

Nucleo-cytoplasmic Trafficking of Metal-regulatory Transcription Factor 1 Is Regulated by Diverse Stress Signals*

Received for publication, October 6, 2000, and in revised form, March 20, 2001
Published, JBC Papers in Press, April 16, 2001, DOI 10.1074/jbc.M009154200

Nurten Saydam‡, Oleg Georgiev‡, Michel Y. Nakano§, Urs F. Greber§, and Walter Schaffner‡¶

From the Institutes of ‡Molecular Biology and §Zoology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

The metal-regulatory transcription factor 1 (MTF-1) is a key regulator of heavy metal-induced transcription of metallothionein I and II and other genes in mammals and other metazoans. Transcriptional activation of genes by MTF-1 is mediated through binding to metal-responsive elements of consensus TGCRNC in the target gene promoters. In an attempt to further clarify the mechanisms by which certain external signals activate MTF-1 and in turn modulate gene transcription, we show here that human MTF-1 has a dual nuclear and cytoplasmic localization in response to diverse stress stimuli. MTF-1 contains a consensus nuclear localization signal located just N-terminal to the first zinc finger that contributes to but is not essential for nuclear import. MTF-1 also harbors a leucine-rich, nuclear export signal. Under resting conditions, the nuclear export signal is required for cytoplasmic localization of MTF-1 as indicated by mutational analysis and transfer to the heterologous green fluorescent protein. Export from the nucleus was inhibited by leptomycin B, suggesting the involvement of the nuclear export protein CRM1. Our results further show that in addition to the heavy metals zinc and cadmium, heat shock, hydrogen peroxide, low extracellular pH (pH 6.0), inhibition of protein synthesis by cycloheximide, and serum induce nuclear accumulation of MTF-1. However, heavy metals alone (and not the other stress conditions) induce a significant transcriptional response via metal-responsive element promoter sequences, implying that nuclear import of MTF-1 is necessary but not sufficient for transcriptional activation. Possible roles for nuclear import under non-metal stress conditions are discussed.

Gene regulation in response to cellular stress is mediated through a variety of signaling pathways. One type of stress response is triggered by heavy metals, such as zinc, cadmium, and copper, and induces transcription of metallothionein genes. Metallothioneins (MTs)¹ are a group of low molecular weight metal-binding proteins that are represented by four isoforms in mammals (1). MT gene transcription is also induced by many different conditions other than heavy metals, including oxida-

tive stress, hypoxia, hormones, and viral infection (2, 3). Most, if not all, other eukaryotes and some prokaryotes also contain metallothioneins, but their primary sequence and their transcriptional regulation may deviate from the situation in vertebrates (4, 5).

The mammalian metal-regulatory transcription factor 1 (MTF-1) is a zinc finger transcription factor that activates the transcription of target genes in response to heavy metal exposure via binding to MREs (6). These sequence motifs of consensus TGCRNC are present in the promoter regions of MT-I, MT-II, and a number of other genes, many of which are involved in stress (7–9). Recently, targeted gene disruption of MTF-1 in the mouse revealed that MTF-1 is essential for liver development during embryogenesis because the null mutant embryos died *in utero* at embryonic day 14 due to liver decay. MTF-1-deficient cells in culture are more susceptible to cadmium and H₂O₂ treatments in comparison with their wild type counterparts (7). Another implication of MTF-1 in cellular stress response came from the work of Dalton *et al.* (10), who found that MTF-1 is activated in response to reactive oxygen species. Recently, hypoxia has also been reported as a condition by which MTF-1 activates MT gene expression through MRE sequences (11). However, the mechanism(s) of MTF-1 activation by heavy metals is only partially understood, and the activation by non-metal stimuli remains to be elucidated.

The rapid cellular response to a multitude of external and internal stress-associated agents is orchestrated by multiple signal transduction pathways. Several transcription factors involved in stress-regulated rapid events, including p53, nuclear factor κ B, and nuclear factor of activated T cells, localize differentially between the cytoplasm and the nucleus in response to DNA damage, growth signals, or environmental stimuli (12, 13). The import of proteins destined for the nucleus requires a nuclear localization signal; the export of large macromolecules from the nucleus is also an active transport process mediated by nuclear export signals (NESs) (12–15). NES sequences are short, leucine-rich motifs that were first identified in the cellular proteins, protein kinase A inhibitor, mitogen-activated protein kinase kinase, and the human immunodeficiency virus Rev protein (16–18). Recognition of NES motifs by the nuclear export receptor CRM1 is specifically suppressed by the antifungal drug leptomycin B (LMB) (19). Here we have addressed the molecular mechanisms of MTF-1 regulation by a variety of stress inducers to explore possible regulatory mechanisms. We demonstrate that cytoplasmic MTF-1 is imported into the nucleus of serum-starved cells by stress inducers, including heavy metals. While this work was in progress, another group also found a cytoplasmic-nuclear translocation of MTF-1 upon zinc and cadmium treatment of cells (20). Here we characterize a nuclear localization signal (NLS) of MTF-1 and show that MTF-1 enters the nucleus not only upon metal load but also upon oxidative stress, heat shock, low pH, inhibition of protein

* This work was supported by the Swiss National Science Foundation and the Kanton Zürich. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 41-1-635-3150/51; Fax: 41-1-635-6811; E-mail: walter.schaffner@molbio.unizh.ch.

¹ The abbreviations used are: MT, metallothionein; MRE, metal-responsive element; NES, nuclear export signal; MTF-1, metal-regulatory transcription factor 1; LMB, leptomycin B; NLS, nuclear localization signal; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BSA, bovine serum albumin.

