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Original Research Communication

Role for Copper in Transient Oxidation and Nuclear Translocation of MTF-1, but Not of NF-κB, by the Heme–Hemopexin Transport System

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ABSTRACT

Heme–hemopexin (2–10 μ*M*) is used as a model for intravenous heme released in trauma, stroke, and ischemia-reperfusion. A transient increase in cellular protein oxidation occurs during receptor-mediated heme transport from hemopexin which is inhibited by the nonpermeable Cu(I) chelator, bathocuproinedisulfonate. Thus, participation of surface redox process involving Cu(I) generation are proposed to be linked to the induction of the protective proteins heme oxygenase-1 (HO-1) and metallothionein-1 (MT-1) by heme–hemopexin. The region (−153 to −42) in the proximal promoter of the mouse MT-1 gene responds to heme- and CoPP–hemopexin in transient transfection assays and contains metal-responsive elements for MTF-1 and an antioxidant-responsive element (ARE) overlapping a GC-rich E-box to which USF-1 and -2 bind. No decreases in DNA binding of the diamide-oxidation sensitive USF-1 and -2 occur upon exposure of cells to heme–hemopexin. MTF-1 and the ARE-binding proteins are relatively resistant to diamide oxidation and are induced approximately eight- and two-fold, respectively, by heme–hemopexin. BCDS prevents the nuclear translocation of MTF-1 by both heme- and CoPP–hemopexin complexes as well as MT-1 mRNA induction by CoPP–hemopexin. Thus, copper is needed for the surface oxidation events and yet the nuclear translocation of MTF-1 in response to hemopexin occurs via copper, probably Cu(I), -dependent signaling cascades from the hemopexin receptor rather than the oxidation *per se*. Antiox. Redox Signal. 2, 739–752.

INTRODUCTION

THE MOLECULAR ACTIONS of heme at the plasma membrane during hemolysis, trauma, and reperfusion following ischemia are ill defined yet are considered to play an instrumental role in the pathology of these conditions. Moreover, the mechanism(s) whereby heme might mediate oxidative damage *in vivo* are not clear, and experimental models are required. The physiological binder and transporter of heme in the circulation is the glycoprotein hemopexin (Sears, 1969; Smith and Morgan, 1978, 1979). This receptor-mediated heme transport system (Alam and Smith, 1989; Smith and Hunt, 1990) provides a specific means to evaluate the biochemical and regulatory consequences to cells in response to a wide range of extracellular concentrations of heme–hemopexin complexes and of free heme. Hemopexin has a key role in ischemia-reperfusion by acting extracellularly as an antioxidant, se-

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questering heme from hemopexin receptor-null cells, and targeting heme via the hemopexin receptor to liver, lymphocytes, and in certain cell types in barrier tissues like the eye and brain.

Exposing cells to 2–10 μM heme–hemopexin, as a model for heme load, increases intracellular heme levels, but is neither toxic nor apoptotic, although there is a period of cell arrest (Eskew *et al.*, 1999). Adaptive responses include induction of heme oxygenase-1 (HO-1; Alam and Smith, 1989), ferritin (Hunt *et al.*, 1996), and metallothionein (MT; Alam and Smith, 1992; Ren and Smith, 1995), which protects against hydroxyl radicals and sequesters zinc and redox active copper. Heme-hemopexin decreases expression of the transferrin receptor (Alam and Smith, 1989; Taketani *et al.*, 1990) and transferrin itself (Smith, 1999). Inflammation follows hemolytic events and is mediated in part by NF-κB (Karin, 1998). The nuclear translocation of NF-κB is stimulated by heme-hemopexin and the activity of the c-Jun amino-terminal kinase (JNK) is increased (Eskew *et al.*, 1999). In contrast, free heme has minimal effects on NF-κB and does not increase phospho-c-Jun levels (Eskew *et al.*, 1999).

