysis of rainbow trout MT-I and MT-II.

	Rainbow trout				Mouse	
Amino acid	MT-I nearest integer	%	MT-II nearest integer	%	MT-I nearest integer[a]	%
Lys	6	9.5	7	11.1	7	11.4
Asx	6	9.5	6	9.5	4	6.6
Thr	4	6.3	4	6.3	5	8.2
Ser	9	14.3	10	15.9	9	14.8
Glx	5	7.9	4	6.3	1	1.6
Pro	2	3.1	2	3.1	2	3.3
Gly	8	12.7	8	12.7	5	8.2
Ala	4	6.3	3	4.8	5	8.2
1/2Cys	14[b]	22.2	18[b]	28.6	20	32.3
Val	2	3.1	1	1.6	2	3.3
Met	1[c]	1.6	t[c]		1	1.6
Ile	t		t			
Leu	1	1.6	t			
Tyr	t		t			
Phe	t		t			
His	t		t			
Arg	1	1.6	1	1.6		
Trp	t		t			
Total	63		63		61	

[a] From Huang et al. (*21*).
[b] Determined as cysteic acid.
[c] Determined as methionine sulfone.

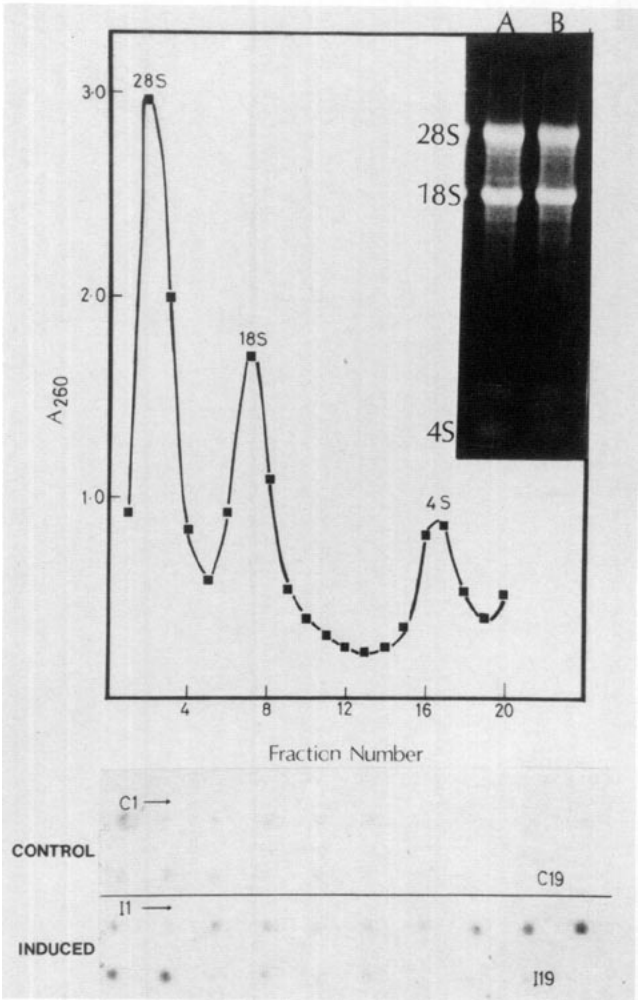

FIGURE 2. Sucrose gradient centrifugation of total RNA and RNA dot hybridization to a mouse MT-I cDNA probe: (A) 10 μg control RNA, (B) 10 μg Cd induced RNA. Total control and cadmium-induced trout liver RNA was fractionated on a linear 5–30% sucrose gradient. Control and Cd induced RNA samples yielded identical profiles. The integrity of the RNA was further demonstrated by methyl mercury agarose gel electrophoresis (inset). Identical control and Cd induced RNA samples from the fractionation were immobilized on nitrocellulose and probed with a [^{32}P] nick translated mouse MT-I cDNA. The numbers represent the fraction number from the control (C) or Cd induced (I) sucrose gradient profiles.

