Chromium(VI) Down-regulates Heavy Metal-induced Metallothionein Gene Transcription by Modifying Transactivation Potential of the Key Transcription Factor, Metal-responsive Transcription Factor 1*

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The robust induction of metallothionein-I and II (MT-I and MT-II) genes by several heavy metals such as zinc and cadmium requires the specific transcription factor metal-responsive transcription factor 1 (MTF1). Chromium (VI), a major environmental carcinogen, not only failed to activate these genes but also inhibited their induction by Zn$^{2+}$ or Cd$^{2+}$. The heavy metal-induced expression of another MTF1 target gene, zinc transporter 1 (ZnT-1), was also down-regulated by Cr$^{6+}$. By contrast, the expression of two MTF1-independent Cd$^{2+}$-inducible genes, heme oxygenase 1 (HO-1) and HSP-70, was not sensitive to Cr$^{6+}$. Cr$^{6+}$ did not also affect the expression of housekeeping genes such as GAPDH or β-actin. Stable cell lines overexpressing variable levels of MTF1, the key transactivator of the MT genes, demonstrated differential resistance toward the inhibitory effect of Cr$^{6+}$, indicating MTF1 as a target of chromium toxicity. The basal and inducible binding of MTF1 to metal response elements was not affected by treatment of cells with Cr$^{6+}$. Transient transfection studies showed that the ability of MTF1 to transactivate the MT-I promoter was significantly compromised by Cr$^{6+}$. The fusion protein consisting of a Gal-4 DNA binding domain and one or more of the three transactivation domains of MTF1, namely the acidic domain, proline-rich domain, and serine-threonine rich domain, activated the GAL-4-driven luciferase gene to different degrees, but all were sensitive to Cr$^{6+}$. MTF1 null cells were prone to apoptosis after exposure to Zn$^{2+}$ or Cd$^{2+}$ that was augmented in presence Cr$^{6+}$, whereas the onset of apoptosis was significantly delayed in cells overexpressing MTF1.

Chromium, a potential human mutagen and carcinogen (1, 2), exists in many different oxidation states in the environ-

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The abbreviations used are: MT, metallothionein; ZnT-1, zinc transporter 1; HSF-70, heat shock protein 70; HO-1, heme oxygenase 1; MRE, metal-responsive element; MTF1, metal-responsive transcription factor 1; TUNEL, terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling; IVGF, in vivo genomic footprinting; PI, propidium iodide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; LM-PCR, ligation-mediated PCR; FITC, fluorescein isothiocyanate; MT3, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMS, dimethyl sulfate.

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teins, MT-III and MT-IV, are expressed in a tissue-specific manner in and in squamous epithelial cells of tongue and skin, respectively. Several metal regulatory elements (MREs) located on the immediate MT promoter mediate its robust expression by recruiting the key transactivation factor metal-responsive transcription factor 1 (MTF1). MTF1 is a 71–84-kDa protein with six zinc fingers of the Cys2–His2 type and three different transactivation domains, all of which function cooperatively (21). In response to heavy metals MTF1 translocates to the nucleus, attains a conformation that binds to the cognate cis-elements, and transactivates the gene. Among the different heavy metals only zinc can directly activate MTF1, whereas other metals like cadmium probably activate it by mobilizing the intracellular zinc pool (22). MTF1 is essential for development as knockout mice die because of liver decay (22).

Recent studies show that MTF1 has several important target genes, such as those for C/EBPα (24), the placental growth factor (PlGF) (25), and zinc transporter-1 (ZnT-1) (23). The molecular mechanism by which MTF1 is activated in response to different stimuli, triggering its DNA binding and subsequent transactivation of the target genes, remains to be elucidated.

Recent studies show that various signal transduction pathways that include protein kinase C, casein kinase II, and tyrosine kinase modulate the transactivation function but not the DNA binding activity of MTF1 (26). Besides MTF1, several other transcription factors like Sp1, USF1, glucocorticoid receptor, and STAT3, are also involved in MT gene expression in response to different physiological and pathological conditions (27, 28). In the present study we have identified potential toxin hexavalent chromium, the predominant form in the industrial waste, that not only fails to induce MT expression but also interferes with its expression in response to other heavy metals by inhibiting the transactivation function of MTF1.

MATERIALS AND METHODS

Cell Culture and Heavy Metal Treatment—HepG2 cells were grown in DMEM with Earle’s salt containing 10% fetal bovine serum. dko-7 cells (MTF1 double knockout mouse fibroblast cells) and MTF1-overexpressing cells were grown as a monolayer in DMEM containing 5% fetal bovine serum. At 80–90% confluency the cells were treated for 2–3 h with either a combination of potassium dichromate with zinc sulfate, cadmium sulfate, or individual heavy metals at concentrations indicated in the respective figure legend. The cells were then washed and harvested for either RNA isolation or nuclear extract preparation as described.

Construction of Retroviral Vector Harboring Human MTF1 and Generation of Stable Cell Lines Expressing MTF1—To construct recombinant human MTF1 cDNA with the FLAG tag at the C terminus, we amplified the MTF1 coding region from pchMTF1 with the primers F, 5′-TAATCCGCTCCTACATAGGAGACC-3′, and R, 5′-ATCTTATCA- TTATCATTTGTTCATCTGCTTGGTTGACTCTTTGGAAGAGCTGGTGTGAG-3′, and ligated the PCR product to the Shab1 site of the pBabe-puro vector (29). pBabe-hMTF1-FLAG was then transfected into HepG2 cells (double knock out for hMTF1), cells and the cells overexpressing MTF1 were selected with DMEM containing puromycin (4 μg/ml) and hMTF1-overexpressing cells were grown in DMEM with Earle’s salt containing 10% fetal bovine serum. dko-7 cells (MTF1 double knockout mouse fibroblast cells) and MTF1-overexpressing cells were grown as a monolayer in DMEM containing 5% fetal bovine serum. At 80–90% confluency the cells were treated for 2–3 h with either a combination of potassium dichromate with zinc sulfate, cadmium sulfate, or individual heavy metals at concentrations indicated in the respective figure legend. The cells were then washed and harvested for either RNA isolation or nuclear extract preparation as described.

