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 CdCl₂ 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 CdCl₂ and ZnCl₂ 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 ¹⁰⁹Cd 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 CdCl₂, 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 ³²P nick-translated mouse MT-I cDNA probe (a gift of Dr. R. Palmiter). The resulting autoradiograph shown in Figure
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-1 from DEAE column; (lane 6), MT-II from DEAE column.

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

| Amino acid | MT-I nearest integer | Rainbow trout | MT-II nearest integer | Mouse | % | % |
|------------|----------------------|---------------|-----------------------|-------|---|---|
| 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     | 14b                  | 22.2          | 18b                   | 28.6  | 20| 32.3 |
| Val        | 2                    | 3.1           | 1                     | 1.6   | 2 | 3.3 |
| Met        | t                    | 1.6           | t                     | t*    | 1 | 1.6 |
| Ile        | t                    | t             | t                     | t     | t | t  |
| Leu        | 1                    | 1.6           | t                     | t     | t | t  |
| Tyr        | t                    | t             | t                     | t     | t | t  |
| Phe        | t                    | t             | t                     | t     | t | t  |
| His        | t                    | t             | t                     | t     | t | t  |
| Arg        | 1                    | 1.6           | 1                     | 1.6   | 1 | 1.6 |
| Trp        | t                    | t             | t                     | t     | t | t  |
| Total      | 63                   | 63            | 61                    | 61    |   |   |

*From Huang et al. (21).

b Determined as cysteic acid.

c Determined as methionine sulfoxide.
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Figure 2. Sucrose gradient centrifugation of total RNA and RNA dot hybridization to a mouse MT-1 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 32P nick translated mouse MT-1 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. Middlogarithmic phase RTH or CHSE cultures were exposed to various concentrations of ZnCl₂ or CdCl₂ for 3 days. Proteins were labeled in vivo with [35S]-cysteine, carboxymethylated, and analyzed by polyacrylamide gel electrophoresis (8) (Fig. 3A). Massive induction of MT synthesis occurred in RTH cells exposed to 100 μM and 200 μM ZnCl₂ (lanes 3 and 4, respectively), and, to a much lesser degree, in cells exposed to 10 μM CdCl₂ (lane 6). Concentrations of metal higher than 10 μM CdCl₂ or 100 μM ZnCl₂ led to appreciable cell death (unpublished observations). CHSE cells did not synthesize MT in response to either ZnCl₂ or CdCl₂ exposure (lanes 7 through 11).

In vitro translation of total nucleic acid (22) isolated from 100 μM ZnCl₂-induced RTH cells revealed a substantial level of MT-mRNA translational activity (Fig. 3B, lanes 5 and 6). Analysis of 10 μM CdCl₂ RNA translates mimicked the protein labeling results (data not shown). RNA extracted from ZnCl₂-treated CHSE cells (lanes 1 and 2) and 10 μM CdCl₂-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 CdCl₂ 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 ZnCl₂ 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 CdCl₂ and ZnCl₂ for 3 days (data not shown). When CHSE cells were grown in media containing 5 μM CdCl₂, 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 ZnCl₂, 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 CdCl₂ exposure was more clearly presented by analyzing the heat soluble fraction of the cell extracts on SDS–polyacrylamide gels (Fig. 5B).

The metal-induced proteins in both cell cultures have been further characterized by gel filtration chromatography. The elution profile for [35S]-cysteine labeled proteins from 10 μM CdCl₂-induced and control CHSE cells consisted of three peaks of radioactivity (Fig. 6A). The first peak contained high molecular weight proteins eluting in the void volume of the column, while the third peak contained free [35S]-cysteine eluting at the total column volume. The middle peak represented the metal induced proteins eluting with a Vₐ/Vₜ 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 ZnCl₂-induced and uninduced CHSE cells, with 109CdCl₂ (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. 6B). This peak of radioactivity coincided with the [35S]-cysteine peak from the in vivo labeling exper-
FIGURE 3. Comparison of cellular proteins and cell-free translation products isolated from control, ZnCl₂- and CdCl₂-treated fish cells. (A) CHSE and RTH cells were grown to semiconfluent monolayers (10⁶ cells for CHSE or 6.5 × 10⁶ cells for RTH) and exposed to either ZnCl₂ (50, 100 or 200 µM) or CdCl₂ (5 or 10 µM) for 3 days. Newly synthesized proteins were labeled with [³⁵S]-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 were fixed in acetic acid/methanol, dried and fluorographed: (lane 1) RTH control; (lane 2) RTH 50 µM ZnCl₂ (lane 3) RTH 100 µM ZnCl₂ (lane 4) RTH 200 µM ZnCl₂ (lane 5) RTH control; (lane 6) RTH 10 µM CdCl₂ (lane 7) CHSE control; (lane 8), CHSE 50 µM ZnCl₂ (lane 9) CHSE 100 µM ZnCl₂ (lane 10) control CHSE; (lane 11) 5 µM CdCl₂ (B) CHSE and RTH cells were exposed to 100 µM ZnCl₂ 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 [³⁵S]-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 ZnCl₂ (lanes 3 and 4) RTH control; (lanes 5 and 6) RTH 100 µM ZnCl₂ (lane 7) control rainbow trout liver RNA; (lane 8) CdCl₂-induced rainbow trout liver RNA.

Discussion

The rainbow trout, like other animals, responds to injections of CdCl₂ 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 proving the mechanisms of MT induction in trout.

Thomas and co-workers (10) as well as Ollson and Haux (18) have shown that two isoforms of rainbow trout MT are induced in response to injections of CdCl₂; 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,
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 CdCl₂ and ZnCl₂ 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
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.

![Gel filtration chromatography of proteins isolated from control, CdCl₂- and ZnCl₂-treated fish cells.](image)

(A) CHSE cells were exposed to 10 µM CdCl₂ for 4 days. Proteins were labeled by the addition of [³⁵S]-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: (●) CdCl₂-exposed CHSE cells; (○) control CHSE cells. (B) CHSE cells were exposed to 100 µM ZnCl₂ for 4 days and lysed; cell extracts were incubated with ¹⁰⁹Cd⁺ (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 CdCl₂ for 4 days. Newly synthesized proteins were labeled and analyzed by gel filtration chromatography as described in (A) above: (●) CdCl₂-exposed RTH cells; (○) control RTH cells. (D) CHSE and RTH cells were exposed to 100 µM ZnCl₂ for 3 days. Newly synthesized proteins in CHSE cultures were labeled with [³⁵S]-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 [³⁵S] and [³⁵S] channels.
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.

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