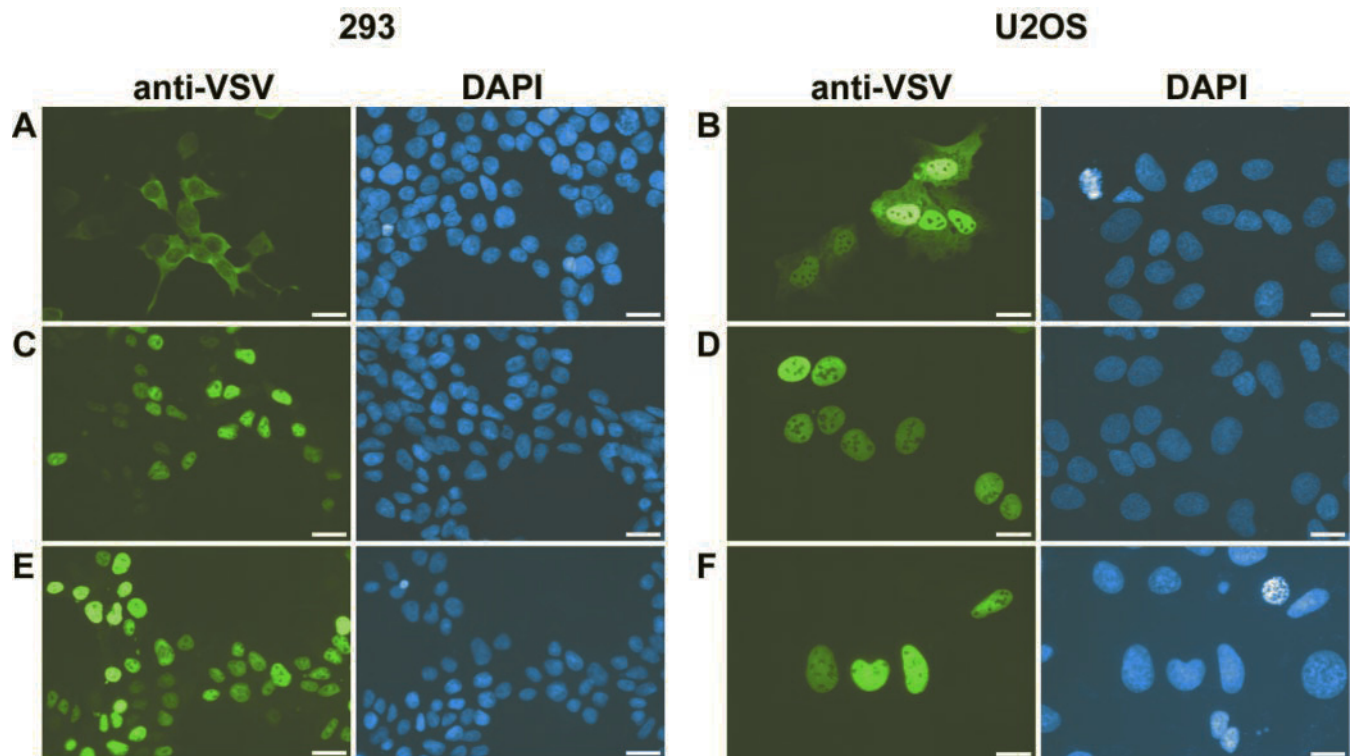


FIG. 1. Subcellular distribution of MTF-1 in 293 and U2OS cells. An expression vector coding for VSV epitope-tagged MTF-1 was used for transfection of human 293 and U2OS cells. To reduce serum content, 24 h after transfection the cells were incubated for another 24 h in a medium containing 0.5% BSA replacing FBS. The cells were either left untreated (A and B) or treated with 200 μ M ZnCl₂ (C and D) or 60 μ M CdCl₂ (E and F), and the cells were then fixed and stained with anti-VSV antibody, followed by fluorescein-labeled secondary antibody (left panels) and DNA staining with 4',6-diamidino-2-phenylindole (right panels). Scale bar, 20 μ m.

synthesis by cycloheximide, and serum factors. Additionally, we find that MTF-1 has a functional NES that is sensitive to LMB and mediates subcellular localization and nuclear-cytoplasmic shuttling. Most importantly, we demonstrate that the nuclear accumulation of MTF-1 *per se* does not result in transcriptional activation via MRE sequences, indicating that nuclear translocation and transcriptional activation functions can be separated.

MATERIALS AND METHODS

Plasmid Construction—The human MTF-1 expression vector was described previously (21). An 11-mer VSV epitope from vesicular stomatitis virus G protein (22) was inserted at amino acid position 744 in the C-terminal end. The NES and NLS mutants (designated MTF-1NES_{mut} and LLI_(133–135)MTF-1-VSV, respectively) were created by site-directed mutagenesis. The oligonucleotides used were as follows (the sense strand is shown; lowercase letters represent mutated nucleotides): (a) NES_{mut}, 5'-GGATACAAATCACTCACCTTGTgCAGTGACgctAGCgcTgcGTCCACAGATTCTG-3'; (b) NES'_{mut}, 5'-CCACAGGAAATTCAGCATCTgcATCTgcTCCAgcTGTAgcGCAACCTGGCCTCTCCG-3'; (c) LLI_(133–135)MTF-1-VSV, 5'-gcagtcggaatgtccGgaacACTaCtGaTgaagtaaagcggtacc-3'. mMTI-Luc was constructed by inserting a blunt-ended fragment encompassing the promoter (−727 to −13) region of the mMTI gene (23) into the *Sma*I site of the pGL3 basic vector (Promega).

The NLS (¹³³KRKEVKR¹³⁹), NES (³³⁶LCLSDLSLL³⁴⁴), and NES' (⁴¹⁷LSLPLVLQPGL⁴²⁷) sequences of human MTF-1 were cloned in the *Hind*III-*Kpn*I sites of double peGFP (peGFP_{2x}), which is located in the C terminus. The peGFP_{2x} vector containing tandemly repeated eGFP coding sequences was a kind gift from Dr. Ulrike Kutay (ETH, Zürich, Switzerland).

Cell Cultures and Transfections—Adenovirus-transformed human embryonal kidney 293 cells, U2OS human osteosarcoma cells, and SV40-transformed MTF-1^{−/−} embryonic stem cells designated as DKO7 cells (24) were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc.) supplemented with 10% fetal bovine serum (ICN). Cells were transfected by the calcium phosphate method (25).

Indirect Immunofluorescence—293 and U2OS cells were plated onto coverslips in 6-well tissue culture dishes in DMEM supplemented with

10% FBS 16 h before transfection. Cells were transfected with 2 μ g/well plasmid DNA using the calcium phosphate method. Sixteen h after transfection, cells were washed and incubated for another 24 h in the medium containing 10% FBS. The cells were switched to the medium in which serum was replaced by 0.5% BSA (DMEM-BSA). After 24 h in the serum-limited condition, the treatments were initiated as indicated. To monitor redistribution of MTF-1 from the nucleus to the cytoplasm after zinc or cadmium withdrawal, cells were pretreated with 100 μ M ZnCl₂ or 60 μ M CdCl₂ for 1 h and washed three times with phosphate-buffered saline and three times with DMEM-BSA. Further incubations were performed in DMEM-BSA as indicated. For treatment of cells with low pH, the pH of DMEM supplemented with 0.5% BSA was adjusted by the addition of HCl from pH ~7.4 to pH 6.0. This pH remained stable throughout the experiment. For fixation, cells were treated with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline for 3 min. After blocking nonspecific binding by incubation in 5% newborn calf serum in phosphate-buffered saline for 10 min, the cells were incubated with anti-VSV antibody (Sigma) diluted 1:250 in 5% newborn calf serum at room temperature for 30 min. After rinsing, cells were incubated with fluorescein-coupled rabbit anti-mouse IgG (Molecular Probes) diluted 1:200 in 5% newborn calf serum at room temperature for 1 h. After three washings, nuclei were stained with 4',6-diamidino-2-phenylindole (0.001 mg/ml) for 2 min, and cells were imaged by using a Reichert-Jung Polyvar upright fluorescence microscope equipped with fluorescein filters (excitation, 475–495 nm; emission, 520–560 nm), 4',6-diamidino-2-phenylindole filters (excitation, 330–380 nm; emission, 420 nm/long pass), and Nomarski optics. Images were recorded through a $\times 40$ objective (oil immersion) and collected on an 8-bit charge-coupled device camera (Hamamatsu C 5405) online to an Apple Macintosh computer, as described previously (26). Digital images were processed using Photoshop software (Adobe).

Determination of MTF-1 Localization—Multiple fields were examined to count at least 200 positive cells, and localizations of MTF-1 were classified as nuclear, cytoplasmic, and both nuclear and cytoplasmic. Mean and S.D. values of the number of cells showing nuclear, cytoplasmic, and both nuclear and cytoplasmic staining of MTF-1 were derived from three independent experiments, each sampling about 200 different cells.