Electrophoretic Mobility Shift Assay—Nucleic extracts used for the DNA binding activities of MTF1 proteins were prepared as described (33), incubated with [γ-32P]ATP-labeled MRE-s duplex probe in the binding buffer (20 mM Hepes (pH 7.9), 75–90 mM KCl, 5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 μg of poly(dI-dC)/poly(dI-dC) of protein, and 10–12% glycerol), and resolved on a 6% non-denaturing polyacrylamide gel (33). The MTF1 antibody used for the supershift experiment was a generous gift from Dr. Walter Saffran. The Sp1 consensus oligonucleotide and anti-STAT3 antibody (sc-842) were from Santa Cruz Biotechnology Inc., Santa Cruz, CA.

In Vivo Genomic Footprinting—In vitro DNA methylation and extraction of the methylated DNA were performed as described (34). The mouse MT-I and human MT-IA promoters were amplified by ligated-mediated PCR (LM-PCR) according to the procedure of Mueller and Wold (35), as modified by Ping et al. (36). Briefly, HepG2 and MTF1–11 cells in DMEM (control or treated with 100 μM zinc sulfate) were exposed to a limited dimethyl sulfate treatment (1 μl/ml, 2 min at room temperature). The genomic DNA was isolated from the cells, purified, and subjected to piperidine cleavage (10%) at 90 °C for 30 min. The purified cleaved DNA (2 μg) was then subjected to LM-PCR to amplify MT-I promoters using primers described by Mueller and Wold (35).

The primers used to amplify human MT-IA promoters are the following: HMT2A/5′-1, 5′-ACCTGTCTGCACTTCCAACC-3′; HMT2A/3, 5′-TATACAAAGATGCTCCGGTTCC-3′; mZnT-1-A, 5′-GGAAGCGGTGTCCATACATTGATG-3′; HMT2A/3, 5′-ATATCCGAGCCCGACGAGAC-3′; HMT2A/3, 5′-TATATCCGAGCCCGACGAGAC-3′; HMT2A/3, 5′-GATGTCAGCGACAGTTCCCGG-3′.

Transfection Assay—HepG2 cells were grown in DMEM with 10% fetal bovine serum. For the transfection assay, 5.0 × 105 cells were plated onto 24-well plates 24 h beforehand and then transfected using the calcium phosphate precipitation method (37). Each transfection mixture contained a total of 8.8 μg of DNA including the reporter plasmid pMT-Luc (38), pRL-TK (Renilla luciferase reporter driven by the TK promoter), Promega pRL-SV40 (Promega internal control (the reporter plasmid), and eukaryotic expression vector harboring the gene of interest described in the respective figure legends. The cells were allowed to incubate in the presence of the transfection mixture in complete medium (DMEM plus 10% fetal bovine serum) for 16 h in a
in HepG2 cells. Total RNA (30 μg) isolated from HepG2 cells treated with Zn\(^{2+}\) (100 μM), Cd\(^{2+}\) (30 μM), Cr\(^{6+}\) (100 μM), Cr\(^{6+}\) (100 μM) plus Zn\(^{2+}\) (100 μM), or Cd\(^{2+}\) (30 μM) for 2 or 4 h. The cells were then washed of the heavy metals and subjected to RT-PCR assay including the manufacturer’s protocol (Roche Applied Science). Each treatment group was carried out in triplicate. Cells lysed with 0.1% Triton X-100 before the addition of RT-PCR reagent served as the negative control. Assay values for untreated cells were taken as 100%, and that of the negative control was assigned as 0%. B. Cr\(^{6+}\) down-regulates heavy metal-induced MT gene expression in HepG2 cells. Total RNA (30 μg) isolated from HepG2 cells treated with Zn\(^{2+}\) (100 μM), Cd\(^{2+}\) (30 μM), and Cr\(^{6+}\) (100 μM) for 3 h (as indicated) were subjected to Northern blot analysis with \(^{32}\)P-labeled mouse MT-I and GAPDH cDNA as probe. For the combination treatments, Cr\(^{6+}\) was added 15 min before the addition of Zn\(^{2+}\) or Cd\(^{2+}\). C. HepG2 cells were treated with Zn\(^{2+}\) (100 μM) and/or Cr\(^{6+}\) (100 μM) for 3 h, and total RNA was subjected to RT-PCR analysis with human MT-IIA and β-actin-specific primers. D. Run-on transcription with isolated nuclei. Nuclei were isolated from (5 × 10\(^5\)) cells either untreated or treated for 2 h with Cr\(^{6+}\) (100 μM), Zn\(^{2+}\) (100 μM), and Cr\(^{6+}\) plus Zn\(^{2+}\), and incubated in transcription buffer along with ATP, GTP, CTP, and [α-\(^{32}\)P]UTP at 30 °C for 5 min. RNA was purified, and identical cpm samples (10\(^4\))/ml were allowed to hybridize with plasmid DNA (pBS/SK, MT-I, and GAPDH) immobilized on Hybond N+ membrane in Rapid Hyb buffer (Amersham Biosciences) at 65 °C for 2 h. The membrane was washed and exposed to x-ray film.

37 °C incubator with 5% CO\(_2\) followed by replacement with fresh medium. Eight hour after removal of the transfection mixture the cells were split into 12 35-mm dishes, and each triplicate set was either left untreated or treated with Zn\(^{2+}\) and/or Cd\(^{2+}\) for 3 h before harvest. After a total of 48 h (and respective treatments) the cells were harvested in 1× lysis buffer (Promega), and luciferase activity was measured using the dual luciferase assay kit (Promega) in a luminometer (Lumat LB 9507; EG&G Berthold, Oak Ridge, TN). The different glutathione S-transferase-MTF1 fusion proteins used in the transfection studies were generous gifts from Dr. Schaffner (21).