After cells are exposed to oxidative stress, *e.g.*, H_2O_2, inflammatory cytokines (transforming growth factor-β1, interleukin-1, or tumor necrosis factor; see Schulze-Osthoff *et al.*, 1995; Schmidt *et al.*, 1996; Muller *et al.*, 1997) and perfusion after ischemia (Meldrum *et al.*, 1997; Tacchini *et al.*, 1997), the DNA binding of AP-1 and NKκB is increased. When heme is transported into cells by hemopexin, there is an increase in cell oxidation state (Eskew *et al.*, 1999), as shown by transient increases in protein carbonyl content, to levels essentially identical to those generated during ischemia-reperfusion injury of brain (Oliver *et al.*, 1990). Heme also increases carbonyl content, but cobalt-protoporphyrin (CoPP)–hemopexin (Eskew *et al.*, 1999) does not. Thus, the hemopexin system enables evaluation of the relative contributions to gene regulation of signaling pathways and cell-surface redox processes activated by hemopexin receptor occupancy and heme transport. Activation of signaling pathways involving reactive oxygen intermediates (ROIs) is linked with increased transcription of the MT-1 gene by heme-hemopexin, and the promoter region between −153 and −42 relative to the transcription start site is required for MT-1 induction (Ren and Smith, 1995). This DNA segment contains binding sites for several oxidation-sensitive transcription factors known to be involved in MT-1 gene regulation by various structurally distinct inducers. The sites include six metal-responsive elements (MRE) to which MRE binding transcription factor (MTF-1) binds, a site for upstream stimulating factor (USF)-1 and USF-2, which bind to part of an overlapping composite E box/anti-oxidant response element (ARE), and an Sp1 site. Deletion analysis and transient transfection assays revealed that the ARE is required for MT-1 gene regulation by heme-hemopexin and that the MREs contribute (Ren and Smith, 1995). For example, deletion of MREs 5′ or 3′ to the E box/ARE composite element drastically diminishes the augmented transcriptional activation by heme-hemopexin and dithiocarbamates, which also act via the MREs (Palmiter, 1994). Glutathione protects against oxidative stress and MTF-1 is also required for synthesis of the heavy subunit of γ-glutamylcysteine synthetase gene, which encodes a key enzyme in glutathione synthesis (Gunes *et al.*, 1998).

Copper plays several roles in the responses of cells when hemopexin binds to its receptor with consequent activation of signaling cascades and MT-1 (Sung, 1999) and HO-1 gene regulation (Sung *et al.*, 2000). Here, we address the induction of the DNA binding of transcription factors USF 1 and 2, the ARE-binding proteins, and MTF-1 in response to heme–hemopexin and the sensitivity of DNA binding to transient cellular oxidation by heme–hemopexin and to chemical oxidation by diamide. Because MTF-1, proposed to be retained in cytosol analogously to NF-κB (Palmiter, 1994), is shown here to be activated by heme–hemopexin, we also determined whether oxidation or activation of signaling pathways upon hemopexin receptor occupancy is the principal mechanism leading to the nuclear translocation of MTF-1. The effects of heme–hemopexin and of CoPP-hemopexin, which allows investigation of signaling pathways in the absence of both oxidation and tetrapyrrole transport (Smith *et al.*, 1993), are compared with those of

free heme and hydrogen peroxide, which also affect cells and increase the protein carbonyl content similarly to heme–hemopexin (Eskew *et al.*, 1999). Finally, the role of redox-active copper on protein carbonyl production, the DNA binding of USF1, USF2, and the ARE-binding proteins, and the nuclear translocation and DNA binding of MTF-1 and NF-κB in response to heme-hemopexin were investigated using the Cu(I)-specific chelator bathocuproinedisulfonate.

MATERIALS AND METHODS

Reagents

Metalloporphyrin (Porphyrin Products, Logan, UT) concentrations were determined spectrophotometrically in dimethylsulfoxide using published procedures (Smith *et al.*, 1993). Dinitrophenylhydrazine (DNPH), guanidine, and bathocuproine disulfonate were obtained from Aldrich (Milwaukee, WI).

