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COORDINATE AMPLIFICATION OF METALLOTHIONEIN I AND II GENE SEQUENCES IN CADMIUM-RESISTANT CHO VARIANTS[1]

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ABSTRACT Cadmium-resistant (Cd^r) variants of the Chinese hamster cell line, CHO, have been derived by stepwise selection for growth in medium containing $CdCl_2$. These variants show coordinately increased production of both metallothionein (MT) I and II and were stably resistant to Cd^{2+} in the absence of continued selection. Genomic DNAs from these Cd^r sublines were analyzed for both MT gene copy number and MT gene organization, using cDNA sequence probes specific for each of the two Chinese hamster isometallothioneins. These analyses revealed coordinate amplification of MT I and II genes in all Cd^r variants which had increased copies of MT-encoding sequences. In situ hybridization of an MT-encoding probe to mitotic chromosomes of a Cd^r variant, which has amplified MT

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genes at least 14-fold, revealed a single chromsomal site of hybridization. These results suggest that the isoMTs constitute a functionally related gene cluster which amplifies coordinately in response to toxic metal stress.

INTRODUCTION

The phenomenon of gene amplification has been observed in both bacteriophage and bacteria [reviewed in (1)] and in eukaryotic cells *in vivo* and in culture [reviewed in (2)]. Specific gene amplification has been reported for developmentally regulated genes (3-6), genes conferring drug resistance (7,8), cellular homologs of viral oncogenes (9), and genes conferring resistance to heavy metal toxicity (10-12).

In the context of resistance to heavy metal toxicity, we have examined the regulation of metallothionein gene expression in the cadmium sensitive (Cd^s) Chinese hamster line, CHO, and in Cd-resistant (Cd^r) variants derived from these cells. Production of high affinity metal-binding proteins, the metallothioneins (MTs), has been shown to be a major factor contributing to the stable Cd^r phenotype (10-16). It is of interest that synthesis of two major isometallothioneins is induced coordinately in Cd^r variants in response to subtoxic exposures to $CdCl_2$ or $ZnCl_2$ (15). In this paper we examine the role of amplification of the genes encoding the two major isoMTs in regulation of isoMT expression and in conferring the Cd^r phenotype. Results of preliminary studies on the chromosomal organization of these genes, examined in one Cd^r variant, may provide insight concerning the coordinate regulation of the isoMTs and the stability of the Cd-resistance phenotype.

RESULTS

Cd^{2+}-resistant CHO sublines have been derived by exposure of the CHO cell in monolayer culture to stepwise increases in $CdCl_2$ levels (13,14). After continuous growth in medium containing stepwise increases in Cd^{2+} concentration, Cd-resistant (Cd^r) variants were cloned and characterized for (a) stability of the Cd^r phenotype in the of selective pressure, (b) Cd^{2+} uptake and intracellular

partitioning, and (c) metallothionein (MT) expression (13, 14, 16). Phenotypic characteristics of the CHO cell and four Cd^r variants resistant to $CdCl_2$ concentrations from 2 to 200 μM in cell culture medium are summarized in Table 1. All of the Cd^r variants are stably resistant to Cd^{2+} during long-term growth (up to 135 population doublings) in the absence of selective pressure.

TABLE I

Phenotypic Characteristics of CHO Cells and Cd^r CHO Variants

Cell Line	Toxic Threshold for $CdCl_2$ Exposure (μM $CdCl_2$)	Basal MT Synthesis Rate[b]	Maximally Induced MT Synthesis Rate[b]
CHO	0.2	--[c]	--[c]
Cd^r2C10	2.0	--[c]	28.3
Cd^r20F4	26	--[c]	60.6
Cd^r30F9	40	--[c]	41.7
Cd^r200T1	145	40	320

[a] Methods for the measurement of toxic threshold for $CdCl_2$ exposure have been published (14).

[b] Relative MT synthesis rate measurement and calculation has been described (15).

[c] MT synthesis was undetectable in these cells under the conditions given.

Increased production of MT in response to Cd^{2+} exposure (both rate of induction and maximal level of expression) is a major factor in development of increased cellular Cd^{2+}-resistance. The Cd^r30F9 variant was an interesting exception to the correlation between increased MT expression and increased Cd^{2+}-resistance (14). Nondenaturing polyacrylamide gel electrophoresis revealed coordinate induction of both major isoMTs in all Cd^r variants (Fig 1, 15). Given the metal-loading capacity and molecular weight of MT, measurements of Cd^{2+} uptake into MT indicate that in maximally-induced Cd 200T1 cells, MT could represent at least 2% of total cytoplasmic protein.