**TUNEL and Annexin V Assays**—For detection of events of apoptosis in cells treated with heavy metals, an *in situ* cell death detection kit, fluorescein (Roche Applied Science Biochemicals), and ApoAlert annexin V kit (BD Sciences) were used. dko-7 and MTF1-12 cells were plated in 8-well LabTek chamber slides (Nalgene NUNC International) at a density of ~30,000 cells per well in DMEM containing 5% fetal bovine serum and puromycin, where appropriate. The cells were allowed to attach to the chamber and grow overnight in an incubator at 37 °C in 5% CO\(_2\). Apoptosis was induced by treating cells with 100 μM Zn\(^{2+}\), 30 μM Cd\(^{2+}\), and 100 μM Cr\(^{6+}\) (as indicated in the Figs. 9 and 10). The cells were then fixed and permeabilized for TUNEL assay. The assay was performed, and the cells were stained with propidium iodide (PI) according to the manufacturer’s protocol. For annexin V assay, the cells were induced with the metals as described and stained with annexin V-FITC and PI according to the protocol provided by the company.

**MTT Assay**—To assess the viability of cells during metal treatment, we performed MTT assay using a kit from Roche Applied Science. MTT reduction was carried out using HepG2 cells. The cells were plated at a density of 0.5 × 10\(^4\) cells/well in a 96-well plate and allowed to grow overnight followed by treatment with metals (100 μM each) for 2 and 4 h. Cells were then washed with medium, and 200 μl of fresh medium was added in each well followed by the addition of 10 μl of MTT reagent. After 4 h of incubation at 37 °C, 200 μl of lysis buffer was added to each well, incubated at 37 °C overnight, and read using an enzyme-linked immunoassorbent assay plate reader at 570 nm.

**RESULTS**

**Hexavalent Chromium Down-regulates Heavy Metal-induced MT-I Gene Expression**—Before we explored the effect of chromium(VI) on the induction of metallothioneins, we determined the duration of heavy metal exposure that exhibited minimal cellular toxicity. MTT assay was used to measure the redox potential of cells (39). Metabolically active cells reduce MTT, turning the yellow dye to purple/blue formazan, whereas the color change was markedly reduced in unhealthy cells. We treated HepG2 cells with either Cr\(^{6+}\) (100 μM), Zn\(^{2+}\) (100 μM), or Cd\(^{2+}\) (30 μM) for 2 or 4 h or pretreated with Cr\(^{6+}\) (100 μM) for 15 min before adding Zn\(^{2+}\) or Cd\(^{2+}\). Cells left untreated for the same length of time were used as control. In cells treated with Cr\(^{6+}\) along with Zn\(^{2+}\) or Cd\(^{2+}\), a marginal decrease in proliferative activity was observed, whereas treatment with individual metals for 4 h had no effect (Fig. 1A). For our subsequent studies we treated the cells for 3 h or less.

To investigate the role of Cr\(^{6+}\) on MT expression, we treated HepG2 cells with K\(_2\)Cr\(_2\)O\(_7\) and performed Northern blots of total RNA with \(^{32}\)P-labeled mouse MT-I cDNA as the probe. The results demonstrated that unlike Cd\(^{2+}\) or Zn\(^{2+}\), Cr\(^{6+}\) by itself could not activate MT gene expression in the cells (Fig. 1B, lanes 2–4). Quantitative analysis showed 45- and 60-fold increases in the MT message in response to Zn\(^{2+}\) and Cd\(^{2+}\), respectively. Surprisingly, MT expression was dramatically inhibited when the cells were exposed to 100 μM Cr\(^{6+}\) for 15 min before treatment with Zn\(^{2+}\) (100 μM) or Cd\(^{2+}\) (30 μM) for 3 h (Fig. 1B, lanes 5 and 6). Hexavalent chromium alone was unable to induce MT gene expression in HepG2 cells at concentrations ranging from 10 to 100 μM (data not shown). A similar inhibitory effect of Cr\(^{6+}\) on heavy metal-induced MT gene expression was also observed when cells were pretreated with Cr\(^{6+}\) for 15 min and removed before Cd\(^{2+}\) or Zn\(^{2+}\) treatment (data not shown). Because MT-IIA is the major species of MT expressed in human cells, we measured its expression in HepG2 cells by RT-PCR using gene-specific primers. Quantitative analysis of the RT-PCR data revealed an 18-fold increase of the basal MT-IIA message after Zn\(^{2+}\) (100 μM) treatment, whereas Cr\(^{6+}\) (100 and 250 μM) did not change the basal MT-IIA expression in this cell line (Fig. 1C, lanes 2–4). As observed earlier, Zn\(^{2+}\)-induced MT-IIA expression was reduced to basal levels upon exposure of the cells to Cr\(^{6+}\) (Fig. 1C, lanes 2, 5, and 6).

Induction of MT genes in response to heavy metals occurs at the level of transcription (22). To investigate whether Cr\(^{6+}\)-affected heavy metal-induced transactivation of MT genes, we performed a nuclear run-on assay with nuclei isolated from...
HepG2 cells either untreated or treated for 2 h with Zn$^{2+}$ (100 μM), Cr$^{6+}$ (100 μM), or Cd$^{2+}$ and Zn$^{2+}$. The results showed 8–10-fold increases in MT transcripts in zinc-treated cells compared with control. When the cells were pretreated with Cr$^{6+}$ for 15 min before the addition of Zn$^{2+}$, the induction decreased by 2–3-fold, whereas Cr$^{6+}$ alone had no effect (Fig. 1D, upper lane). The GAPDH level in each lane was comparable, demonstrating that an equal amount of RNA was used in each sample (Fig. 1D, lower panel). These results demonstrate that the inhibitory effect of Cr$^{6+}$ on Zn$^{2+}$- or Cd$^{2+}$-induced expression of MT occurs primarily at the level of transcription.