Hemopexin isolation and preparation of heme-hemopexin complexes

Intact rabbit hemopexin was purified (Morgan *et al.*, 1993) and stoichiometric 1:1 heme–hemopexin complexes (90–95% saturation) prepared by addition of 0.9–1.1 molar equivalents of heme, with final dimethyl sulfoxide (DMSO) concentration less than 5% vol/vol, followed by dialysis to remove the DMSO. The complex concentration and saturation was determined using extinction coefficients ($A \cdot M^{-1} \cdot cm^{-1}$) of 1.1×10^5 at 280 nm for apo-hemopexin; 1.2×10^5 at 280 nm, and 1.3×10^5 at 405 nm for rabbit mesoheme-hemopexin. Cobalt–protoporphyrin IX–hemopexin complexes were similarly made using published extinction coefficients (Smith *et al.*, 1993).

Cultured cells

Mouse hepatoma cells (Hepa) were cultured in Dulbecco's minimal essential medium (DMEM) containing 5% fetal bovine serum (FBS) as described (Smith and Ledford, 1988). Cells were seeded (4×10^6 cells per T-150) and, after 48 hr of growth, rinsed in HEPES-buffered DMEM, pH 7.4 (equilibrated overnight in 5%CO_2) and then incubated for 1 hr with 2–10 μ*M* heme–hemopexin, 2–10 μ*M* CoPP–hemopexin, or 1–5 μ*M* free heme in HEPES-buffered DMEM. For certain experiments the cells were incubated with hemopexin complexes together with 25 μ*M* bathocuproine disulfonate (BCDS), a specific Cu(I) chelator. Nuclear extracts were prepared, and nuclear MTF-1 levels were assessed by Western immunoblotting.

Assay of the protein carbonyl content of cells

After the usual subculture, cells (6×10^6 cells per T-75) were first incubated for 1 hr in LSD-MEM (low serum DMEM contains 0.5% FBS) and then for an additional hour in the presence or absence of 2–10 μ*M* heme–hemopexin, 2–10 μ*M* CoPP–hemopexin, 0.5–10 μ*M* free heme, or 400 μ*M* H_2O_2 (stock concentration was determined spectrophotometrically using EmM of 43.6 M^{-1} cm^{-1} at 240 nm). The cellular carbonyl content of oxidatively modified proteins in cell extracts was determined (Levine *et al.*, 1990), and contaminating nucleic acids were removed from the cell extracts (1 mg protein) by precipitation upon addition of streptomycin sulfate (1% wt/vol). The protein carbonyl content of the final washed pellet, dissolved in 6 *M* guanidine-HCl, pH 2.3 (600 μl), was calculated from the maximum absorbance (360–390 n*M*) using a molar absorption coefficient of 22 mM^{-1} cm^{-1}. To minimize variation, the amount of protein recovered in the final pellet after solubilization in guanidine was quantitated using the bicinchoninic acid assay (Pierce, Rockford, IL) and the carbonyl content of cell samples expressed per milligram of protein.

Detection of DNA binding of USF, ARE-binding proteins, NF-κB, and MTF-1 in nuclear extracts by electrophoretic mobility-shift assays

Forty eight hours after seeding (4×10^6 cells/T150 flask), Hepa cells were rinsed and incubated for 1 hr in serum-free HEPES-buffered DMEM, pH 7.4, supplemented with heme–hemopexin (50 n*M*–10 μ*M*). Nuclear extracts were then prepared from these cells essentially as described previously for HeLa cells (Dignam *et al.*, 1983). Electrophoretic mobility-shift assays (EMSA) were carried out using