rainbow trout hepatoma (RTH) (*15*) and Chinook salmon embryo (CHSE) (*16*) cell lines. Material obtained from the previous rainbow trout *in vivo* investigations were used as standards throughout these experiments. Mid-logarithmic phase RTH or CHSE cultures were exposed to various concentrations of $ZnCl_2$ or $CdCl_2$ for 3 days. Proteins were labeled *in vivo* with [^{35}S]-cysteine, carboxymethylated, and analyzed by polyacrylamide gel electrophoresis (*8*) (Fig. 3*A*). Massive induction of MT synthesis occurred in RTH cells exposed to 100 μM and 200 μM $ZnCl_2$ (lanes 3 and 4, respectively), and, to a much lesser degree, in cells exposed to 10 μM $CdCl_2$ (lane 6). Concentrations of metal higher than 10 μM $CdCl_2$ or 100 μM $ZnCl_2$ led to appreciable cell death (unpublished observations). CHSE cells did not synthesize MT in response to either $ZnCl_2$ or $CdCl_2$ exposure (lanes 7 through 11).

In vitro translation of total nucleic acid (*22*) isolated from 100 μM $ZnCl_2$-induced RTH cells revealed a substantial level of MT-mRNA translational activity (Fig. 3*B*, lanes 5 and 6). Analysis of 10 μM $CdCl_2$ RNA translates mimicked the protein labeling results (data not shown). RNA extracted from $ZnCl_2$-treated CHSE cells (lanes 1 and 2) and 10 μM $CdCl_2$-treated cells (data not shown) was unable to direct the synthesis of MT. For comparison, RNA translates from control and Cd-induced rainbow trout liver (*8*) were run in lanes 7 and 8, respectively.

The time course for MT induction in RTH cultures maintained in 10 μM $CdCl_2$ is shown in Figure 4. Synthesis of MT above basal level was evident by 12 hr, increased to a maximum at 72 hr, and remained elevated at 96 hr. The time course under 100 μM $ZnCl_2$ exposure differed in that synthesis of MT peaked at 72 hr and thereafter declined (data not shown).

The total spectrum of metal-induced proteins has been analyzed by SDS-PAGE (*17*). A 14,000-dalton metal-inducible protein (MIP) was synthesized in CHSE cells exposed to various concentrations of $CdCl_2$ and $ZnCl_2$ for 3 days (data not shown). When CHSE cells were grown in media containing 5 μM $CdCl_2$, increased synthesis of MIP was evident after 12 hr (Fig. 5, lane 2). Maximal synthesis appeared to occur at 4 days and thereafter declined towards basal level by 7 days. Similar results were observed when CHSE cells were exposed to 200 μM $ZnCl_2$, although maximal synthesis of MIP occurred within 2 days of induction (data not shown). Further experiments have demonstrated that, like MT, the MIP is heat-stable. The time course for synthesis of MIP following $CdCl_2$ exposure was more clearly presented by analyzing the heat soluble fraction of the cell extracts on SDS–polyacrylamide gels (Fig. 5*B*).

The metal-induced proteins in both cell cultures have been further characterized by gel filtration chromatography. The elution profile for [^{35}S]-cysteine labeled proteins from 10 μM $CdCl_2$-induced and control CHSE cells consisted of three peaks of radioactivity (Fig. 6*A*). The first peak contained high molecular weight proteins eluting in the void volume of the column, while the third peak contained free [^{35}S]-cysteine eluting at the total column volume. The middle peak represented the metal induced proteins eluting with a V_e/V_o of 1.6. Analysis of this peak of radioactivity on an SDS–polyacrylamide gel showed one distinct band which co-migrated with the 14,000-dalton MIP (Fig. 7, lane 4).