Hexavalent Chromium Does Not Affect the Expression of Two MTF1-independent, Cadmium-inducible Genes HSP-70 and Heme Oxygenase 1 (HO-1)—We next addressed whether the inhibitory effect of Cr$^{6+}$ was due to a global decline in gene expression during the treatment regimen. We did not observe any inhibitory effect of Cr$^{6+}$ on housekeeping genes like GAPDH and β-actin (Fig. 1, B and C). To investigate this issue further, we selected two other heavy metal-inducible genes, namely, heat shock protein 70 (HSP-70) and heme oxygenase-1 (HO-1). For this purpose, HepG2 cells were treated for 3 h either with Zn$^{2+}$ (100 μM)/Cd$^{2+}$ (30 μM) or Cd$^{2+}$ (100 μM) alone or in combination. Total RNA isolated from these cells was subjected to Northern blot analysis with $^{32}$P-labeled HSP-70 and HSP-70-MT-I cDNA. The basal expression of HSP-70 was relatively high in these cells and was not affected by Zn$^{2+}$ or Cd$^{2+}$ (Fig. 2A, upper panel, lanes 1, 3, and 4). The expression of HSP-70 increased 5-fold after Cd$^{2+}$ treatment, and it was not blocked significantly by Cr$^{6+}$ pretreatment (compare lanes 2 and 6). However, as shown earlier (Fig. 1), both Cd$^{2+}$-and Zn$^{2+}$-induced MT expression was abolished by the presence of 100 μM Cr$^{6+}$ (Fig. 2A, middle panel). Unlike HSP-70, the basal level of hemeoxygenase-1 (HO-1) was undetectable in HepG2 cells. After treatment of these cells with Cd$^{2+}$, the HO-1 level was, however, robustly elevated, whereas Cr$^{6+}$ exposure did not alter HO-1 expression (Fig. 2B, lanes 1–4). As observed with HSP-70, Cd$^{2+}$-induced expression of HO-1 remained unaffected upon exposure to 50 or 100 μM Cr$^{6+}$ (Fig. 2B, lanes 5 and 6). These results clearly demonstrate that the drastic reduction of Zn$^{2+}$/Cd$^{2+}$-mediated MT expression by Cr$^{6+}$ is not a global phenomenon.

Overexpression of MTF1 Can Overcome the Inhibitory Effect of Hexavalent Chromium on Heavy Metal-induced Expression of MT Genes—Inhibition of Zn$^{2+}$- or Cd$^{2+}$-induced expression of MT by Cr$^{6+}$ and not of HSP-70 or HO-1 suggested that the transcription factor MTF1, specific for MT genes, might be a potential target of chromium. MTF1 is essential for both basal and heavy metal-induced expression of the MT genes (30). It was logical to investigate whether overexpression of MTF1 could counteract the inhibitory effect of Cr$^{6+}$. To address this issue, we used a mouse fibroblast cell line (dko-7), from which both copies of endogenous MTF1 were deleted (23). We generated several stable cell lines (puromycin-resistant) expressing variable levels of FLAG-tagged human MTF1 by transfection with a recombinant retroviral vector (pBabe-hMTF1-FLAG). The level of MTF1 expression in these cell lines was determined by Western blot analysis with anti-FLAG antibodies that detected a specific polypeptide of 110 kDa only in cells expressing recombinant MTF1 (MTF1–11 and MTF1–12) but not in the parental dko-7 cells (Fig. 3A, lanes 1–9). We also measured DNA binding activity in whole cell extracts from these cell lines with a $^{32}$P-labeled MRE-s oligonucleotide corresponding to the specific binding site for MTF1 (30). A specific DNA-protein complex could only be detected in MTF1-expressing cell lines but not in dko-7 cells (MTF1 null, Fig. 3B, lanes 1–3). Quantitation of the $^{32}$P signal in the MRE-s MTF1 complex revealed that MTF1 DNA binding activity in MTF1–12 cells was 5-fold higher than that of MTF1–11 cells, which correlated well with the expression level of MTF1 in these cells. The identity of the protein bound to MRE-s as MTF1 was confirmed by competition of the complex with a 100-fold molar excess of unlabeled MRE-s oligo (lanes 4–6) but not with Sp1 consensus oligo (lanes 7–9). Also, a supershift of the DNA-protein complex with anti-MTF1 antibody but not with STAT3 antibody (Fig. 3C) confirmed that the complex was indeed MTF1.

Next we investigated whether the basal and heavy metal-induced MT expression was higher in MTF1–12 cells compared with MTF1–11 cells. As expected, Northern blot analysis showed that both basal and zinc-induced MT-I expression was significantly (7–8-fold) higher in MTF1–12 cells than that observed in MTF1–11 cells (Fig. 4A, lanes 1 and 3 and 5 and 7, and B). We next investigated whether Cr$^{6+}$ had a differential effect on MT-I induction in these cells. As observed in HepG2 cells, Cd$^{2+}$ alone could not activate MT-I expression in either cell line (Fig. 4, A, lanes 2 and 6, and B), but it inhibited zinc-mediated MT-I induction in both cell lines. The Cr$^{6+}$-mediated inhibition was more pronounced in MTF1–11 cells (80%) than in MTF1–12 cells (Fig. 4, A, lanes 4 and 8, and B). These data suggest that MTF1 is indeed one of the targets of Cr$^{6+}$, Cd$^{2+}$-induced MT expression was also inhibited in a dose-dependent manner by Cr$^{6+}$ in MTF1–12 cells (Fig. 4, C and D). MT mRNA levels increased ~65-fold after exposure to Cd$^{2+}$ (lane 2) and was reduced to 20-fold and 4-fold by co-treatment with 100 and 200 μM Cr$^{6+}$, respectively (Fig. 4, C, lanes 4 and 5, and D). Higher concentrations of Cr$^{6+}$ were needed to achieve significant inhibition of MT-I induction in MTF1–12...
cells (200 μM versus 100 μM used in control cells, Fig. 1, A and B), as these cells contain relatively high levels of MTF1. These results reinforce the notion that MTF1 protects the Zn^{2+}- and Cd^{2+}-induced MT expression from the inhibitory effect of Cr(VI).

