Variation in the amounts of hepatic copper, zinc and metallothionein mRNA during development in the rat

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Amounts of hepatic metallothionein mRNA were assessed in RNA from foetal and neonatal rat livers by using dot-blot hybridization. Metallothionein mRNA began to increase about day 15 of gestation and reached a foetal maximum of 5-fold higher than adult values between 18 and 21 days of gestation. The amounts fell significantly for the first 3 days after parturition, and rose again to 6-fold above adult values 6 days after birth. By 15 days after birth the metallothionein mRNA had declined to adult amounts. In comparison, amounts of ornithine transcarbamoylase mRNA did not vary greatly during development. Hepatic zinc concentrations increased from day 14 of gestation to a maximum just before birth, and remained above adult values until 30 days after birth. From 14 days of gestation to 8 days after birth, hepatic copper concentrations were about 4-fold higher than in the adult, but a substantial increase (to about 9-fold higher than in the adult) occurs between 10 and 15 days after birth. CdCl₂ administered to pregnant rats on day 18 of gestation was shown to block placental transfer of zinc, and we found decreased foetal hepatic zinc concentration after the CdCl₂ treatment, but this failed to cause a significant decrease in metallothionein mRNA, suggesting that zinc may not be the primary inducer of hepatic metallothionein mRNA during foetal life.

INTRODUCTION

Metallothioneins are low- M_r cysteine-rich proteins which bind heavy metals and are generally isolated as copper/zinc-containing proteins (Kägi & Nordberg, 1979). Various lines of evidence suggest that metallothioneins may play an important role in the homeostasis of copper and zinc as well as protecting against toxic effects of heavy metals (Kägi & Nordberg, 1979; Brady, 1982).

Hepatic metallothionein concentrations have been found to be elevated during the foetal and neonatal period in various mammals (Bremner *et al.*, 1977; Wong & Klaasen, 1979; Riordan & Richards, 1980; Mason *et al.*, 1981). In the rat, metallothionein protein amounts rise towards the end of gestation, reaching a maximum value around the day of birth, then commence a decline leading to the low values characteristic of the adult by about day 20 of postnatal life (Wong & Klaasen, 1979; Mason *et al.*, 1981). It has been suggested that metallothioneins in the foetal and neonatal liver provide a store of zinc and copper required for the rapid growth period after birth (Bremner *et al.*, 1977; Wong & Klaasen, 1979) and, interestingly, Mehra & Bremner (1984) have found very high plasma metallothionein-I concentrations in the neonatal rat. It is also possible, however, that metallothioneins are increased to protect against potentially toxic concentrations of zinc and copper (Bakka & Webb, 1981).

The mechanism of metallothionein-gene regulation in the foetal and neonatal liver is of interest in its own right, but also it may shed light on the role of these proteins in the developing liver. In the present paper we determine the concentrations of hepatic metallothionein mRNA, zinc and copper in the livers of rats during

development, and we find two peaks of mRNA, one late-foetal and the other early-neonatal, with a decrease around the time of birth. Foetal hepatic zinc concentrations rise rapidly during late gestation, but blockage of this rise did not significantly alter amounts of metallothionein mRNA, suggesting that zinc may not be the primary inducer of foetal metallothionein mRNA.

MATERIALS AND METHODS

Materials

Guanidine hydrochloride (ultra-pure; Bethesda Research Laboratories) was used for all RNA isolations. L-[4,5-³H]Leucine (131 Ci/mmol), L-[³⁵S]cysteine (1100 Ci/mmol) and [α -³²P]dCTP (3000 Ci/mmol) were supplied by Amersham International, Amersham, Bucks., U.K. Nitrocellulose (0.4 μ m pore size) was obtained from Schleicher and Schuell. All other chemicals were of analytical grade.

