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Calcium Phosphate-Mediated Transfection Alters Metallothionein Gene Expression in Response to Cd^{2+} and Zn^{2+}

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The level of expression of a transfected metallothionein (MT)-IGcat fusion gene in response to cadmium differed from that of the endogenous MT-IG gene. Atomic absorption analysis indicated that the total cellular content of cadmium and zinc increased upon calcium phosphate-mediated transfection. Thus, changes in the influx/efflux of metals may regulate the level of MT gene expression.

Metallothioneins (MTs) are a ubiquitous family of proteins which bind transition-state heavy metals (5, 7). In humans, the two isoformic classes of MT, MT-I and MT-II, are represented by one MT-II and at least five variant MT-I polypeptides (8). These proteins are encoded by a multigene family which contains one functional MT-II gene (9) and at least five functional MT-I genes (3, 6, 14, 17, 19). The MT-I genes have been shown to be differentially induced by heavy metals and expressed in a cell type-specific manner (3, 6, 14–16, 19).

In order to elucidate the molecular mechanisms which govern the differential inducibility of the MT-IG gene (3), S1 nuclease studies were used to investigate the transient expression of a MT-IGcat fusion gene in calcium phosphate-transfected Hep-G2 cells (10). Cells were maintained, transfected, and induced by heavy metals (2 μM $CdCl_2$, 150 μM CuCl, and 100 μM $ZnCl_2$) as previously described (3, 4). S1 nuclease analysis of total cytoplasmic RNA (2) illustrates that 215 base pairs (bp) of the 380-bp 5′-end-labeled *Pvu*II probe are protected by MT-IGcat mRNA upon cadmium, copper, and zinc induction (Fig. 1). A low basal level of expression could be detected upon a longer exposure. Of the three inducers, cadmium induces the highest level of MT-IGcat mRNA. However, comparison of the S1 nuclease analysis of the endogenous MT-IG gene in Hep-G2 cells depicted in Fig. 2A with that of the MT-IGcat fusion gene in Fig. 1 indicates that the differential metal inducibility of the endogenous MT-IG gene differs from that of the transfected MT-IGcat fusion gene. In the in vivo situation, copper and zinc induced the highest level of MT-IG mRNA, while in transfected Hep-G2 cells, cadmium induced the highest level of MT-IGcat mRNA.

To investigate the possibility that the calcium phosphate transfection procedure alters the metal inducibility of the transfected MT-IGcat fusion gene, we examined the expression of the endogenous MT-IG gene in mock-transfected Hep-G2 cells. S1 nuclease analysis indicated that the MT-IG gene is expressed at a low basal level and can be induced to its highest level by cadmium and zinc (Fig. 2B). Basal level expression could be detected upon a longer exposure (data not shown). Thus, both the endogenous MT-IG gene and the MT-IGcat fusion gene exhibit identical differential induction patterns in calcium phosphate-transfected cells. It is reasonable to speculate that the transfection process may alter MT-IG expression by disrupting the influx/efflux of heavy metals during induction. Interestingly, Hep-G2 cells that were exposed to 20 μM A23187 for 20 min before metal induction exhibited a similar response to heavy metals. The calcium ionophore A23187 has been reported to increase the ability of divalent cations to diffuse through cellular membranes (13). S1 nuclease analysis indicates that the MT-IG gene is expressed at a low basal level that can be induced to its highest level by cadmium and zinc (Fig. 2C).

Atomic absorption analysis was used to investigate whether calcium phosphate transfection or A23187 exposure alters the total cellular content of cadmium, copper, or zinc. Hep-G2 cells were grown on 225-cm^2 cell culture flasks or on 150-cm^2 plates until almost confluent. The cells on the 150-cm^2 plates were mock transfected, and those on the 225-cm^2 flasks were exposed to A23187; all cells were then induced with cadmium, copper, and zinc. Cells were har-