a 4% (80:1 acrylamide/bis-acrylamide) polyacrylamide gel after incubation of nuclear extracts (3 μg) for 20 min on ice using blunt ended oligonucleotide probes of similar lengths. Oligonucleotides were radiolabeled using T_4 polynucleotide kinase (Promega, WI) and [γ-^{32}P]ATP (DuPont-NEN, specific activity 10 μCi/μl) and 0.035 pmol of probe were incubated with nuclear extracts for 20 min on ice before electrophoresis. For competition studies, the non-radiolabeled oligonucleotide was added to the nuclear extracts 10 min before the radiolabeled probe. The following oligonucleotides: for the transcription factor NF-κB (5'-AGTTGAGGGGACTTTCCCAGGC-3', Promega, WI); for the overlapping E-box/ARE from the mouse metallothionein proximal promoter (MT-1ARE, 5'-AGCTTGGGCGCGT-GACTATGCGTGGGCTG-3', 29-mer); for a USF binding site (AdMLP, 5'-AATTCCTGGC-CACGTGACCGCAGCTGT-3', 27-mer; Vostrov *et al.*, 1995); for the rat glutathione reductase Ya ARE (YaARE, 5'-ATTGCTAATGGTGACAA-AGCAACTTTCGCA-3', 30-mer, Nguyen *et al.*, 1994). The identity of the specific NF-κB–DNA complex was confirmed by separate competition studies with 50 molar excess of the unlabeled NF-κB consensus oligonucleotide (Promega, Madison, WI) as well as by supershift assays (Sung *et al.*, 2000) using a rabbit polyclonal anti-p65 NF-κB antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The identity of USF 1 and 2 was determined by supershift assays with antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) added to the nuclear extracts on ice for 10 min before addition of the radiolabeled oligonucleotide. Because the MRE that confers the strongest response to Zn, MREd, contains an overlapping binding site for Sp1, we used an oligonucleotide, MRE-s (5'-GATCCAGGGAGCTCTGC-ACACGGCCCGAAAAGTA-3', 34-mer) based on a probe designed by Schaffner and colleagues (Radtke *et al.*, 1993) and employed by others (Dalton *et al.*, 1996). EMSA with this probe were carried out using published procedures, including the addition of 100 μM $ZnSO_4$ to the buffers (Radtke *et al.*, 1993; Dalton *et al.*, 1996). Antibodies to MTF-1 were a generous gift from Dr. Walter Schaffner (University of Zurich, Zurich, Switzerland) and were used to assess the nuclear concentrations of MTF-1 by Western immunoblotting because these antibodies are not suitable for super-shift assays.

Detection of MTF-1 in nuclear extracts of Hepa cells

MTF-1 was detected after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of nuclear extracts (15 μg protein determined by the BCA assay) on 8% polyacrylamide gels and transfer to PVDF membranes (BIORAD, Hercules, CA). Polyclonal monospecific antibodies raised against the carboxy-terminal peptide (amino acids 377–524) of mouse MTF-1 were used at a dilution of 1:4,000. Anti-MTF-1 antibodies were detected using donkey anti-rabbit IgG-horseradish peroxidase (1:2,000) and the ECL system (Amersham, Arlington Heights, IL) and quantitation using the NIH Image program. Some variation in the nonspecific immunoreactivity is apparent in different preparations of nuclear extracts and in different batches of antisera. Each experiment was repeated two–three times using different extracts, and the increase in MTF-1 by a particular stimulus was very similar. There was no correlation between an increase in MTF-1 with an increase in intensity of the nonspecific immunoreactive proteins. These latter were generally the same in the extracts from the control and experimental cells.

RESULTS

Binding of redox-sensitive transcription factors USF 1, USF 2, the ARE-binding proteins, and MTF-1 elements from the proximal promoter of the mouse MT-1 gene: constitutive expression, the effect of heme–hemopexin, and differential sensitivity to oxidation

To examine the level of constitutive expression of nuclear proteins binding to the composite E-box-like/ARE element (MT-1ARE; see Fig. 1A), EMSAs were used with a radiolabeled MT-1ARE oligonucleotide and extracts from control cells (Fig. 1B). Two DNA–protein species were detected and competition studies with non-radiolabeled oligonucleotides containing either the glutathione reductase ARE