Rat breeding and injection of CdCl₂

Sprague-Dawley rats were maintained on a diet of Mecon rat cubes containing 29.1 μ g of copper/g and 115 μ g of zinc/g. Foetal ages were estimated from the time of mating; males were placed with females overnight and the following morning was taken as day 0 of gestation. Foetal ages are referred to by G (for gestation) followed by the number of days post conception. Pregnant rats were injected with 0.5 or 1.0 mg of Cd/kg as CdCl₂ in iso-osmotic saline (0.9% NaCl) into the tail vein on day 18 of pregnancy. Pups were removed on day 20 of pregnancy. Control rats were injected with 0.9% NaCl.

Abbreviation used: OTC, ornithine transcarbamoylase (EC 2.1.3.3).

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RNA dot-blot hybridizations

Total RNA (0.5 or 1.0 μg) was dotted on to nitrocellulose presoaked in $10 \times \text{SSC}$ (1.5 M-NaCl/0.15 M-sodium citrate) in volumes of 5 μl , containing 2.5 M-formaldehyde and $6 \times \text{SSC}$, by the method of White & Bancroft (1982). The amount of total RNA used was within the linear range for quantification purposes up to 1 μg /dot (results not shown), and the loadings were checked by Methylene Blue staining. The filter was air-dried, baked for 2 h at 80°C under vacuum, then pre-hybridized for 16 h at room temperature in a hybridization mixture which contained 50% (v/v) formamide, $5 \times \text{SSCE}$ (0.75 M-NaCl, 0.075 M-sodium citrate, 25 mM-EDTA, pH 8.0), $1 \times$ Denhardt's (2 mg each of polyvinylpyrrolidone, Ficoll and bovine serum albumin/ml) and 250 μl of denatured herring sperm DNA/ml. Filters were then hybridized for 24 h at 42°C in a fresh solution of the hybridization mixture, which also contained 100 μg each of poly(U) and poly(C)/ml, as well as 100 ng of the 400-base-pair mouse metallothionein-1 cDNA, which included the entire coding sequences and all the 3'-untranslated region of the mRNA (Durnam *et al.*, 1980) labelled to a specific radioactivity of $>10^8$ c.p.m./ μg of DNA by nick translation (Rigby *et al.*, 1977) with $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$. After hybridization, the filter was washed in $2 \times \text{SSC}/0.1\%$ SDS for 30 min at 20°C , then in $0.5 \times \text{SSC}/0.1\%$ SDS for 1 h at 55°C . These hybridization and washing conditions were expected to remove any cross-hybridization with metallothionein-2 mRNA (Searle *et al.*, 1984); however, since we have not directly tested this, our results are shown as amounts of metallothionein mRNA rather than metallothionein-1 mRNA. After autoradiography to detect the dots, they were excised, and radioactivity was measured by dissolving the nitrocellulose in methoxyethanol and counting with a toluene-based scintillation fluid in a Packard liquid-scintillation counter. Counts were corrected relative to an internal standard.

Copper and zinc determinations

Livers were removed with scissors and forceps that had been soaked in 2% EDTA, then rinsed with distilled water to remove traces of copper and zinc. Livers were stored at -20°C until analysis. Copper and zinc concentrations were determined by flame atomic-absorption spectrometry, with a Perkin-Elmer model 5000 atomic-absorption spectrophotometer.

RESULTS

Variation in amount of metallothionein mRNA during development and comparison with ornithine transcarbamoylase mRNA

Fig. 1(a) shows the amount of metallothionein mRNA expressed relative to an adult RNA standard; each point represents the mean $\pm$ S.E.M. for five measurements. At day G14 (of gestation), metallothionein mRNA amounts are possibly somewhat elevated (almost 1.5-fold relative to adult); by day G15, the amounts are rising rapidly, reaching a foetal peak of 5-fold relative to the adult, from days G18 to G21. The amounts fall at birth, and start to rise again on day 3 of postnatal life, reaching a second peak on day 6. Subsequently the metallothionein mRNA decreases, reaching adult values by day 15. To assess the

Fig. 1. Hepatic concentrations of metallothionein mRNA, zinc and copper during development