The metal-binding ability of CHSE-MIP was studied by incubating unlabeled cell extracts, prepared from 100 μM $ZnCl_2$-induced and uninduced CHSE cells, with $^{109}Cd^{2+}$ (0.2 μCi). When the incubation was carried out at 21°C for 24 hr, a major Cd-binding peak was observed in metal-induced cells that was absent from uninduced cells (Fig. 6*B*). This peak of radioactivity coincided with the [^{35}S]-cysteine peak from the *in vivo* labeling exper-

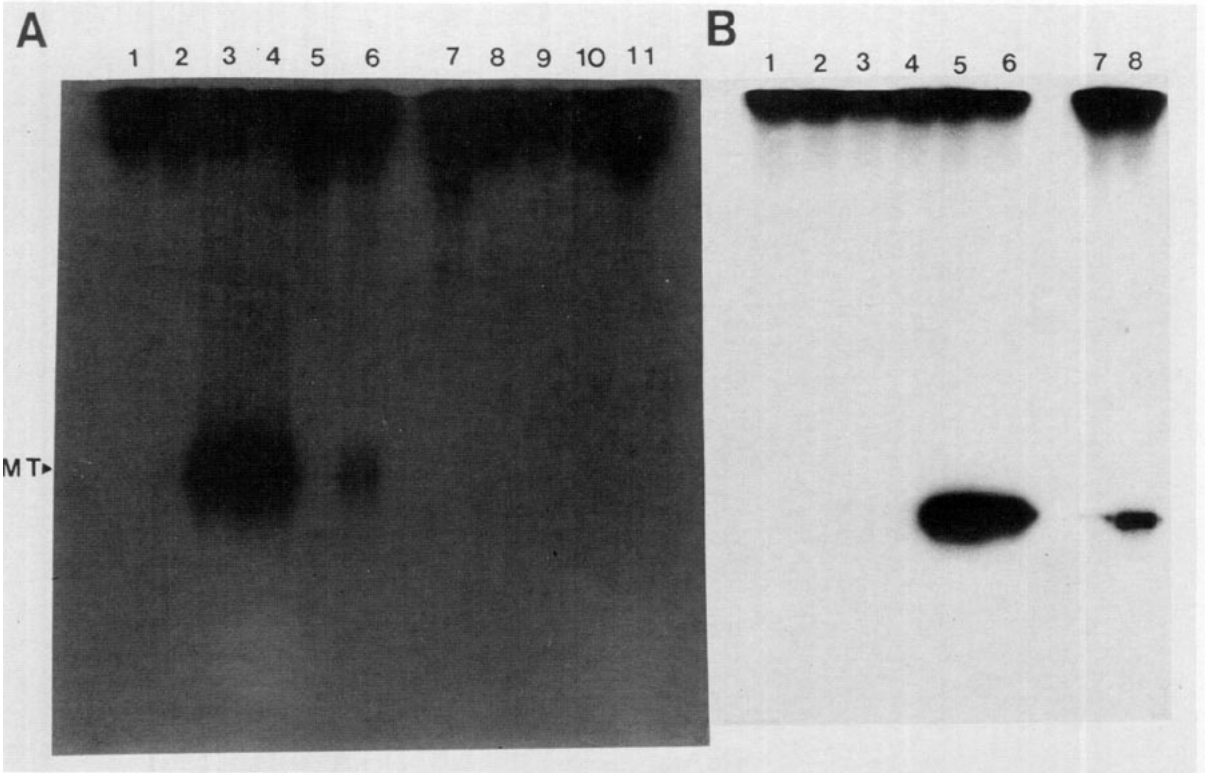

FIGURE 3. Comparison of cellular proteins and cell-free translation products isolated from control, $ZnCl_2$- and $CdCl_2$-treated fish cells. (*A*) CHSE and RTH cells were grown to semiconfluent monolayers (10^6 cells for CHSE or 6.5×10^5 cells for RTH) and exposed to either $ZnCl_2$ (50, 100 or 200 μM) or $CdCl_2$ (5 or 10 μM) for 3 days. Newly synthesized proteins were labeled with [^{35}S]-cysteine (50 μCi/mL) during the final 2 hr of incubation. Following lysis, aliquots containing equal amounts of acid-insoluble radioactivity were reduced and carboxymethylated. The carboxymethylated samples were applied to a 20% polyacrylamide slab gel with a 5% stacking gel. Electrophoresis was carried out in the absence of SDS. The gels with fixed in acetic acid/methanol, dried and fluorographed: (lane 1) RTH control; (lane 2) RTH 50 μM $ZnCl_2$; (lane 3) RTH 100 μM $ZnCl_2$;(lane 4) RTH 200 μM $ZnCl_2$; (lane 5) RTH control; (lane 6) RTH 10 μM $CdCl_2$; (lane 7) CHSE control; (lane 8), CHSE 50 μM $ZnCl_2$; (lane 9) CHSE 100 μM $ZnCl_2$; (lane 10) control CHSE; (lane 11) 5 μM $CdCl_2$. (*B*) CHSE and RTH cells were exposed to 100 μM $ZnCl_2$ for 3 days and total nucleic acids were extracted, 10 μg was translated in the BRL wheat germ cell-free system in the presence of [^{35}S]-cysteine. Translations were terminated by chilling. Aliquots containing equal amounts of acid insoluble radioactivity were carboxymethylated and analyzed by PAGE as described in (*A*). Gels were fixed with acetic acid/methanol, dried and fluorographed: (lanes 1 and 2) CHSE 100 μM $ZnCl_2$; (lanes 3 and 4) RTH control; (lanes 5 and 6) RTH 100 μM $ZnCl_2$; (lane 7) control rainbow trout liver RNA; (lane 8) $CdCl_2$-induced rainbow trout liver RNA.