(Ya ARE) or a USF-binding site (5′-CACGTGAC-3′; AdMLP) as well as supershift assays with antibodies specific for USF 1 and 2 (Fig. 1B–E) show that the USF–DNA complex migrates more slowly than the ARE–DNA complex (Fig. 1B–D), confirming published studies (Dalton *et al.*, 1996). Thus, USF 1 and 2 can recognize the E-box-like sequence (5′-CGCGTGAC-3′) in the MT1 proximal promoter. Both sequence and Mg(II) concentrations affect the affinity of USF for various E-box (CANNTG; Fig. 1A) and E-box-like sequences (Bendall and Molloy, 1994). Maximal binding to consensus E-box sequences (*e.g.*, AdMLP) requires a high Mg(II) concentration (4 m*M*; Fig. 1D), whereas at 1 m*M* Mg(II) or in the absence of Mg(II), USF binding is less discriminating (Bendall and Molloy, 1994). AdMLP is an effective competitor for both DNA–protein species generated by MT-1ARE (at 1 m*M* Mg(II); Fig. 1B), providing evidence that the ARE-binding proteins bind to the AdMLP probe.

MTF-1 plays a principal role in MT-1 regulation by oxidative stress. H_2O_2 increases the DNA binding of MTF-1 about eight-fold, but this requires 2.5 m*M* H_2O_2 in the presence of 2% serum (Dalton *et al.*, 1996) (see Fig. 2A), whereas the induction in serum-free medium by 500 μM H_2O_2 is considerably less (data not shown). Low concentrations of a specific DNA–MTF-1 complex, designated "MTF1-MRE-s1," are constitutively expressed in Hepa cells. Heme–hemopexin (2–10 μM) rapidly increased the nuclear levels of MTF-1 seven- to 11-fold as shown by functional DNA binding using EMSAs (Figs. 2B and 3A) and by Western immunoblotting of nuclear extracts (Fig. 3B,C). Additional specific DNA–protein complexes are produced by increasing concentrations of heme-hemopexin, designated s2 and s3, which migrate more slowly in the native gel. These s2 and s3 complexes are not seen in response to H_2O_2, even after prolonged exposure of the autoradiograph. The amount of these complexes was not decreased by competition with a mutated MREd nor by a non-radiolabeled MT-1ARE, confirming the specificity of these interactions.

One means to affect the relative occupancy of proteins that bind to a composite element would be differential sensitivity to oxidation. USF is known to be extremely sensitive to chemical oxidation by diamide (azodicarboxylic acid bis [*N,N*-dimethylamide] oxidation) (Pognonec *et al.*, 1992), and the effects of a range of diamide concentrations (0.1–5 m*M*), to mimic increases in intracellular oxidation, on DNA binding to the MT-1ARE oligonucleotide were investigated. The DNA binding of USF 1 and 2 decreased by more than 80% by 0.1 m*M* diamide and was abolished by 0.25 m*M* (Fig. 2C,D), whereas 5 m*M* diamide caused only about a 30% decrease in ARE binding (Fig. 2C). This oxidation is reversible for both transcription factors upon reduction with 30 m*M* β-mercaptoethanol (Fig. 2C). Thus, the ARE-binding proteins are quite resistant to oxidation by diamide, consistent with their proposed function in activating protective genes in response to oxidative stress.

MTF-1, like the ARE-binding proteins, is resistant to diamide oxidation. Detectable loss of DNA binding of MTF-1, about a 30% decrease, required 5 m*M* diamide, which was fully restored upon reduction with β-mercaptoethanol (Fig. 2F). Overall, these observations extend published studies on these constitutively expressed transcription factors (Dalton *et al.*, 1996) and provide new information about the characterization of these DNA proteins using *in vitro* assays.