(a) Dot-blot estimations of amounts of hepatic metallothionein and OTC mRNA during development. Total RNA (0.5 and 1.0 μg) was dotted on to nitrocellulose and hybridized to a nick-translated mouse metallothionein probe as described in the Materials and methods section. After washing and autoradiography to locate the dots, they were excised, and their radioactivity was determined by liquid-scintillation counting. For each set of dots, values were expressed relative to an adult-rat standard, and the means values $\pm$ S.E.M. for five determinations were plotted. The numbers of litters and rats (in parentheses) used at each age were as follows: G14, 1 litter (4 rats); G15, 1 (5); G17, 2 (18); G18, 2 (22); G20, 2 (21); G21, 2 (17); day 0, 3 (24); day 1, 2 (11); day 3, 3 (24); day 4, 2 (6); day 6, 3 (9); day 9, 3 (9); day 10, 1 (5); day 13, 1 (3); day 15, 2 (7); day 21, 1 (2); day 29, 2 (8); adult (A), 1 (1). G refers to gestational age and 0 is the day of birth. The broken line from G18 to G20 shows the foetal hepatic metallothionein mRNA concentration after CdCl_2 (Cd) or saline (S) administration to pregnant rats at G18 (average of five litters for the Cd treatment and one litter for the saline control). The OTC mRNA data are from previous work (McIntyre *et al.*, 1985) and were obtained for the same RNA samples. (b) Variation of hepatic zinc and copper during rat development. Livers were excised from foetal and neonatal rats and analysed individually for copper and zinc as described in the Materials and methods section. Mean values ($\pm$ S.E.M.) are plotted with 2–4 litters and 5–17 animals per point, except for G15, G18, day 17, day 19 and day 23, for which only one litter (3–5 animals) was used. The points marked 'Cd' are the results from pregnant rats injected at G18 with CdCl_2 and foetal livers analysed at G20 (average of 4 litters, 17 animals). The points marked 'S' refer to the results from one litter of saline-injected animals.

significance of the fall in mRNA around birth we compared the data from days G18, G20 and G21 as a group with those from days 0, 1 and 3 after birth. The median relative mRNA concentration in the foetal group was 5.09, which was significantly different ($P < 0.0001$) from the median for the immediate postnatal group (3.21) by the Mann-Whitney test (Gibbons, 1976). Similarly, when the median relative mRNA concentration at days 0, 1 and 3 were compared with those at days 4, 6 and 9 (median 4.61), the difference was also significant ($P < 0.02$) by the Mann-Whitney test.

To ensure that the variation in metallothionein mRNA was not simply reflecting a generalized change in all mRNA species during development, we compare in Fig. 1(a) the amounts of OTC mRNA during development, previously assessed with the same RNA preparations (McIntyre *et al.*, 1985). Although the OTC mRNA shows some changes in amount from day G14 to adult, with a rise in the late-foetal period and a decrease around birth, the variation is much less than that with metallothionein mRNA.

Copper and zinc concentrations in the foetal and neonatal liver

Copper and zinc concentration in livers from rats of various ages were determined by atomic-absorption spectrometry. Fig. 1(b) shows that foetal hepatic copper concentrations are significantly elevated (about 4-fold) compared with the adult, and there is no significant change in the newborns until after day 10, when a pronounced increase occurs, reaching a maximum around day 14 and subsequently declining to adult concentrations around day 30. A similar increase in hepatic copper concentrations around day 14 in Wistar rats has been noted by Mason *et al.* (1981). Hepatic zinc concentrations are much higher than those of copper during foetal life and rapidly increase from day G14 to a maximum just before birth, possibly with a slight decrease around birth, followed by a rise at days 3 and 4 with a subsequent decline to near-adult values at day 30.