iment. When the extract from 100 μM $ZnCl_2$-induced cells was incubated with $^{109}Cd^{2+}$ at 60°C for 20 min, no binding of Cd^{2+} to the 14,000-dalton protein was observed. Therefore, although MIP had been shown to be stable to heating at 60°C, the binding of the metal to the protein was not.

The chromatographic profile for [^{35}S]-cysteine-labeled proteins from 10 μM $CdCl_2$ induced RTH cells also contained three peaks of radioactivity (Fig. 6*C*). The second peak represented a metal-induced protein with a V_e/V_o of 1.9, which was characteristic of MTs isolated from various organisms including rainbow trout (*8*). In order to demonstrate the unique elution properties of RTH-MT and CHSE-MIP, both lines were exposed to 100 μM $ZnCl_2$ for 3 days. RTH proteins were labeled *in vivo* with [^{3}H]-serine, while CHSE proteins were labeled with [^{35}S]-cysteine. Radiolabeled proteins were extracted from both cell cultures, mixed, and applied to a Sephadex G-75 column. The induced [^{3}H]-serine peak eluted at a V_e/V_o of 2.0, while the major [^{35}S]-cysteine induced peak eluted at a V_e/V_o of 1.6 (Fig. 6*D*).

Discussion

The rainbow trout, like other animals, responds to injections of $CdCl_2$ by producing MT. Amino acid analysis of the two trout MT isoforms revealed the unusual and unique amino acid composition characteristic of all true metallothioneins. The observation that a mouse MT cDNA will hybridize weakly but significantly to fractions known to contain high levels of MT-mRNA translational activity is further confirmation that the similarity of the MT system from species to species extends even to the nucleic acid sequence level. Unfortunately, the weakness of the hybridization implies the mouse probe is of little value in probing the mechanisms of MT induction in trout.

Thomas and co-workers (*10*) as well as Olsson and Haux (*18*) have shown that two isoforms of rainbow trout MT are induced in response to injections of $CdCl_2$; interestingly, Ley et al. (*9*) found only one form following Zn induction. When Thomas (*10*) studied the response of the fish to environmental Cd, however, the metal appeared to be sequestered by two non-MT-like proteins. Thus,

