Cadmium-induced Accumulation of Metallothionein Messenger RNA in Rat Liver*

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Multiple injections of nontoxic levels of cadmium to a rat result in much higher level of metallothionein (MT) production in the liver than does the single injection. In order to understand the underlying mechanisms we have quantitated and compared the metallothionein-specific messenger RNA contents in the livers following the two induction regimens. Cell-free translation assays coupled with specific immunoprecipitation of MT revealed that MT-mRNA activity in livers of animals multiply injected with Cd is 7- to 10-fold higher than that in livers 4 h after a single Cd-induction.

By oligo(dT)-cellulose chromatography, sucrose density gradient centrifugation, and methylmercuric hydroxide-agarose gel electrophoresis this mRNA has been enriched approximately 100-fold from the total RNA. The size of the mRNA is about 400 nucleotides.

Hybridization assays with a complementary DNA probe synthesized against the enriched MT-mRNA showed a 4-fold difference in the level of MT-mRNA between the two induction regimens in agreement with the results obtained by the cell-free translation assays. The possible mechanisms for these observations in consideration of the short lived nature of MT-mRNA are discussed.

The transition class II B metal cadmium (Cd) is known to induce an accumulation in the rat liver of thionein protein which binds strongly to this and other metals of this class (1, 2). The exact mechanism for such an induction is not clear, although increases in the level of mRNA have been suspected. Induction with a single injection of Cd resulted in a rapid appearance of mRNA for Cd-thionein. The level of this mRNA peaked 4–5 h after the induction and decreased thereafter, indicating that this RNA has a definite and relatively short half-life (3-5).

Cd-thionein accumulates after the appearance of its mRNA (3-5). The half-life of metallothionein in the liver is 3½ days (6-8). Cd released from the degraded protein may further induce a new round of transcription.

Animals which are chronically exposed to nontoxic doses of Cd develop a tolerance toward subsequent exposure to toxic levels of this metal. Repetitive injections of Cd resulted in elevated levels of Cd-thionein in the rat liver as compared to a single injection (9, 10). To date, however, little information is available as to the transcriptional state elicited by the multiple induction. For example, extrapolating the above observation made with the single dose injection, 24-h interval, multiple induction should not be expected to result in a high level of mRNA because of the short lived nature of thionein mRNA despite more accumulation of thionein.

In this study, we have given single and repeated injections of Cd to rats and compared the amount of thionein mRNA in the livers. Our results show, contrary to the expectation mentioned above, that the thionein mRNA is amplified with the repeated induction regimen.

EXPERIMENTAL PROCEDURES

Preparation of anti-rat MT antibody. The MT- and MT-A, B, C, D, E purified were mixed in an equal ratio and polymerized with a glutaraldehyde solution to cross-link the protein before subchromatography as well as intracellular reaction. The serum was collected after a month, and further sera were collected every over a three month period, and two of these gave positive antiserum in the charcoal-laboratory assay.

nDNA purification. The total cytoplasmic RNA was prepared using the chloroform-aqueous phenol technique and purified with a chloroform solution to cross-link the protein before subchromatography as well as intracellular reaction. The serum was collected after a month, and further sera were collected every over a three month period, and two of these gave positive antiserum in the charcoal-laboratory assay.

Preparation of antisera to MT antibody. The MT- and MT-A, B, C, D, E, purified were mixed in an equal ratio and polymerized with a glutaraldehyde solution to cross-link the protein before subchromatography as well as intracellular reaction. The serum was collected after a month, and further sera were collected every over a three month period, and two of these gave positive antiserum in the charcoal-laboratory assay.

Portions of this paper (including "Experimental Procedures," Figs. 1, 2, 3, and 5, and Tables 1 and II) are presented in miniprint prepared by the authors. Miniprint is easy to read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014, Request Document No. 80M-1766, cite authors), and include a check or money order for $8.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
solution at 40-50°C for 2 min. The average RNA yield using this method is 2 mg per g of wet liver.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Poly(A)-containing mRNA; SDS, sodium dodecyl sulfate.