Effect of block of zinc entry into foetal liver on metallothionein mRNA concentrations

To assess the effects of decreased zinc entry into the foetal liver on metallothionein mRNA concentrations, we made use of the observation by Bakka *et al.* (1981) that injection of CdCl_2 into pregnant rats on day 18 of gestation caused a placental block of zinc entry into the foetus. Injection of 1 mg of Cd^{2+} /kg body wt. as CdCl_2 , as used by Bakka *et al.* (1981) with Wistar rats, to pregnant Sprague-Dawley rats at G18 proved lethal to all fetuses by G19 or G20. We decreased the dose to 0.5 mg/kg at G18, and all pups survived to G20. From four litters ($n = 17$), the mean hepatic zinc concentration was $184 \pm 14 \mu\text{g/g}$ (see point 'Cd' on the Zn curve in Fig. 1b). A saline-injected control litter had a mean zinc concentration of $371 \pm 35 \mu\text{g/g}$ (point 'S' on the Zn curve in Fig. 1b), not significantly different from the control value. The decrease in hepatic zinc was in accord with that found by Bakka *et al.* (1981), and negligible cadmium was found in the foetal livers, $1.8 \pm 0.8 \mu\text{g/g}$ for the saline-injected litter and $1.9 \pm 0.8 \mu\text{g/g}$ for the CdCl_2 -injected litters. Measurements below $5 \mu\text{g/g}$ are below the limit of reliable estimation. The copper

concentrations in the saline- and CdCl_2 -treated livers were the same as in the untreated group.

The amounts of hepatic metallothionein mRNA at G20 after CdCl_2 injection at G18 (average of five treated litters) were slightly decreased compared with the untreated controls, and were identical with the values in a saline-injected control litter (see broken lines in Fig. 1a). Analysis of variance of the three groups (untreated, Cd and saline) showed no significant difference between any group ($P > 0.05$), and this was substantiated by a Friedman two-way analysis of variance ($P = 0.174$) (Gibbons, 1976).

DISCUSSION

The metallothionein mRNA concentrations that we have found in the developing rat liver differ from those reported by Andersen *et al.* (1983). They found that amounts of metallothionein mRNA remained significantly above those of the adult as late as 40 days of age, whereas Piletz *et al.* (1983) found that metallothionein synthesis rates had declined to adult values by 21 days. This discrepancy between mRNA amounts and synthesis rates was interpreted by Andersen *et al.* (1983) as being due, at least in part, to translational control of metallothionein genes expressed in the neonatal period. Our results are reasonably consistent with the synthesis rates found by Piletz *et al.* (1983), and were obtained by using a cDNA probe rather than the less reliable translation assays. In addition, our translation analysis (J. F. B. Mercer & A. Grimes, unpublished work) agrees completely with the dot-blot measurements, so we consider that the translational-control hypothesis is not supported by our data.

A striking feature of our metallothionein mRNA measurements is the apparent biphasic nature of the mRNA amounts, with foetal and neonatal peaks and a pronounced decrease in the 0–3-day period. Since we chose to pool the livers from many individuals for each RNA preparation, we do not have an estimate of the inter-animal variation, and hence the significance of the decrease must be assessed with caution. We did, however, show by statistical analysis using a Mann-Whitney test that amounts in the 0–3-day-old group were significantly lower than in the G18–G21 and the 4–9-day-old groups, and, considering that we used many animals from two or three litters for each important time point, it seems likely that this decrease will be biologically significant.

Since the RNA measurements depend on loading a given quantity of total RNA (mainly rRNA) on nitrocellulose, the apparent variation of metallothionein mRNA amounts could have arisen if either the amount of total mRNA were to change relative to rRNA during the development period, or if some RNAs were partially degraded. If either of these factors was operating, however, all mRNA species would have been affected. The amounts of OTC mRNA only varied between 0.5 and 1.5 relative to the adult, compared with the 5–6-fold relative rise in metallothionein mRNA, suggesting that the major changes in the latter are specific. Amounts of OTC mRNA did, however, decrease on day G21 and on days 0, 1 and 3 after birth. When examined by Northern blots, there was no evidence of RNA degradation in these samples (results not shown), so it is possible that some decrease in mRNA relative to rRNA could be occurring

around the time of birth. Analysis of a number of different mRNA species in individual animals will be required to substantiate this conclusion.