Metallothionein Gene Amplification

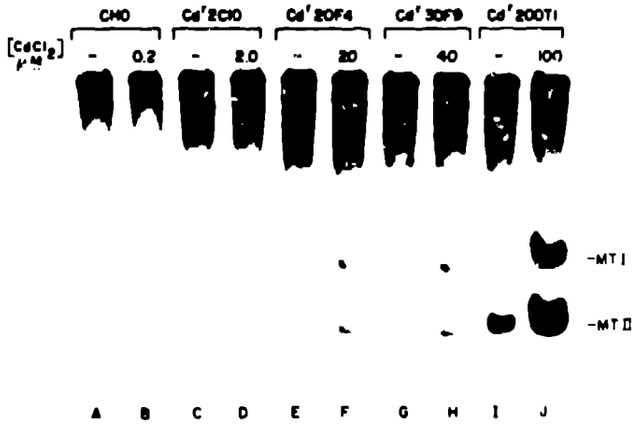

FIGURE 1. Nondenaturing polyacrylamide gel electrophoresis of cytoplasmic fractions from CHO cells and Cd^r variants. Cells were exposed to indicated concentrations of $CdCl_2$ and pulse-labeled with ^{35}S-cysteine for 30 minutes prior to the time of maximal MT synthesis (14-16).

To determine whether the MT overproduction phenotype of of the Cd^r variants was a consequence of genotypic alteration in the genes encoding either or both of isoMTs, genomic DNA from CHO cells and each of the Cd^r variants was purified and analyzed for MT gene organization and copy number. These analyses used specific MT-encoding and 3' non-coding sequence probes derived from recombinant cDNA clones for the two major Chinese hamster isoMTs [pCHMT1 and pCHMT2, (17)]. Figure 2 diagrams the strategy for derivation of DNA sequence probes for MT protein encoding regions and for 3' non-coding regions specific for MTI and MTII genes. In control experiments, filter hybridization analyses of linearized plasmid pCHMT1 and pCHMT2 DNAs using the MT protein coding region probe derived from pCHMT2 provided cross-hybridization with pCHMT1 under conditions of high stringency ($\geq$ 80% homology cut-off) as expected from the 81% nucleotide sequence homology in the protein coding region. In contrast, under the same hybridization stringency conditions, probes derived from the 3'-untranslated regions of pCHMT1 and pCHMT2 showed hybridization to their homologous plasmids but no cross-hybridization to their nonhomologous plasmids. The properties of these pCHMT1- and pCHMT2-derived cDNA probes permitted analysis of both the organization and dosage of MT genes in the CHO cells and in the Cd^{2+}-resistant variants.

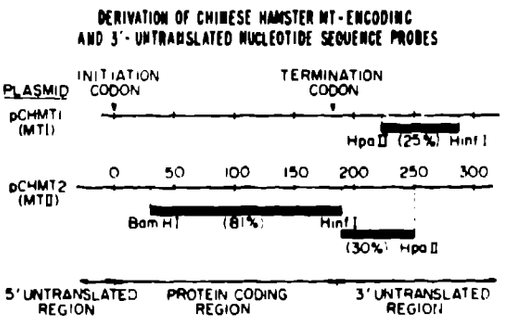

FIGURE 2. Strategy for derivation of Chinese hamster MT-encoding and 3'-untranslated nucleotide sequence probes. Heavy solid lines indicate regions used for probe construction. Numbers in parentheses under heavy lines represent sequence homology between pCHMT1 and pCHMT2 in the designated regions (17).

Sequence representation of MT structural genes in CHO cells and the CHO Cd^r variants were estimated 1) by nucleic acid reassociation kinetic analyses using the MTII-encoding structural gene probe (Fig. 2) as tracer driven by genomic DNAs isolated from the different cell lines, and 2) by Southern blotting and filter hybridization analysis using the same probe and DNAs from the same variants. The reassociation kinetics of Chinese hamster pCHMT2 DNA with total genomic DNA from the CHO cell and each of cadmium resistant variant cells is shown in Fig. 3. Similar results were been obtained using an indeperdently purified set of genomic DNAs and a 63 nucleotide HinfI-HpaII restriction fragment from pCHMT2 (Fig. 2) as tracer. When genomic DNA was omitted from the reaction mixes, the rate of pCHMT2 DNA self-reassociation was slow relative to that measured in the presence of CHO DNA indicating that tracer self reassociation does not significantly affect the hybridization kinetics observed in Fig. 3. Based upon extrapolation from Cot 1/2 values of the respective hybridization reactions, MT-like gene sequences are amplified approximately 1x, 7x, 3x, and 14x in the genomes of Cd^r2C10, Cd^r20F4, Cd^r30F9, and Cd^r200T1 respectively, relative to their abundance in the genome of the cadmium-sensitive, parental CHO cell. By comparison with the rate of reaction of the slowest kinetic component in 300 nucleotide long total, genomic Chinese hamster DNA (10), we estimate that the complement of MT genes in CHO cells is near single-copy levels.

