

# A role of metallothionein in zinc regulation after oestradiol induction of vitellogenin synthesis in rainbow trout, *Salmo gairdneri*

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The regulation of metallothionein (MT) biosynthesis in rainbow-trout liver was studied after a single intraperitoneal injection of oestradiol-17 $\beta$ . Sampling was performed after 2, 7, 14, 21, 28 and 35 days. Following induction of vitellogenin synthesis in the liver, liver somatic index (LSI) rose from 1.25 to 2.00 in 14 days. Associated with the increase in LSI was an elevation of hepatic vitellogenin mRNA and zinc concentrations. The vitellogenin mRNA concentrations peaked at 7 days after treatment. The zinc concentrations increased to a peak at day 14. MT was analysed by using differential pulse polarography and a rainbow-trout MT RNA probe. The MT mRNA concentrations rose after 14 days and remained elevated at 21 and 28 days. The MT concentrations increased after 14 days and remained elevated throughout the experimental period. The concentrations of MT-bound zinc increased in association with the elevation in MT concentrations in the oestradiol-treated rainbow trout. These findings indicate that MT is involved in the regulation of zinc during the period of vitellogenin induction and that MT may function by maintaining the pool of available zinc at an appropriate concentration.

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## INTRODUCTION

Metallothionein (MT) was first discovered as a cadmium- and zinc-binding protein in horse renal cortex (Margoshes & Vallee, 1957). The existence of MT has since been demonstrated in a wide variety of organisms and organs (Hamer, 1986). The function of MT is uncertain, but it has been suggested that MT may play a role in zinc and copper metabolism and in the detoxification of group IB and IIB metal ions (Webb, 1979). These functions are indicated by the observations that heavy-metal ions both bind to MT and induce MT synthesis. MT gene transcription can also be induced in some mammalian cell types by other factors, such as glucocorticoids, interferon and stress conditions (Oh *et al.*, 1978; Karin *et al.*, 1980; Friedman *et al.*, 1984).

Zinc is required for the catalytic properties of many enzymes (Vallee & Wacker, 1970). In addition, zinc is also required for the stability of membranes and polyribosomes, for many components of the protein-synthesizing process, and possibly for the allosteric regulation of some enzymes (Chvapil, 1973; Sandstead, 1975). It has been proposed that MT may be a storage protein for zinc (Chen *et al.*, 1974) and that MT serves as a temporary storage protein for zinc and copper during early development in neonatal mammals (Panemangalore *et al.*, 1983). Further, MT appear to be involved in zinc regulation during periods of thymic growth in mice (Olafson, 1985). Together these studies suggest an involvement of MT in zinc regulation during periods of growth and development.

The period of exogenous vitellogenesis is characterized

by large metabolic changes in female fish. There is a marked increase in plasma concentrations of oestradiol-17 $\beta$  and testosterone, which is accompanied by hypertrophy of the liver and the gonads (van Bohemen *et al.*, 1981). Administration of oestradiol to juvenile fish results in the appearance of vitellogenin in the plasma (DeVlaming *et al.*, 1980). In a recent study hepatic MT concentrations were shown to rise in female rainbow trout during exogenous vitellogenesis (Olsson *et al.*, 1987). The rise in metallothionein concentration occurred at the time of ovulation, when the production of vitellogenin in the liver had ceased. It occurred concomitantly with a redistribution of zinc from the nuclear and mitochondrial fractions to the cytosolic fractions. These results suggested that MT is involved in the intracellular regulation of zinc during the period of exogenous vitellogenesis. In the present study we have induced vitellogenin synthesis by giving juvenile rainbow trout intraperitoneal injections of oestradiol-17 $\beta$ .

The objective of the study was to determine the time course for the appearance of hepatic MT mRNA after the induction of vitellogenin synthesis, and to investigate the relationship between MT synthesis and hepatic zinc and vitellogenin mRNA concentrations.