**Hexavalent Chromium Also Inhibits MTF1-dependent Expression of ZnT-1**—We next investigated whether Cr(VI) could down-regulate the expression of another MTF1 target gene in response to heavy metals. The gene for the plasma membrane zinc transporter (ZnT-1) also harbors an MRE on its proximal promoter, and its transcription is induced by zinc (23). ZnT-1 exports Zn^{2+} from the intracellular pool to maintain zinc homeostasis in the cell (40). To study the effect of Cr(VI), cell lines expressing a differential level of MTF1 (MTF1-11 and MTF1-12) were treated with Zn^{2+} (100 μM) and/or Cd^{2+} (100 μM), and total RNA isolated from these cells was subjected to RT-PCR with mouse ZnT-1-specific primers. Both cell lines induced ZnT-1 in response to Zn^{2+}, and the level of expression was 3-fold higher in MTF1-12 cells compared with the MTF1-11 cells, whereas the β-actin level remained unaltered in both cell lines (Fig. 5, compare lanes 2 and 6). Like MT-I, ZnT-1 expression was not up-regulated by treatment with Cr(VI) alone (lanes 3 and 7), whereas pretreatment of the cells with Cr(VI) resulted in drastic reduction in the Zn^{2+}-induced ZnT-1 levels in both cell lines (compare lanes 2 and 4 with lanes 6 and 8). It is noteworthy that the inhibition in MTF1-12 cells (45%) was less pronounced than that in MTF1-11 (72%). These data demonstrate that Cr(VI) down-regulates Zn^{2+}-activated expression of at least two MTF1 target genes and reemphasizes the inverse correlation between the MTF1 levels and the inhibitory effect of Cr(VI).

**Hexavalent Chromium Does Not Inhibit Zinc-induced Occupancy of MT-IIA and MT-I Promoters in Vivo**—In response to heavy metals such as Zn^{2+} or Cd^{2+}, distinct footprints at MREs, MLTF/ARE, and Sp1 binding sites on the MT-I promoter in vivo and concurrent transcriptional activation have been observed (34). MTF1 is activated after treatment with heavy metals and binds to multiple cis elements (MREs) on the MT-I promoter. Because MTF1 is a transcription factor with six zinc fingers in its DNA binding domain, we reasoned that the inhibitory effect of chromium is probably mediated through inactivation of these fingers. To address whether Cr(VI) inter-
Cr\textsuperscript{6+} selected two different cell lines (HepG2 and MTF1) in vivo formed MT-IIA promoters (Fig. 5). DMS treatment, and DNA was prepared (see "Materials and Methods" for details). Human MT-IIA immediate upstream promoter harbors various cis elements including MREs, AP-1, Sp1 and glucocorticoid response element (Fig. 6A). Treatment of HepG2 cells with zinc induced footprinting at G residues encompassing the MRE-f site and the glucocorticoid response element site on the lower strand of the MT-IIA promoter (Fig. 6B). MTF1 harbors six zinc fingers, whereas glucocorticoid receptor has two zinc fingers in its DNA binding domain and are activated by zinc (41), which explains the zinc induced footprinting of these factors on the MT-IIA promoter. There was no apparent footprinting at the AP-2/AP-1 site in control or treated cells. IVGF analysis of the upper strand of the MT-IIA promoter revealed constitutive footprinting at the MRE-a site, where a G residue within the cis element was hypersensitive and three G-residues flanking the MRE-a were protected from DMS-induced methylation (Fig. 6C). There was a slight change in the MRE-a footprinting profile after zinc treatment, when a G residue within the cis element was hypersensitive (Fig. 6C). All the footprints detected on MT-IIA promoter in control and zinc-treated cells remained unaltered when cells were treated with Cr\textsuperscript{6+} alone or before exposure to Zn\textsuperscript{2+}. None of the zinc-induced footprints detected in the upper or lower strand of the MT-IIA promoter were observed in the Cr\textsuperscript{6+} treated cells, where the G-ladder remained identical to control DNA. These results also rule out occupancy of a negative element in the immediate promoter region by a potential repressor upon chromium exposure. To our knowledge this is the first IVGF study of human MT-IIA promoter, which revealed zinc-inducible occupancy of not only MREs but also glucocorticoid response element. We also measured DNA binding activity of MTF1 in the nuclear extract prepared from HepG2 cells treated with zinc, chromium, or both. The results showed that zinc-induced activation of MTF1 was not blocked by pretreatment with chromium, which itself could not activate MTF1 binding (data not shown). These results clearly demonstrate that neither translocation of MTF1 to the nucleus nor its binding to the promoter in response to zinc is compromised by chromium pretreatment.

To analyze in vivo footprinting of the mouse MT-I promoter (Fig. 6D) we subjected MTF1–11 cells to the same treatment regimen as described for HepG2 cells. Treatment with Zn\textsuperscript{2+} induced distinct footprinting at MRE-a, -b, -c, and -d sites on the lower strand in MTF1–11 cells. Some of the G residues at the MRE-a, -b, -c, and -d sites were protected, and some at the MRE-c' site were hypersensitive to DMS treatment due to binding of a transcription factor (Fig. 6E). Zn\textsuperscript{2+}-induced footprinting was also observed at MRE-c' and MRE-e in the upper strand of the MT-I promoter (Fig. 6F). In untreated MTF1–11 cells, the upper strand of the MT-I promoter was occupied at the MLTF/ARE, Sp1, and MRE-d/Sp1 binding sites. All the constitutive footprints observed in the control cells remained unaffected in the Cr\textsuperscript{6+}-treated cells. No inducible footprints appeared in the Cr\textsuperscript{6+}-treated cells, as observed in Zn\textsuperscript{2+}-exposed cells (Fig. 6, E and F, lanes 2 and 4). The footprinting profiles of the MT-I promoter in cells treated with Zn\textsuperscript{2+} and Cr\textsuperscript{6+} were identical to that in response to Zn\textsuperscript{2+} alone (Fig. 6, D and F, lanes 3 and 5). Zn\textsuperscript{2+}-induced occupancy of MREs and other sites remained intact, and no novel footprinting appeared in either the MT-I or MT-IIA promoter after Cr\textsuperscript{6+} treatment. These data confirmed that Cr\textsuperscript{6+} did not alter the interaction of transacting factors (basal or inducible) with cognate cis elements on the MT-I or MT-IIA promoters.