Of the known regulators of metallothionein transcription, heavy metals (Durnam & Palmiter, 1981) and glucocorticoid hormones (Hager & Palmiter, 1981) could be influencing the amount of mRNA in the foetal and neonatal period. We attempted to assess the role of zinc and copper in metallothionein-gene regulation during rat development by comparing the hepatic zinc and copper concentrations with amounts of metallothionein mRNA. There are severe limitations in this analysis; nevertheless we consider that the data do provide support for the hypothesis that zinc is not the primary regulator of foetal metallothionein genes. The limitations of the data arise from two problems. (1) There was considerable inter-animal variation in zinc and copper concentrations. The relationship between zinc and metallothionein mRNA would have been better assessed in the same animal; however, our methods of RNA isolation were not readily applicable to the large numbers that this analysis would have required, and zinc analysis of only part of a liver gave even more variable results (J. F. B. Mercer & A. Grimes, unpublished work). (2) Measurement of total metal concentration does not yield information about whether the zinc (or copper) is free or bound. Metallothionein induction will presumably only occur when entry of metal into the liver is rapid enough to result in a build up of non-bound metal ions, which then will induce metallothionein-gene expression. Thus in the 14–22-days-old animals zinc concentrations are still elevated relative to the adult, but metallothionein mRNA is not, presumably because all the zinc is bound in a non-inducing form.

In the period G14–G20, zinc concentrations in the liver are increasing, so an increase in metallothionein mRNA might be expected, owing to entry of free zinc, and indeed this is found. However, zinc concentrations continue to rise from G18 to G21, but the metallothionein mRNA concentrations remain at a constant, but still elevated, value. The lack of response to the zinc influx in the late-foetal period could suggest that sufficient metallothionein is present to bind immediately much of the zinc, or that zinc is not the principal inducer of metallothionein. The blockage of placental transit of zinc with CdCl_2 caused a rapid decrease in foetal hepatic zinc; however, the slight decrease in metallothionein mRNA was the same as in saline controls. If zinc were the sole regulator of metallothionein mRNA, the prevention of zinc entry should have caused a rapid decrease in metallothionein mRNA amounts, since the mRNA appears to have a half-life of about 4 h in rat liver (estimated from the data of Wake & Mercer, 1985). We consider that this result, taken together with the lack of response metallothionein mRNA concentrations to the influx of zinc in the period G18–G21, suggests that zinc may not be the principal regulator of amounts of foetal-liver metallothionein mRNA. This conclusion is supported by the observation that in foetal transgenic mice containing a growth-hormone gene linked to a metallothionein promoter, growth-hormone concentrations are not elevated (C. J. Quaife & R. D. Palmiter, personal communication). Since this gene construct is inducible by zinc but not by glucocorticoids (Palmiter *et al.*, 1982), this was taken as evidence that zinc (and copper) were not inducing foetal metallothionein genes.

Foetal corticosterone is maximally increased in late-gestation foetal rats (Greengard, 1975; Martin *et al.*, 1977), and metallothionein genes are induced by glucocorticoids, so it is likely that this hormone makes some contribution to the elevation of foetal-liver metallothionein concentrations. Andrews *et al.* (1984), however, reported that metallothionein mRNA concentrations were quite high in foetal mouse liver before a significant rise in corticosterone, which may suggest that foetal-liver metallothionein-gene expression is being regulated by some other unidentified factors.

In the neonatal rat, hepatic metallothionein concentrations remain elevated until about 15 days after birth, and so corticosterone is unlikely to be involved in metallothionein-gene regulation over this period, since the concentrations of the hormone decrease rapidly after birth (Greengard, 1975; Martin *et al.*, 1977). Zinc concentrations remain high, although steadily decreasing, so it is possible that amounts of metallothionein mRNA are responding to an influx of zinc from the milk, and it would be of interest to assess the amounts in animals on a zinc-deficient diet.

In conclusion, we have demonstrated that metallothionein mRNA concentrations in foetal and neonatal rat liver are substantially elevated above the adult values from 15 days gestation to about 10 days *post partum*. There is a possible decrease around the time of birth. The analysis of the changes in hepatic zinc and metallothionein mRNA in untreated animals and after placental block of zinc entry suggests that this metal is not the principal regulator of metallothionein mRNA in the foetal liver, but further analysis will be necessary to substantiate this conclusion and to assess further the nature of metallothionein gene regulation in the neonatal liver.

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