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Amplification of Metallothionein-1 Genes in Mouse Liver Cells *in Situ*: Extra Copies Are Transcriptionally Active (42737)

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Abstract. We have reported previously (J. Koropatnick *et al.*, Nucleic Acids Res **13**:5423–5439, 1985) that metallothionein-1 (MT-1) genes in adult mouse liver undergo a two- to three-fold increase in average copy number within 6 hr of treatment of mice with high levels of cadmium salts. The extra copies persist for at least 3 weeks in the absence of subsequent doses of cadmium. We report here that amplified MT-1 genes, which are relatively nuclease resistant early (6 hr) after induction, undergo a change in chromatin structure that renders them nuclease sensitive within 3 days. The change in chromatin structure is accompanied by an increase in the rate of transcription of MT-1 genes to a level approximately twofold higher than that maximally inducible in mouse liver with low MT-1 gene copy number. These data indicate that extra copies of MT-1 genes induced to appear in adult mouse liver cells *in situ* are, like their counterpart in cultured somatic cells, transcriptionally competent and inducible. However, post-transcriptional events (possibly specific degradation of MT-1 mRNA) have an adverse effect on the level of gene expression at the mRNA level. Two possible mechanisms to explain the appearance of amplified MT-1 genes in organs *in situ* after heavy metal treatment are discussed. First, *de novo* amplification of extra MT-1 genes in all, or a subset, of mouse liver cells may be responsible. Alternatively, a portion of mouse liver cells that already possess extra MT-1 genes might be selected for preferential DNA replication. © 1988 Society for Experimental Biology and Medicine.

In mice, metallothioneins [small cysteine-rich metal-binding proteins involved in resistance to heavy metal toxicity, reaction to stress, and metal homeostasis (1–3)] are induced by a variety of factors, including heavy metals and hormones (2), and are developmentally regulated in liver and yolk sac (4, 5). The two predominant genes in vertebrates are designated MT-1 and MT-2, and have been cloned from mouse as well as human, rat, Chinese hamster, and monkey cells (2). MT-1 gene copy number is variable amongst species—mice have one copy each of MT-1 and MT-2 per cell, while primates have multiple copies (6).

Stepwise selection for cadmium resistance in cultured cells (including mouse) results in amplification of the MT-1 gene and resistance to cadmium toxicity (7–9). We have shown that *in vivo* treatment of mice with high levels of cadmium results in a tissue-specific two- to three-fold increase in MT-1 gene dosage in mouse liver DNA in relation to both AFP (α-fetoprotein) gene dosage and the bulk of DNA (3). While this increase in copy number is small in relation to induced amplification of other genes in cultured cells, it has been argued that low-level amplification (less than five-fold) may be biologically important in cellular resistance to chemical toxicity in humans (10). However, there is no significant increase in the amount of MT-1 mRNA associated with extra MT-1 gene copies. It is possible that extra MT-1 genes are (a) present but transcriptionally incompetent, (b) transcriptionally competent and induced, but mRNA is not accumulated due to uncharacterized post-transcriptional events, or (c) a combination of the above, in that extra copies are initially untranscribed, but become transcriptionally competent after a period of time and begin to transcribe mRNA. In order to test these possibilities, genomic and cDNA nucleic acid probes were used to (a) assess the relative average copy number and chromatin conformation of MT-1 and α-fetoprotein (AFP) genes in cells with amplified MT-1 gene copies, (b) measure MT-1 mRNA levels in relation to those of control genes [for actin and dihydrofolate reductase (dhfr)], and (c) measure relative MT-1 transcription rate in liver cell nuclei in

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relation to that of a control gene (actin) at various times after the appearance of extra MT-1 gene copies.

Materials and Methods. *Animals.* Male CD-1 mice between 3 and 5 months of age (30–50 g) were obtained from Charles River Laboratories (Laprairie, Quebec).

Cadmium treatment. Control mice were injected ip with 1 ml of 0.9% saline in water and killed humanely, as were all mice, by cervical dislocation and exsanguination at various times. Induced mice were injected ip with $CdSO_4$ in 1 ml 0.9% saline (1 mg cadmium/kg mouse) and killed 4 hr later; Superinduced mice were injected ip with 1 mg cadmium/kg mouse at 0 and 72 hr, and were killed 4 hr after the second cadmium dose. Amplified mice were injected with 10 mg cadmium/kg (in 1 ml 0.9% saline) and killed 72 hr later. Amplified/Induced mice were injected ip with 10 mg cadmium/kg mouse (in 1 ml 0.9% saline) at 0 hr, with 1 mg cadmium/kg mouse at 72 hr, and were killed 4 hr after the second injection.

Four mice were used for each of the five experimental categories. Livers were excised and pooled, and separate aliquots were taken for (a) isolation of nuclei for nuclease digestion, (b) isolation of nuclei for run-off gene transcription experiments, (c) isolation of total tissue RNA, and (d) total tissue RNA/DNA ratio measurement.