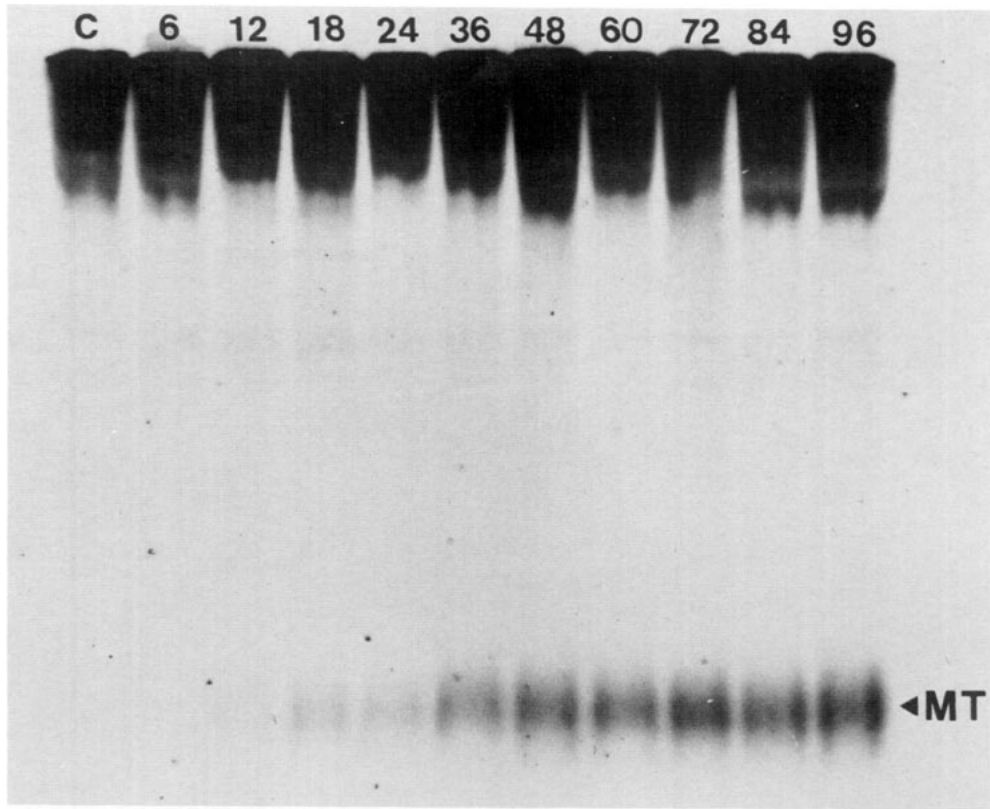

FIGURE 4. Time course of metallothionein protein induction during exposure of RTH cells to $CdCl_2$. RTH cells were exposed to 10 μM $CdCl_2$ for periods of time ranging from 6h to 4 days. [^{35}S]-Cysteine (30 μCi/mL) was added for the final 4 hr of incubation. Cell lysis, electrophoresis, and fluorography were performed as described in the legend to Figure 3 *A*: (lane 1) control; (lane 2) 6 hr of $CdCl_2$ exposure; (lane 3) 12 hr $CdCl_2$); (lane 4) 18 hr $CdCl_2$; (lane 5) 24 hr $CdCl_2$; (lane 6) 36 hr $CdCl_2$; (lane 7) 48 hr. $CdCl_2$; (lane 8) 60 hr $CdCl_2$; (lane 9) 72 hr $CdCl_2$; (lane 10) 84 hr $CdCl_2$; (lane 11) 96 hr $CdCl_2$.

the situation in trout is complicated by the type of metal inducer as well as by its mode of introduction.

The study of heavy metal induced gene expression in fish was extended by investigating the RTH and CHSE cell cultures. Exposure of RTH to both $CdCl_2$ and $ZnCl_2$ resulted in induction of MT and MT-mRNA. However, CHSE cells did not synthesize MT under any experimental conditions employed in this study. In addition, *in vitro* translations failed to detect MT-mRNA activity in any CHSE RNA preparations. To our knowledge, the CHSE cell line is only the third example (*19,20*) of a cell line or organism that fails to produce MT-mRNA in response to heavy metals. It is interesting to note that the CHSE cell line is embryonic in origin. We speculate that the MT genes are in an uncommitted state in the CHSE cells, suggesting developmental regulation of MT genes in fish. The failure of two mouse cell lines to synthesize MT-mRNA in response to metals was correlated with hypermethylation of the MT genes (*19,20*). We are currently investigating the role of methylation in MT-mRNA induction in CHSE cells.

While CHSE cells failed to produce MT, they were observed to synthesize a low molecular weight (14,000-dalton), heat-stable, cadmium-binding protein (MIP) in response to heavy metal treatment. This protein had previously been observed by Heikkila and co-workers (*22*) and was identified as a MT-like protein due to its physical properties. However, in addition to the protein synthesis and translation data mentioned above, the elution properties of CHSE-MIP were shown to be distinct from those of RTH-MT. Although the CHSE-MIP does appear to bind cadmium, it was shown to lose this characteristic when heated to 60°C; this is in contrast to MT which readily binds cadmium under these conditions (*8*).