Identification and Characterization of Metallothionein (MT)

The unique physical and chemical properties of metallothionein allow for its rapid and definite identification. In this study, tissue homogenates were centrifuged and the supernatant was isolated from the supernatant and fractionated on a Sephadex G-75 column. The profile depicted in Fig. 1 shows that 100 Cd binds specifically to protein fractions of MT, ~ 10,000 and the peak of these fractions runs as a major band on SDS-gel electrophoresis (Fig. 1B, lane 4) with a mobility also corresponding to this molecular weight. These peak fractions were further separated into two isospecies of metallothionein, MT-A and MT-B by DEAE-Sephadex A-50 column chromatography (Fig. 2). Upon electrophoresis on non-SDS gels, MT-A and MT-B show different mobilities (Fig. 2B). Thus, our preparations of MT were identified and quantitated on the basis of absorbance (ε_{250} = 1.78 \times 10^4 \text{ liter m}^{-1} \text{ cm}^{-1})

2 The abbreviations used are: MT, metallothionein; [A']mRNA, poly(A)-containing mRNA; SDS, sodium dodecyl sulfate.

Accumulation of Metallothionein in Rat Liver in Response to Dose and Frequency of Induction

The ability of the liver to accumulate the Cd-binding protein, metallothionein, in response to the exposure to a nontoxic level of cadmium has been well recognized (2). Our results, included in Table 1, are in general agreement with those of others. The comparison made in our study shows that daily injections for 4 days with 1 day of recuperation after the final booster (hereafter referred to as multiple induction regimen) gives the highest yield of MT among all of the conditions tested.
Quantitation of MT-mRNA Induced by Single and Multiple Induction Regimens by Cell-free Translation Assays

Preparation of Anti-MT Antibody—In order to characterize and quantitate metallothionein synthesis in cell-free translation systems, anti-MT antibody against an equimolar mixture of purified rat liver MT-A and MT-B (see Fig. 2) was elicited in rabbits. From the Ouchterlony assay shown in Fig. 3, it is apparent that the antisera reacted specifically not only with the glutaraldehyde-polymerized antigen but also with native MT-A and MT-B. As a control it is shown that rat serum albumin did not react with the antiserum (Fig. 3). Neither did nonimmune sera react with MT under similar assay conditions (data not shown).

![Fig. 3. Ouchterlony assay of rabbit anti-rat MT antibody.](image)

Cell-free Synthesis of MT and Quantitation of MT-mRNA Activity—The anti-MT antisera were used to react with the *in vitro* translation products in order to identify MT synthesis. With this assay method, MT-mRNA activities of the rat livers induced by two different induction regimens were examined and compared. Using the same amount of template (50 μg/ml) and within their linear response range, it can be seen in Fig. 4 that the mRNA from rat liver after 5 days of multiple Cd-induction directed the synthesis of a metallothionein much more strongly than did the mRNA from 4-h Cd-induced liver. By densitometric tracing, we estimate that the MT-mRNA activity of multiply Cd-induced liver preparation is 6.6- and 10.7-fold higher in wheat germ extract and reticulocyte lysate systems, respectively, than that of 4-h Cd-induction. The products banded at the same position as the authentic MT, and the banding patterns were essentially the same between the wheat germ extract and reticulocyte lysate systems.

![Fig. 4. Cell-free synthesis of MT and quantitation of MT-mRNA activities of two induction regimens by the cell-free translation assays.](image)
**Purification of MT-mRNA**

Total cytoplasmic RNA of Cd-induced rat livers was separated into poly(A)-containing and -lacking (deficient) RNA species using oligo(dT)-cellulose affinity chromatography. The [A']mRNA was then fractionated by size in a sucrose gradient and the MT-mRNA-enriched fractions were localized by the cell-free translation assay coupled with immunoprecipitation (Fig. 5, A and B). Applying the relationship derived by Burgi and Hershey (22), the relative sedimentation coefficient of metallothionein mRNA is calculated to be 9.4 S, equivalent to an average molecular weight of 139,000 or about 400 nucleotides.

Methylmercuric hydroxide-agarose gel electrophoresis further enriched the MT-mRNA (Fig. 6). The enriched fractions also measured about 9 S (Fig. 6B) and were active in directing *in vitro* synthesis of metallothionein. Shown also in Fig. 6 is the degree of purification of MT-mRNA in each purification step. Comparing lanes 2 through 5 in Fig. 6, it is apparent that MT-mRNA was progressively enriched among the 9 S mRNA species.