**Transient Overexpression of MTF1 Can Alleviate the Inhibitory Effect of Cr\textsuperscript{6+} on Zn\textsuperscript{2+}- and Cd\textsuperscript{2+}-induced MT-I Promoter Activity**—If Cr\textsuperscript{6+} affects the transactivation potential of MTF1, transient overexpression of MTF1 should alleviate the inhibitory effect of Cr\textsuperscript{6+} on MT-I promoter activity in transfection studies. To test this possibility, HepG2 cells were transiently transfected with a MT-I promoter/luciferase reporter plasmid (pMT-Luc) (38) and treated with Zn\textsuperscript{2+}, Cd\textsuperscript{2+}, and/or different concentrations of Cr\textsuperscript{6+}. Both Zn\textsuperscript{2+} and Cd\textsuperscript{2+} activated the MT-I promoter when the cells were treated with the heavy metals for 3 h before harvest. Cr\textsuperscript{6+} inhibited the basal promoter activity by 40 and 75% at a concentration of 50 and 100 µM, respectively, whereas 10 µM Cr\textsuperscript{6+} had no detectable effect (Fig. 7A). Both the Zn\textsuperscript{2+} and Cd\textsuperscript{2+}-induced promoter activities were inhibited significantly by 100 µM Cr\textsuperscript{6+} (65 and 70%, respectively). To determine the effect of MTF1 overexpression, HepG2 cells were next co-transfected with pMT-Luc with pRL-TK as an internal control along with different amounts of the human MTF1 expression vector (pchMTF1). As observed earlier (38), the basal MT-I promoter activity increased with increasing amounts of cotransfected hMTF1 expression vector (Fig. 7B, lanes 1, 4, 7, and 10). The addition of Zn\textsuperscript{2+} further enhanced this promoter activity, although the differences between the basal and Zn\textsuperscript{2+}-induced activity diminished with increased expression of hMTF1 (lanes 2, 5, 8, and 11). The addition of Cr\textsuperscript{6+} (100 µM) before Zn\textsuperscript{2+} inhibited the promoter activity sig-
significantly (lanes 3, 6, 9, and 12). This could be reversed by overexpression of MTF1 protein. In the absence of MTF1 overexpression Zn\textsuperscript{2+}-induced MT-I promoter activity was inhibited by 71% in the presence of Cr\textsuperscript{6+} (Fig. 7B, lanes 2 and 3). The inhibition decreased to 65% when 6 \textmu M of pchMTF1 was cotransfected in HepG2 cells with pMT-Luc (lanes 4 and 5). This recovery from the Cr\textsuperscript{6+}-induced effect was even more pronounced when 8 \textmu M (lanes 8 and 9) and 10 \textmu M (lanes 11 and 12) of the pchMTF1 were cotransfected (45 and 20% inhibition, respectively). These data further confirm the notion that MTF1 overexpression indeed protects MT-I promoter activity from the inhibitory effect of Cr\textsuperscript{6+}.

Hexavalent Chromium Inhibits the Function of all Three Transactivation Domains of MTF1—IVGF analysis and electrophoretic mobility shift assay (data not shown) clearly demonstrated that the DNA binding activity of MTF1 was not compromised in cells treated with Cr\textsuperscript{6+}. We therefore hypothesized that the transactivation domains of MTF1 could possibly be the targets of Cr\textsuperscript{6+}. MTF1 has three different transactivation domains, namely acidic domain (Ac), the proline-rich domain (P), and the serine-threonine rich domain (ST) (Fig. 8). A complex interaction between these domains is required for MTF1 activity (21). To explore which functional domain of MTF1 is the target of Cr\textsuperscript{6+}, we used fusion proteins consisting of the DNA binding domain of the heterologous yeast factor Gal4 and different regions of the mouse MTF1 protein (21). HepG2 cells were cotransfected with a luciferase reporter plasmid that contained five Gal4 binding sites on the promoter (pG5-Luc, Promega) and different Gal4/MTF1 fusion proteins. The Gal4 fusion proteins used in this experiment are [Gal4]Ac/P/ST, [Gal4]Ac/P, [Gal4]Ac, [Gal4]ST, and [Gal4]P. The cells were treated with Zn\textsuperscript{2+} and/or Cr\textsuperscript{6+} 3 before harvest. The expression of [Gal4]Ac/P/ST and [Gal4]Ac resulted in similar basal pG5-Luc promoter activity, which remained comparable after Zn\textsuperscript{2+} treatment (Fig. 8B, lanes 1 and 9, and 3 and 11). This insensitivity to Zn\textsuperscript{2+} can be attributed to the lack of a zinc finger domain in the MTF1 fusion proteins, as the rest of the domains responded minimally to Zn\textsuperscript{2+} (21). When the [Gal4]ST fusion protein was overexpressed, pG5-Luc activity was decreased to 30% that observed in presence of [Gal4]Ac/P/ST (compare lanes 5 and 7 to lanes 1 and 3), whereas [Gal4]Ac/P and [Gal4]P showed less than 10% of the activity (compare lanes 13, 15, and 17, 19 to lanes 1 and 3). When exposed to Cr\textsuperscript{6+}, cells expressing the five different fusion proteins exhibited on
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average a 60–70% reduction in the activity of the pG5-Luc promoter. A similar degree of inhibition was observed irrespective of the presence or absence of Zn$^{2+}$. These results suggest that the functions of all three transactivation domains of MTF1 are susceptible to the inhibitory effect of Cr$^{6+}$.

