

# Selection of mouse cells with amplified metallothionein genes retaining their glucocorticoid inducibility

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Two new mouse cell mutants, resistant to either 80 or 100 mM CdCl<sub>2</sub>, were isolated to study the regulation of transcription by the glucocorticoid hormones. Their metallothionein *mt*-1<sup>+</sup> and *mt*-2<sup>+</sup> genes were amplified coordinately to a maximum of 30 copies per cell. By Southern blot analysis, no gross rearrangement was detectable near the *mt*<sup>+</sup> loci. Contrary to other mutants previously isolated, the metallothionein-specific mRNAs of these mutants are inducible by dexamethasone.

Metallothionein; Cadmium; Glucocorticoid; Gene amplification; Heavy metal

## 1. INTRODUCTION

Mouse metallothioneins (MTs) are 6 kDa proteins that bind heavy metals [1]. They are encoded by two genes, *mt*-1<sup>+</sup> and *mt*-2<sup>+</sup>, about 6 kbp apart, whose transcription is coordinately inducible by cadmium (Cd) and dexamethasone (Dex) [2–5]. With Dex, the glucocorticoid–receptor complex binds to a glucocorticoid-responsive DNA sequence (GRE) to enhance transcription [6,7]. With the mouse metallothionein genes, however, transfections did not allow the identification of functional GREs because the transfected genes, although inducible by cadmium, were not inducible by Dex [3,8]. Similarly, Cd-resistant (Cd<sup>r</sup>) hamster and mouse cells whose *mt*<sup>+</sup> genes had been amplified were also not inducible by Dex [9,10]. These are unusual results. We report here that absence of induction in the mouse is not a general rule and we describe the selection of new Cd<sup>r</sup> LMTK mouse cells with amplified *mt*<sup>+</sup> genes that are still inducible by Dex.

## 2. MATERIALS AND METHODS

### 2.1. Cell culture

Wild-type (WT) LMTK cells were grown at 37°C in DMEM plus 10% bovine serum. L80 and L100 mutant cells were grown in the presence of 80 or 100 µM CdCl<sub>2</sub>, respectively, except when indicated. Serum was dialysed 2 times against 100 vols. of 0.15 M NaCl.

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*Abbreviations:* MT(s), metallothionein(s); Dex, dexamethasone; GRE, glucocorticoid responsive element; Cd<sup>r</sup>, cadmium resistant; WT, wild-type.

### 2.2. DNA and RNA purification

DNA was purified [11] and its concentration was determined by fluorescence [12]. DNA digests were analysed by Southern blots. Cytoplasmic RNA from subconfluent cell populations was purified [11] and the concentration was determined by absorption at 260 nm. Absence of degradation and the RNA concentration of different preparations were verified by formaldehyde–agarose gel electrophoresis followed by staining with Acridine orange. RNA was then denatured with formaldehyde and applied directly to nitrocellulose filters for dot blot analysis [13].

### 2.3. DNA probes

The 221 bp *Sma*I fragment of the mouse *mt*-1<sup>+</sup> cDNA [14] and the 158 bp *Rsa*I fragment of mouse *mt*-2<sup>+</sup> cDNA [8] were labelled to a specific activity of 10<sup>9</sup> cpm/µg. Each one was specific and did not cross-hybridize [15]. DNA and RNA hybridizations were at 42°C and washes were in 0.1 × SSC at 65°C [13]. Autoradiograms were scanned using a Chromoscan 3 (Joyce Loebel).

### 2.4. MT synthesis

Proteins were labelled with [<sup>35</sup>S]cysteine, carboxymethylated, and electrophoresed on 20% SDS–polyacrylamide gels [15,16]. The gels were fluorographed and autoradiographed.

## 3. RESULTS AND DISCUSSION

### 3.1. *mt*<sup>+</sup> gene amplification

LMTK mouse cells were re-cloned twice. Two clones were selected and grown in parallel and at high cell concentration, first in 15, then in 50 and then in either 80 or 100 µM CdCl<sub>2</sub>. After 7 months, one independent clone in each selection was isolated and named L80 and L100, respectively. The LD<sub>50</sub> (defined as the level of Cd required for 50% inhibition of clone formation) of the mutant and the WT cells were determined from their inactivation curves (Table 1) [15]. The stability of their Cd<sup>r</sup> phenotype was assessed, and after 3 months both cell lines showed a marked decrease in their LD<sub>50</sub> (Table I) and the amount of MT proteins, indicating that the

Table I  
Characteristics of Cd<sup>r</sup> cells

| Cell line | LD <sub>50</sub> <sup>a</sup> (μM) | LD <sub>50</sub> <sup>b</sup> | Chromosome number <sup>c</sup> |       |
|-----------|------------------------------------|-------------------------------|--------------------------------|-------|
|           |                                    |                               | Average                        | Range |
| LMTK      | 12                                 | 12                            | 39 ± 1 <sup>d</sup>            | 30-47 |
| L80       | 95                                 | 40                            | 50 ± 4                         | 30-93 |
| L100      | 115                                | 30                            | 41 ± 1                         | 33-44 |

<sup>a</sup>CdCl<sub>2</sub> concentration necessary to inhibit the formation of colonies by 50%

<sup>b</sup>LD<sub>50</sub> of cell populations grown for 3 months in the absence of exogenous Cd

<sup>c</sup>Standard deviation (*n* = 50)

<sup>d</sup>Normal 2*n* = 40.