## MATERIALS AND METHODS

### Fish holding conditions and sampling

Juvenile rainbow trout, *Salmo gairdneri*, about 1 year old and with a body weight of about 100 g, were obtained from a local fish hatchery (Antens Fiskodling AB,

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Abbreviations used: MT, metallothionein; LSI, liver somatic index.

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Göteborg, Sweden). The fish were acclimated in the laboratory for 1 week in basins with filtered, aerated and recirculating tap-water at a temperature of 8 °C. The fish were transferred to two 1 m<sup>3</sup> aquaria at the start of the experiment. Fifty fish were kept in each aquarium, which received filtered and aerated tap-water at a temperature of 8 °C. The experiment was started by giving each fish one intraperitoneal injection of oestradiol-17 $\beta$  (Sigma Chemical Co.), which was finely dispersed in peanut oil in an ultrasonic bath. The controls received peanut oil only. The dose was 10 mg of oestradiol/kg body wt. in a volume of 0.2 ml. The fish were sampled 2, 7, 14, 21, 28 and 35 days after the injection. The fish were not fed during the experiment.

At sampling, the fish were stunned with a blow to the head and weighed. The liver of each fish was excised and weighed. The liver somatic index (LSI) was calculated as the percentage of the total body weight (liver weight  $\times$  100/body weight).

#### MT determination and metal analysis

A 200 mg sample of each liver was homogenized in 1.0 ml of ice-cold 10 mM-Tris/HCl buffer, pH 7.6, and divided into four 0.25 ml aliquots, of which two were used for MT determination and the remaining two were prepared for heavy-metal analysis.

MT was determined by differential pulse polarography at 20 °C (PARC model 174 analyser, PARC/EG & G model 303 SMDE) by using a modification of the Brdicka (1933) procedure as previously described (Olsson *et al.*, 1987). The specificity of the polarographic technique for determination of MT has been ascertained by Sephadex G-75 and Mono-Q column chromatography of heat-denatured material (Olsson, 1987).

The analysis of copper and zinc by air/acetylene-flame atomic absorption spectrophotometry was performed as described by Olsson *et al.* (1987).

#### Chromatography

Liver samples from two or three fish were pooled and homogenized in ice-cold 10 mM-Tris/HCl buffer, pH 8.0, in a Teflon/glass homogenizer. The homogenates were centrifuged at 20000 *g* for 30 min at 4 °C. The supernatant was subsequently chromatographed on a Sephadex G-75 column (1.5 cm  $\times$  50 cm) equilibrated with the same buffer. Fractions (5 ml) were collected and copper and zinc contents were determined in each fraction by atomic absorption spectrophotometry.

#### Isolation of total RNA

For the measurement of metallothionein and vitellogenin mRNA concentrations, total RNA was extracted according to the method of Auffrey & Rougeon (1980) with few modifications. About 100 mg of liver samples was homogenized at 4 °C in 2 ml of extraction solution consisting of 6 M-urea/3 M-LiCl. The homogenates were kept overnight at 4 °C for precipitation of RNA and centrifuged at 10000 *g* for 15 min at 4 °C. The RNA pellets were resuspended in 500  $\mu$ l of 100 mM-sodium acetate buffer, pH 5.2, extracted several times with phenol at 60 °C, precipitated with ethanol and quantified by measuring absorbance at 260 nm.

#### Northern-blot and slot-blot analysis

For Northern-blot analysis, 10  $\mu$ g of total RNA was subjected to electrophoresis with the use of the glyoxal

method (Thomas, 1980) and transferred to diazobenzoyloxymethyl-paper (Transabind; Schleicher and Schuell) by the procedure of Alwine *et al.* (1979). Slot-blot analysis was performed to quantify MT and vitellogenin mRNA. Nitrocellulose (Schleicher and Schuell) filters were soaked in water for 15 min followed by 30 min in 10  $\times$  SSC (1  $\times$  SSC is 0.15 M-NaCl/15 mM-sodium citrate buffer, pH 7.0). RNA samples (10  $\mu$ g) were prepared by glyoxylation (Thomas, 1983) and applied in duplicate to the filters. The slots were then washed twice with 3  $\times$  SSC and the filters were allowed to dry before being baked for 2 h in a vacuum oven at 80 °C.

