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Regulation of metallothionein gene expression in Cd- or Zn-adapted RK-13 cells**

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Abstract. We explored the molecular genetics underlying the massive induction of isoMTs by Zn^{2+} or Cd^{2+} in metal tolerant rabbit kidney (RK-13) sub-line cells, using band shift assays and Southern blotting analysis. In sub-line cells accommodated to intermediate metal concentrations (100 μM Zn^{2+}; 1–20 μM Cd^{2+}) evidence suggested that the increase in the capacity for isoMT synthesis is brought about by an increased binding activity of the nuclear transcription factors MTF-1 and Sp1. Using quantitative band shift analysis with a mouse MRE-d oligonucleotide probe, the binding of both transcription factors was found to be enhanced two to three times over the binding activity measured in the unexposed parental RK-13 cells. Their increase in binding activity is probably the cause of the overexpression of MT genes and the development of metal tolerance in these cells. In cells tolerant to the highest concentrations of metal the analysis of Southern blot signals revealed MT gene amplification to be the most probable cause of the increased MT production. Thus, in cells of sub-lines growing in the presence of 350 μM Zn^{2+}, two of the isoMT genes were coordinately triplicated and in cells tolerant to 150 μM Cd^{2+} one isoMT gene was amplified two-fold.
Key words. Metallothionein; isometallothioneins; gene expression; rabbit kidney cell-line; cadmium adaptation; zinc adaptation.

Metallothioneins (MTs**) are unique cysteine-rich proteins capable of binding heavy metal ions in metal-thiolate clusters. Their biosynthesis is inducible both by physiologically essential metal ions Zn^{2+} and Cu^{2+} as well as by toxic metals such as Cd^{2+}, Hg^{2+}, Bi^{3+} and others. Thus, MTs are thought to be involved both in metal homeostasis and in cellular protection from metal toxicity. The MT gene promoters are structurally complex. Although the fine structure of MT gene promoters varies significantly, all MT gene promoters contain multiple cis-acting DNA sequences termed metal response elements (MREs)[1–3]. They apparently represent binding sites for trans-acting factors (proteins) regulating the level of transcription of the MT gene in response to environmental metal concentrations[4,5]. Another interesting aspect of the MT genes is their ability to be preferentially amplified by heavy metal administration[6,7]. Both activation of gene transcription and gene amplification have been reported to play roles in the overexpression of MTs in cellular adaptation to metal exposure.

The ability of rabbit kidney cells (RK-13) to become resistant to high metal concentrations in the culture medium and to respond with the production of large quantities of MT (ref. 8) implies the existence of specific adaptive mechanisms. As reported previously for other cell lines, such adaptation can occur by different means:

1) by changes in the efficiency of MT gene expression at the level of transcription or translation[9,10],
2) by an increase in gene copy number (gene amplification)[6,11], or
3) by switching on silent genes (gene commitment)[12].

In order to shed light on the adaptive response of RK-13 cells, we monitored and compared both the binding activity of the regulatory transcription factors MTF-1 and Sp1 known to bind within the mouse MT-1 promoter, and the actual volume of the MT gene amplification in parental and in various metal tolerant sub-line cells. To this end we determined the relative quantities of the two transcription factors in nuclear extracts of parental and of metal tolerant RK-13 sub-line cells, using band shift assays with appropriate DNA probes. The number of MT gene copies was monitored by Southern blot hybridization. The studies revealed significant changes in the binding activities of the two nuclear factors MTF-1 and Sp1 when the cells were exposed to low and intermediate metal concentrations, which might enhance the efficiency of the transcription of MT genes. Cells adapted to high metal concentrations showed substantial MT gene amplification.

Materials and methods

Cell lines and cell culture. RK-13 cells and cultures of Cd-resistant and of Zn-resistant sub-lines and the culture conditions used were as described previously[8]. For genomic DNA isolation and nuclear extracts confluent cell cultures were used.

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** A full list of abbreviations is given at the end of the article.