Hexavalent Chromium Can Enhance Zinc and Cadmium-induced Apoptosis, Which Can Be Counteracted by MTF1 Overexpression—Exposure of cells in culture to heavy metals such as Cr$^{6+}$ (14), Cd$^{2+}$ (42), and Zn$^{2+}$ (43) induces apoptosis, particularly at an early stage, which is eventually followed by necrosis if the heavy metals are not scavenged by detoxifying proteins like metallothioneins. Because Cr$^{6+}$ inhibits the heavy metal-induced MT expression and increased MTF1 expression counteracted the inhibitory effect, it was of interest to investigate how MTF1 null cells respond to heavy metal-induced apoptosis in comparison to overexpressing cells. For this purpose, we first used annexin V staining to analyze the onset of apoptosis under different treatment conditions (see “Materials and Methods” for details) using dko-7 (MTF1 null) and MTF1–12 cells (MTF1-overexpressing) (Fig. 3). The cells were either left untreated (control) or treated with 100 μM Zn$^{2+}$, 100 μM Cr$^{6+}$, or 100 μM each Zn$^{2+}$/Cr$^{6+}$ for 2 h followed by annexin V staining for 15 min. The cells were counter-stained with PI. Treatment with Zn$^{2+}$ and/or Cr$^{6+}$ for a short time period (2 h) induced apoptosis in dko-7 cells (MTF1 null) under all three treatment regimens (Fig. 9, panel A). Phosphatidylserine residues that are translocated from the inner to the outer layer of the plasma membrane at an early stage of apoptosis after membrane damage are detected by annexin V binding. As apoptosis progresses, the membrane loses its integrity, and a halo of green stain appears within the entire cell. Precise analysis and comparison of the fluorescent profile showed that the entire cell was stained green when dko-7 cells were exposed to Zn$^{2+}$ alone or along with Cr$^{6+}$ (Fig. 9A, 2a and 4a). In the Cr$^{6+}$-treated cells, green specks on the plasma membrane were distinctly visible with concurrent staining of the cytoplasm (Fig. 9A, 3a). This observation suggested that MTF1 null cells offered minimum resistance to heavy metal-induced apoptosis. When MTF1–12 cells were subjected to identical treatment, annexin V-FITC staining was barely detectable in cells treated for 2 h with Zn$^{2+}$ or Cr$^{6+}$ alone (Fig. 9B, 2a and 3a). However, simultaneous treatment of MTF1–12 cells with both heavy metals induced onset of apoptotic cell death, as was evident from the appearance of green specks on the membrane periphery (Fig. 9B, 4a). The most logical explanation for this observation is that MTF1–12 cells resist Zn$^{2+}$-induced apoptosis by inducing MTs to scavenge intracellular Zn$^{2+}$ and by inducing ZnT-1 that effluxes excess intracellular Zn$^{2+}$. The ability of MTF1–12 cells to resist Cr$^{6+}$-induced apoptosis is likely due to the higher basal levels of MT-1 and ZnT1 expression in these cells (Figs. 4 and 5). dko-7 cells that lack MTF1 cannot express these proteins and are prone to apoptosis when challenged with metals. Although MTF1–12 cells could resist zinc treatment for at least 2 h, exposure to Cr$^{6+}$ before zinc treatment augmented the toxic effect of zinc and led to onset of apoptosis. This deleterious effect of Zn$^{2+}$ and Cr$^{6+}$ on MTF1–12 cells can be attributed to decreased expression of MT and ZnT-1 in the presence of Cr$^{6+}$ (Figs. 4 and 5). Strong PI staining was visible in metal-treated dko-7 cells (Fig. 9A, 2b–4b). Because the dye can only penetrate cells where the membrane integrity is lost, it can be assumed that apoptosis is prevalent in these cells. Minimal PI staining in MTF1–12 cells implicates resistance of these cells toward these heavy metals under the experimental conditions used in this study.

We also analyzed the induction of apoptotic cell death in dko-7 and MTF1–12 cells by heavy metals using the TUNEL assay. This assay detects cellular endonuclease-mediated ordered DNA fragmentation, a late event in apoptotic cell death. Both dko-7 and MTF1–12 cells were treated with Zn$^{2+}$ and/or Cr$^{6+}$ for 2 h and the TdT (terminal deoxynucleotidyltransferase) assay was performed following the manufacturer’s protocol. Insignificant levels of FITC staining were observed in the untreated dko-7 cells, whereas in cells treated with heavy metals alone or in combination (this is the same treatment done for annexin staining) TUNEL-positive FITC staining was observed (Fig. 10, panel A). On the other hand, when MTF1–12 cells were treated with Zn$^{2+}$, Cd$^{2+}$, or Cr$^{6+}$, TUNEL-positive cells were not detected irrespective of the treatment condition (Fig. 10, panel B). This observation supports the data from the annexin V assay, where onset of apoptosis in MTF1–12 cells was not observed in the presence of Cr$^{6+}$ or Zn$^{2+}$ alone. Unlike dko-7 cells TUNEL-positive cells were not detected in MTF1–12 cells exposed to Cr$^{6+}$ before Zn$^{2+}$ or Cd$^{2+}$ treatment. We have seen earlier (by annexin V staining) that a combination of Zn$^{2+}$ and Cr$^{6+}$ in 2 h can only lead to the onset of apoptosis in MTF1–12 cells. Because TUNEL
detects a late event in the apoptosis, we did not expect to detect significant TUNEL-positive staining in MTF1–12 cells under this treatment regimen. Positive PI staining was observed in both dko-7 and MTF1–12 cells, as cells were permeabilized before performing TUNEL assay. This set of data suggests that Cr\textsuperscript{6+} augments the toxic effect of heavy metals such as Zn\textsuperscript{2+} and Cd\textsuperscript{2+}. Also, MTF1 null cells (dko-7) are more vulnerable to heavy metal-induced apoptotic cell death and increased expression of MTF1 can protect the cells from a heavy metal insult.

**DISCUSSION**

The three heavy metals studied here, namely cadmium, zinc, and chromium, pose differential levels of toxicity and health hazards. Unlike cadmium, zinc and trivalent chromium are considered essential nutrients. However, high levels of zinc and chromium in its hexa- or pentavalent states cause many toxic manifestations. For example, uptake of excess zinc interferes with iron and copper metabolism in the body (44). Excessive exposure to extracellular zinc can damage the central nervous system (45). Chromium in its hexavalent state is considered a serious health hazard due to its carcinogenic effect (7). Our study demonstrates that exposure to multiple heavy metals containing hexavalent chromium, a toxin that is quite common in the environment, has deleterious effects on humans due to the impairment of detoxification mechanisms, namely MT and zinc efflux induction.

