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ONTOGENICALLY REGULATED EXPRESSION OF METALLOTHIONEIN AND ITS MESSENGER RNA IN CHICK LIVER

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Summary: By electrophoretic and immunological assay the concentration of hepatic metallothionein in new born chick liver was found to be ontogenically modulated, reaching a peak accumulation per gram liver in fourth day of hatching and declining below the detection limit after second week postnatal. The protein was undetectable upto second week of incubation in egg-embryonic stage. The concentration of metallothionein mRNA shows drastic change during first few days after hatching. The greatest accumulation of metallothionein mRNA was detected in the one day new born chicks, which declined rapidly there after, and reduced to a barely detectable level. Metallothionein was also detected in the *in vitro* translated product of one day neonatal chick hepatic poly(A)$^+$ RNA by ^{35}S-cysteine labelling and immunoprecipitation. The naturally occuring new-born chick liver metallothionein was found to be a zinc-metallothionein and the concentration of hepatic zinc in new-born chick was found to undergo drastic modulation during development, unlike some other chick tissues. Endogenous zinc ion mobilization can thus play a significant role in the developmental regulation of chick metallothionein expression.

Introduction: Changes in gene expression are frequently associated with development and differentiation. Metallothioneins are generally considered as house keeping proteins because a low level of these proteins is expressed in a variety of tissues (1). Nevertheless, the pattern of metallothionein (MT) gene expression is fairly complex and subject to many hormonal, physiological and environmental stimuli (1,2). Recently, both qualitative as well as quantitative variation in the expression of MT genes during development has been observed (3-7). Although MT occurs ubiquitously among eukaryotes, the status of MT gene expression in young animals from non-mammalian species has not been investigated so far. The exact biological role of MT is still not known (8) but association of MT with rapid growth and proliferation in both transformed or tumor cells (9) as well as in regenerating liver (10)

Abbreviations Used: ABTS, 2,2'-azino-di-(3-35hylbenzthiazoline sulphonate); BSA, Bovine serum albumin; ELISA, Enzyme linked immunosorbent assay; MT, Metallothionein; PAGE, Polyacrylamide gel electrophoresis; SDS, Sodium dodecyl sulphate.

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has been pointed out. In this perspective the study of MT gene expression in rapidly growing young animals like chicken is of interest. Moreover, the chicken contains only a single form of MT in both heavy metal induced state and in new born liver (11) in contrast to multiple forms of MT in mammals (12), which provides a simple model for this kind of study. Hence, this study was conducted to ascertain the status of MT gene expression in chick liver at both protein and mRNA level and to examine its relationship with the endogenous heavy metal concentration.

Materials and Methods: Fertilized eggs of different days of incubation and the newborn white leghorn chicken of different ages were obtained from the State Poultry Farm, Tollygounge, Govt. of West Bengal. All the chemicals used were of analytical grade and obtained from Sigma, USA. The reticulocyte lysate translation mixture (N-90), ^{35}S-cysteine and (α ^{32}P)-dCTP were obtained from Amersham. The DNase I, DNA polymerase and restriction enzymes were purchased from BRL. The reagents for ELISA was obtained from Amersham, U.K.

SDS-PAGE and Silver Staining: The total cytosolic proteins of developing chick livers (20,000 x g supernatant) were carboxymethylated with 150 mM iodoacetate (13) and analyzed on 20% SDS-PAGE according to the method of Laemmli et al., (14) with minor modification (15). The gels were silver stained to detect MT (16). The proteins were measured by Bradford's dye binding assay (17) using BSA as standard.

Antibody Production and Immunological Assay: Hepatic MT was purified from cadmium injected adult chicken as described earlier (11). The immunization procedure was adopted from Granger and Lazarides (18). The antibody titre in immunized sera was tested by Ouchterlony double diffusion (11,19) as well as by enzyme linked immunosorbent assay (ELISA) using horse radish peroxidase linked protein A conjugate and ABTS. The concentrations of MT in tissues were obtained by competitive inhibition assay (20). The MT from developing chick liver and other tissues for immunological detection and estimation was purified by heat treatment and acetone-precipitation (11). The metal estimation and analysis was performed in 6(N) nitric acid digested tissue or protein (120°C, 20 hrs.) using Varian AA575 atomic absorption spectrophotometers.