Nuclear isolation and digestion. Livers were rapidly excised, placed on ice, and minced with sterile scissors. Samples from four identically treated mice were pooled to produce an average population of nuclei for each preparation. Nuclei were prepared in resuspension buffer (RSB) + $CaCl_2$ as described previously (3) at a concentration of 1 mg DNA (as nuclei)/ml, determined by absorbance at A_{260} in 1% sodium dodecyl sulfate and 1 mM EDTA. This method precludes the need for pelletting nuclei on a sucrose cushion, which tends to enrich for larger nuclei from relatively fragile cells and excludes smaller stromal hemopoietic and hematopoietic cell nuclei of insufficient density to sediment through 2.4 M sucrose (11, 12). We find a far greater variability in nuclear size after exclusion of the sucrose sedimentation step (confirmed by microscopic examination). At this point, three separate aliquots of 3×10^7 nuclei (untreated with nuclease) were taken, the concentration of nuclei was measured independently in each by hemocytometer counting, and DNA was isolated as described below to determine the average DNA content per nucleus. The amount of DNA isolated was measured by fluorescence enhancement with Hoechst 33258 stain, using the Hoefer TKO mini-fluorometer (Hoefer Technical Bulletin No. 119). In addition, DNA was directly immobilized from isolated nuclei by quick-blotting in 12.2 m sodium iodide, passage through a nitrocellulose filter, and acetylation of residual protein with acetic acid (13, 14). This allowed subsequent hybridization with a radiolabeled mouse satellite DNA probe (11) as an indicator of DNA content. Progressive nuclease digestion of the remaining nuclei was done as described previously (3). One-milliliter aliquots of nuclei, on ice, had added to them sufficient micrococcal nuclease (Boehringer-Mannheim) from a stock solution of 2 units/μl to bring them to 100 units of nuclease/mg DNA. Nuclei were pelleted by centrifugation at 1500 rpm at 0°C for 5 min and the supernatant was separated from pelleted nuclei. Pelleted nuclei contained chromatin fragments that were more resistant to nuclease digestion than total chromatin or chromatin in supernatants (3). After 5, 10, 15, and 20 min of micrococcal nuclease treatment, digestion in supernatant and pellet samples was stopped by the addition of EDTA to 20 mM and sodium dodecyl sulfate to 1%. Control samples, to determine the action of endogenous nuclease, were allowed to sit on ice for appropriate times without the addition of micrococcal nuclease. EDTA and SDS were added to them in the same fashion as to test samples. DNA was isolated by incubation with RNase A, Tl RNase, and proteinase K, extraction with chloroform/isoamyl alcohol, and precipitation in 95% ethanol as described previously (3). Two- and five-microgram aliquots of DNA were immobilized, in triplicate, on GeneScreen Plus paper (New England Nuclear) using a Schleicher & Schuell 96-place microsample filtration manifold as previously described (19), and were hybridized to gene probes and analyzed as described below. For some samples, DNA was cleaved to completion with

*Hind*III using the conditions recommended by the manufacturer (Boehringer-Mannheim) and separated by electrophoresis through a 0.8% nondenaturing agarose gel followed by transfer to GeneScreen Plus paper as described previously (3).

MT-1 mRNA measurement. Total cellular RNA from an aliquot of liver tissue representing four mice was purified using the guanidinium isothiocyanate/hot phenol method (15). Fifteen micrograms of total cellular RNA was electrophoresed on a 0.8% formaldehyde agarose gel and Northern blotted to nylon paper (15). Alternatively, 1 to 5 μg of total RNA was immobilized on filters by RNA dot blotting as described previously (16). Northern transfers and RNA dot blots were hybridized to gene probes and analyzed as outlined below.

Hybridization to labeled probes. DNA dot blots and Southern transfers were hybridized to a nick-translated mouse MT-1 genomic DNA probe containing exons 2 and 3—a gift from Dr. R. Palmiter (17)—under stringent conditions as described previously (3, 16). All probes used in this study had a sp act $> 1 \times 10^8$ cpm/μg. Filters were autoradiographed overnight at $-70°$C without allowing them to dry. They were then washed at 85°C in hybridization buffer with 0.01 *M* NaCl for 16 hr to remove hybridized MT-1 probe. Complete removal was checked by autoradiography, and the filters were rehybridized to a nick-translated mouse AFP probe containing exons 9 to 13, previously described as the *Eco*B fragment (13), and autoradiographed overnight. To determine the relative concentration of AFP and MT-1 genes, densitometer scans through the center of each dot were taken, the area under each peak was integrated, and the data were reduced to arbitrary absorbance/μg DNA dotted for each 2 and 5 μg DNA dot hybridized (a total of six dots per DNA sample). The MT-1 or AFP gene concentration in DNA dots (mean densitometer absorbance/μg DNA, $\pm$standard error) was plotted. For Southern transfers, a densitometer scan was made through the center and right and left edges of each autoradiograph band of interest. The average of the three intensities (area under the peak of the migration vs absorbance graph) was calculated and taken as a measure of gene concentration in DNA. RNA dot blots and Northern transfers were hybridized to an MT-1 cDNA probe [400 bp, including 16 bp of pBR322 DNA—a gift from Dr. R. Palmiter (18)]. Hybridizations and radioautography were done under the same conditions as DNA hybridizations. MT-1 probe was eluted from the filters by the procedure recommended by the manufacturer (NEN) and rehybridized to a mouse dhfr cDNA probe (the gift of Dr. W. Flintoff), and subsequently to a mouse α-actin cDNA probe (1350 bp in pBR322) that cross-hybridized to nonmuscle actin mRNAs (19). Mouse Taper liver tumor hepatoma satellite DNA was isolated as described previously (11), radiolabeled, and used as a probe for DNA content in liver cell nuclei. To reduce probe hybridization levels from optical density/μg total RNA to optical density per cell, total nucleic acids (TNA) were isolated from an aliquot of livers from each category of mice by deproteinization with sodium dodecyl sulfate and proteinase K, followed by phenol/chloroform extraction (20). TNA concentration was accurately measured at A_{260}. An aliquot was then heated to 80°C for 20 min to 0.2 *M* NaOH, followed by neutralization with 1 *M* Tris–Cl (pH 7.0) and 2 *M* HCl and precipitation with 2.5 vol of ethanol. RNA was hydrolyzed to alcohol-insoluble fragments by hot alkali (data not shown); hence precipitated nucleic acids were assumed to be DNA. The ratio of RNA to DNA was found to be quite constant for all liver samples, both control and cadmium treated (RNA/DNA = 5.5–5.8). DNA isolated from a known number of nuclei (measured by hemocytometer counting) yielded a constant DNA content per cell (4.7 to 5.6 $\times 10^{-12}$ g per nucleus) for all samples of nuclei. Total cellular RNA was found to be 3 $\times 10^{-11}$ g per cell.

MT-1 gene transcription rate. Nuclei for run-off transcription were isolated from mouse liver by the method of Mulvihill and Palmiter (21) and were stored at $-70°$C in 40% glycerol. It was found that nuclei from mice treated with cadmium showed a marked decrease in efficiency of transcription after 2 weeks of storage (data not shown), so all stored nuclei were used within 1 to 2 days of isolation.