We suggest that both MT and the CHSE-MIP may function in detoxification of heavy metals. Interestingly, Thomas and co-workers (*10*) have described a 14,000-dalton, non-MT metal-binding protein that is induced in rainbow trout liver following environmental exposure to cadmium. This protein fraction, like MIP, loses its metal binding properties when heated. It seems possible that this protein and the MIP are the same since we have observed an apparently identical protein induced in RTH (results not shown). We are currently purifying the MIP from CHSE and RTH and will test this hypothesis through amino acid analysis comparisons and possibly through antibody cross-reactivity. If the MIP is similar to the environmentally induced protein, it may well be a better indicator of environmental heavy metal toxicity than MT.

Thus, although MT is probably the major product of heavy metal abuse in fish it is important to note that a

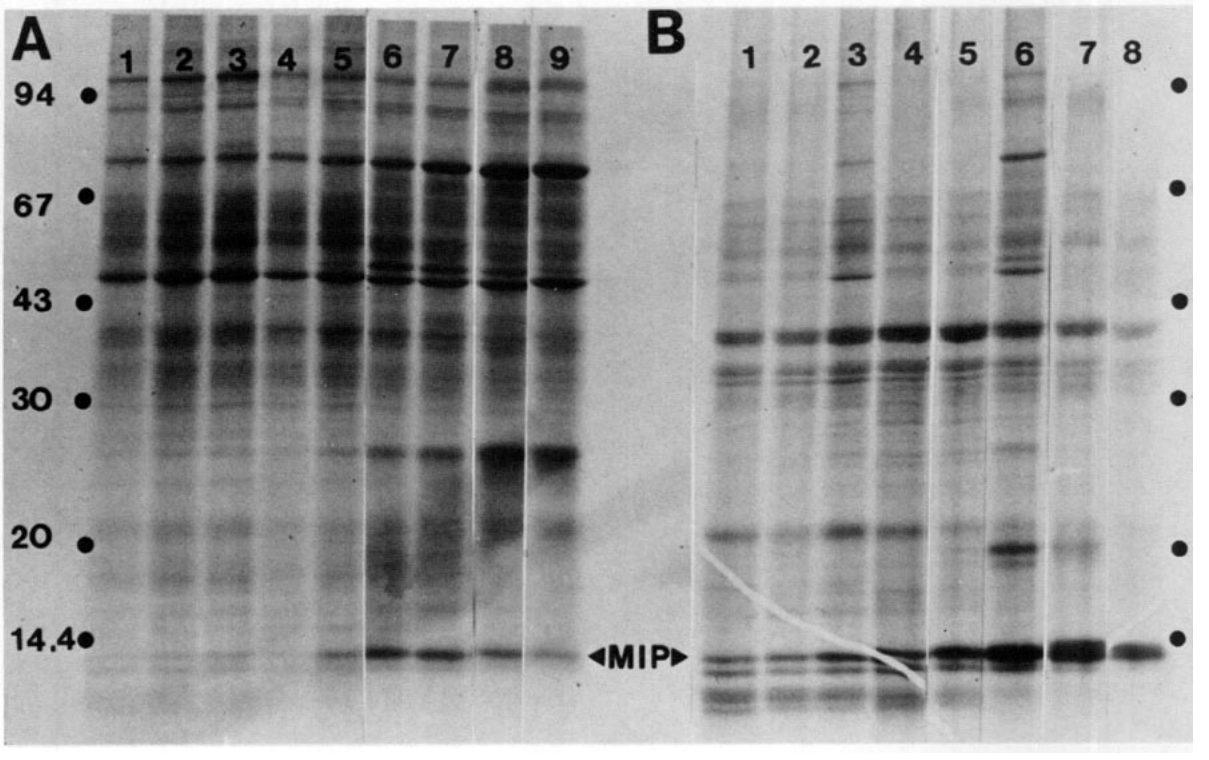

FIGURE 5. Time course of metal-induced protein (MIP) synthesis during exposure of CHSE cells to $CdCl_2$. (*A*) CHSE cells were exposed to 5 μM $CdCl_2$ for periods of time ranging from 12 hr to 7 days. [^{35}S]-cysteine (30 μCi/mL) was added for the final 2 hr of incubation. Aliquots containing equal amounts of acid-precipitable radioactivity were applied to a 7 to 17% polyacrylamide, 0.1% SDS slab gel with a 5 % stacking gel, using the discontinuous buffer system of Laemmli (*17*). Electrophoresis was carried out in the presence of SDS and gels were fluorographed as described in the legend to Figure 3 *A*. Molecular weights of polypeptide markers are expressed in kilodaltons: (lane 1) control; (lane 2) 12 hr of $CdCl_2$ exposure; (lane 3) 1 day $CdCl_2$; (lane 4) 2 days $CdCl_2$; (lane 5) 3 days $CdCl_2$; (lane 6) 4 days $CdCl_2$; (lane 7) 5 days $CdCl_2$; (lane 8) 6 days $CdCl_2$; (lane 9) 7 days $CdCl_2$. (*B*) Samples identical to those analyzed in (*A*) were heated to 60°C for 20 min following cell lysis. Heat-soluble fractions containing equal amounts of acid-precipitable counts were analyzed as described in (*A*). Lanes 1 to 8 are exactly as described in the legend to Figure 5*A*.