Isolation of RNA: Total nucleic acids were isolated from hepatic tissues by proteinase K/SDS digestion followed by phenol, chloroform, isoamyl alcohol (50:50:1) extraction and ethanol precipitation and RNA was precipitated selectively by 2 M LiCl (21). The poly(A)$^+$RNA was isolated by using oligo-dT cellulose column (22).

Northern-blot Analysis: Poly(A)$^+$ RNA (10 μg) was electrophoresed in 2%-Agarose, formaldehyde gel and blotted to nitrocellulose (23). Nick translated PstI-BglII fragment of mouse MT-I cDNA cloned in pBR322 (24) was used as a probe for hybridization (25).

Translation of RNA and Immunoprecipitation: The poly(A)$^+$ RNA from one day neonatal and cadmium-induced chicken liver were translated in nuclease treated rabbit reticulocyte lysate according to manufacturers protocol using ^{35}S-cysteine. Immunoprecipitation was carried out (26) using chicken anti-MT sera (IgG fraction).

Results:

Developmental Regulation of Hepatic MT in Chick Liver: The silver stained SDS-PAGE of the unfractionated cytoplasmic proteins from developing chick

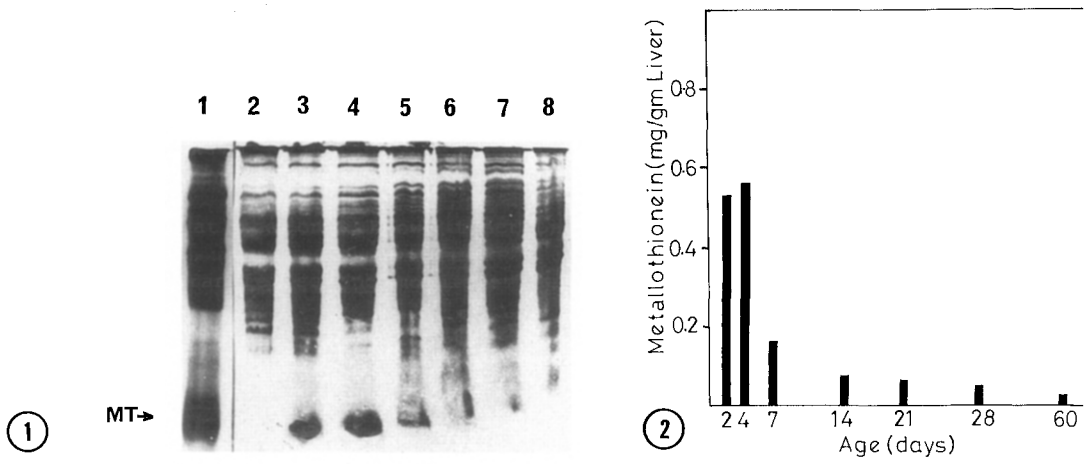

Fig.1. SDS-PAGE of developing chick hepatic cytosolic proteins. Cytosolic proteins (200 µg) from developing chick liver homogenized in 10 mM Tris-HCl pH 8.6 (10% w/v, 20,000 g) were carboxymethylated (13) and analyzed in 20% SDS-PAGE (15) and silver-stained (16). Samples were from heavy metal induced adult (Lane 1); 14 day embryonic liver (Lane 2); 2,4,8,14, 3 week and 4 week old neonatal chick liver (Lane 3-8) respectively. The position of MT (Rf 0.8) is marked which correspond to the position of purified chicken MT run in this system.