293 cells plated on coverslips were transfected with 2 μ g of the eGFP

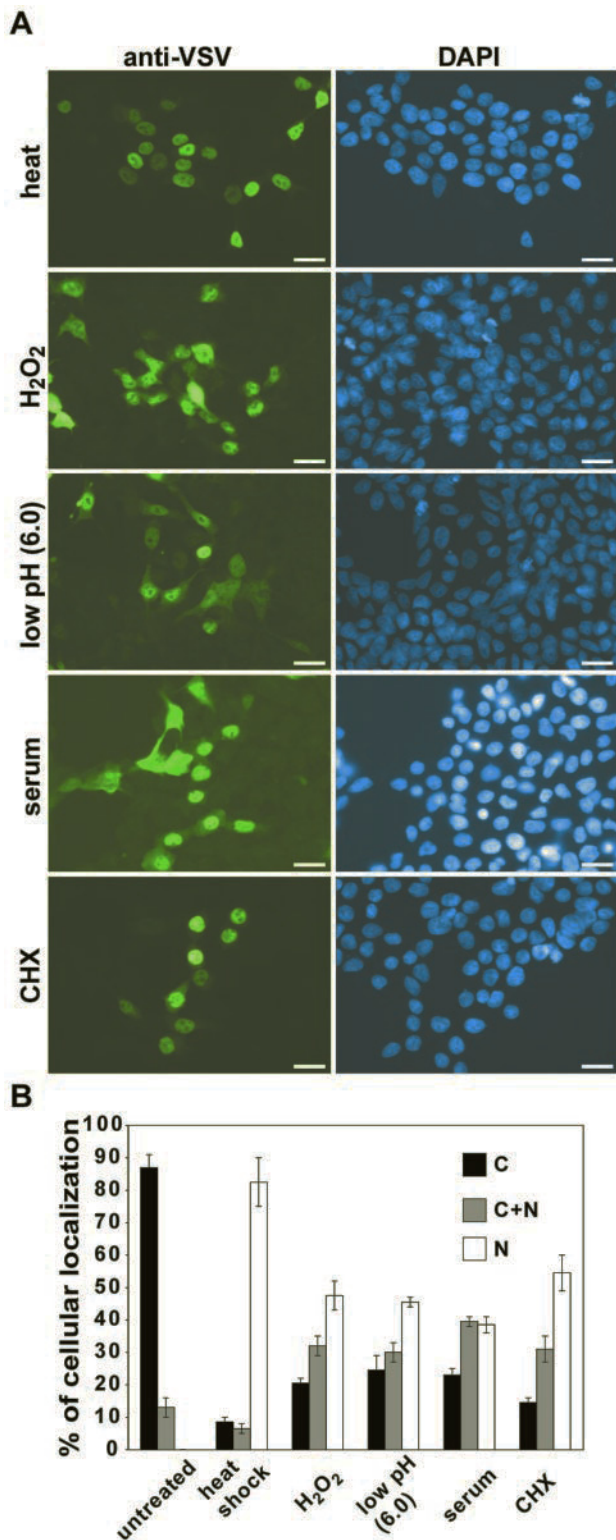


FIG. 2. Heat shock and other stress conditions also induce nuclear import of MTF-1. Subcellular localization of MTF-1-VSV exposed to various stress conditions was examined. 293 cells transiently transfected with MTF-1-VSV were treated with heat (43 °C, 1 h), hydrogen peroxide (800 μ M, 3 h), low pH (pH 6.0, 1 h), serum (10% dialyzed FBS, 3 h), and cycloheximide (10 μ g/ml, 3 h) (A). Control cells were processed in parallel as described in the Fig. 1 legend, and MTF-1 was visualized by fluorescence microscopy. Scale bar, 20 μ m. B, about 200 MTF-1-positive cells were counted to quantify nuclear versus cytoplasmic distribution of MTF-1, and the results were classified as exclusively cytoplasmic (C), exclusively nuclear (N), and both cytoplasmic and nuclear (C+N). The mean values of cells with exclusively nuclear MTF-1 were calculated from three different experiments using ~200 cells, with the corresponding S.D. (B).

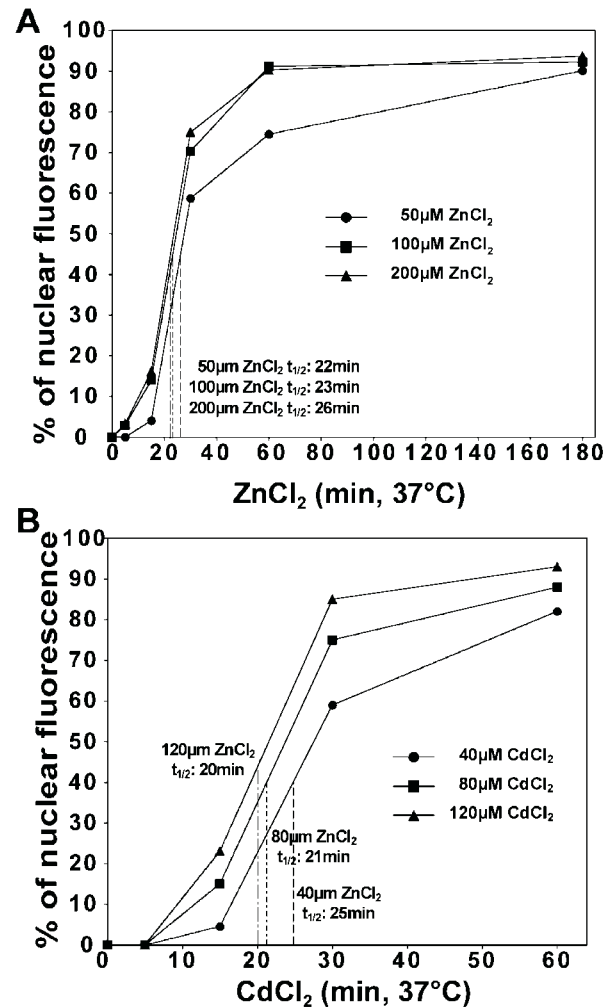


FIG. 3. Time course of nuclear import of MTF-1 in the presence of zinc or cadmium. Serum-starved 293 cells were incubated with different concentrations of zinc chloride (A) or cadmium chloride (B) for the indicated times at 37 °C and analyzed for MTF-1 localization by indirect immunofluorescence. The fraction of cells with exclusively nuclear staining was plotted for each time point. $t_{1/2}$ indicates the time of half-maximal nuclear transport of each condition. The values show the average of three independent experiments.

plasmid constructs. 24 h after transfection, cells were fixed with 4% paraformaldehyde for 15 min and analyzed by fluorescence microscopy as described above.

Luciferase Reporter Gene Assay—293 and DKO7 cells were cultured in DMEM supplemented with 10% FBS. Transient transfections were carried out in 100-mm tissue culture plates with 5 μ g of reporter plasmid, 3 μ g of LacZ expression vector, and 5 μ g of each MTF-1 expression vector by the calcium phosphate method. 16 h after transfection, the medium was removed, and cells were fed with medium containing 10% FBS. After 24 h, media were changed to 0.5% BSA-supplemented medium, and cells were serum-starved in that medium by incubation for another 24 h. Cells were then harvested and assayed for luciferase activity as described previously (27). The results were normalized to the LacZ expression from the cotransfected reference gene.

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assay was performed as described previously (6). Binding reactions were performed by incubating 2–5 fmol of an end-labeled, 31-base pair-long oligonucleotide containing a strong MRE consensus sequence (MRE-s), with nuclear extracts obtained according to the protocol of Schreiber *et al.* (28). For competition experiments, 2 pmol of unlabeled oligonucleotides were added to the reaction mixture before the addition of the extracts.

RESULTS

MTF-1 Shows Stress-inducible Nuclear Import—To determine the effects of zinc and cadmium on the intracellular

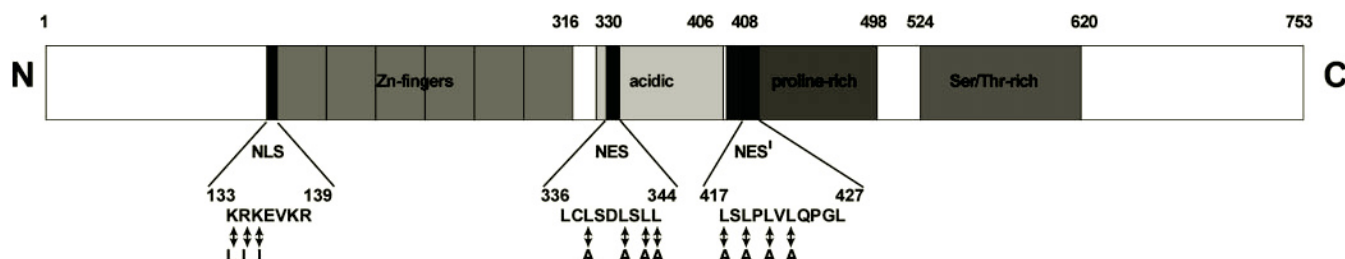


FIG. 4. Mutagenized amino acids in the putative NLS and NES motifs of human MTF-1. Schematic representation of the primary structure of human MTF-1 showing a putative NLS (residues 133–139) and two putative NES sequences (NES, residues 336–344; NES', residues 417–427) with the mutation sites indicated.