Quantification of MT. MT was quantified using high performance liquid chromatography (HPLC) described elsewhere[8].

Preparation of nuclear extracts from RK-13 and Cd- or Zn-resistant sub-line cells. Nuclear extracts from parental RK-13 cells, from Cd-resistant and from Zn-resistant sub-line cells, were prepared for MRE-d and octamer band shift assays according to Schreiber et al.[13] with the following modifications: to reduce metal ion losses only 0.1 mM EDTA was used in the nuclear extraction buffer. The protein concentration of the nuclear extracts was determined by Bio-Rad protein assay (Bio-Rad Laboratories, CA, USA). The nuclear extracts were diluted with extraction buffer C to obtain a final protein concentration of 5 µg/µl or 10 µg/µl.

Band shift assay. The band shift assay was performed according to Westin and Schaffner[5] with minor modifications. The 32 bp oligonucleotide of the mouse MRE-d element, that contains a MTF-1 and an overlapping Sp1 binding site, was employed. Binding reactions were carried out by incubating 5–10 fmoles of the end-labelled 32 bp oligonucleotide with 20 µg of nuclear protein and 4 µg poly (dI-dC) in a buffer containing 25 mM Hepes, pH 7.9, 15% glycerol, 100 mM KCl, 12.5 mM MgCl, 3 mM DTT, 1 mM PMSF, 5 µg/ml leupeptine, 0.5 µg BSA and 10 µM $ZnSO_4$, in a total volume of 20 µl. After 20 min incubation at room temperature the reaction mixture was loaded onto a 4% polyacrylamide (38:2 crosslink) gel in 0.25 × TBE buffer at a final concentration of 100 µM $ZnSO_4$, and electrophoresed at 10 V/cm at room temperature for 2.5 h. For competition experiments 1 pmole of MRE-d or SpHSV oligonucleotides were added to the reaction mixture prior to the addition of the extract.

The octamer band shift assay (according to Kemler et al.[14] with minor modification) was used as the internal standard in this work. The binding reaction was carried out by incubation of 1–10 fmoles (about 3000 cpm) end-labelled octamer oligonucleotide with 1 µg non-specific competitor DNA (poly dI-dC) in a buffer containing 4% Ficoll, 20 mM Hepes, pH 7.9, 30 mM KCl, 1 mM EDTA, 1 mM DTT, 4 mM $MgCl_2$, 1 µg bovine serum albumin (BSA, Boehringer Mannheim, Germany) and 10 µg nuclear extract protein in a total volume of 15 µl. After 15 min incubation at room temperature the samples were directly loaded onto a 4% polyacrylamide gel (38:2 crosslink) and electrophoresed at 10 V/cm in 0.25 × TBE for 2.5 h.

The intensity of the autoradiographic signals of Sp1, MRE-d and octamer complexes were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, USA). The intensity ratios of both Sp1 complex/octamer complex and MRE-d complex/octamer complex in nuclear extracts from the parental RK-13 cells and in nuclear extracts from metal resistant sub-line cells were compared.

Preparation of genomic DNA from RK-13 cells and Cd- or Zn-resistant sub-line cells. Cells of parental RK-13 and of Cd-resistant and of Zn-resistant sub-lines collected from five confluent dishes (10 cm diameter) each were used to isolate genomic DNA. The monolayer cells were harvested using a rubber policeman. Total DNA was prepared according to Sambrook et al.[15]

Genomic DNA restriction enzyme digestion and transfer to nylon membrane. Purified genomic DNA was digested with EcoRI and HindIII endonucleases (Boehringer Mannheim, Germany) at 37 °C for 4 h; 2.5 units restriction enzyme per 1 µg of DNA was used. The digested genomic DNA was separated by electrophoresis on a 0.8% agarose gel and transferred to nylon membranes (Biodyne B, 0.45 µm, Pall, New York, USA) by using capillary transfer[15] under neutral conditions (20 × SSC).