Single-stranded RNA probes corresponding to the coding regions of rainbow-trout metallothionein B (Bonham *et al.*, 1987) and rainbow-trout tubulin (a gift from Dr. G. H. Dixon, University of Calgary) cDNAs were used for hybridizations. These probes were synthesized by using [ $\alpha$ -<sup>32</sup>P]CTP (Amersham) according to the protocols provided by Promega Biotec. In the case of vitellogenin cDNA (a gift from Dr. M. P. R. Tenniswood, University of Ottawa) DNA fragments were labelled with [ $\alpha$ -<sup>32</sup>P]dCTP by using the random hexanucleotide primer method of Feinberg & Vogelstein (1983). Filter hybridization and post-hybridization washes were performed at 68 °C by the procedure of Varshney & Gedamu (1984). The final washing was carried out in 0.1  $\times$  SET (1  $\times$  SET is 0.15 M-NaCl/1 mM-EDTA/30 mM-Tris/HCl buffer, pH 7.8) at 68 °C. The filters were autoradiographed by using Kodak XAR5 film with intensifying screen (Dupont Cronex) at -80 °C. Hybridization intensity of the bands was measured by densitometer scanning (Gelman DcD16), and statistical analysis of the data was performed by the Mann-Whitney *U* test (Siegel, 1956). Significant correlations were established at the *P* < 0.05 level.

## RESULTS

The effect of the oestradiol injections on liver hypertrophy was monitored by recording the LSI (Table 1). The LSI increased to a maximum of about 2.0 at day 14. Statistically significant elevations in LSI were observed at days 7, 14 and 21. The LSI returned to control levels after 28 days and remained low up to 35 days after treatment.

Accumulation of MT and vitellogenin mRNA was analysed by hybridizing total RNA with respective homologous single-stranded RNA and cDNA probe. The concentration of each transcript was monitored by densitometer scanning of autoradiograms of slot blots. There was no increase in total RNA or tubulin mRNA concentrations in response to the oestradiol treatment. The variations in RNA amounts applied were therefore corrected by hybridizing the filters with a trout tubulin anti-sense RNA probe as an internal standard. The specificities of the probes were ascertained by Northern-blot analysis. There were no detectable amounts of vitellogenin mRNA in the control animals. In the induced fish, vitellogenin mRNA was detectable at all sampling times and the maximum concentration was obtained at day 7 (Fig. 1).

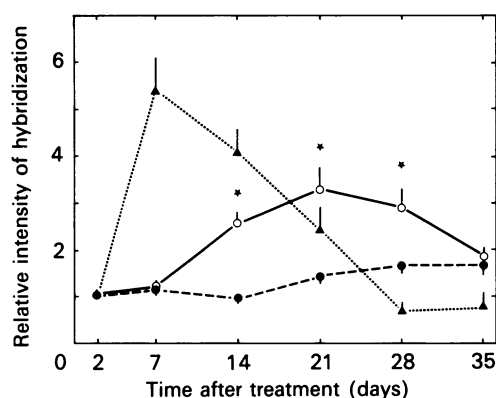
The zinc, copper and MT concentrations per g of liver are presented in Table 1. The massive production of vitellogenin following oestradiol treatment led to increased LSI. In order to correct for this change in LSI,

**Table 1. LSI and liver zinc, copper and MT concentrations of control and oestradiol-treated fish**

For experimental details see the text. Values are given as means  $\pm$  S.E.M. ( $n = 6$ ).