localization of MTF-1, 293 (adenovirus-transformed human embryonal kidney) cells and U2OS (human osteosarcoma) cells were transiently transfected with an expression vector encoding a VSV-tagged MTF-1. This allowed for easy identification of functional MTF-1 (data not shown). 24 h after serum deprivation, subcellular localization of the MTF-1-VSV fusion protein was determined by indirect immunofluorescence. Under normal growth conditions in serum-containing cell culture medium, a considerable fraction of MTF-1 localizes to the nucleus (Ref. 24; data not shown). Here, we have addressed this point and find that MTF-1 stays almost exclusively in the cytoplasm in serum-starved 293 cells (Fig. 1A). In U2OS cells, however, the fluorescence signal of tagged MTF-1 was detected in both the cytoplasm and the nucleus (Fig. 1B). Exposure of the cells to 200 μ M zinc chloride (ZnCl_2) (Fig. 1, C and D) or 60 μ M cadmium chloride (CdCl_2) (Fig. 1, E and F) for 1 h resulted in a striking nuclear accumulation of MTF-1 in both 293 and U2OS cells. In addition, the treatment of 293 cells with heat shock (43 $^{\circ}\text{C}$, 1 h), H_2O_2 (800 μ M, 3 h), low pH (pH 6.0, 1 h), cycloheximide (10 μ g/ml, 3 h), and serum factors (10% FBS, 3 h) caused nuclear translocation of MTF-1 (Fig. 2A). The calculated percentages of nuclear accumulation upon each different treatment are shown in Fig. 2B. The cells were also treated with substances that induce nuclear translocation of other transcription factors: tumor necrosis factor α (50 ng/ml, 1 h) and 12-O-tetradecanoylphorbol-13-acetate (100 ng/ml, 1 h), which activate nuclear factor κ B by inducing the nuclear translocation of RelA (29), and sorbitol (400 mM, 1 h) which is known to stimulate the nuclear translocation of signal transducer-activators of transcription in mammalian cells (30) and HOG1 mitogen-activated protein kinase in yeast (31); however, these treatments did not induce any obvious change in the subcellular localization of MTF-1 (data not shown). This indicates that the nuclear translocation of MTF-1 in response to stress conditions is a specific property of this transcription factor.

Heavy Metal-induced Nuclear Translocation of MTF-1 Is Concentration- and Time-dependent—293 cells transfected with a plasmid encoding VSV-tagged human MTF-1 were treated with different concentrations of zinc chloride or cadmium chloride, and MTF-1 localization was analyzed at several time points by indirect immunofluorescence staining. Rapid translocation of MTF-1-VSV was obtained at 100 μ M zinc chloride, with a half-maximal nuclear transport time ($t_{1/2}$) of 23 min (Fig. 3A). Nuclear translocation was nearly complete after 30 min of exposure. A higher zinc concentration (200 μ M) had little accelerating effect (Fig. 3A). Similarly, increasing concentrations of cadmium decreased $t_{1/2}$ from 25 min (40 μ M) to 21 min (120 μ M cadmium chloride) (Fig. 3B).

Analysis of the Nuclear Translocation of MTF-1—Human MTF-1 contains near the first zinc finger the motif ($^{133}\text{KRKEVKR}^{139}$) that fits the consensus of a classical NLS sequence. To evaluate the contribution to nuclear accumulation of this motif, we performed site-directed mutagenesis to replace the three basic amino acids Lys 133 -Arg 134 -Lys 135 with Leu-Leu-

Ile (Fig. 4). The mutated protein (LLI $_{(133-135)}$ MTF-1-VSV) was localized exclusively in the cytoplasm of resting cells. Nevertheless, when the LLI $_{(133-135)}$ MTF-1-VSV-expressing cells were treated with either 200 μ M zinc chloride or 60 μ M cadmium chloride for 1 h, 72% of the transfected cells had fluorescent nuclei (Fig. 5A). However, the kinetics of nuclear localization upon zinc treatment was delayed, with a pronounced extension of the initial lag phase (Fig. 5B). The most pronounced difference was observed in a short-term incubation (30 min) with 200 μ M zinc chloride in which 75% of the cells transfected with wild type MTF-1 showed nuclear staining, but only 6.5% of the cells contained nuclear NLS-mutated MTF-1. At 60 min of zinc treatment, this difference had almost leveled out in that wild type and mutant MTF-1 were localized in the nucleus in 90.5% and 72% of the cells, respectively. Thus, it was not unexpected to find only marginal differences in metallothionein promoter activation by wild type compared with NLS mutant MTF-1, as measured during a 3-h time period. In these experiments, the first time point with clearly elevated reporter gene transcripts was 2.5 h after stress induction for both wild type and NLS mutant (Fig. 5C). However, a strong effect of the NLS mutant was observed in other stress conditions. Translocation of NLS mutant MTF-1 to the nucleus by administration of hydrogen peroxide, low pH, cycloheximide, and serum factors was severely impaired, reaching only about 10% of wild type MTF-1 levels, even after extended incubation times of up to 4 h (data not shown).

LMB Induces Nuclear Accumulation of MTF-1 without Affecting Nuclear Import—To define the mechanism(s) responsible for the steady-state cytoplasmic retention of MTF-1, we decided to use LMB, a specific inhibitor of the nuclear export protein CRM1 (19, 32). Upon LMB treatment of MTF-1 transfected cells for 3 h, 63 \pm 6% of the transfected cells showed nuclear accumulation (Fig. 6). The shift from the cytoplasm to the nucleus was detectable within 30 min of LMB administration and was nearly complete after 6 h (data not shown). Treatment with LMB at 4 $^{\circ}\text{C}$ did not cause any nuclear MTF-1 accumulation (data not shown). To test the effect of LMB on the metal-induced nuclear accumulation of MTF-1, 293 cells were pretreated with 10 ng/ml LMB for 15 min and incubated at 37 $^{\circ}\text{C}$ for 60 min in the presence of 200 μ M zinc chloride or 60 μ M cadmium chloride. As expected for an export blocker, simultaneous treatments with LMB and heavy metals did not impair the nuclear import of MTF-1 (data not shown).

MTF-1 Has a Functional Nuclear Export Signal Sequence—Examination of the sequence of human MTF-1 revealed two candidate NES sequences (tentatively designated NES and NES'), $^{336}\text{LCLSDLSLL}^{344}$ (NES) and $^{417}\text{LSLPLVLQPGL}^{427}$ (NES'), respectively (Fig. 4). The putative MTF-1 NES motifs are similar in sequence to the well-established leucine-rich NES sequences in other proteins such as human immunodeficiency virus Rev (18), protein kinase A inhibitor (16), and mitogen-activated protein kinase kinase (17) (Fig. 7). This figure also shows a comparison to MTF-1 NES candidate motifs