Preparation of ^{32}P-labelled deoxyoligonucleotide for Southern blot hybridization. For Southern hybridization a 19mer deoxyoligonucleotide (SCC: GCTGCTGCTC-CTGCTGCCC) was used as a probe. Its sequence is homologous to the 5′ end of the cysteine-rich third exon of rabbit MT-1 (ref. 16) and has more than 95% identity with all functional mammalian MT gene DNA sequences recorded in the Gene Bank.

The oligonucleotide was synthesized on an ABI Model 394-08 DNA synthesizer using the β-cyanoethylphosphoramidites method. After desalting on a PD-10 column (Pharmacia, Sweden) it was labelled according to Sanbrook et al.[15] with ^{32}P-γ-ATP using bacteriophage T_4 polynucleotide kinase (Boehringer Mannheim, Germany).

Southern blot hybridization and gene copy number determination. 40 µg EcoRI/HindIII-digested DNA from parental RK-13 cells, from Cd-resistant or from Zn-resistant RK-13 sub-line cells were immobilized onto a nylon membrane after electrophoresis and blotting. Hybridization was performed by using a quaternary alkylammonium solution[15] containing 2 pmoles ^{32}P end-labelled SCC oligonucleotide at 50 °C for 40 h. The intensities of the autoradiographic bands were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, USA) and normalized to the intensities of the bands at 1.3 kb and 1.2 kb.

Results

MTF-1 and Sp1 binding to MT gene promoter in metal-tolerant RK-13 sub-line cells. Using the two transcription factors MTF-1 and Sp1, two protein-DNA complexes were observed with RK-13 nuclear extracts (fig. 1). They displayed the same electrophoretic mobilities as those observed previously with HeLa nuclear extracts. By competition experiments using unlabelled SpHSV and MRE-d oligonucleotides (results not shown), the slower migrating band was identified as the