Nuclei were used for *in vitro* transcription in the presence of α-^{32}P]UTP by the procedure of McKnight and Palmiter (22), except that 300 μl of nuclei (containing 250 to 300 μg of DNA) was transcribed in the presence of 300 μCi of radiolabeled UTP. The resulting radiolabeled RNA was isolated under conditions that utilized DNase I digestion and was hybridized to plasmid DNA immobilized on nitrocellulose filter disks. Each hybridization reaction (40 μl) contained (a) one filter of pBR325 DNA and one filter of pBR322 DNA (0.2 μg each) to measure background binding, and (b) one filter of MT-1 cDNA in pBR325 and one filter of actin cDNA in pBR322 (0.2 μg each). The efficiency of hybridization was estimated by hybridization of nick-translated MT-1 or α-actin cDNA to immobilized cold probe in parallel reactions. α-Amanitin (1 μg/ml) or unlabeled MT-1 and α-actin cDNA (400 ng each) were added to separate parallel hybridization reactions to assess the specificity of hybridization to (a) transcribed sequences and (b) MT-1 and actin mRNA, respectively.

Results. *Increased MT-1 gene content after heavy metal treatment.* Figure 1 illustrates the increased MT-1 gene concentration in Amplified and Amplified/Induced mouse liver in comparison with Control, Induced, and Superinduced liver, which is in agreement with, and extends, our previously reported results (3). Since this analysis is close to the limit of detection visually, and photographic reproduction can be quantitatively misleading, band intensity was quantified by densitometer tracing (Fig. 2). Both Amplified and Amplified/Induced livers exhibit an approximately 1.75-fold increase in MT-1 gene concentration in comparison with Control, Induced, and Superinduced samples. No significant increase in AFP gene concentration (as indicated by the optical density of the 1.3-kb band) was observed. DNA content per nucleus was essentially unchanged in all samples measured (Table I). It appears, therefore the MT-1 gene concentration is elevated at 3 days after high-level cadmium induction, in relation both to the bulk of DNA and to the specific gene for AFP.

Nuclease sensitivity of amplified MT-1 gene copies. The MT-1 gene sequences in adult mouse liver are relatively nuclease-resistant 6 hr after their appearance (3). We have previously shown that nuclease resistance of the MT-1 gene in mouse liver is negatively correlated with induction of expression of the gene (11, 16). As MT-1 genes in mouse liver are transcribed, resistance to micrococcal nuclease digestion is decreased.

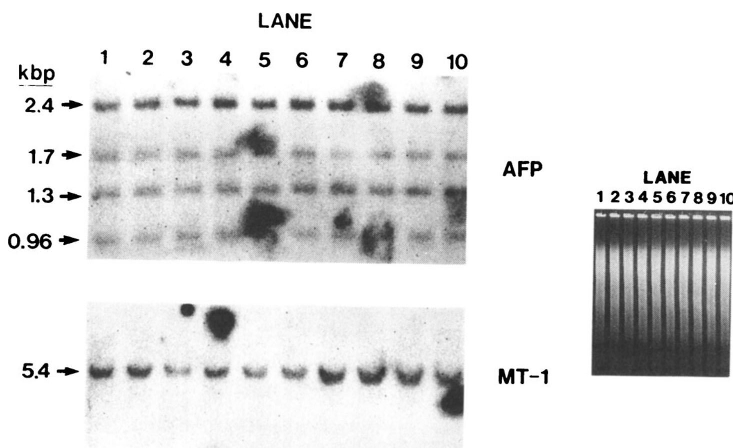

FIG. 1. Metallothionein-1 gene concentration in mouse liver. DNA was prepared from various mouse liver cell nuclei samples, cleaved with *Hin*dIII, subjected to gel electrophoresis, transferred to a nylon filter membrane, and hybridized to MT-1 and AFP genomic DNA probes as outlined under Materials and Methods. Lanes 1–2, Control; lanes 3–4, Induced; lanes 5–6, Superinduced; lanes 7–8, Amplified, lanes 9–10, Amplified/Induced. A photograph of the ethidium bromide-stained gel before transfer indicates relative bulk DNA loaded per lane.

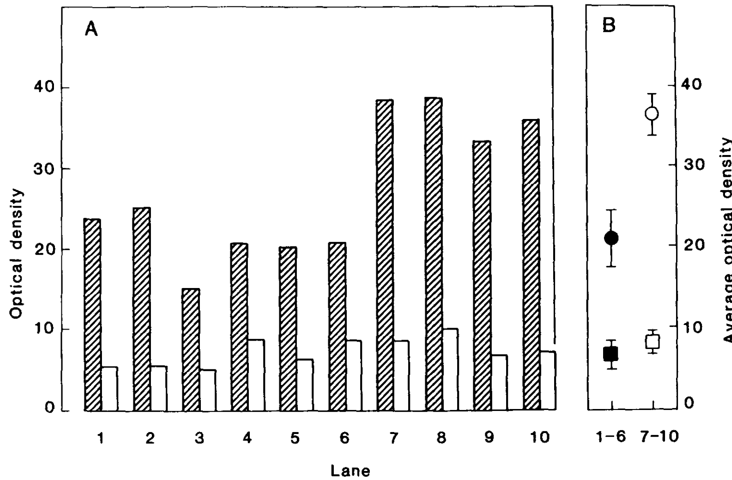

FIG. 2. Quantification of MT-1 gene concentration. Densitometer scans of the AFP and MT-1 probes hybridized to the filter shown in Fig. 1 are graphed. The average optical density ±SD of autoradiograph bands from Control, Induced, and Superinduced mice (●, ■) and Amplified and Amplified/Induced mice (○, □) is plotted. (●, ○) 5.4-kb MT-1 band; (■, □) 1.3-kb AFP band.

It is possible that the initial nuclease resistance of the amplified MT-1 genes is related to lack of transcription. If nuclease resistance is lost at some time later than 6 hr after cadmium treatment, it may be correlated with increased transcriptional competence of the extra MT-1 gene copies (see Introduction). To test this, liver cell nuclei from mice treated with cadmium were digested with micrococcal nuclease for up to 20 min as described under Materials and Methods. DNA was purified from precipitated, nuclease-resistant nuclear chromatin and total chromatin, dot blotted onto nitrocellulose filters, and hybridized to a genomic MT-1 probe and then to a genomic AFP DNA probe (an intervening washing step removed hybridized labeled MT-1 sequences). Results are plotted in Fig. 3.