It should be emphasized that the inhibitory effect of Cr\textsuperscript{6+} on heavy metal-induced MT gene expression is not merely a result of global toxicity imposed by Cr\textsuperscript{6+}, as Cd\textsuperscript{2+}–induced expression of stress response genes, namely HSP-70 and HO-1, remained unaltered under this condition. We have also observed unabated expression of housekeeping genes such as GAPDH and \(\beta\)-actin under the same treatment regimen. It should also be noted that at a concentration similar to that of Zn\textsuperscript{2+} (100 \(\mu\)M) and/or Cr\textsuperscript{6+} (100 \(\mu\)M) for 2 h, the cells were then treated with annexin V–FITC and PI according to manufacturer’s protocol. The cells were then viewed under a fluorescence microscope.
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It has been hypothesized that Cd²⁺ and other heavy metals act by releasing Zn²⁺ from intracellular zinc storage proteins, leading to activation of MT-I (22). Because in a wide range of concentrations Cr⁶⁺ (10–250 μM, Fig. 1B, and some data not shown) could not activate the MT promoter, one can speculate that unlike other heavy metals, it fails to mobilize Zn²⁺ from the intracellular storage sites. However, this notion does not explain why Cr⁶⁺ blocks activation of MT expression in response to other metals. The DNA binding activity of MTF1, a Zn²⁺-mediated process is not compromised in cells treated with Zn²⁺ and Cr⁶⁺. Indeed, the data presented here point to an inhibitory effect at the transactivation stage subsequent to DNA binding by MTF1. This conclusion was supported further by the observation that stable or transient overexpression of MTF1 in mouse MTF1 null cells and HepG2 cells can significantly protect the ability of these cells to respond to Zn²⁺ or Cd²⁺ by expressing the MT gene. The extent of this protection correlates directly with the level of MTF1 expression. Because there is no dramatic increase in MTF1 transcript levels in response to heavy metals that normally leads to robust transcription of the MT genes, MTF1 probably undergoes significant post-translational modification in this process (26, 50). We have not, however, detected any significant change in MTF1 phosphorylation/dephosphorylation when MTF1–12 cells were treated with either Cr⁶⁺ alone or in combination with Zn²⁺ or Cd²⁺ (data not shown).

The present study has also shown that Cr⁶⁺ interferes with the function of all three different activation domains of MTF1 in concert. This finding is consistent with an earlier observation (21) that demonstrated the role of all three domains namely, N-terminal, C-terminal, and middle domains, of MTF1 in the activation of the MT-I promoter. Cr⁶⁺ may inhibit interaction between the MTF1 transactivation domains with a co-activator(s) or other general transcription factor by an as yet unknown mechanism. Identification of co-activators or general transcription factors with which MTF1 interacts is a big challenge to the field and is beyond the scope of the present study.

Both Zn²⁺- and Cd²⁺-induced apoptosis was observed at an early stage in MTF1 null cells (dko-7), whereas MTF1-overexpressing cells (MTF1–12) were resistant to these metals. It has been reported that zinc can be internalized through the mitochondrial uniport, leading to generation of reactive oxygen species and induction of apoptosis (51). Unlike MTF1–12 cells, the lack of MT-I and ZnT-1 expression in dko-7 cells would result in increased accumulation of free intracellular zinc, facilitating the cell death process. From all the data gathered so far it is obvious that pretreatment with Cr⁶⁺ would augment the toxic effect. This has been nicely demonstrated in MTF1–12 cells, where a combination of Zn²⁺ and Cr⁶⁺ resulted in annexin V-sensitive membrane disintegration, whereas the cells treated with Zn²⁺ alone showed no sign of apoptosis. From this data it is evident that suppression of MT and ZnT1 expression in the presence of Cr⁶⁺ led to an imbalance in the intracellular...
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zinc pool, resulting in onset of apoptosis. However, the observed apoptosis in MTF1−/− cells showed significant delay compared with dko-7 cells. This observation reemphasizes the role of MT-I and ZnT-1 in scavenging toxic heavy metals and, consequently, the need for functional MTF1, required for induction of the above proteins.

It is important to realize that the effects of MTF1 dysfunction on the cellular process caused by Cr⁶⁺ are multifaceted. Uninterrupted expression of the zinc transporters as well as the zinc-metallothionein storage proteins is essential for the maintenance of Zn²⁺ homeostasis in cells. Of the four zinc transporters, ZnT-1 is the only one present on the plasma membrane, where it functions as a zinc effluxor (40). ZnT-1 is expressed ubiquitously, and the homozygous knockout is diminished in the presence of Cr⁶⁺ (54, 55). We speculate that the overall capacity of cells to respond to stress (54, 55) and proper liver development (56). It is logical to speculate that the capacity of the cells to maintain Zn²⁺ homeostasis is severely impeded. Our previous study demonstrates that the MT-I gene is highly induced by heavy metals and probably plays a protective role when mice are exposed to restrained stress (53) or viral infection (28). It is, therefore, conceivable that the ability to cope with infection and stress where zinc plays a protective role will be hindered upon exposure to Cr⁶⁺. The liver-enriched transcription factor C/EBPα is also a candidate target gene for MTF1 (24). This gene is important for cellular stress response (54, 55) and proper liver development (56). It is logical to speculate that the overall capacity of cells to respond to stress will be diminished in the presence of Cr⁶⁺. Because the C/EBPα family of proteins is critical for liver development, it would be of interest to explore the role of Cr⁶⁺ in mammalian development. Future studies will address this and related issues.

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Chromium(VI) Down-regulates Heavy Metal-induced Metallothionein Gene Transcription by Modifying Transactivation Potential of the Key Transcription Factor, Metal-responsive Transcription Factor 1

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