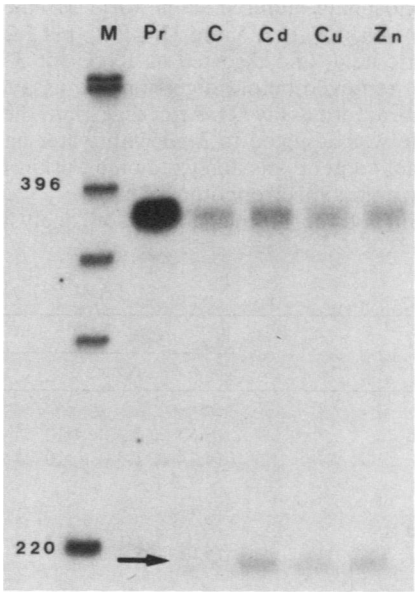

FIG. 1. Transient expression of the MT-IGcat fusion gene in Hep-G2 cells. S1 nuclease analysis of total cytoplasmic RNA isolated from uninduced (C), cadmium-induced (Cd), copper-induced (Cu), and zinc-induced (Zn) cells transfected with MT-IGcat. The arrow indicates the 215-bp S1-resistant fragment. Pr, 380-bp probe; M, ^{32}P-labeled *Hin*fI fragments of pBr322.

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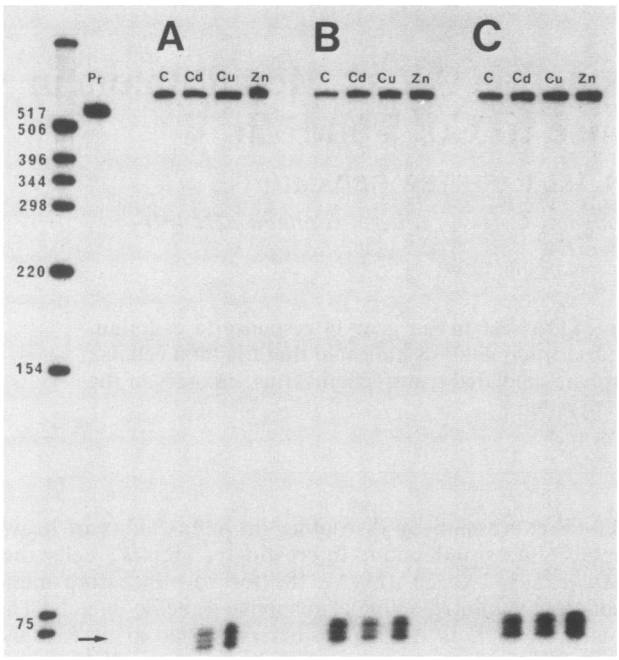

FIG. 2. Expression of the MT-IG gene in Hep-G2 cells. S1 nuclease analysis of total cytoplasmic RNA isolated from control (A), calcium phosphate mock-transfected (B), and A23187 (20 μM)-exposed (C) cells in response to no inducer (C), cadmium (Cd), copper (Cu), and zinc (Zn). For each analysis the transcription initiation site of the MT-IG gene was mapped as described earlier (3). The 65-bp S1-resistant fragment is indicated by an arrow. The appearance of multiple bands is characteristic of the total cytoplasmic mRNA preparation (2), since repeated S1 analysis of total nucleic acids detects only one MT-IG transcription initiation site (3). Pr, 630-bp *Apa*I-*Ava*I probe; M, ^{32}P-labeled *Hin*fI digest of pBR322.

vested by trypsinization, and the cell pellet was suspended in 200 μl of phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2), added to 2 ml of nitric acid, and digested at 100°C for 3 h. A 10-μl portion was removed before digestion and assayed for protein by the Bradford assay (1). After digestion, the volume of each sample was adjusted to 3 ml with water and the total cellular metal content was analyzed on a Perkin Elmer 5000 atomic absorption spectrophotometer.

The results of the atomic absorption analysis are presented in Fig. 3 and Table 1. These data indicate that cadmium- and zinc-induced and uninduced cells of the control, calcium phosphate, and A23187 treatment groups contained relatively the same amount of total cellular copper. However, the total cellular copper content increased about 10-fold upon copper induction for all three groups. Thus, neither calcium phosphate transfection nor A23187 exposure increases the total cellular content of copper in Hep-G2 cells.