In liver cell nuclei from Control mice, progressive nuclease digestion from 0 to 15 min tends to concentrate MT-1 genes, both in nuclear and in total chromatin (3, 11). This arises from the indirect nature of the assay: chromatin containing uninduced MT-1 genes is relatively nuclease resistant under these conditions (16). Progressive cleavage of the relatively nuclease-sensitive chromatin that does not contain MT-1 genes results in the subsequent loss of those degraded sequences during DNA isolation. The result is that DNA purified from chromatin after nuclease digestion has a higher concentration of MT-1 sequences than does DNA purified beforehand. Conversely, 4 hr after induction with 1 mg cadmium/kg mouse (Induced) [sufficient to maximally induce MT-1 gene transcription (23)] MT-1 genes became sensitive to nuclease digestion. Preferential cleavage of chromatin containing MT-1 genes degraded that DNA to the point of

TABLE I. AVERAGE DNA PER LIVER CELL NUCLEUS

Sample	Relative mouse satellite DNA content[a] (cpm ± SD)	DNA content[b] ($g \times 10^{-12}$ ± SD)
Control	327 ± 12	7.1 ± 0.5
Induced	309 ± 11	6.9 ± 0.3
Superinduced	253 ± 42	6.8 ± 1.1
Amplified	291 ± 19	6.6 ± 0.4
Amplified/induced	278 ± 33	6.9 ± 0.2

[a] Mouse satellite DNA probe was hybridized to liver cell nuclei immobilized on nitrocellulose paper by the NaI quick-blot method as explained under Materials and Methods. Hybridization to individual dot blots (1000 nuclei/dot) was measured by scintillation counting six identical dots.

[b] DNA was isolated from 3×10^7 liver cell nuclei and DNA per nucleus measured by fluorescence enhancement using Hoechst 33258 stain as described under Materials and Methods.

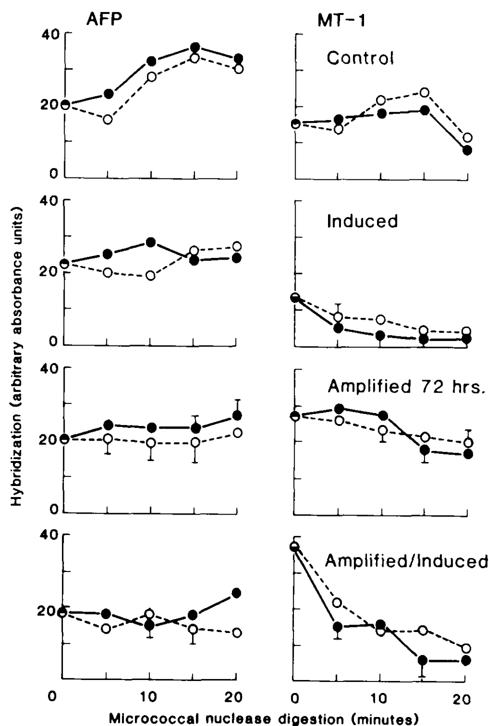

FIG. 3. Nuclease sensitivity and relative concentration of AFP and MT-1 genes in liver cell nuclei from mice treated as outlined in Fig. 1. Nuclease digestion and relative hybridization were determined as outlined under Materials and Methods. (●) Total chromatin (in supernatant plus pelleted nuclei); (○) chromatin in pelleted nuclei alone. Decreased hybridization with increasing digestion indicates increased nuclease sensitivity. All standard errors are less than ±4 arbitrary absorbance units, unless specifically noted.

nonhybridizability. This resulted in the depletion (with increasing time of nuclease digestion) of MT-1 gene concentration in total chromatin and pelleted nuclear chromatin alone. No increase in initial MT-1 gene concentration (time 0, Induced) was observed (3, 4, 11).

By 72 hr after treatment with 10 mg cadmium/kg (Amplified), average MT-1 gene copy number is elevated (0 min digestion datum point is 1.8 times greater than Control liver). This is in close agreement with the 1.74-fold increase in concentration illustrated in Fig. 2. In addition, MT-1 gene copies in total chromatin, and chromatin from pelleted nuclei alone, are cleaved by nuclease in a fashion very similar to that seen in Control liver samples. That is, neither heightened nuclease sensitivity (comparable to Induced samples) nor heightened nuclease resistance [as seen in pelleted nuclear chromatin 6 hr after cadmium treatment (3)] is apparent. Therefore, it appears that extra copies of the MT-1 gene maintain a chromatin structure, similar to that seen in Control mice, that is indicative of relative transcriptional quiescence (11). However, nuclease sensitivity may now be induced in these genes in the same way as seen in Induced mice. Four hours after induction of transcription of these genes by low level cadmium treatment (1 mg cadmium/kg mouse) (Amplified/Induced), there is a decrease in nuclease-resistance of *all* MT-1 genes present—both in total chromatin and in MT-1 genes in pelleted nuclei. Hybridizable AFP gene sequences in the same DNA dots do not show changes in gene copy number (i.e., 0-time digestion points in all left-hand panels of Fig. 3 do not differ significantly from each other). AFP genes in Control DNA are relatively nuclease resistant in comparison with the bulk of DNA (20 min of nuclease cleavage results in a concentration of AFP sequences in total chromatin by 50%), while any cadmium treatment results in some diminution of resistance (20 min of nuclear cleavage results in approximately 25% decrease in AFP gene concentration after 20 min of digestion, depending upon tissues sampled). The heightened nuclease sensitivity is similar to that observed previously (3, 16) and may be due to the contribution of endogenous nuclease activity in cells subjected to stress. However, the variations are minor in comparison with those observed for MT-1 genes, and AFP genes are never preferentially cleaved over the bulk of DNA. AFP hybridization within the same dotted DNA provides a useful internal control to measure MT-1 gene concentration variation in relation to another low copy number gene, as well as the bulk of dotted DNA.