Fig.2. Concentration of MT per gram chick liver as obtained from ELISA using monospecific chicken anti MT sera (Ig fraction) and horse-radish-peroxidase linked protein-A conjugate and ABTS as substrate (Amersham). Microtiter plates were coated with 20 µg/ml MT overnight a 4 C in Tris-HCl (pH 9.5). Non-specific absorption was blocked with 1% BSA in PBS and anti MT antibodies diluted to 1:1,000 in PBS was allowed to bind with the coated antigen at 37° C for 1 hr. After through washing proteinA-enzyme conjugate at 1:10,000 dilution was allowed to hybridized at 37° C for 1 hr. After washing ABTS was applied in each well (1 mM, 100 µl) as substrate. The colour development was monitored at 410 nm. All washings were done with PBS-Tween-20. From competitive inhibition curve the concentration of MT in the livers at different developmental period were calculated (20).

liver couldbe directly analyzed to detect any ontogenic change in MT concentration (Fig.1). In this gel system, metallothionein separates well from all other hepatic proteins as a fast migrating band of Rf value 0.8 and aids such detection (15). High amounts of MT were detected in two and four day old new born chick liver (Fig.1, Lane 3 & 4) and this protein was found to decrease sharply after eight days relative to other hepatic proteins (Fig.1, Lane 5-8). In 4 week old chick liver as well as in 14 day embryo before hatching, MT was undetectable (Fig.1, Lane 2 & 8). The enzyme linked immunosorbant assay (ELISA) was used to calculate the amount of MT in developing chick liver. The result of the estimation of MT in developing chick liver by competitive binding assay is shown in Fig.2. The four day old chick liver shows greatest accumulation of MT per gram liver.

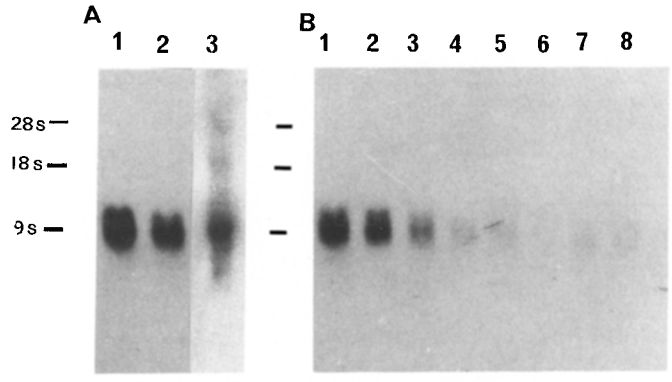

Fig.3. Northern analysis for the detection of MT mRNA separated in 2% Agarose formaldehyde gel (23,25). (A) poly(A)$^+$ RNA (10 µg) from Cd induced mouse and chick liver were electrophoresed in Lane 1 and Lane 2 respectively. In Lane 3 20 ug chick liver total RNA was loaded. After transfer to the nitrocellulose the blots were hybridized with mouse MT-I cDNA probe (5 x 10^6 cpm). The Lane 3 was exposed for a longer period for detection. Marker rRNA band positions were visualized in Agarose gel by acridine organge staining. (B) The poly(A)$^+$ RNA (10 µg) from 1, 2, 4, 7, 14, 21, 28 and 35 days old chicks (Lane 1-8) respectively was electrophoresed, transferred to nitrocellulose and hybridized with 10^7 cpm of mouse MT-I cDNA. Prehybridization and hybridization were done at 37°C 40% formamide (25) for 1 and 20 hours respectively followed by washing with 2 x SSC/0.1% SDS at room temperature for 30 min. and twice more with 0.2 x SSC/0.1% SDS at 50°C (15 min). Filters were dried and exposed with X-ray films (Kodak) at -70°C in presence of intensifying screen for 7 days.

Change in MT mRNA Concentration in Developing Chick Liver: Poly(A)$^+$ RNA was isolated from the developing chicken liver at different stages. The nick translated mouse MT probe was found to cross-hybridize with both mouse and chicken MT mRNA under fairly stringent condition (37° C, 40% formamide) in northern blot to produce a single band, of about 9S in size in 2% agarose-formaldehyde gel (Fig.3A). For the measurement of the relative abundance of MT mRNA in developing chick liver, poly(A)$^+$ RNA from these stages were subjected to northern analysis. One day old chick liver shows maximum accumulation of MT mRNA, which decreased on 2nd and 4th day and from 7th day onward became very low (Fig.3B).