The total cellular contents of cadmium in the control, calcium phosphate, and A23187 treatment groups increased 6-, 50- and 30-fold, respectively, over those of the copper- and zinc-induced and uninduced cells of the same treatments upon cadmium induction (Fig. 3A; Table 1). These results indicate that the total cellular content of cadmium in cadmium-induced calcium phosphate- or A23187-treated cells exceeded that of the cadmium-induced control group by 6.6- and 4.1-fold, respectively. This observation explains why cadmium is such a good inducer of MT-IGcat and MT-IG expression in calcium phosphate-transfected and A23187-exposed cells. Excess intracellular metal could elevate the level of MT-IG or MT-IGcat induction through a putative cadmium-specific *trans*-acting factor and the metal-responsive elements (18).

Total cellular zinc in the control, calcium phosphate, and A23187 treatment groups increased by approximately 1.75-, 2.62-, and 2.29-fold, respectively, over that of the cadmium- and copper-induced and uninduced cells of the same treatments upon zinc induction. In this case, the total cellular zinc contents of zinc-induced calcium phosphate- and A23187-treated cells exceeded those of zinc-induced cells of the control group by approximately 1.89- and 1.74-fold, respectively. Interestingly, the total cellular zinc contents of cadmium- and copper-induced and uninduced cells of calcium phosphate-transfected and A23187-exposed groups exceeded those of the control group by 1.25- and 1.33-fold, respectively. Obviously, the source of this zinc must be the media. Considering that a 1.75-fold increase in total cellular zinc results in considerable induction of MT-IG mRNA in response to zinc induction (Fig. 2A), the increase in total cellular zinc upon calcium phosphate transfection or A23187 exposure is significant. This increase in total cellular zinc would explain the basal level of MT-IG expression observed in uninduced mock-transfected and A23187-treated cells (Fig. 2B and C). The mechanism for this induction probably

TABLE 1. Metal contents of treated and control cells

Metal and cell type[a]	Total metal content (mol/mg of protein)[b] of cells with treatment		
	None	Calcium phosphate	A23187
Cadmium			
Uninduced	$(6.85 \pm 1.38) \times 10^{-12}$	$(5.45 \pm 3.16) \times 10^{-12}$	$(5.74 \pm 1.83) \times 10^{-12}$
Induced	$(4.14 \pm 1.33) \times 10^{-11}$	$(2.72 \pm 0.83) \times 10^{-10}$[c]	$(1.70 \pm 0.35) \times 10^{-10}$[c]
Copper			
Uninduced	$(6.04 \pm 1.79) \times 10^{-10}$	$(6.45 \pm 2.09) \times 10^{-10}$	$(7.23 \pm 1.62) \times 10^{-10}$
Induced	$(5.08 \pm 0.12) \times 10^{-9}$	$(7.47 \pm 2.67) \times 10^{-9}$	$(6.49 \pm 0.61) \times 10^{-9}$
Zinc			
Uninduced	$(1.38 \pm 0.21) \times 10^{-9}$	$(1.73 \pm 0.23) \times 10^{-9}$[c]	$(1.83 \pm 0.23) \times 10^{-9}$[c]
Induced	$(2.41 \pm 0.43) \times 10^{-9}$	$(4.54 \pm 0.84) \times 10^{-9}$[c]	$(4.19 \pm 0.37) \times 10^{-9}$[c]

[a] Values in rows labeled "uninduced" are for uninduced and other-metal-induced cells ($n = 9$); for induced cells, $n = 3$.
[b] Mean ± standard deviation. All differences between values for uninduced and induced cells are statistically significant within each treatment (t test; $P < 0.05$).
[c] Significantly different from corresponding control value (t test; $P < 0.05$).