Metallothionein Gene Amplification

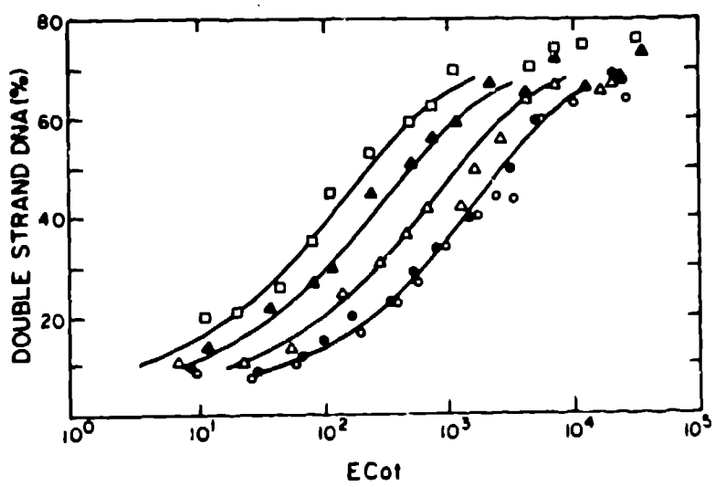

FIGURE 3. Nucleic acid reassociation kinetic analyses of pCHMT2 with genomic DNAs from CHO (○), Cd^r2C10 (●), Cd^r20F4 (▲), Cd^r30F9 (△), and Cd^r200T1 cells (□). Methods for extraction, shearing, annealing, and hydroxylapatite chromatography of Chinese hamster cell DNA have been described (10).

To investigate further how the amplified MT genes are organized in the genomes of the Cd^r variants, electrophoretically-resolved, restriction endonuclease digests of genomic DNA from CHO cells and each of the Cd^r variants were analyzed by filter hybridization with the MT-coding region probe (Fig. 2). Two major bands of hybridization, at ~2.3 kb and ~17-19 kb, were observed in the HindIII digests of Cd^r20F4, Cd^r30F9 and Cd^r200T1. This result suggested that these two hybridization bands may represent MTI and II genes since MTI and II coding regions share extensive homology (17). Further, the results in Fig. 4 indicate that the MT genes are amplified coordinately.

Metallothionein Gene Amplification

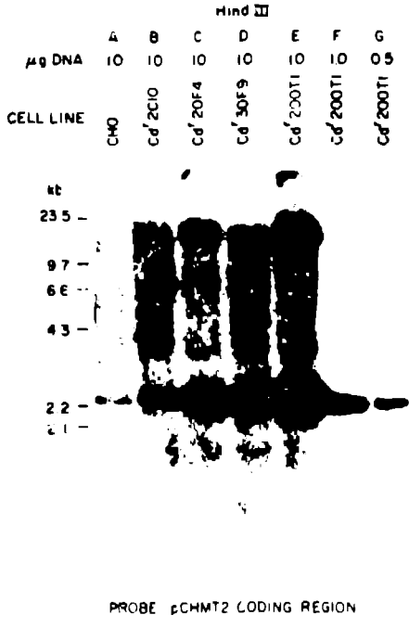

FIGURE 4. Filter hybridization analyses of genomic DNAs from CHO cells and Cd^r variants. DNA was digested with HindIII according to conditions specified by the supplier, electrophoretically resolved in a 1% agarose gel, transferred to Gene Screen filters (New England Nuclear Corp.), and hybridized with the pCHMT2 protein-coding region probe (Fig. 2). Hybridization was for 24-48 hr at 60°C according to procedures specified for Gene Screen by New England Nuclear Corp. Filter washes were also performed according to procedures specified by the supplier. In independent digests the high molecular weight fragment (17-19 kb) is also observed in CHO and Cd'2C10 cells. The lower intensity observed for the 17-19 kb fragment may reflect comparative specificity of the pCHMTII probe for the 2.3 kb fragment. Alternatively, less efficient transfer of high molecular weight DNA fragments may explain this result.