**Comparative Quantitation of MT-mRNA with cDNA Probe**

MT-mRNA enriched by sucrose gradient fractionation was used as a template for complementary DNA (cDNA) synthesis in the presence of [α-32P]dATP. This radiolabeled DNA was used as a probe to titrate the MT-mRNA content of RNA preparations from samples taken after 4-h single dose induction and after 5-day, multiple dose induction by Cd. The results are shown in Fig. 7 and Table II. It can be seen, first, that fractionation of total poly(A)+ RNA by sucrose density gradient yielded 9 S RNA which hybridized with the cDNA probe 10-fold more than the unfractionated poly(A)+ RNA and approximately 100-fold more than the total RNA (Table II). RNA without purification or poly(A)+ RNA alone contained insufficient quantities of MT-mRNA sequences to show hybrid reactivity as detected in this autoradiograph (Fig. 7).
Metallothionein mRNA

Fig. 7. Titration by Northern hybridization assay of MT-mRNA in various RNA preparations. The DBM-paper was spotted with RNA preparations as follows: A and a, total cytoplasmic RNAs from rat liver after 4 h and 5 days Cd-induction, respectively; B and b, poly(A)\(^+\) mRNA; C and c, sucrose gradient 9 S region of poly(A)\(^+\) mRNA; D and d, poly(A)\(^-\) RNA fractions; E and e, water alone. 1\(\times\) and 2\(\times\) refer to the amount of RNA spotted. 1\(\times\) = 0.8 \(\mu\)g of RNA, except that the amount of the sucrose gradient 9 S region of poly(A)\(^+\) mRNA (C and c) was one-tenth of that of other RNAs. The filters containing RNA were incubated with \(^{32}\)P\(\cdot\)DNA probe for 6 days at 37°C. R\(d\) for 1\(\times\) RNA = 2.5 \(\times\) 10\(^{-1}\) mol s\(^{-1}\) liter\(^{-1}\). Autoradiography resulted after 48 h of exposure.

Secondly, since the titration was made in the linear range of the hybridization reaction (compare 1\(\times\) to 2\(\times\)), the results also show that in RNA prepared from 5-day multiple induction there is an approximately 4-fold higher level of MT-mRNA compared with the RNA from 4-h-induced rat liver.

DISCUSSION

In this study we have shown that poly(A)-containing 9.4 S RNA from rat liver with an apparent molecular weight of 1.39 \(\times\) 10\(^{6}\) directed the \textit{in vitro} synthesis of metallothionein. The product was identified by specific immunoprecipitation. While other studies also reported a similar observation (14), this is the first to provide a more positive identification of the translation product. We have produced antibody to the metallothionein and used it to specifically precipitate the \textit{in vitro} translation products. Metallothionein, a small protein of only 61 residues, is a poor antigen unless polymerized to elicit antibody formation (11). The immunoassay used here, having not been previously available, is of special value to this study.

We have also taken advantage of the more recently developed Northern hybridization technology (21) to immobilize the RNA in question on diazotized papers for solid state DNA-RNA hybridization, thus allowing both visual and quantitative examination of the various stages of RNA preparation for their enrichment in MT-mRNA. This procedure enabled us to estimate that the specific activity of MT messenger is at least 4-fold higher in the liver RNA preparations after multiple induction with Cd over a 5-day period than that in RNA obtained after 4 h with a single induction shot, which corroborates results obtained by the cell-free translation assays (Fig. 4). It is significant to note this difference since previous studies have shown that MT-mRNA is short lived, having a half-life of only a few hours (3).

The high level of MT-mRNA in the multiply induced liver may be explained in several ways: Greater numbers of the liver cells may be recruited to respond to the multiple induction. Preliminary studies with indirect immunoassay of liver sections show, however, the increase in reactivity with MT-specific antibody is uniformly distributed among most, if not all, cells. On the other hand, repetitive induction by Cd may, 1) stabilize this mRNA; 2) enhance the rate of specific MT gene transcription; or 3) stimulate selective amplification of the MT gene. In the first case, the RNA found in the liver induced after 5 days would consist of both pre-existing and newly synthesized species and represent a true accumulation of mRNA. On the other hand, if the rate of transcription were enhanced by multiple induction but that of the mRNA decay remained unchanged, most if not all of the RNA found on day 5 would be newly synthesized. As to the possibility that genes for metallothionein are selectively multiplied during the period of multiple induction, and the mRNA is short lived, one would also observe an elevated level of mRNA as well as amplified DNA sequences encoding the MT gene. These possibilities are being examined kinetically and quantitatively with the aid of cloned MT genes and antibody-staining techniques.

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Table II

| RNA fractions | 5 days induction | 4 hours induction |
|---------------|-----------------|------------------|
| RNA from      | 2x               | 1x               |
| Total RNA     | 9                | 0                |
| [\(^{32}\)P]RNA | 170              | 98               |
| Denslow gradient 9 S region | 197 | 532 |
| [\(^{32}\)P]RNA | 0                | 21               |

* The amounts of RNA applied onto the DNB-paper were 1/10 of the other fractions. See also Figure 8.