Therefore, we observe that preexisting MT-1 genes in mice uninduced with cadmium are relatively nuclease resistant with respect to the bulk of chromatin. After low level cadmium induction (1 mg cadmium/kg), nuclease resistance of MT-1 genes de-

creases. With high-level cadmium induction (10 mg cadmium/kg), MT-1 gene concentration is elevated by approximately 1.74- to 1.8-fold in liver. At 3 days after induction, the concentration of MT-1 genes remains high in liver, and extra MT-1 genes now lose their nuclease resistance upon low-level cadmium induction in the same way that the normal complement of MT-1 genes in mouse does (11,16).

MT-1 mRNA concentration. We have previously observed that the extra MT-1 gene copies appearing in mouse liver at 6 hr after high-level cadmium induction did not produce a higher MT-1 mRNA concentration than that induced by 1 mg cadmium/kg mouse (3). Since the extra MT-1 gene copies were associated with a pool of relatively nuclease-resistant chromatin that appears at approximately the same time as extra MT-1 gene copies, and nuclease resistance is negatively correlated with transcription, I speculated that MT-1 mRNA concentration may be inducible to levels that are greater than that possible in Induced mice only when micrococcal nuclease resistance disappears in extra MT-1 genes. In order to test this, total liver RNA was isolated and the concentration of MT-1 mRNA transcripts was measured by hybridization of Northern transfers and RNA dot blots to an MT-1 cDNA probe followed by hybridization to α-actin and dhfr cDNA probes, as outlined under Materials and Methods. The results are illustrated in Fig. 4.

Induced mice have MT-1 mRNA levels 35- to 40-fold higher than Control mice. However, MT-1 mRNA accumulation was significantly lower in Superinduced mice—only a 20-fold increase was apparent. Amplified mice have a MT-1 mRNA concentration that was close to Control levels despite an elevated MT-1 gene concentration. However, Amplified/Induced mice have the same level of accumulated MT-1 mRNA seen in Superinduced mice—only *half* the level seen in Induced mice. This holds true for MT-1 mRNA levels from 2 to 6 hr after induction (Fig. 5) and for several inducing concentrations of cadmium (Fig. 6). This suggests that the extra MT-1 gene copies do *not* contribute to elevated accumulation of MT-1 mRNA at 3 days after their production, even though relative nuclease sensitivity (indicative, possibly, of transcriptional activity) appears by that time. Variation in the amount of total cellular RNA immobilized in dot blots and variation in the hybridizability of that RNA cannot account for the differences seen, since dhfr and actin mRNAs in the same RNA dot blots show uniform hybridization in each of the samples. Therefore, either MT-1 mRNA accumulation is specifically decreased in Amplified/Induced samples, or MT-1 gene transcription is half that observed in Induced samples.

A formal criticism of measurement of amplified MT-1 gene copy number in cells induced to transcribe MT-1 mRNA is the possibility that contamination of DNA with MT-1 mRNA results in falsely elevated hybridization of immobilized DNA to radiolabeled gene probes. However, Amplified mice have an approximately 2-fold higher MT-1 gene content in liver than Induced or Superinduced mice, even though MT-1 mRNA accumulation is 10- to 11-fold less (Figs. 1,2,4). In addition, Southern transfers of *Hin*dIII-cleaved DNA show the same increase in gene concentration as DNA dot blots (Figs. 1,2 in comparison with Fig. 3). Therefore, the possibility of false high estimates of MT-1 gene content due to contamination of DNA with MT-1 mRNA appears to be remote.

Transcription of MT-1 genes. Since increased nuclease sensitivity of MT-1 genes does not correlate with elevated MT-1 mRNA concentration in cells with elevated MT-1 gene concentration, the question of whether extra MT-1 genes were contributing to an increased rate of transcription was addressed. Run-off MT-1 gene transcription was measured in isolated mouse liver cell nuclei in comparison with actin gene transcription, as outlined under Materials and Methods. Results are shown in Table II and graphed in Fig. 7. Denatured, unlabeled MT-1 or α-actin cDNA added to hybridization mixtures effectively reduced hybridization to background levels or lower (Table II), indicating that hybridization is specific for MT-1 or actin sequences, respectively. The α-actin probe cross-hybridizes with genes for nonmuscle β- and γ-actins presents in cytoplasmic microfilaments (19). Hybridization