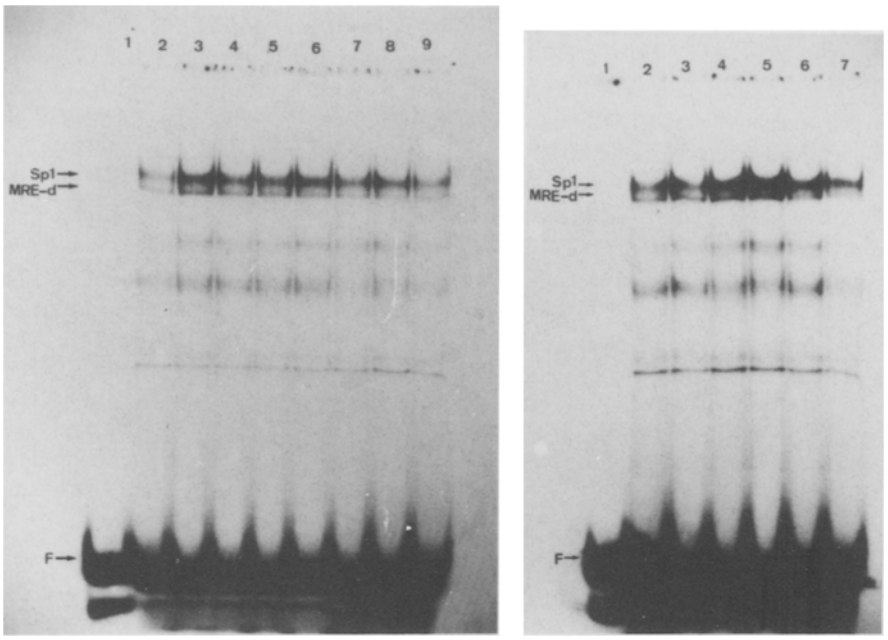

Figure 1. Band shift analysis of nuclear extracts obtained from various Cd-resistant (left hand side) and Zn-resistant (right hand side) RK-13 sub-line cells. *Left*: lane 1: no extract; lane 2: parental RK-13 cells; lane 3: Cd1; lane 4: Cd5; lane 5: Cd10; lane 6: Cd20; lane 7: Cd60; lane 8: Cd100; lane 9: Cd150. *Right*: lane 1: no extract; lane 2: parental RK-13 cells; lane 3: Zn10; lane 4: Zn100; lane 5: Zn150; lane 6: Zn250; lane 7: Zn350. For each assay 5–10 fmol of end-labelled MRE-d oligonucleotide were incubated with 20 µg of nuclear protein and 4 µg of poly(dI-dC) for 20 min at room temperature. Electrophoresis was performed for 2.5 h in a 4% polyacrylamide gel at 10 V/cm. The intensities of the corresponding octamer band shifts were 3.03 ± 0.18 for Cd^{2+}-adapted and 1.32 ± 0.15 for Zn^{2+}-adapted sub-line cells.

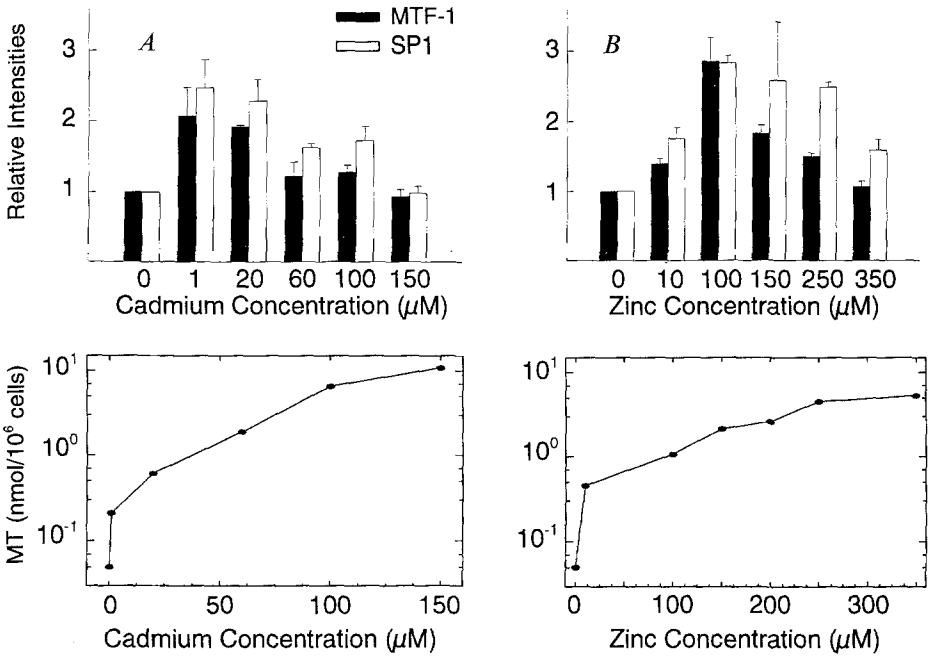

Figure 2. *Top*: Quantification of MTF-1 and Sp1 complexes (fig. 1) in *A* Cd^{2+}- and *B* Zn^{2+}-adapted sub-line cells. The intensities are normalized to the intensities of the corresponding octamer band (fig. 1) and are calculated relative to those obtained in the parental RK-13 (0 µM Cd^{2+}) cells. The data shown represent mean values obtained from at least three independent experiments. *Bottom*: MT in *A* Cd^{2+}- and *B* Zn^{2+}-adapted sub-line cells. Total MT was quantified by summing up the amounts of all isoforms as determined by HPLC[8].

Sp1/MRE-d complex and the faster migrating band as the MTF-1/MRE-d complex[5].