Metallothionein Gene Amplification

To test the hypothesis of coordinate amplification, genomic DNA from the Cd^r200Tl was digested with different restriction endonucleases (HindIII, BamHI, and EcoRI) and analyzed by filter hybridization with both MT-encoding structural gene probe and the MTI- and MTII-specific cDNA probes obtained from the 3' untranslated regions of pCHMT1 and pCHMT2 inserts. The results of these filter hybridization analyses (Fig. 5A) reveal two primary bands of hybridization (of approximately equal intensity) with the MTII protein-encoding region probe in each of the digests described above. In contrast, when digested with Hind III or BamHI Cd^r200Tl DNA yielded two fragments which hybridized differentially to the MTI- and MTII-specific 3' untranslated region probes (Fig. 5B) probes. These fragments were of the same apparent size as the HindIII and BamHI fragments which hybridized with MT coding region probes (Fig. 5A). The two fragments generated by Eco RI did not show differential hybridization to the isoMT-specific probes. The results with HindIII and BamHI demonstrate that the genomic DNA sequences encoding MTI and MTII are amplified coordinately in the Cd^r200Tl subline. Further the similarity of fragment sizes hybridizing the MT-protein coding region probe in both HindIII (Fig. 4) and BamHI (data not shown) digests of all Cd^r variants indicates that the MTI and II genes are amplified coordinately in all of these cell lines. In independent filter hybridization measurements, comparison of hybridization of the MTII encoding probe with CHO genomic DNA and dilutions of Cd^r200Tl genomic DNA revealed that the MT gene copy number in Cd^r200Tl may be as high as 40-50 fold greater than the copy number in the CHO cells (Fig. 4).

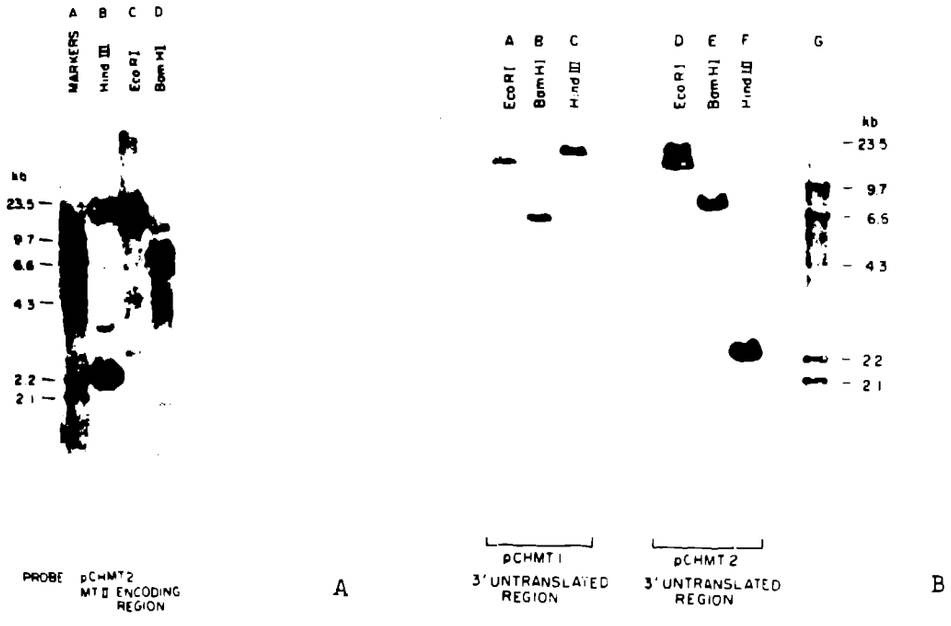

FIGURE 5. Analyses of genomic DNA from Cd^r200Tl digested with the indicated restriction endonuclease, electrophoretically resolved in a 1% agarose gel, transferred to filter and hybridized with MT coding region probe (A, lanes B-D) or 3' untranslated region probe from pCHMT1 (B, lanes A-C) or 3' untranslated region probe from pCHMT2 (B, lanes D-F). Lane C contains ^{32}P-labeled HindIII digested markers. Filter hybridization and wash procedures were described in the legend to Fig. 4.