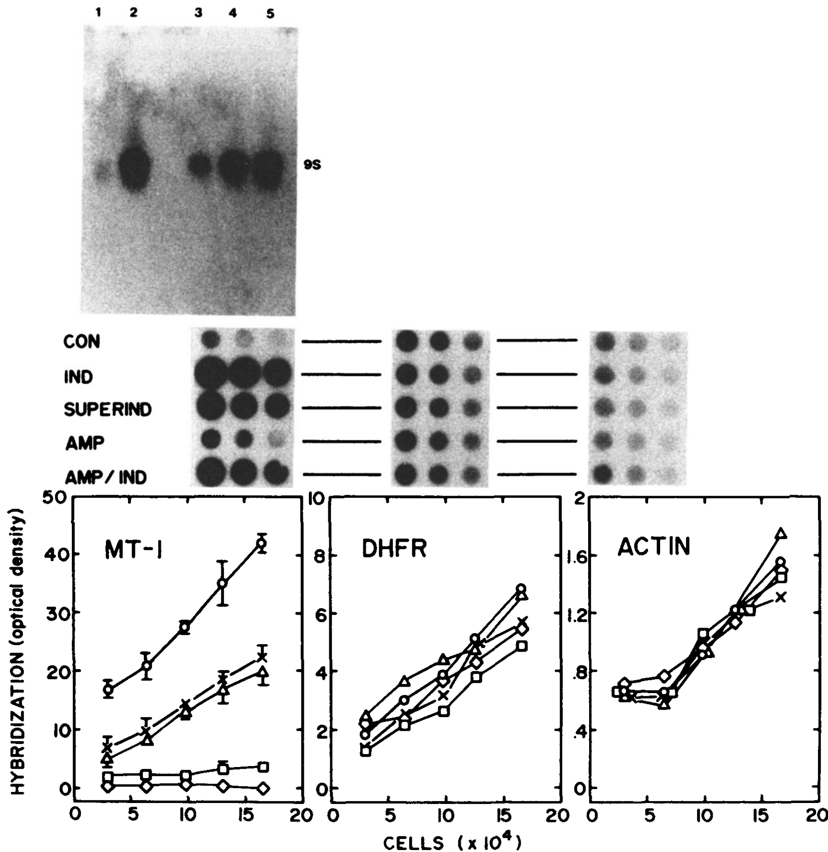

FIG. 4. Induction of MT-1 mRNA in livers of cadmium-treated mice. Total tissue RNA was isolated, dot blotted to nylon paper, and sequentially hybridized to MT-1, dhfr, and α-actin cDNA probes as outlined under Materials and Methods. A Northern blot was hybridized to MT-1 cDNA only. Representtive autoradiographs of hybridization to the rabiolabeled probes are shown (5,2.5, and 1.25 μg total tissue RNA per dot) and the optical density of autoradiographs from another series of hybridized RNA dots is graphed below. The mean optical density from four dots ± SE is plotted. In dhfr and actin dot hybridizations SE never exceeded 0.5 and 0.1 arbitrary O.D. units, respectively. (Lane 1 and ◇) Control; (lane 2 and ○) Induced; (lane 3 and ×) Superinduced; (lane 4 and □) Amplified; (lane 5 and △) Amplified/Induced.

due to transcription alone was measured, since the addition of α-amanitin reduced hybridization of radiolabeled MT-1 mRNA to background levels (Table II). Control mice exhibit an MT-1 gene transcription rate of 13 ppm relative to total transcription. Maximal induction of MT-1 genes without increased MT-1 gene concentration (Induced and Superinduced mice) yielded a 50-fold increase in relative transcription rate to approximately 400 ppm, which is in agreement with literature reports (7, 24). Seventy-two hours after induction of elevated MT-1 gene concentration (Amplified) the relative transcription rate was reduced to lower, but not control, levels (153 ppm). However, subsequent induction with 1 mg cadmium/kg mouse (Amplified/Induced) resulted in a relative rate of transcription of 855 ppm within 4 hr. This was twice the relative transcription rate attainable in mice without elevated MT-1 gene concentration in liver, and was in accordance with the heightened sensitivity of extra MT-1 gene copies in chromatin to digestion with micrococcal nuclease (Fig. 3). One possible explanation for the heightened relative transcription rate of MT-1 genes in Amplified/Induced mice would be that total

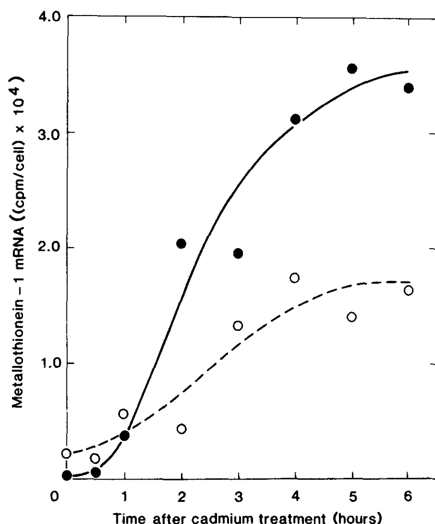

FIG. 5. Time course of MT-1 mRNA induction in mice with elevated liver MT-1 gene concentration. Induced (●) and Amplified/Induced (○) mice were killed at various times after treatment with 1 mg cadmium/kg, and assessed for relative MT-1 mRNA concentration in total cellular RNA as for Fig. 4.

incorporation of radiolabeled [^{32}P]UTP is decreased in those mice, while MT-1 gene transcription remains unaffected. In this case, an increase in run-off transcription rate would be apparent rather than real, due to the relative nature of such transcription measurements, and would require that incorporation of radiolabeled UTP into Induced cell nuclei would be twice that observed for Amplified/Induced nuclei. Measurement of such incorporation demonstrates that this is not the case (Fig. 8). Therefore, it appears that the extra MT-1 gene copies that appear after high-level cadmium treatment contribute to a relative rate of MT-1 gene transcription that is greater than that possible in mice with the normal complement of MT-1 gene copies.

Discussion. In this study, we have extended our initial observation that metallothionein-1 genes increase approximately 2-fold in concentration in mouse liver after high-level cadmium treatments (3). We have observed that the extra MT-1 gene copies are present when relative MT-1 mRNA transcription rate is increased approximately 2.0- to 2.3-fold over that possible in mice with the basal complement of MT-1 genes. In mice with amplified MT-1 genes, low-level cadmium treatment induced nuclease sensitivity in those genes in a manner similar to that seen in mice with basal MT-1 gene concentration (11, 16) (Fig. 3), indicating that all MT-1 genes, including extra copies, may be recruited into transcription by 3 days after high-level cadmium treatment. Although this suggests that extra MT-1 genes are contributing to enhanced MT-1 expression in the same way that amplified MT-1 genes in cultured CHO (24, 25) and mouse (7) cells do, it was initially surprising to find that MT-1 mRNA concentrations in the livers of mice with increased MT-1 gene concentration were approximately one-half that observed in maximally induced livers of mice with low MT-1 gene copy number (Fig. 4). Data derived from RNA dot blots hybridized to a MT-1 cDNA probe correlated well with Northern transfers of RNA hybridized to the same probe. This suggests that changes in mRNA stability may account for the lack of correspondence between gene concentration and transcription rate on the one hand and mRNA accumulation on the other. Discrepancies in specific mRNA accumulation and

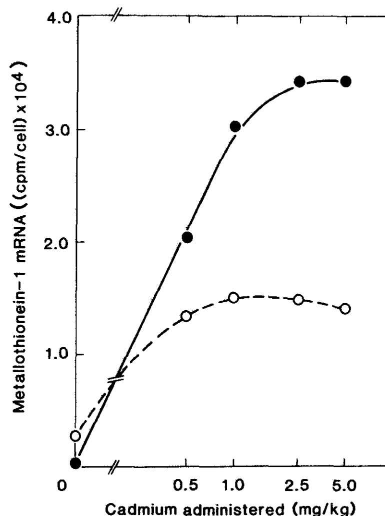

FIG. 6. Relationship of inducing cadmium level with MT-1 mRNA induction in mice with elevated liver MT-1 gene concentration. Induced (●) and Amplified/Induced (○) mice were killed 4 hr after induction with 0 to 5 mg cadmium/kg and assessed for relative MT-1 mRNA expression in total cellular RNA as for Fig. 4.