The autoradiographic intensities of both protein-DNA complexes, normalized to the intensity of the corresponding octamer band shift[14] of the same nuclear extracts are shown in figure 2. The signal intensities of MTF-1/MRE-d complexes formed with nuclear extracts from the series of Cd-resistant sub-line cells (fig. 2A,

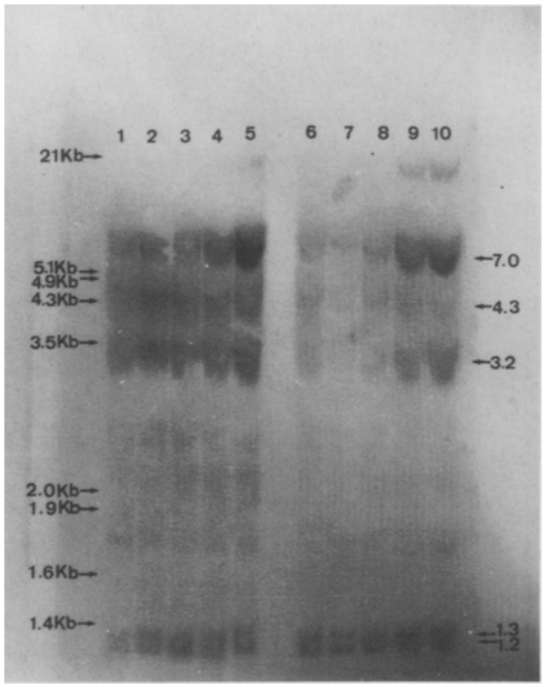

Figure 3. Southern blot analysis of genomic DNA from parental RK-13, Cd^{2+}- and Zn^{2+}-adapted sub-line cells. Lane 1: parental RK-13 cells; lane 2: Cd20; lane 3: Cd60; lane 4: Cd100; lane 5: Cd150; lane 6: Zn100; lane 7: Zn150; lane 8: Zn200; lane 9: Zn250 and lane 10: Zn350. 40 µg of genomic DNA from each cell line was digested with EcoRI/HindIII and run on an 0.8% agarose gel, blotted, and probed with oligonucleotide SCC. The numbers on the right hand side indicate the positions of the MT gene-containing fragments. The numbers on the left hand side indicate the positions of the λ EcoRI/HindIII fragments.

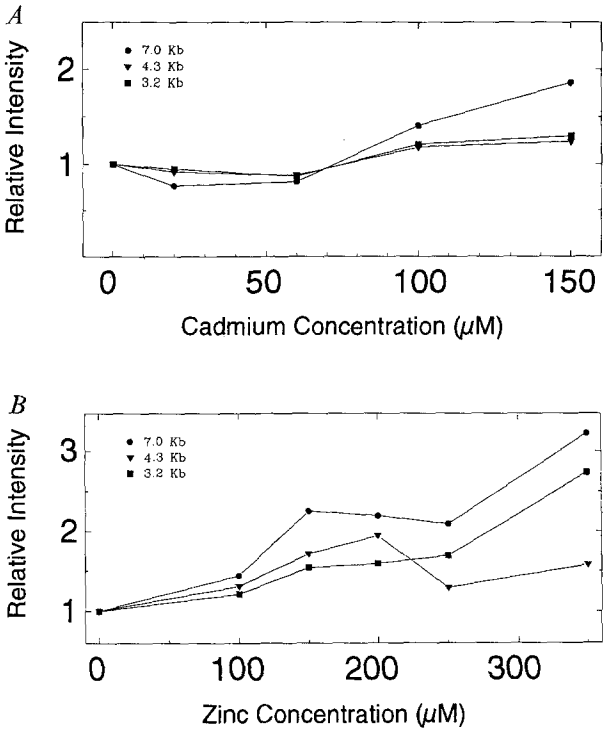

Figure 4. Quantification of the band intensities at 7.0, 4.3 and 3.2 kb (fig. 3) in *A* Cd^{2+}- and *B* Zn^{2+}-adapted sub-line cells in a representative experiment. The intensities of the three bands were normalized to the corresponding bands at 1.3 and 1.2 kb and calculated relative to those obtained in parental RK-13 (0 µM Cd^{2+}) cells.