variety of other genes may be activated, including MIP. We have previously shown that another set of fish genes, coding for the heat shock or stress proteins, is transcriptionally activated in response to heavy metals (*22,23*). These proteins appear to be produced independently of MT and MIP.

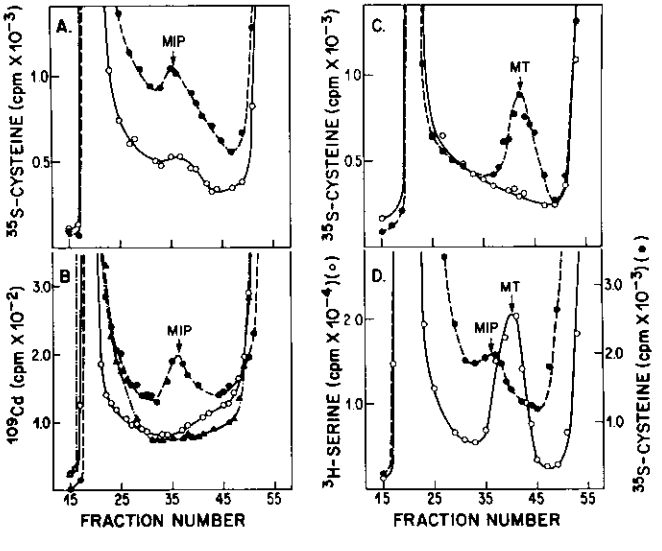

FIGURE 6. Gel filtration chromatography of proteins isolated from control, $CdCl_2$- and $ZnCl_2$-treated fish cells. (*A*) CHSE cells were exposed to 10 μM $CdCl_2$ for 4 days. Proteins were labeled by the addition of [^{35}S]-cysteine (30 μCi/mL) for the final 4 hr of incubation. Samples were applied to a Sephadex G-75 column (2 × 70 cm) equilibrated with elution buffer (50 mM Tris-HCl, pH 8.6, 5 mM β-mercaptoethanol, 0.2% sodium azide) at 4°C; 2-mL fractions were collected, and a portion was analyzed by liquid scintillation counting: (●) $CdCl_2$-exposed CHSE cells; (○) control CHSE cells. (*B*) CHSE cells were exposed to 100 μM $ZnCl_2$ for 4 days and lysed; cell extracts were incubated with $^{109}Cd^{2+}$ (0.2 μCi) for (●) 24 hr at 21°C or (○) for 20 min at 60°C and analyzed by gel filtration on a Sephadex G-75 column as described in (*A*) above; (▲) cell extracts from uninduced CHSE cultures were also incubated for 24 hr at 21°C. Fractions were collected and counted on an LKB Universal gamma counter. (*C*) RTH cells were exposed to 10 μM $CdCl_2$ for 4 days. Newly synthesized proteins were labeled and analyzed by gel filtration chromatography as described in (*A*) above: (●) $CdCl_2$-exposed RTH cells; (○) control RTH cells. (*D*) CHSE and RTH cells were exposed to 100 μM $ZnCl_2$ for 3 days. Newly synthesized proteins in CHSE cultures were labeled with [^{3}H]-serine (30 μCi/mL). Radioactive polypeptides were extracted from both cell cultures, mixed, and applied to a Sephadex G-75 column. Fractions were collected and analyzed on both [^{3}H] and [^{35}S] channels.