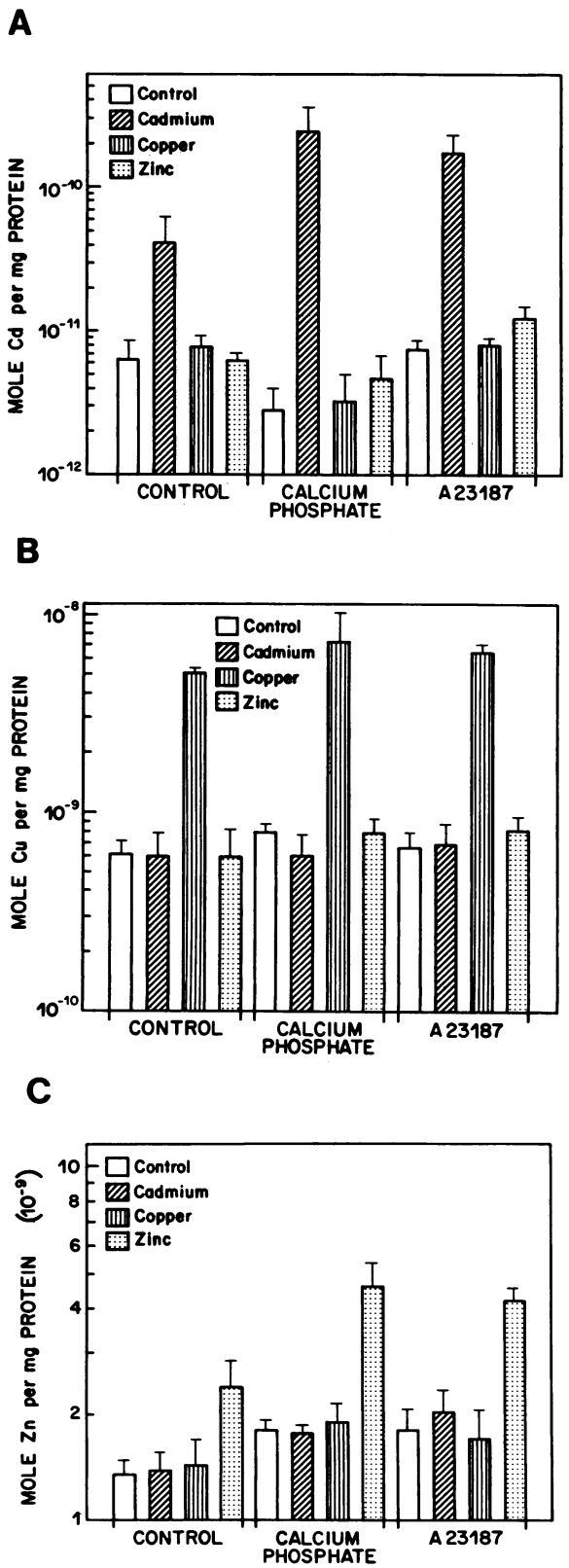

FIG. 3. Cellular metal content of Hep-G2 cells. Moles of cadmium (A), copper (B), and zinc (C) per milligram of cellular extract was determined by atomic absorption as described in the text. Metal content was measured in control, calcium phosphate mock-transfected, and A23187-treated cells in response to no inducer (C), cadmium (Cd), copper (Cu), and zinc (Zn). Each determination was made in triplicate.

involves a zinc-specific *trans*-acting factor and the metal-responsive elements (20).

Analysis of the transient expression of calcium phosphate-mediated transfected chimeric fusion genes has become a useful tool for monitoring gene expression. However, we show here that calcium phosphate-mediated transfection alters MT-IG expression in response to cadmium and zinc. During metal induction it allows 6.6- and 1.9-fold increases in total cellular cadmium and zinc, respectively, over levels in cadmium- and zinc-induced samples of the control group. These results suggest that the differential expression of MT genes in cultured cells may in part be regulated by changes in the influx/efflux of heavy metals. Recently, Pine et al. have shown that calcium phosphate-mediated transfection activates the expression of interferon-stimulated and *fos* genes (12). If calcium phosphate-mediated transfection were to increase the total cellular content of calcium, then it is possible that this technique could also alter the expression of such genes as *fos* and MT-IIA, which are regulated by this secondary messenger.

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