Time after treatment (days)	LSI		Zinc concn. ( $\mu\text{g/g}$ )		Copper concn. ( $\mu\text{g/g}$ )		MT concn. (nmol/g)	
	Control	Oestradiol-treated	Control	Oestradiol-treated	Control	Oestradiol-treated	Control	Oestradiol-treated
2	1.21 $\pm$ 0.06	1.33 $\pm$ 0.03	22.0 $\pm$ 0.09	29.1 $\pm$ 3.1	125 $\pm$ 22	128 $\pm$ 15	23.4 $\pm$ 4.6	18.9 $\pm$ 4.3
7	1.33 $\pm$ 0.09	1.88 $\pm$ 0.08*	28.5 $\pm$ 3.1	22.2 $\pm$ 1.3	108 $\pm$ 5	95 $\pm$ 10*	18.6 $\pm$ 3.2	26.3 $\pm$ 4.7
14	1.31 $\pm$ 0.07	2.05 $\pm$ 0.14*	20.9 $\pm$ 1.5	25.0 $\pm$ 0.9	107 $\pm$ 8	87 $\pm$ 10*	23.4 $\pm$ 8.8	50.7 $\pm$ 11.6
21	1.09 $\pm$ 0.05	1.37 $\pm$ 0.07*	23.1 $\pm$ 1.2	29.6 $\pm$ 1.8	139 $\pm$ 11	130 $\pm$ 16	21.8 $\pm$ 9.1	32.7 $\pm$ 5.1
28	1.14 $\pm$ 0.05	1.14 $\pm$ 0.11	22.3 $\pm$ 1.2	26.5 $\pm$ 1.7	153 $\pm$ 19	164 $\pm$ 30	26.7 $\pm$ 4.1	68.1 $\pm$ 7.9*
35	1.12 $\pm$ 0.05	1.09 $\pm$ 0.07	20.3 $\pm$ 1.7	24.4 $\pm$ 1.0	160 $\pm$ 22	179 $\pm$ 10	33.7 $\pm$ 7.1	75.0 $\pm$ 6.5*

\* Statistical significant difference at the  $P < 0.05$  level (Mann-Whitney  $U$  test).

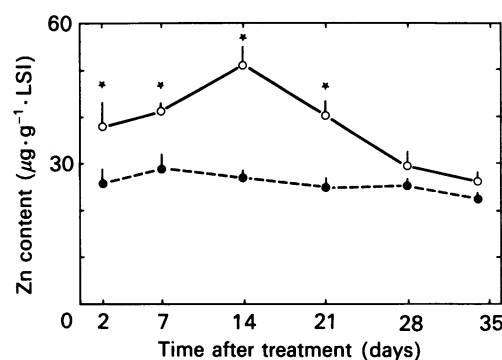


**Fig. 1. Detection of vitellogenin mRNA and MT mRNA in rainbow-trout liver**

Duplicate samples were analysed by slot-blot hybridization on nitrocellulose filters by using  $^{32}\text{P}$ -labelled rainbow-trout vitellogenin cDNA and MT-B anti-sense RNA probes. The results are presented as relative amounts of vitellogenin mRNA ( $\blacktriangle$ ) and MT mRNA ( $\bullet$ , controls;  $\circ$ , oestradiol-treated) (means  $\pm$  S.E.M.,  $n = 6$ ) and were corrected for variations in applied amount of RNA by comparing the band intensities after hybridization with  $^{32}\text{P}$ -labelled rainbow-trout tubulin anti-sense RNA probe. Vitellogenin mRNA controls are not shown since no vitellogenin mRNA could be detected in the control samples. \*Statistical significant difference at the  $P < 0.05$  level (Mann-Whitney  $U$  test).