The coordinate amplification of the MTI and MTII encoding genes in Cd^r200Tl, coupled with th observed stability of the Cd^r phenotype suggested that the amplification event(s) may be localized to a specific chromosome(s). *In situ* hybridization (21,22) of the MTII protein coding region probe with mitotic chromosomes from the Cd^r200Tl cell line revealed a single chromosomal site of hybridization (Fig. 6). This observation supports the proposal that MT genes are closely linked. Interestingly, further cytogenetic analyses have shown that the region of hybridization in these cells corresponds to a translocation breakpoint on a large rearranged chromosome in the Cd 200Tl karotype. Further molecular genetic and cytogenetic analyses are directed toward revealing the genomic arrangement(s) and chromosomal location(s) of the isoMT genes in the Chinese hamster.

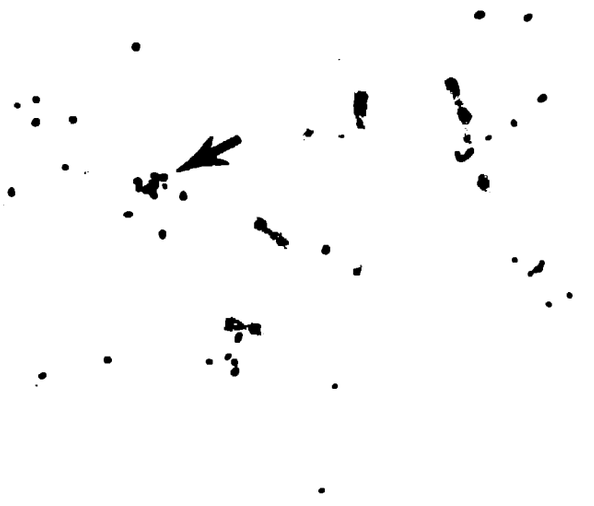

FIGURE 6. In situ hybridization of mitotic chromosomes from the Cd^r 200T1 cell with an MTII-encoding sequence probe. ^{125}IdCTP-labeled MTII-encoding probe was hybridized with the Cd^r 200T1 metaphase plates according to established procedures (21,22). Arrow designates consistently labeled region on a large, rearranged chromosome.

DISCUSSION

Specific gene amplification is one mechanism by which eukaryotic cells respond to metabolic stress [reviewed in (2)]. In the cases studied most extensively, inhibitors of specific target enzymes have been used to select cells containing increased copies of the gene(s) encoding the target enzyme (7,8). In other cases, cellular resistance to metabolic inhibitors arises through amplification of genes encoding products not directly related to the action of the inhibitor (18-19). Cellular resistance to heavy metal toxicity falls into the latter category.

Increased production of metallothionein in Cd-resistant cells has been identified as a major factor in conferring the resistant phenotype (13-16). By virtue of their high affinity Cd-binding capacity, cytoplasmic MTs sequester

the toxic Cd^{2+} ion, thereby reducing access of this inhibitory heavy metal to intracellular targets (14, 16). In this study, increased production of both major isoMTs was observed in Cd^r Chinese hamster cells. In the clonal variants resistant to 20-200 M Cd^{2+} (Cd^r20F4, Cd^r30F9, Cd^r200T1), coordinate amplification of both MTI- and MTII-encoding genes occurred at levels ~7-, 3-, and 14-fold, respectively, above the gene copy number in CHO by reassociation kinetics analyses, and up to 50-fold in Cd^r200T1 by filter hybridization analyses. Paradoxically, both the degree of gene amplification, as well as MT induction capacity, do not correspond quantitatively with increased Cd-resistance suggesting that other mechanisms, possibly metal-inducible (20), operate in conferring Cd^{2+}-resistance (14,16).

The stability of the Cd^r phenotype reported here for Cd^r-CHO variants suggested that the amplified MT genes were stably chromosome-associated (2). In all Cd^r variants which maintain amplified MT genes, the G1 DNA content is indistinguishable from the parental CHO cell (data not shown). This finding is in contrast to that of Gick and McCarty (12) who demonstrated that Cd^r CHO cells with amplified MT genes also displayed an abundance of tetraploid cells, as well as partial instability of the Cd^r phenotype. In the context of the relationship between stability of the Cd^r phenotype and chromosomal or extrachromosomal location of amplified genes, the *in situ* hybridization analyses shown here indicate a chromosomally-integrated site for the amplified MT genes. This localization of MT-encoding sequences to a single chromosome is consistent with our observation of the coordinate amplification of Chinese hamster MTI and II genes suggesting their close linkage. Further analyses of the organization of the unit(s) of amplification, the linkage of the MT genes, and the chromosomal location of the MT genes in both CHO and euploid Chinese hamster cells are in progress.

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