Cd<sup>r</sup> phenotype of L80 and L100 is unstable. These cells behave very much like Cd<sup>r</sup> mouse S180 cells [9] which also lose their amplified *mt-1*<sup>+</sup> genes and their resistance to Cd after growing for 4 months in the absence of Cd. We then determined if such instability in L80 and L100 could be revealed by the presence of small acentromeric, double-minute chromosomes [17,18]. At a magnification of ×500 no such structures on giemsa-stained metaphase spreads [19] were detectable (not shown). The mutant and WT DNAs were then analysed and Fig. 1 shows the hybridization of the DNA digests. From the results we determined that (i) the restriction patterns of

LMTK, L80 and L100 were similar; (ii) the size of the DNA fragments agreed with the restriction map of the mouse *mt*<sup>+</sup> loci [8,20,21]; and (iii) the restriction fragments encoding *mt-1*<sup>+</sup> and *mt-2*<sup>+</sup> had been amplified unaltered, as part of a larger amplification unit, about 30 times for L100 and 15 times for L80. Thus, we conclude that within the amplified unit no major rearrangement had occurred in the vicinity of the *mt*<sup>+</sup> loci.

### 3.2. Metallothionein synthesis

As shown in Fig. 2, increased synthesis of MTs and the Cd resistance phenotype are associated. Both Cd<sup>r</sup> cells show an 8-12-fold increase in total MTs over the WT induced by 15 μM CdCl<sub>2</sub>.

### 3.3. MT mRNA expression

It has been reported that mouse and hamster mutants in which *mt*<sup>+</sup> genes have been amplified do not synthesize more MT mRNAs in the presence of Dex than the WT [9,10]. This is not the case with L80 and L100. The expression of MT-1 and MT-2 mRNAs in both mutants, induced with either cadmium or Dex, was determined by dot blot analysis. Prior to induction, the cells were first grown for 5 days in the absence of Cd and in 10% dialysed fetal bovine serum since Cd inhibits the accumulation of nuclear glucocorticoid receptors [9]. Cytoplasmic RNAs (10 μg) were spotted onto nitrocellulose filters and hybridized. Fig. 3 shows that the syn-