Translation of poly(A)$^+$ RNA from Neonatal and Cadmium Induced Chicken Liver: Since MTs are highly rich in cysteine residues (30%) the poly(A)$^+$ RNA from developing chicken liver can be tested for its ability to stimulate the synthesis of MT in in vitro translation system in presence of ^{35}S-cysteine. The poly(A)$^+$ RNA (10 µg) from one day old newborn chick liver and cadmium treated chickens were translated in vitro. The translation product was immunoprecipitated using chicken MT antisera and also with preimmune sera as control. The total translation product as well as the immunopre-

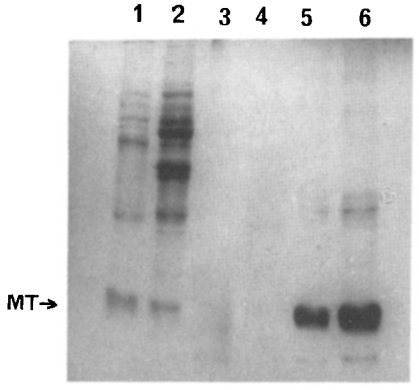

Fig.4. In vitro translated product of poly(A)$^+$RNA (10 µg) from cadmium induced chick liver (Lane 1) and one day neonatal chick liver (Lane 2) labelled with ^{35}S-cysteine and electrophoresed in 20% SDS-PAGE (15). The lane 5 and 6 show the immunoprecipitated translated product corresponding to lane 1 and 2 respectively with the chick anti MT antisera. The lane 3 and 4 show the controls with preimmune serum from the same rabbits applied on cadmium induced and neonatal poly(A)$^+$RNA translation product. The position of the MT in this gel system obtained from paralelly runs with cold purified chicken MT is marked.

cipitates were carboxymethylated and analyzed on 20% SDS-PAGE followed by fluorography (Fig.4). The presence of MT was easily detected in the translation product (Fig.4, Lane 1 & 2) which was further confirmed by the immunoprecipitation (Fig.4, Lane 3 to 6).

MT Level in Neonatal Chicken Liver and its Relationship with Cellular Zinc-ion Concentration: The cytosolic and MT bound zinc and copper concentration in developing chick tissues(liver, kidney, intestine, brain, muscle) were estimated by atomic absorption spectrophotometry. Both the total cytosolic zinc concentration and the content of MT bound zinc show remarkable change during early developmental period (Fig.5). The copper content in both these fractions did not show any significant variation (data not shown). At the early neonatal stage (1-4 day after hatching) the cytosolic and MT bound zinc in liver was found to be high and about 80% of this cytosolic excess zinc was found to be associated with MT. The zinc content of kidney, brain, heart or muscle did not show any significant pattern of change (Fig.5). In intestine the zinc content was slightly higher in newborn animals but it was less than that of liver.

Discussion: Two lines of evidence suggest ontogenic modulation of MT concentration in neonatal chick liver. The SDS-PAGE analysis combined with silver-staining and the ELISA, using monospecific chicken MT antibody (Fig.2). The mouse MT-I cDNA, capable of hybridizing with both mouse and