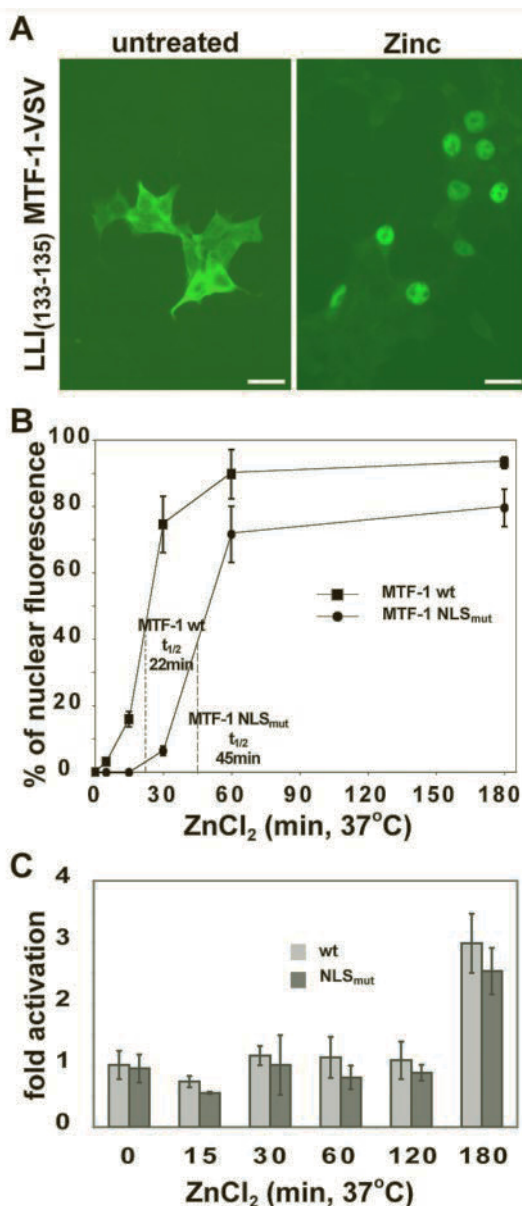


FIG. 5. Nuclear accumulation of MTF-1 in response to zinc or cadmium is delayed by mutations in the NLS-like basic amino acid cluster. 293 and MTF-1-negative DKO7 cells were transfected with either the expression plasmids coding for MTF-1-VSV or the LLI₍₁₃₃₋₁₃₅₎MTF-1-VSV. 24 h after transfection, cells were serum-starved for another 24 h and treated with zinc for the indicated times. LLI₍₁₃₃₋₁₃₅₎MTF-1-VSV-expressing 293 cells were either untreated or treated with 200 μ M ZnCl₂ for 1 h and visualized by fluorescence microscopy (A). Scale bar, 20 μ m. The kinetics of zinc-induced nuclear import of the wild type or the NLS mutant MTF-1 was determined in 293 cells (B). After treatment with 200 μ M ZnCl₂ for 5, 15, 30, 60, and 180 min, cells were fixed and processed for indirect immunofluorescence. The percentages of the cells showing exclusively nuclear staining from three independent experiments are presented graphically with the S.D. The kinetics of zinc-induced transcriptional activity of the wild type and the NLS mutant MTF-1 was analyzed by luciferase assay using a natural mouse metallothionein I promoter as a reporter in 293 cells (C). After treatment with 200 μ M ZnCl₂ for 15 min, 30 min, 1 h, 2 h, and 3 h, cells were harvested and processed for luciferase assays. Results were normalized to cotransfected β -galactosidase activity. The data from the three independent experiments are shown with the S.D.

from three different species (human, mouse, and Japanese puffer fish (*Fugu rubripes*)). To determine whether the putative NES sequences are functional, we generated three human MTF-1 mutants in which the four most critical leucine residues in either NES, NES', or both were replaced by alanines (MTF-

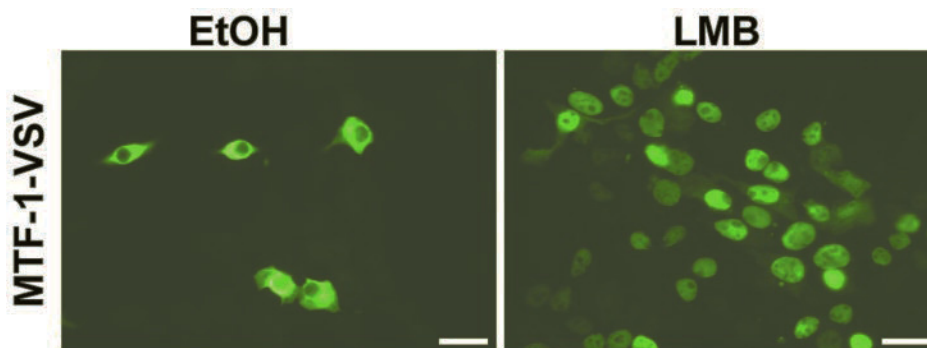
1NES_{mut}, MTF-1NES'_{mut}, and MTF-1NESNES'_{mut}, respectively) (Fig. 4). VSV-tagged versions of these mutants were transfected into 293 cells in parallel to wild type MTF-1. The subcellular distribution of each protein was determined by indirect immunofluorescence staining. The wild type MTF-1 and the MTF-1NES'_{mut} localized exclusively to the cytoplasm of serum-starved cells. In contrast, MTF-1NES_{mut} and MTF-1NESNES'_{mut} localized to the nucleus, with $92 \pm 3\%$ nuclear staining (the remaining cells showed both cytoplasmic and nuclear fluorescence) (Fig. 8). These data indicate that NES but not NES' is required for efficient cytoplasmic localization of MTF-1.

An MTF-1-derived NES Directs Nuclear Export of a Heterologous Protein, Most Likely via Interaction with the Nuclear Export Protein CRM1—To test whether the activities of the NLS and NES sequences of MTF-1 are transferable to another protein, we transfected the NLS- or NES-eGFP_{2x} constructs into 293 cells and observed their localizations 24 h after transfection. The tandem duplication of GFP protein is suitable for such studies because it apparently distributes almost equally to cytoplasm and nucleus (Fig. 9A). In agreement with a role in nuclear export for the NES motif, NES-eGFP_{2x} was detected exclusively in the cytoplasm (Fig. 9C). By contrast, the NLS and NES' sequences of MTF-1 did not change the cellular localization of the double GFP protein (Fig. 9, B and E versus A). To test for a possible involvement of CRM1 in the MTF-1 nuclear export, we analyzed the effect of leptomycin B on the subcellular localization of NES-eGFP_{2x} fusion proteins in 293 cells. Treatment with leptomycin B abolished nuclear export of the fusion protein containing the NES sequence (Fig. 9D). These results show that the NES of MTF-1 is a functional nuclear export signal that may be recognized by CRM1.

Nuclear Accumulation of MTF-1 per se Is Not Sufficient for Transcriptional Activation of the Mouse Metallothionein Gene Promoter—To determine whether nuclear accumulation of MTF-1 is sufficient for transcriptional activation, we performed transcription assays on native 293 cells treated with heavy metals, H₂O₂, low pH (pH 6.0), heat shock, cycloheximide, serum, or LMB. The effect of MTF-1 on gene expression was measured with a luciferase reporter gene driven by the metallothionein I promoter. As expected, zinc treatment resulted in a strong, 5-fold induction of luciferase activity, but all other conditions tested showed markedly lower luciferase activities (Fig. 10A). The export blocker LMB, which also induces nuclear accumulation of MTF-1, had no effect on transcription, confirming the notion that nuclear localization of MTF-1 per se is not sufficient for transcriptional activity. In addition, nuclear localization is not sufficient for DNA binding activity. Nuclear extracts of MTF-1 null mutant cells (DKO7) that had been transfected with wild type MTF-1 and exposed to non-metal stress including LMB or H₂O₂, heat shock, serum, low extracellular pH, and cycloheximide did not show any significant DNA binding activity as measured by electrophoretic mobility shift assay (data not shown).

Next, we determined the transcriptional activity of the NES mutants of MTF-1. NES mutant MTF-1 did not activate transcription from the mMTI promoter, even after zinc treatment. However, cells transfected with NES' mutant MTF-1 vector activated transcription in response to zinc, similar to wild type MTF-1 transfected cells. As expected, the double mutant (NESNES') MTF-1 behaved in a manner similar to that of the NES mutant MTF-1 (Fig. 10B). NES, NES', and NESNES' mutants did not show any DNA binding activity in the absence of zinc *in vitro*, but a relatively strong DNA binding activity was observed upon zinc treatment of cells (Fig. 10C). Together, our results demonstrate that neither nuclear localization of

FIG. 6. Nuclear export of MTF-1 is sensitive to LMB. The transfected and serum-starved 293 cells were treated with either LMB in ethanol (final concentration, 10 ng/ml and 0.1% v/v ethanol) or ethanol alone (0.1% v/v) for 3 h at 37 °C. Cells were then fixed and stained with anti-VSV antibody. Quantification of this experiment revealed that MTF-1 accumulated in the nucleus in 63% of LMB-treated cells. Scale bar, 20 μ m.