Heme–hemopexin causes a transient increase in cellular oxidation state as shown by the increase in protein carbonyl content from 3 nmol/mg protein to 7 nmol/mg protein effected by 10 μM heme–hemopexin, which is essentially equivalent to that produced by 400 μM H_2O_2 (Eskew *et al.*, 1999) (see Fig. 4). However, decreases in the levels of redox-sensitive USF 1 and 2 binding were not reliably observed using EMSAs; but the amount of DNA–ARE-binding protein complexes detected doubled after a 1-hr incubation of cells with heme–hemopexin (Fig. 2D,E).

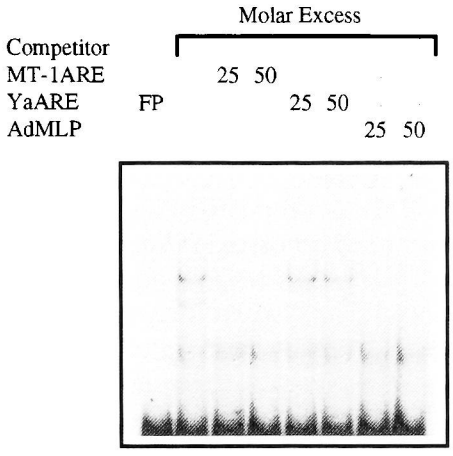
Effects of receptor occupancy on the DNA binding and nuclear translocation of the metal responsive element binding protein, MTF-1

Hemopexin receptor occupancy alone with 5 and 10 μM CoPP–hemopexin also increased the DNA binding of MTF-1 within 1 hr (Fig. 3A) and, as with heme-hemopexin, a slower-mi-

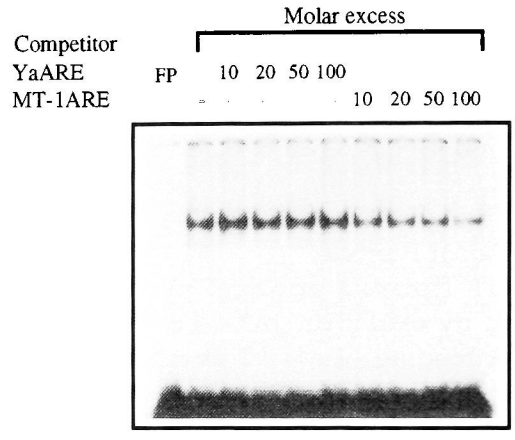
A

T G A C TCA	Consensus TRE (AP-1 SITE)
G T G A C NNN GC	Consensus ARE
GCGGGG C G C G T G A C TAT GC	Mouse MT-1 Promoter
agcttGGG C G C G T G A C TAT GC GTGGGCTG	MT-1ARE29mer probe
ATTCGTA A T G G T G A C AAA GC AACTTTCGCA	Rat GSTYaARE [YaARE]
TAGAGTC A C A G T G A C TTG GC AAAATCTGAG	Rat Quinone reductase ARE
C A N N T G	E-box
C A G C T G A C	USF determinant+AC
AATTCCTGGC C A C G T G A C CGC AGCTGT	AdMLP Probe
C A C G T G A C CCG	Human HOTF1/USF
CCAC C A C G T G A C TCG AG	Rat HOTF1

B



C



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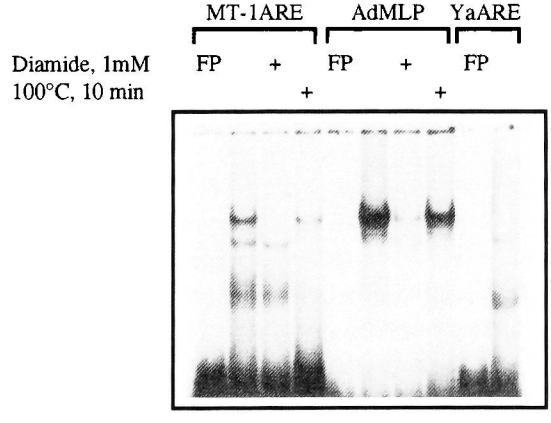