top) showed that the binding activity of MTF-1 was increased 2.1-fold when the cells were adapted to Cd^{2+} concentrations of 1 µM, and decreased again to the original level as the cells became tolerant of higher Cd^{2+} concentrations. The Sp1/MRE-d complex displayed very similar changes with a 2.5-fold increase in sub-line cells adapted to 1 µM Cd^{2+}, and a decrease to the original level at higher Cd^{2+} concentrations. In contrast, the synthesis of MT (fig. 2A, bottom) increased progressively from 0.05 to 10.7 nmol/10^6 cells with the adaptation of the cells to increased concentrations of Cd^{2+}. The increase in autoradiographic band intensity observed in the Zn-resistant RK-13 sub-line series (fig. 2B, top) revealed that the binding activity of MTF-1 increased 2.9-fold when the cells became adapted to an environmental Zn^{2+} concentration of 100 µM and decreased again in cells tolerant to yet higher Zn^{2+} concentrations. The autoradiographic quantification of the change in the binding activity of Sp1 after adaptation to Zn^{2+} indicated similar tendencies. In cells resistant to metal concentrations higher than 100 µM Zn^{2+} the relative band shift intensity of Sp1 was 1.6 to 2.6-fold higher than that measured in the parental cells. As for Cd^{2+} adapted sub-line cells the MT production increased from 0.05 to 5.3 nmol/10^6 with increased concentration of Zn^{2+} (fig. 2B, bottom).

MT gene content of metal-tolerant RK-13 sub-line cells. In order to assess and compare the gene content in parental RK-13 and various Cd- or Zn-resistant RK-13 sub-line cells, genomic DNA was digested with restriction enzymes EcoRI/Hind III and analyzed by Southern blot hybridization. Figure 3 shows that each cell sub-line produced five major bands, corresponding to fragments of 7.0 kb, 4.3 kb, 3.2 kb, 1.3 kb and 1.2 kb, that hybridized to the synthetic oligonucleotide SCC (see 'Materials and methods' section). A sixth fragment of about 1.6 kb was visible upon prolonged exposure. The intensities of the bands of 1.3 kb and 1.2 kb were similar in all sub-lines and were, therefore, used as internal references to normalize the intensities of the other three bands. Quantification of the relative band intensities showed that the sub-line cells adapted to 100 µM Cd^{2+} (Cd100) displayed 1.4 times the amount of the 7.0 kb MT gene-containing fragment; Cd150 sub-line cells contained 1.9 times the amount found in the parental RK-13 cells (fig. 4A). The relative amounts of the two bands at 4.3 and 3.2 kb were only slightly increased (1.2 times in the Cd100 and 1.3 times in Cd150 sub-line cells). In Zn-adapted sub-line cells the relative amounts of the MT gene fragment at 7.0 kb increased from 1.4 to 3.2 with the adaptation of the cells from 100 to 350 µM Zn^{2+} (fig. 4B). Similarly, the amount of the 3.2 kb band increased by 1.2 to 2.7 times

the amount found in the parental RK-13 cells. In contrast, the band at 4.3 kb showed a 1.9-fold increase at 200 μM Zn^{2+} but its relative intensity decreased again in sub-line cells adapted to 250 μM Zn^{2+} (Zn250) and Zn350 sub-line cells.

Discussion

The regulation of MT gene expression by heavy metals is believed to be mediated by nuclear transcription factors, which recognize short DNA motifs, the so-called MREs[17–19]. It therefore stands to reason that increased cellular tolerance to metal toxicity could also be brought about by enhancing the efficiency of transcriptional regulation through an increased supply or activation of such transcriptional factors. Gel retardation assays[5] afford a convenient means to identify and quantify the nuclear factors MTF-1 and Sp1 in nuclear extracts of the various Cd- or Zn-resistant RK-13 sub-line cells binding to the mouse MRE-d element (fig. 1). The PhosphorImager scans of the two signals showed that both factors are normal constituents of RK-13 cells and that their levels increased up to three-fold upon adaptation of the RK-13 sub-lines to elevated metal concentrations (fig. 2, top). The two nuclear proteins undergo a parallel increase in binding activity until they reach a maximum, after which the level diminishes again in the cell sub-lines adapted to still higher metal concentrations.