MAPKK (33-43)	LQKKLEELELD
HIV Rev (75-84)	L-PPLERLTLD
PKI α (39-47)	L--KLAGLDIN
hMTF-1NES (336-344)	L--CLSDL SLL
mMTF-1NES (335-343)	L--YLSELGLL
fMTF-1NES (356-364)	L--CLSDISLI
hMTF-1NES' (417-427)	L--SLP-LVLQPGL
mMTF-1NES' (416-426)	L--SLP-LVLQSGI
fMTF-1NES' (432-442)	S--LIS-FSLDPTA

FIG. 7. MTF-1 contains two putative conserved NESs. A comparison of the two putative NES sequences (NES and NES') of human, mouse, and *F. rubripes* MTF-1 with the NES consensus sequence of human immunodeficiency virus Rev, protein kinase A inhibitor, and mitogen-activated protein kinase kinase (MAPKK) is shown. Important hydrophobic residues (leucine and isoleucine) in the sequences are boxed.

MTF-1 nor an ability to bind DNA *in vitro* is sufficient to ensure transcriptional activation of target genes.

DISCUSSION

We have analyzed the subcellular localization of MTF-1 in cultured cells under resting conditions and also under various stress conditions. We demonstrate that this transcription factor localizes to the cytoplasm of resting cells but rapidly translocates to the nucleus under a variety of stress conditions, including exposure to heavy metals, heat shock, hydrogen peroxide, low pH, inhibition of protein synthesis by cycloheximide, and serum factors. The dual localization of MTF-1 is conferred by an N-terminal classical NLS that facilitates nuclear localization and a NES that promotes nuclear export of MTF-1 and is also functional on a reporter protein. Whereas nuclear localization of MTF-1 is necessary for transcriptional activation of metal-responsive genes, it is not sufficient. This was indicated by DNA binding experiments and transcription assays of cells exposed to stress conditions that did not directly involve heavy metals.

A major question concerns the mechanism(s) of MTF-1 induction in response to stress. The zinc fingers of MTF-1 are required for DNA binding, and it was noted early on that this DNA binding required higher zinc concentrations than other zinc finger transcription factors, such as Sp1. In a recent study, Bittel *et al.* (33) have further characterized this zinc induction and found zinc finger 1 to play a crucial role, whereas other zinc fingers behaved "conventionally" as constitutive DNA binding domains. Whereas this property of MTF-1 nicely explained the induction by zinc, it fell short of explaining induction by other heavy metals, let alone by other stress conditions. Indeed,

attempts to induce DNA binding with other heavy metals failed (34), suggesting that they could not substitute for zinc. To make things worse, any stress condition other than zinc load led to a loss of MTF-1 DNA binding (34).² Recently, we were able to develop in our laboratory a cell-free transcription system that depends on MTF-1 and yields DNA binding and transcriptional activation by stress conditions such as cadmium and copper, provided that these conditions could release zinc from zinc storage proteins.³ These findings suggest a unifying mechanism of MTF-1 activation via elevated intracellular zinc concentration. The data presented here now indicate an additional level of complexity, namely, a regulation of MTF-1 activity by subcellular compartmentalization. We found that various stress conditions such as exposure to heavy metals (zinc and cadmium), heat shock, H₂O₂, low pH, and inhibition of protein synthesis by cycloheximide cause translocation of MTF-1 from the cytoplasm to the nucleus. Thus MTF-1 joins a growing list of transcription factors that are regulated at the level of nuclear import in response to a variety of signals, including stress conditions (35).

MTF-1 harbors, immediately N-terminal to the first zinc finger, a putative SV40 T antigen-like NLS. Mutational analysis showed that the candidate NLS facilitates nuclear accumulation of MTF-1 but that it is not essential for import *per se*. However, the NLS failed to induce nuclear translocation when fused to a reporter protein (eGFP_{2x}). One possible explanation could be that the basic sequence motif studied here is incomplete and part of a larger, perhaps bipartite, NLS. MTF-1 may also cooperate with a cofactor that contains an NLS of its own. However, such a cofactor would only be operative under heavy metal stress because nuclear translocation of a candidate NLS-mutated MTF-1 under the non-metal conditions was very poor. Alternatively, MTF-1 might be specifically modified by heavy metal stress, possibly exposing additional nuclear targeting signals. These open questions seem to warrant further experiments on the mechanism of MTF-1 nuclear import.

The situation with regard to the NES appears to be more straightforward. We have characterized two candidate nuclear export signal sequences (NES and NES') that resemble leucine-rich NES sequences of other proteins. NES but not NES' turned out to be functional in nuclear export. Inhibition of the export activity by LMB or amino acid mutations in NES changed MTF-1 localization from the cytoplasm to the nucleus in 293 cells, indicating that the cytoplasmic accumulation of MTF-1 under basal conditions is accomplished by the NES element. Furthermore, the transfer of the NES sequences from MTF-1 to a eGFP_{2x} protein revealed that one copy of NES (but not of NES') is sufficient to export the reporter protein from the nucleus to the cytoplasm. Like export of MTF-1, export of

² B. Zhang, N. Saydam, O. Georgiev, and W. Schaffner, unpublished data.

³ B. Zhang, O. Georgiev, and W. Schaffner, unpublished data.

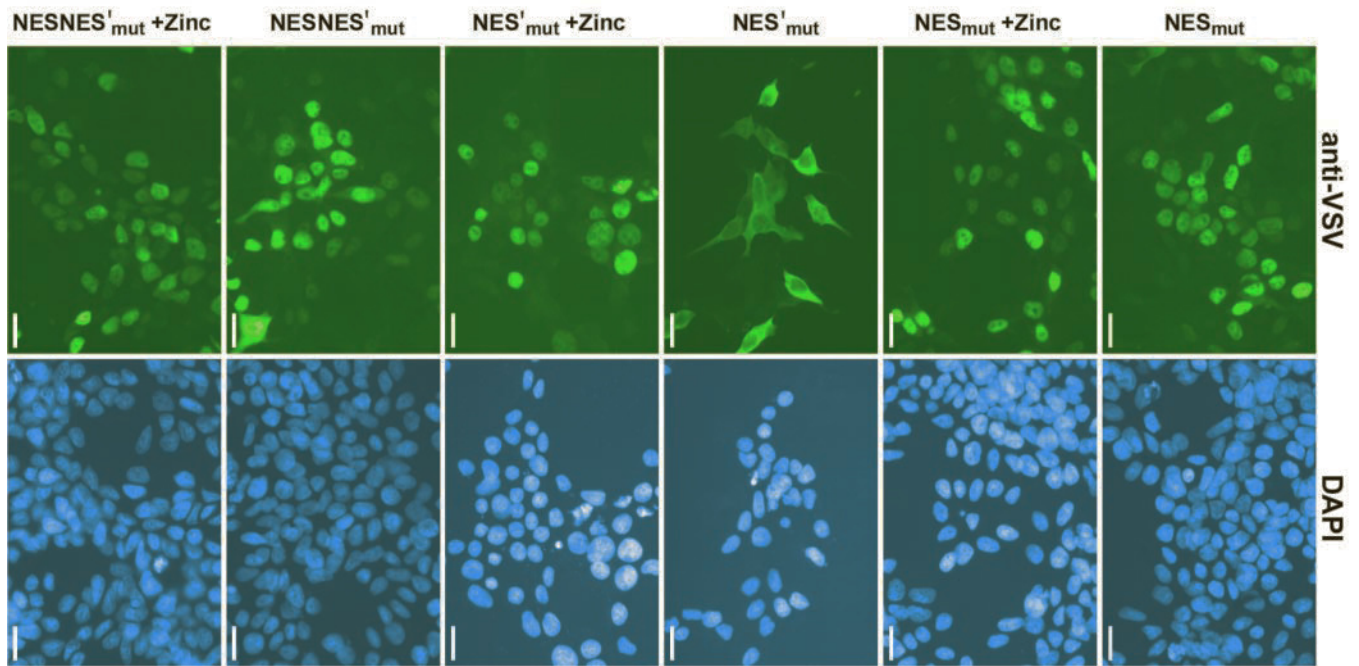


FIG. 8. **Cytoplasmic localization of MTF-1 requires nuclear export mediated by NES but not by NES'.** 293 cells were transfected with the expression vectors encoding MTF-1 wild type, NES_{mut}, NES'_{mut}, or NESNES'_{mut} proteins. After 24 h of serum starvation, the cells were treated with 200 μ M ZnCl₂ for 1 h and processed for indirect immunofluorescence. Scale bar, 20 μ m.