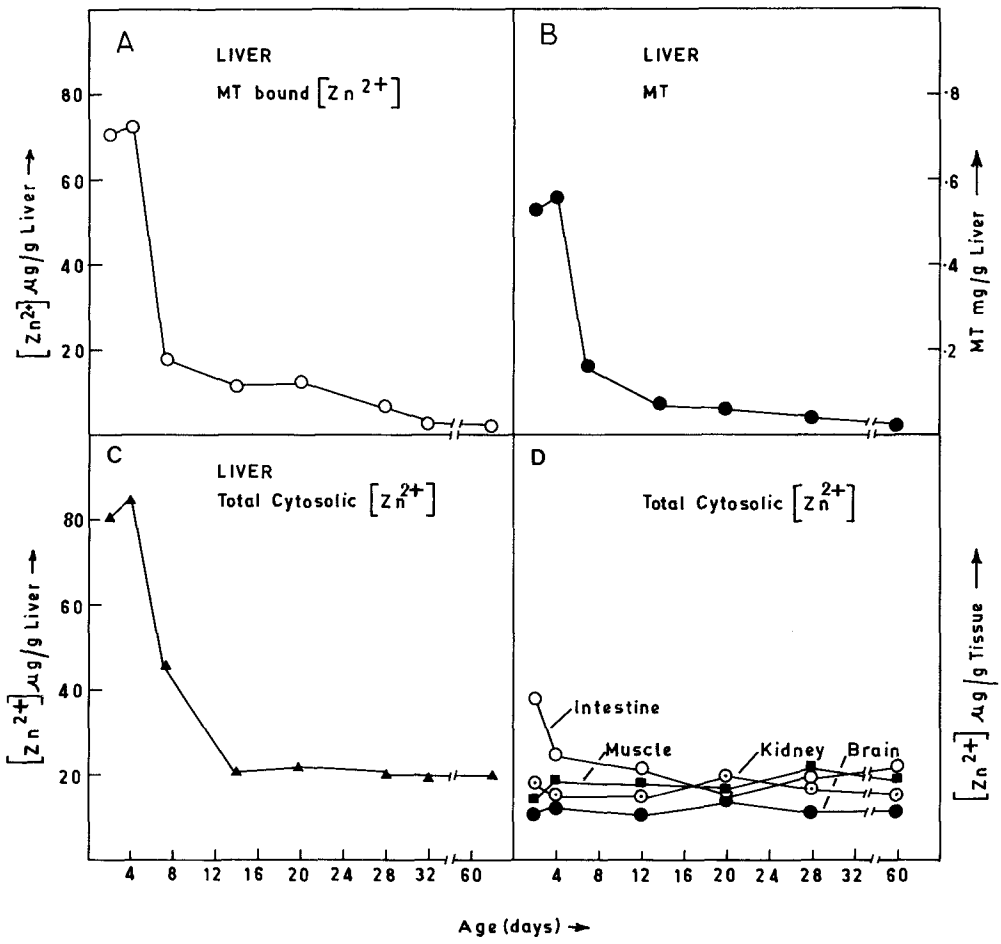

Fig.5. Concentration of zinc in developing chick tissue and hepatic MT fractions determined by atomic absorption spectrophotometry at 213.9 nm after digestion in 6(N) HNO_3, compared with standard solutions of zinc.

chick MT mRNA (Fig.3A) when used as a probe, helped our assay of the MT mRNA level in developing chick liver (Fig.3B). This result indicates that the variation of the amount of MT in chick liver (Fig.1 & 2) is probably controlled by a change in MT mRNA concentration, possibly triggered by the enhanced transcription of MT gene. The identity of MT mRNA size from cadmium induced chicken liver and neonatal chick liver (Fig.3A) and the immunoprecipitated translated product of MT by them (Fig.4) suggest that same MT mRNA species are involved in both these processes. Metabolism of endogenous heavy metals like zinc and copper in mammalian liver undergoes profound alteration during development (27-30). The MT-gene expression in developing chick liver may atleast in part be controlled by the mobilization of zinc ion during early development (Fig.5). The MT genes are susceptible to many different type of inducers, including steroid hormones and the regulatory mechanism of

MT gene expression during development is not definitely understood, Glucocorticoid regulationof MT has been suggested to occur during murine development (31) based on indirect evidence, while in sea-urchin it is probably induced by zinc (6,7). However, in mammalian species, the feotal-neonatal early development is intimately linked with the maternal system and is subjected to complex maternal-feotal interactions. In avian species like chicken embryonic development is free from such direct and continuous maternal influence. What is evident is that the concentration of hepatic Zn in new born chick undergoes a drastic modulation during development. The zinc in embryonic liver is primarily mobilized from the egg yolk (data not shown). Precisely, how the concentration of Zn is regulated in chick liver is yet to be answered.

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