The total MT content increases progressively with the adaptation of the cells to increased concentrations of the metal (fig. 2, bottom). The pronounced augmentation of the MT content in sub-line cells adapted to lower metal concentrations is in good agreement with the highest level of the MTF-1 and Sp1 activities. However, the synthesis of even higher amounts of MT at high metal concentrations, at which the activities of both transcription factors are reduced again, indicates that these sub-line cells respond to the metal challenge with additional mechanisms other than transcriptional regulation alone.

Amplification of MT genes is an important factor in bringing about cellular tolerance to metal toxicity. Some investigators have shown that cells selected for resistance to cadmium toxicity can exhibit MT gene amplification[6,7] and there is also some evidence that an increase in gene copy number occurs in the liver of mice exposed to Cd^{2+} (ref. 20). To delineate the contributions of gene amplification to the metal resistance acquired by RK-13 cells the MT gene copy content was monitored in parental RK-13 and in sub-line cells adapted to increasing concentrations of Cd^{2+} or Zn^{2+}. While the significant increase in relative intensity of only one MT gene fragment in Cd^{2+}-adapted sub-line cells (fig. 4A) is in contrast to previous studies[8] Zn^{2+}-adapted sub-line cells exhibited a good correlation between gene amplification (fig. 4B) and the enhanced synthesis of different isoMTs[8]. Surprisingly, however, at the highest Zn^{2+} concentration the abundance of the 4.3 kb MT gene fragment was selectively lowered again. This change, which may be related to the earlier reported disappearance of the gene product MT-2d at extreme Zn^{2+} exposure[8], supports the view that the amplification of the various genes in Cd^{2+}- and Zn^{2+}-adapted sub-line cells is regulated differentially and in a metal-specific manner.

The present data show clearly that the adaptation of RK-13 cells to increased doses of Zn^{2+} or Cd^{2+} is a multistep process. The augmentation of cellular MT as cells become tolerant may be viewed as the result of a progressive adjustment at the different hierarchic levels controlling MT gene expression, from the promotion of transcription of the MT genes by metal activation of constitutive MTFs, to a quantitative enhancement in the occupation of DNA by MTF (or of their MRE-binding) (fig. 1), and finally to a multiplication of the number of gene copies (fig. 3). Increased DNA-binding activities of the transcription factors MTF-1 and Sp1 in cells adapted to moderately elevated metal concentrations (fig. 2) could reflect either increased synthesis of these proteins or their forced liberation from an inactive complex[21]. The reduction in DNA-binding by these transcription factors observed in sub-line cells accommodated to yet higher metal concentrations suggests that in these conditions, where the cellular MT content is increased by another order of magnitude, modulation of MT expression at the transcriptional level is completely replaced by gene amplification and perhaps other as yet unidentified adaptive mechanisms. Since the extent of gene amplification seems insufficient to account for the observed MT production, especially in the Cd^{2+}-tolerant cells, changes at the translational level, for example in the stability of mRNA, might also be operative.

Abbreviations

Cd60: RK-13 sub-line cells resistant to Cd^{2+} in the culture medium. The number indicates the Cd^{2+} concentration to which the cells were adapted.
HPLC: high performance liquid chromatography
MT: metallothionein
MTF-1: metal transcription factor
MRE: metal responsive element
PMSF: phenylmethyl sulfonyl fluoride
RK-13: rabbit kidney cell line
SCC: a 19 mer deoxyoligonucleotide based on the sequence of beginning of the third exon of the rabbit MT-1 gene
Sp1: a transcription factor of RNA polymerase II common to all vertebrates
Zn100: RK-13 sub-line cells resistant to Zn^{2+} in the culture medium. The number indicates the Zn^{2+} concentration to which the cells were adapted.

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