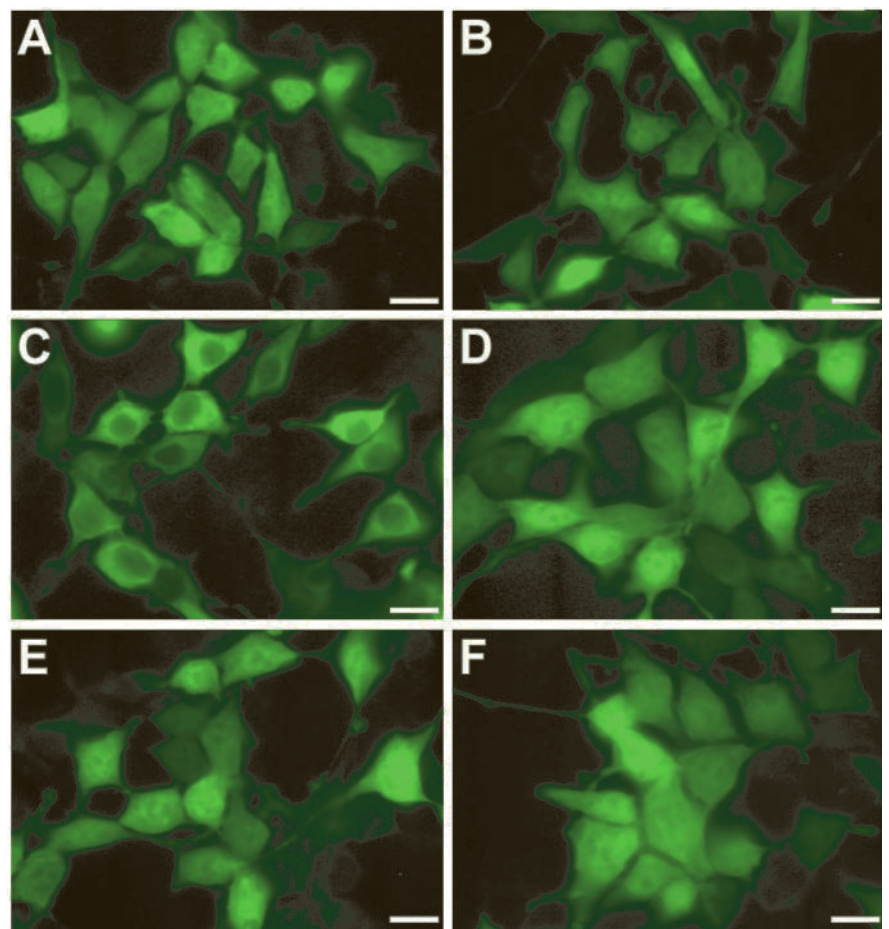


FIG. 9. **MTF-1 NES induces nuclear export of a heterologous protein.** 293 cells were transfected with the GFP vectors encoding the double GFP (eGFP_{2x}) alone (A), NLS-eGFP_{2x} (B), NES-eGFP_{2x} (C and D), and NES'-eGFP_{2x} (E and F). After 24 h, the indicated samples were treated with 10 ng/ml LMB for 6 h (D and F), and cells were fixed and analyzed by fluorescence microscopy. Scale bar, 20 μ m.

eGFP_{2x}-NES was sensitive to LMB, suggesting that NES is responsible for the cytoplasmic accumulation of MTF-1 under resting conditions and that export is carried out through an interaction between NES and the export protein CRM1. In

transcription assays, mutation of NES but not of NES' abolished the heavy metal-dependent activity. It is possible that loss of activity of NES_{mut} MTF-1 was due to an impaired function of the strong acidic MTF-1 activation domain in which

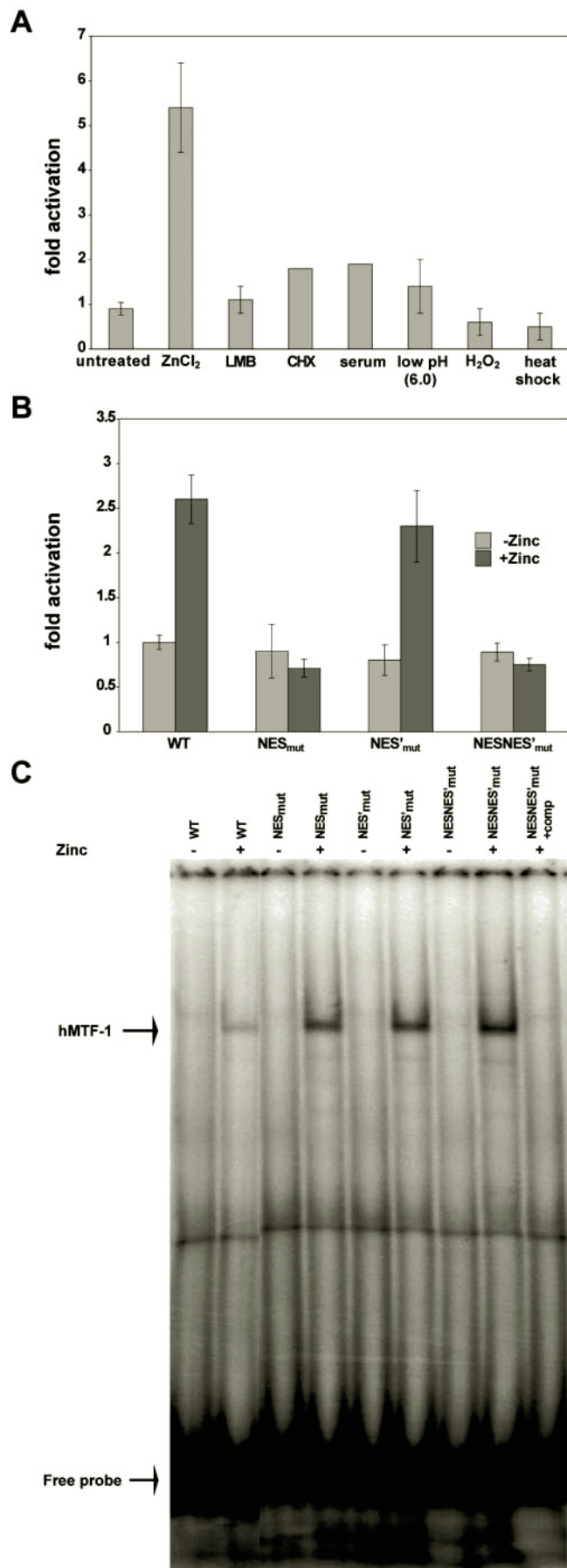


FIG. 10. Transcriptional activation and DNA binding activities of NES mutants. 293 cells transfected with mMTI-Luc and CMV-LacZ were starved for 24 h and then treated with ZnCl₂ (200 μ M), leptomycin

NES is embedded. Alternatively, nucleo-cytoplasmic shuttling of MTF-1 might be required to ensure correct inducibility, perhaps by allowing for essential posttranslational modification(s) in the cytoplasm. Furthermore, shuttling of MTF-1 between two compartments may, on one hand, ensure removal of MTF-1 from its target genes and, on the other hand, still allow for a rapid transcriptional response.

Although the activity of a number of transcription factors is regulated by cytoplasmic-nuclear transport, it is also clear that the mere presence of a factor within the nucleus is not necessarily sufficient for transcriptional activation. Rather, the factor has to be further activated for optimal DNA binding and/or transactivation by protein modifications, such as phosphorylation or acetylation (36, 37), and/or released from an inhibitory protein (38–40). Consistent with a need for posttranslational modification, our preliminary data suggest that phosphorylation is involved in signal-dependent activation of MTF-1.⁴ The characterization of posttranslational modifications of MTF-1 will be necessary for a full understanding of stress-mediated gene regulation.