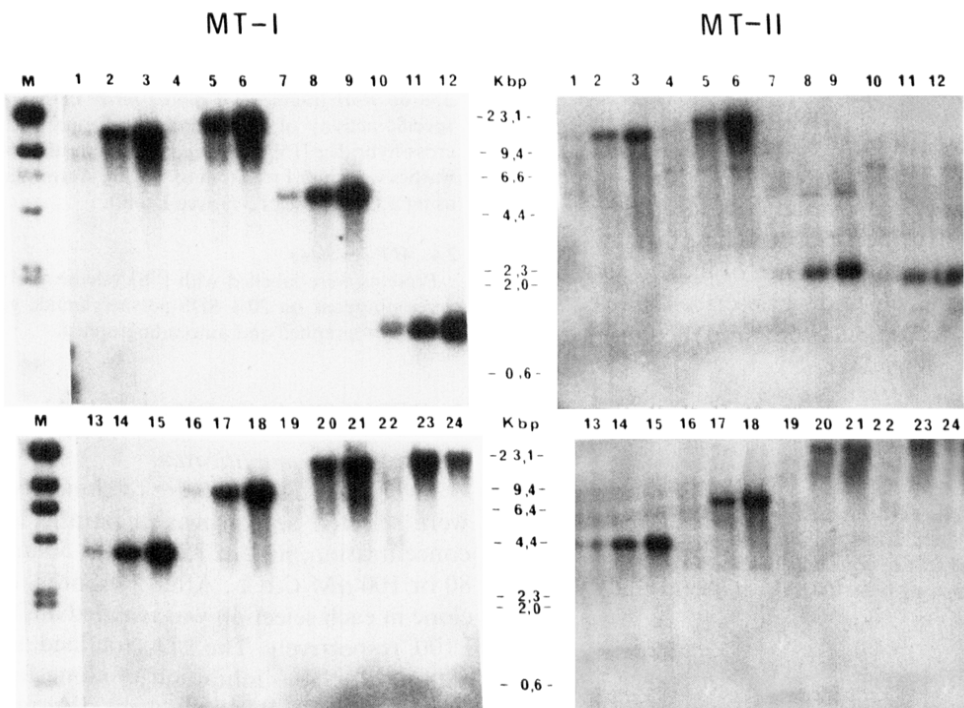


Fig. 1. Amplification of the *mt-1*<sup>+</sup> and *mt-2*<sup>+</sup> genes. The WT and mutant DNAs were digested with 8 restriction enzymes, electrophoresed and blotted onto nylon membranes. The filter was hybridized with the *mt-1*<sup>+</sup> cDNA probe. After autoradiography, the filter was washed in 0.4 M NaOH for 30 min at 45°C to remove the probe. The filter was then prehybridized and re-hybridized with the *mt-2*<sup>+</sup> probe. The molecular weight maker (M) is a *Hind*III digest of phage lambda DNA. Lanes 1, 4, 7, 10, 13, 16, 19, 22, LMTK; lanes 2, 5, 8, 11, 14, 17, 20, 24, L80; lanes 3, 6, 9, 12, 15, 18, 21, 23, L100. DNAs were cut with *Bam*HI (lanes 1-3), *Eco*RV (lanes 4-6), *Hind*III (lanes 7-9), *Pst*I (lanes 10-12), *Eco*RI (lanes 13-15), *Kpn*I (lanes 16-18), *Xho*I (lanes 19-21) or *Sal*I (lanes 22-24).



Fig. 2. Induction of metallothioneins. [ $^{35}$ S]Cysteine-labelled cytoplasmic proteins were electrophoresed on 20% SDS-denaturing acrylamide gels. 100,000 cpm were loaded in each lane. Lane 1, uninduced LMTK; lane 2, LMTK induced by 15  $\mu$ M CdCl<sub>2</sub>; lane 3, L100, 100  $\mu$ M; lane 4, L80, 80  $\mu$ M.

thesis of both MT-1 and MT-2 mRNAs are inducible by Dex or Cd in L80, L100 and WT cells. In WT cells the levels of both MTs mRNAs were 6- and 4-fold greater after Cd and Dex induction, respectively. In the mutant cells the induction of both MTs mRNAs is also coordinate. For L80 cells, the mRNAs levels were increased 7- and 6-fold after induction with Cd and Dex, respectively, and for L100 cells, the induction was about 5-fold. This increase in MT mRNA is most probably due to activation of gene transcription, as already shown with human MT-IIA [4,22,23] and mouse *mt-1*<sup>+</sup> and *mt-2*<sup>+</sup> genes [8,24]. This is, to our knowledge, the first report of mutant cells with amplified mouse *mt*<sup>+</sup> genes which are still inducible by Dex. There are two possible explanations why these mutants are inducible by Dex while the others were not. First, GREs located far from the transcription signals could have been amplified in L80 and L100 but not in the other mutants. The absence of such elements in the vicinity of mouse WT *mt*<sup>+</sup> genes is supported by the absence of new DNase 1 sensitivity sites after glucocorticoid induction [25]. Also, transfected genes were inducible by Cd but not by Dex in experiments using 10 kbp of genomic DNA comprising both mouse *mt*<sup>+</sup> genes [8,9]. GREs could be further away since the amplified mouse *mt-1*<sup>+</sup> genes from Cd<sup>r</sup> S180 cells do not respond to Dex even though at least 20 kbp of DNA were co-amplified on each side of the *mt-1*<sup>+</sup> gene [26]. Thus, in our mutants, the amplicons are large enough to include distant GREs. Also, GREs in LMTK cells may be closer to the *mt*<sup>+</sup> genes because of frequent rearrangements occurring in heteroploid cells. The use of our amplification mu-

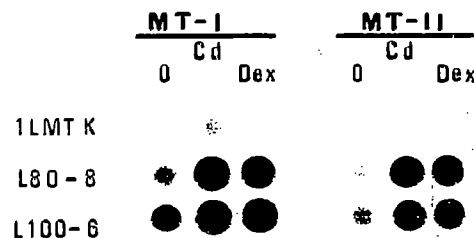


Fig. 3. Induction of mRNA in response to cadmium and dexamethasone. 10  $\mu$ g of RNA was spotted onto nitrocellulose in duplicate. Filters were hybridized with the *mt-1*<sup>+</sup> or *mt-2*<sup>+</sup> probe and autoradiography was overnight on XAR-5 Kodak film. 0, cells grown in Cd-free medium for 5 days; Cd, cells induced for 8 h with 15  $\mu$ M CdCl<sub>2</sub> (1 LMTK), 120  $\mu$ M CdCl<sub>2</sub> (L80) or 140  $\mu$ M CdCl<sub>2</sub> (L100); Dex, all cells induced for 8 h with 100 nM Dex.

tants in the mapping of DNase 1 hypersensitive sites might uncover discrete chromatin structure alterations around the *mt*<sup>+</sup> genes upon glucocorticoid induction.

A second explanation is that the structure of the DNA or chromatin is important for the *mt*<sup>+</sup> induction. This has been proposed to explain why transfected or injected glucocorticoid-responsive genes maintained either episomally or integrated at different sites, lose their capacity to be induced by Dex [3,8,27–32]. Karin et al. [22,23] also found that in some transfections the hMT-IIA regulatory region did not respond to Dex. Recently, it was shown that the organization of DNA in nucleosomes affects the affinity of the glucocorticoid receptor for its target, and that activation of the MMTV long terminal repeat results from disruption of a nucleosome specifically positioned over the regulatory region [33,34]. As for the WT cells, the chromatin structural requirements for induction by Dex of the *mt*<sup>+</sup> genes would thus have been maintained in L100 and L80 cells.

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