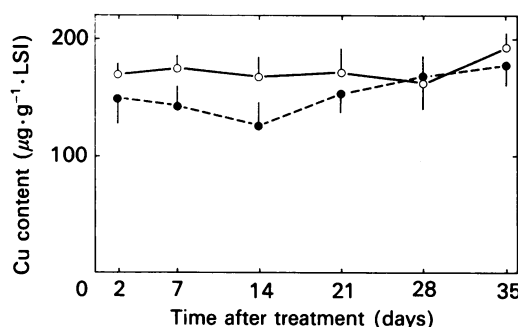
both the metal and the MT concentrations were determined per total liver. When calculated per g of liver  $\times$  LSI the zinc content increased in the oestradiol-treated fish (Fig. 2). The zinc concentrations were elevated in a statistically significant manner compared with the controls at 2 days, continued to increase until day 14 and fell at day 21. The control concentrations were  $25 \mu\text{g}$  of  $\text{Zn} \times (\text{g of liver})^{-1} \times \text{LSI}$  and maximum concentrations of  $50 \mu\text{g}$  of  $\text{Zn} \times (\text{g of liver})^{-1} \times \text{LSI}$  were reached after 14 days in the oestradiol-treated fish. There were no statistically significant differences in copper concentrations between oestradiol-treated and control fish when calculated on the basis per g of liver  $\times$  LSI (Fig. 3).

There was a 2–3-fold increase in hepatic MT mRNA



**Fig. 2. Hepatic zinc concentrations in control ( $\bullet$ ) and oestradiol- $17\beta$ -treated ( $\circ$ ) juvenile rainbow trout**

The results are presented as means  $\pm$  S.E.M. ( $n = 6$ ). \*Statistical significant difference at the  $P < 0.05$  level (Mann-Whitney  $U$  test).



**Fig. 3. Hepatic copper concentrations in control ( $\bullet$ ) and oestradiol- $17\beta$ -treated ( $\circ$ ) juvenile rainbow trout**

The results are presented as means  $\pm$  S.E.M. ( $n = 6$ ).

concentrations after 14 days in oestradiol-induced fish (Fig. 1). The MT mRNA concentrations remained elevated at 21 and 28 days after oestradiol treatment. The hepatic MT concentrations of the oestradiol-injected rainbow trout were elevated above the controls after 14 days (Fig. 4).

Column chromatography revealed that the hepatic MT was a Cu-thionein in control trout. More than 95% of the metal found in the MT peak of control fish was

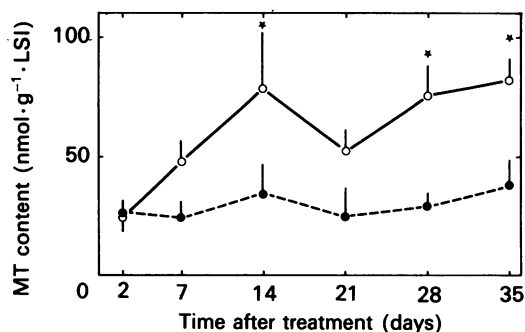


Fig. 4. Hepatic MT concentrations in control (●) and oestradiol-17 $\beta$ -treated (○) juvenile rainbow trout

The results are presented as means  $\pm$  S.E.M. ( $n = 6$ ). \*Statistical significant difference at the  $P < 0.05$  level (Mann-Whitney  $U$  test).

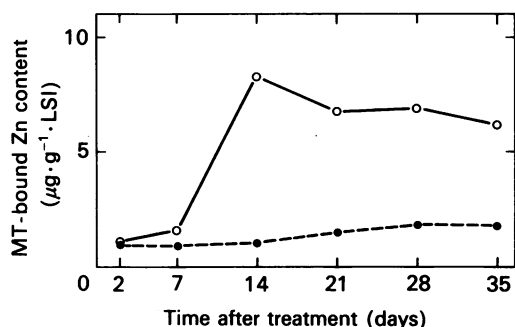


Fig. 5. Zinc concentrations in the MT peak after Sephadex G-75 chromatography of livers from control (●) and oestradiol-17 $\beta$ -treated (○) juvenile rainbow trout

The results are presented as mean of two or three chromatograms performed on pooled (two or three) liver samples.

copper. Less than 5% of the total hepatic zinc was found in the MT peak of these fish. However, 14 days after the oestradiol treatment 10% of the zinc was found in the MT peak and after 35 days 23% of the total zinc was found in the MT peak. No differences in copper distribution was observed between the two groups during the experimental period. From the chromatographic analysis, the amount of zinc in MT was calculated and corrected for changes in liver size. The results are presented as  $\mu\text{g}$  of MT-bound Zn  $\times$  LSI (Fig. 5). It is noteworthy that the zinc concentrations in the MT peak increase abruptly in association with the induction of MT.