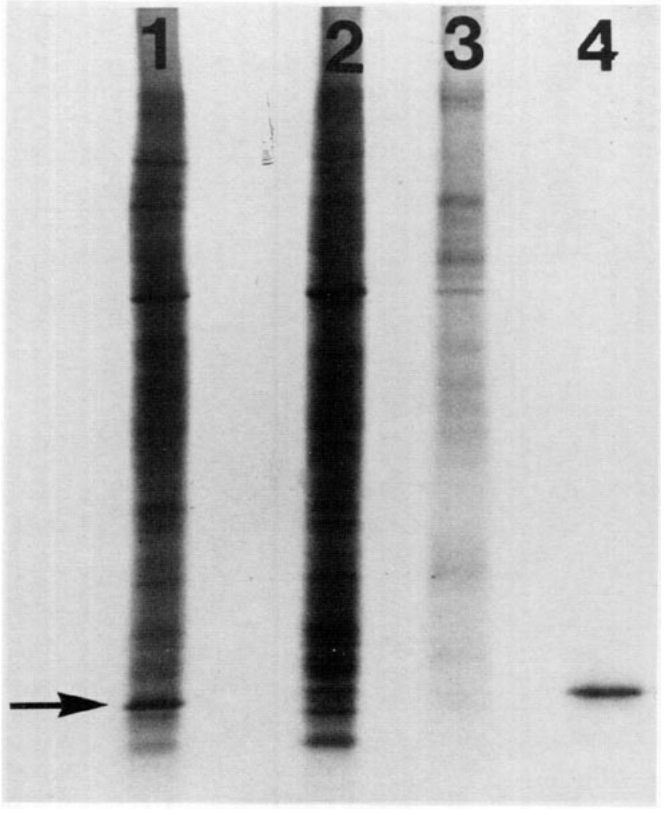

FIGURE 7. Purification of metal-induced protein (MIP) isolated from $CdCl_2$-treated CHSE cells. CHSE cells were exposed to 5 μM $CdCl_2$ for 4 days and newly synthesized proteins were labeled by the addition of [^{35}S]-cysteine (30 μCi/mL) for 2 hr. Cell extracts were analyzed by gel filtration chromatography on a Sephadex G-75 column. Fractions from the first peak, which consisted of high molecular weight proteins, and the second peak, containing the metal-induced protein were separately pooled. Aliquots were analyzed in parallel with total $CdCl_2$-induced and uninduced proteins on a 7 to 17% gradient SDS–polyacrylamide slab gel. Electrophoresis and fluorography were performed as described in the legend to Fig. 5*A*: (lane 1) total cellular proteins from $CdCl_2$-induced CHSE cells; (lane 2) total cellular proteins from uninduced cells; (lane 3) material obtained from the first peak of a G-75 column; (lane 4) material obtained from the second peak of a G-75 column.

To understand further the mechanisms of heavy metal-induced gene expression, our long-term aim is to clone the fish genes of both MT and MIP. At this time we are classifying a number of metal-induced cDNAs obtained from a cadmium-induced trout cDNA library.

This work was supported by grants from the Natural Sciences and Engineering Research Council (NSERC) of Canada and the Alberta Heritage Foundation for Medical Research (AHFMR) to L. Gedamu. Keith Bonham and Janet Price-Haughey are recipients of AHFMR Studentships. We wish to thank Beverly Culham and Dr. N. W. Shworak for their contribution during the preliminary investigation of the metal-induced protein and helpful suggestions for this work.

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