TABLE II. TRANSCRIPTION RATE OF MT-1 AND ACTIN GENES IN ISOLATED LIVER CELL NUCLEI

Sample	α-Amanitin (mg/ml)	Input cpm × 10^3	MT-1[a] cDNA (ng)	Actin[a] cDNA (ng)	MT-1 mRNA[b] hybridization (dpm)	Actin mRNA[c] hybridization (dpm)	Relative rate of mRNA synthesis (ppm) MT-1	Relative rate of mRNA synthesis (ppm) Actin
Control	0	1059	0	0	22	147	13	132
	0	530	0	0	15	77		
	1	265	0	0	−28[d]	−5	<0	<0
	0	530	400	400	−10	6	<0	11
Induced	0	976	0	0	540	156	380	109
	0	488	0	0	355	103		
	1	244	0	0	−31	1	<0	5
	0	488	400	400	−5	−2	<0	<0
Superinduced	0	1351	0	0	638	118	414	95
	0	676	0	0	358	54		
	1	338	0	0	−11	−21	<0	<0
	0	676	400	400	−19	−4	<0	<0
Amplified	0	783	0	0	138	170	153	117
	0	392	0	0	78	124		
	1	196	0	0	−15	−4	<0	<0
	0	392	400	400	3	−8	7	<0
Amplified/Induced	0	879	0	0	903	142	855	82
	0	440	0	0	527	106		
	1	220	0	0	−10	N.D.[e]	<0	N.D.
	0	440	400	400	−7	−12	<0	<0

[a] Unlabeled competitor DNA (without plasmid vector) added to the hybridization mixture.
[b] ^{32}P-MT-1 mRNA hybridized to the filter, corrected for background (57 ± 4 dpm), and efficiency of hybridization (37–42%).
[c] ^{32}P-actin mRNA hybridized to the filter, corrected for background (33 ± 6 dpm), and efficiency of hybridization (29–37%).
[d] Negative numbers indicate hybridization that is less than background (i.e., dpm hybridized to a filter containing plasmid vector only).
[e] Not done.

transcription have been reported by other workers during MT-1 gene amplification in cultured CHO cells [a 3-fold increase in MT-1 gene copy number results in a 2.9-fold increase in relative gene transcription rate, but an approximately 14-fold increase in accumulated MT-1 mRNA (7)]. However, the whole animal system differs in that mRNA accumulation was diminished rather than increased in the presence of amplified MT-1 genes. Based on the kinetics of MT-1 mRNA accumulation (Fig. 5), the half-life of cytoplasmic MT-1 mRNA in Induced mice (which transcribe MT-1 genes at the highest inducible level in the absence of amplified MT-1 gene copies) can be estimated to be 1.5–3.5 hr (26), which is in agreement with published half-life estimates of MT-1 mRNA mouse liver cells *in vitro* (7) and *in vivo* (24). If the transcription rate in Amplified/Induced mice was doubled, then half-life would have to be reduced by 75% to reduce MT-1 mRNA accumulation to one-half that observed in Induced mice, resulting in a half-life of 20 to 50 min. However, measurement of half-maximal cytoplasmic mRNA accumulation at various times after induction shows no such decrease (Fig. 5). It is possible that MT-1 mRNA is degraded in the nucleus, rather than the cytoplasm. However, the only conclusion that may be drawn from these data is that post-transcriptional events must be responsible for the lack of correspondence between MT-1 gene transcription

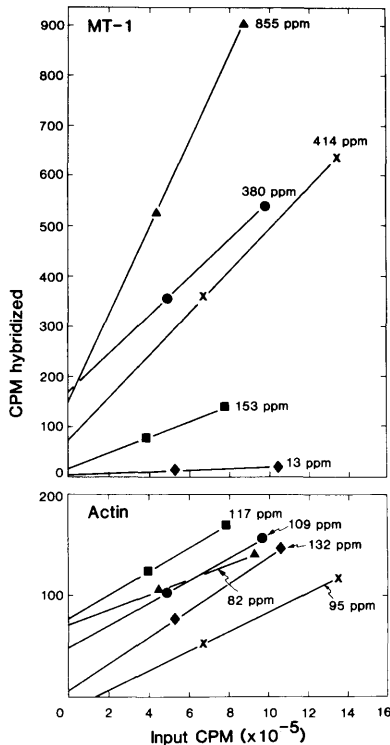

FIG. 7. Nuclear transcription of MT-1 genes in Control (♦), Induced (●), Superinduced (×), Amplified (■), and Amplified/Induced (▲) mouse liver. Nuclei were isolated and transcribed *in vitro* as described under Materials and Methods. Varying amounts of ^{32}P-RNA were hybridized to immobilized MT-1 cDNA in pBR325 or α-actin cDNA in pBR322. The parts per million (ppm) indicated were determined from the slope of each line. Graphed hybridization values are corrected for background and hybridization efficiency. Table I presents numerical data.

rate and mRNA accumulation. The post-transcriptional events must be specific to MT-1 mRNA (or a pool of which MT-1 mRNA is a part) since dhfr mRNA concentration remained constant (Fig. 4) and, for actin genes, both mRNA accumulation and relative transcription rate remained constant (Figs. 4 and 7, Table II). It is not clear from these data what caused the selective decrease in MT-1 mRNA accumulation, although stress effects related to high cadmium dose may play a role. It is apparent, however, that the phenomenon is relatively specific to MT transcripts. In addition, MT-1 mRNA accumulation in Superinduced mice was half that observed in Induced mice despite approximately equal transcription rates (Table II, Fig. 4). Therefore, decreased accumulation of MT-1 mRNA was not peculiar to transcripts from amplified MT-1 gene copies, but could occur in mice with low MT-1 gene copy number.