## DISCUSSION

The results of the present study suggest that the role of MT during vitellogenin synthesis in the liver of rainbow trout is to maintain homeostasis of hepatic zinc concentrations.

Zinc has been implicated as an essential element in DNA, RNA and protein synthesis (Sandstead, 1975). In a previous study it was demonstrated that the hepatic zinc concentrations increased during the onset of exogenous vitellogenesis (Olsson *et al.*, 1987). This suggests that zinc is necessary for the increased activity in

the liver during the period of exogenous vitellogenesis. During this period the fish undergo large metabolic changes, and there is a marked increase in plasma steroids that is accompanied by hypertrophy of the liver and gonads (van Bohemen *et al.*, 1981). Large quantities of vitellogenin are produced in the liver when the plasma concentrations of oestradiol-17 $\beta$  increase during the onset of exogenous vitellogenesis (Haux & Norberg, 1984).

It has previously been shown that the hepatic zinc concentrations decrease at the end of the period of exogenous vitellogenesis and that this is followed by an increase in hepatic MT concentrations (Olsson *et al.*, 1987). The increase in MT concentrations of the livers correlated to a period of zinc redistribution from the microsomal and mitochondrial fractions of the liver to the cytosolic fraction (Olsson *et al.*, 1987). In the present study it was observed that the increase in MT concentrations was associated with a redistribution of zinc from high- $M_r$  proteins to MT. Whereas the increase in vitellogenin mRNA is a direct effect of oestradiol treatment of the fish, the rise in hepatic MT mRNA concentrations comes from a secondary effect of the oestradiol treatment. The increase in MT mRNA concentrations occurs in connection with lowered metabolic activity in the liver. This lowered activity is manifested by a decrease in LSI as well as a decrease in vitellogenin mRNA and zinc concentrations. These results demonstrate that MT synthesis is induced after the cessation of vitellogenin synthesis in the liver of rainbow trout. At this time, 14 days after treatment, a massive increase of zinc in MT was observed. It is therefore likely that the function of MT during this phase is to control the zinc homeostasis of the liver.

Administration of oestrogen, to mammals, has been known for many years to produce an increased plasma concentration of copper, mainly through the induction of caeruloplasmin (Henkin, 1980). It has been reported that increases in renal tissue copper occur following oestrogen treatment of rats (Henkin, 1980). This suggests that some redistribution of copper occurs following the administration of exogenous oestrogen. Oestrogens have been shown to increase zinc in the liver and erythrocytes and to increase copper in plasma, liver and tibia of mammals (McBean *et al.*, 1971; Lei *et al.*, 1976). The copper concentrations are kept at a constant level throughout the period of exogenous vitellogenesis (Olsson *et al.*, 1987). This was also the case in the present study, where the copper concentrations were found to be stable throughout the experiment, when calculated per g of liver  $\times$  LSI.

In summary, we suggest that the function of MT during the period of exogenous vitellogenesis is to maintain homeostasis of hepatic zinc. The induction of vitellogenin mRNA synthesis by oestradiol-17 $\beta$  is followed by an elevation of the total hepatic zinc concentrations. This increase is necessary to maintain a high activity of protein synthesis. When the metabolic activity decreases, as seen by lowered LSI in this experiment, the need for zinc as a cofactor of many enzymes is decreased and this leads to a redistribution of the metal within the liver. Zinc is a potent inducer of MT, and a redistribution of zinc from enzymes to MT is one way to maintain low free intracellular zinc concentrations. The induction of MT mRNA and MT thus constitutes an important factor in zinc regulation during

the period of exogenous vitellogenesis in rainbow trout.

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