Thus far, the metallothionein genes are the best characterized target genes of MTF-1 in heavy metal signaling. Further target genes with MRE motifs in their promoters were recently identified (9). Why MTF-1 is imported into the nucleus under non-metal stress (heat shock, H₂O₂, low pH, cycloheximide, and serum factors), conditions that failed to activate MRE-containing promoters in our assays, remains an open question. It is interesting to note that, at least in some species, heat induces metallothionein transcription, and, conversely, heavy metals can induce heat shock gene transcription. It is possible that novel target genes for non-metal stress response are activated (or repressed) through MTF-1 via sequence motifs different from the classical MREs, for example, in cooperation with other transcription factors. Within the framework of such a model, we have begun to screen a random collection of oligonucleotides for sequences that specifically bind MTF-1 in absence of zinc load.⁵ Besides activation of novel target genes under non-metal stress, one can also envisage other scenarios. One straightforward reason for nuclear import under many conditions may be to remove MTF-1 from a cytoplasmic “danger zone” into the better protected nuclear environment. Whereas this seems reason enough to transport MTF-1 to the nucleus, preliminary findings also point in another direction and suggest a synergistic activation of metallothionein promoters by a combination of metal and nonmetal stress.⁴

Acknowledgments—We thank Dr. Minoru Yoshida (University of Tokyo, Tokyo, Japan) and Dr. Ulrike Kutay for the generous gifts of leptomycin B and eGFP_{2x} construct, respectively.

REFERENCES

- Vasak, M., and Hasler, D. W. (2000) *Curr. Opin. Chem. Biol.* **4**, 177–183
- Liu, X. D., and Thiele, D. J. (1997) *Methods* **11**, 289–299
- Moffatt, P., and Denizeau, F. (1997) *Drug Metab. Rev.* **29**, 261–307
- Thiele, D. J. (1992) *Nucleic Acids Res.* **20**, 1183–1191
- Zhao, H., Butler, E., Rodgers, J., Spizzo, T., Dueterhoeft, S., Eide, D. (1998) *J. Biol. Chem.* **273**, 28713–28720
- Radtke, F., Heuchel, R., Georgiev, O., Hergersberg, M., Gariglio, M., Dembic,

⁴ N. Saydam and W. Schaffner, unpublished data.

⁵ Y. Wang and W. Schaffner, unpublished data.

B (10 ng/ml), cycloheximide (10 μ g/ml), low pH (pH 6.0), serum (10% dialyzed FBS), H₂O₂ (800 μ M) for 4 h, and heat shock (43 °C) for 1 h. The cells were harvested and processed for luciferase assays (A). The data presented are the mean values from three independent experiments with the S.D. Wild type or NES mutant MTF-1 expression vectors were transfected into mouse DKO7 cells lacking MTF-1. Serum-starved cells were either left untreated or treated with 200 μ M ZnCl₂ for 4 h before luciferase assays (B) or electrophoretic mobility shift assays (C).

- Z., and Schaffner W. (1993) *EMBO J.* **12**, 1355–1362
7. Günes, C., Heuchel, R., Georgiev, O., Müller, K. H., Lichtlen, P., Bluthmann, H., Marino, S., Aguzzi, A., and Schaffner W. (1998) *EMBO J.* **17**, 2846–2854
8. Langmade, S. J., Ravindra, R., Daniels, P. J., and Andrews, G. K. (2000) *J. Biol. Chem.* **275**, 34803–34809
9. Lichtlen, P., Wang, Y., Belser, T., Georgiev, O., Certa, U., Sack, R., and Schaffner, W. (2001) *Nucleic Acids Res.* **29**, 1514–1523
10. Dalton, T. P., Li, Q., Bittel, D., Liang, L., and Andrews, G. K. (1996) *J. Biol. Chem.* **271**, 26233–26241
11. Murphy, B. J., Andrews, G. K., Bittel, D., Discher, D. J., McCue, J., Green, C. J., Yanovsky, M., Giaccia, A., Sutherland, R. M., Laderoute, K. R., and Webster, K. A. (1999) *Cancer Res.* **59**, 1315–1322
12. Komeili, A., and O'Shea, E. K. (2000) *Curr. Opin. Cell Biol.* **12**, 355–360
13. Kaffman, A., and O'Shea, E. K. (1999) *Annu. Rev. Cell Dev. Biol.* **15**, 291–339
14. Görlich, D., and Kutay, U. (1999) *Annu. Rev. Cell Dev. Biol.* **15**, 607–660
15. Nakielnny, S., and Dreyfuss, G. (1999) *Cell* **99**, 677–690
16. Wen, W., Meinkoth, J. L., Tsien, R. Y., and Taylor S. S. (1995) *Cell* **82**, 463–473
17. Fukuda, M., Gotoh, I., Gotoh, Y., and Nishida, E. (1996) *J. Biol. Chem.* **271**, 20024–20028
18. Fischer, U., Huber, J., Boelens, W. C., Mattaj, I. W., and Luhrmann, R. (1995) *Cell* **82**, 475–483
19. Kudo, N., Matsumori, N., Taoka, H., Fujiwara, D., Schreiner, E. P., Wolff, B., Yoshida, M., and Horinouchi, S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 9112–9117
20. Smirnova, I. V., Bittel, D. C., Ravindra, R., Jiang, H., and Andrews, G. (2000) *J. Biol. Chem.* **275**, 9377–9384
21. Heuchel, R., Radtke, F., Georgiev, O., Stark, G., Aguet, M., and Schaffner, W. (1994) *EMBO J.* **13**, 2870–2875
22. Soldati, T., and Perriard, J. C. (1991) *Cell* **66**, 277–289
23. Glanville, N., Durnam, D. M., and Palmiter, R. D. (1981) *Nature* **292**, 267–269
24. Radtke, F., Georgiev, O., Müller, H., Brugnera, E., and Schaffner, W. (1995) *Nucleic Acids Res.* **23**, 2277–2286
25. Graham, F. L., and van der Eb, A. J. (1973) *Virology* **52**, 456–467
26. Greber, U. F., Suomalainen, M., Stidwill, R. P., Boucke, K., Ebersold, M. W., and Helenius, A. (1997) *EMBO J.* **16**, 5998–6007
27. Ausubel, F. M., Roger, B., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., and Struhl, K. (1987) *Current Protocols in Molecular Biology*, John Wiley & Sons, New York
28. Schreiber, E., Matthias, P., Müller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17**, 6419
29. Mercurio, F., and Manning, A. M. (1999) *Oncogene* **18**, 6163–6171
30. Gatsios, P., Terstegen, L., Schliess, F., Häusinger, D., and Kerr, I. M. (1998) *J. Biol. Chem.* **273**, 22962–22968
31. Ferrigno, P., Posas, F., Koepp, D., Saito, H., and Silver, P. A. (1998) *EMBO J.* **19**, 5606–5614
32. Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997) *Nature* **390**, 308–311
33. Bittel, D. C., Smirnova, I. V., and Andrews, G. K. (2000) *J. Biol. Chem.* **275**, 37194–37201
34. Bittel, D., Dalton, T., Samson, S. L.-A., Gedamu, L., and Andrews, G. (1998) *J. Biol. Chem.* **273**, 7127–7133
35. Ferrigno, P., and Silver, P. A. (1999) *Oncogene* **18**, 6129–6134
36. Gonzalez, G. A., and Montminy, M. R. (1989) *Cell* **59**, 675–680
37. Gu, W., and Roeder R. G. (1997) *Cell* **90**, 595–606
38. Pratt, W. B., and Toft, D. O. (1997) *Endocr. Rev.* **18**, 306–360
39. Helin, K., Harlow, E., and Fattaey, A. R. (1993) *Mol. Cell. Biol.* **13**, 6501–6508
40. Minet, E., Mottet, D., Michel, G., Roland, I., Raes, M., Remacle, J., and Michiels, C. (1999) *FEBS Lett.* **460**, 251–256