Post-transcriptional modification events resulting in decreased mRNA accumulation could, conceivably, result in underestimation of the transcription rate of MT-1 genes in Amplified/Induced mice in relation to the transcription rate in Induced mice. On the other hand, cellular regulators of MT-1 mRNA accumulation may operate primarily in cytoplasm, which would leave measurement of run-off transcription in isolated nuclei relatively unaffected. In either case, overestimation of MT-1 gene transcription rate in mouse liver with elevated MT-1 gene copy number was unlikely.

How extra MT-1 genes in mouse liver appear within 6 hr (3) of high level cadmium induction remains a subject of speculation. It is possible that one to two extra MT-1 genes in each cell, or multiple MT-1 gene copies in a subset of cells, are produced in liver after high-level cadmium induction, possibly by overreplication of specific regions of DNA, as reported for developmental amplification of *Drosophila* chorion protein genes (27) or

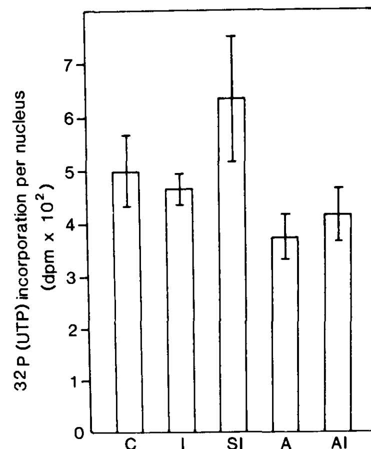

FIG. 8. Total transcription in liver nuclei samples. The recovery of ^{32}P-RNA per nucleus after run-off transcription of isolated nuclei is plotted as the mean of values from three experiments ± SE. C, Control; I, Induced; SI, Superinduced; A, Amplified; AI, Amplified/Induced.

hypothesized for induced amplification of dhfr gene sequences in CHO cells (28). Although a long period of time is generally required for the gradual accumulation of resistance to toxic agents mediated by specific amplification of MT-1 genes (8, 9) and other genes (22) in cultured cells, the frequency of gene amplification can be enhanced in cultured cells by transient perturbation of DNA synthesis during S phase. Treatment with hydroxyurea (29, 30), methotrexate (31, 32), uv light, and carcinogenic chemicals (33) promotes overreplication of a large portion of the genome, and treatment of this type substantially increases cellular DNA content (34). For the increase in MT-1 gene copy number reported here, it is therefore puzzling to observe that only MT-1 genes, and not AFP genes, show increased concentration in liver cell DNA (Figs. 1–3), even at the earliest time that extra MT-1 gene copies appear (3). In addition, average liver cell nuclear DNA content (measured directly after purification of DNA from nuclei, and inferred from hybridization of satellite DNA sequences in a given number of nuclei) was not increased at the time that extra MT-1 gene copies appeared (Table I). These observations are inconsistent with the overreplication of DNA that occurs during enhancement of gene amplification in cultured cells, and suggests that *de novo* amplification of MT-1 genes does not occur in response to high-level cadmium induction. An alternative explanation, consistent with the observations made here, is that MT-1 gene content is heterogeneous in normal mouse liver cells untreated with cadmium. After induction of transcription of MT-1 genes with high cadmium dose, a subset of liver cells with high MT-1 gene copy number (and, possibly, greater resistance to cadmium toxicity) might be induced to enter S phase (we have observed that DNA replication is induced by 10 mg cadmium/kg mouse, but not 1 mg cadmium/kg mouse [3]). If other liver cells are inhibited in their ability to successfully replicate their DNA by toxic cadmium treatment, then an overall increase in apparent MT-1 gene copy number per cell in mouse liver would be observed in conjunction with preferential replication of DNA in a subset of cells. It is important to note that cytokinesis (which is unlikely to be completed by 6 hr after induction of DNA replication) would not need to occur in order to see increased MT-1 gene concentration in a population of cells—only DNA replication is required. In such a situation, a *de novo* increase in the MT-1 gene copy number per liver cell need not be invoked, but a preexisting MT-1 gene amplification in selected cells would contribute to an overall increase in MT-1 gene concentration in bulk liver DNA by means of preferential cellular DNA replication alone.

This could account for the approximately two-fold increase in average MT-1 gene copy number observed, provided that cells with endogenous high MT-1 gene copy number are a sufficiently well-represented subpopulation. It has already been observed that rat liver cells induced to express MT are positive for binding to antimetallothionein antibody only in the epithelial cell population, and that only a fraction of epithelial cells are positive (17, 35). Such variation in expression might reflect variable gene copy number, especially as a wide variety of relatively undifferentiated cultured cell systems are capable of induced MT expression (36). With respect to other gene systems, cells with amplified dhfr genes occur spontaneously and at high frequency (8×10^{-4} to 3×10^{-2} events per cell division) in cultured CHO cells in the absence of treatment with methotrexate (23) and spontaneous variations in specific gene copy number may occur widely in other animal cells (37–40). Cells of this type would be good candidates for selective DNA replication.

These data support the hypothesis that extra MT-1 gene copies appearing soon after induction of MT-1 gene transcription in mouse liver by high cadmium doses are persistent and contribute to increased MT-1 transcription rate. This suggests, in turn, that heterogeneity in specific gene copy number is a mechanism for controlling gene expression not only in cultured cells, but also in normal animal tissues. Post-transcriptional events that are relatively specific to MT-1 transcripts (i.e., do not include actin and dhfr mRNA) accompany the increased relative transcription rate. The nature of those events is not known. We are exploring two possibilities to explain induced MT-1 copy number

change *in vivo:* (a) like spontaneous heterogeneity in gene copy number in cultured somatic cells and tumor cell lines (36), normal mouse liver cells are heterogeneous in their complement of MT-1 genes, and high-level cadmium treatment induces selective replication of DNA in cells with high copy number, or (b) *de novo* gene amplification, possibly enhanced by cell damage and perturbation of DNA replication by toxic cadmium treatment, might result in a small, but very rapid, increase in MT-1 gene copy number in mouse liver cells *in vivo.*

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