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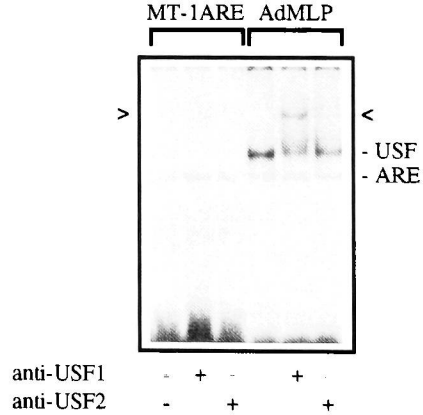

grating species (s-3) is apparent. The levels of MTF-1 in the nucleus are increased up to 14-fold in response to increasing concentrations of heme-hemopexin from 2 to 10 μM, as shown by Western immunoblotting analysis. These levels are significantly higher than in response to 100 μM $ZnSO_4$ (Fig. 3B), used as a positive control because zinc is an effective inducer of MT genes via MTF-1 binding to the MRE. Increased nuclear concentrations of MTF-1 in response to CoPP–hemopexin were also detected by immunoblotting of nuclear extracts (Fig. 3C). Free heme increases protein carbonyl content to levels similar to that seen with heme-hemopexin but is a poor inducer of MT-1 (Ren and Smith, 1995). The effects of heme on MTF-1 were barely detectable, causing only a slight increase DNA binding to MRE-s (data not shown) without detectable changes in nuclear levels of MTF-1 (Fig. 3D), and, thus, were insignificant compared with the extensive induction by heme–hemopexin.

Effect of the specific Cu(I) chelator bathocuproine disulfonate on cellular oxidation state and the nuclear translocation of MTF-1 in response to heme-hemopexin

Evidence is accumulating for more than one role for extracellular or surface copper in the induction of MT-1 and HO-1 (Sung *et al.*, 2000) mRNA by hemopexin. Bathocuproine disulfonate (BCDS) prevents the increase in cellular oxidation state, *i.e.*, protein carbonyl content by heme–hemopexin (Fig. 4A). Both heme–hemopexin and CoPP–hemopexin are shown here to cause the nuclear translocation of MTF-1 and increased DNA binding. BCDS also inhibited the nuclear translocation of MTF-1 in response to both heme- and CoPP–hemopexin complexes, implicating an effect of copper for MTF-1 regulation transmitted from the receptor rather than the transient oxidation. In contrast to these inhibitory effects of the Cu(I) chelator on MTF-1, nuclear levels of Rel/NF-κB remain elevated (Fig. 4C), and there is no detectable effect of BCDS on the DNA binding of USF and ARE-binding proteins (data not shown).

DISCUSSION

When hemopexin binds heme with its redox active central iron atom rather than the far less redox-active heme analog with cobalt, there is a transient increase in cellular oxidation (Eskew *et al.*, 1999). This is considered to be associated with endocytosis and heme release from hemopexin and uptake because free heme also increases cellular oxidation. CoPP–hemopexin activates MT-1 transcription in intact hepatoma cells and in transient transfection assays, implicating signals derived from the hemopexin receptor for MT-1 gene regulation. Both heme- and CoPP–hemopexin complexes rapidly activate the JNK signaling cascade, and essentially all of the cellular c-Jun is phosphorylated within 30 min (Eskew *et al.*, 1999). Inhibition of MT-1 and HO-1 mRNA induction by the impermeable copper chelator BCDS supports a role for extracellular or surface-chelatable Cu(I) in these gene regulation pathways. By analogy with the role of reduction of iron for uptake and consistent with the inhibitory effects of

FIG. 1. Mobility-shift assay of complexes formed between USF and ARE-binding proteins with overlapping recognition sites in a composite element present in the mouse MT-1 proximal promoter. (**A**) Sequences of a consensus TRE, ARE, a primary determinant for USF binding recognized by basic helix-loop-helix transcription factors including c-Myc and Max, the E-box consensus sequence CANNTG; AREs from the rat Ya glutathione *S*-transferase gene and the mouse MT-1 gene; and USF binding sites (AdMLP) and from the proximal promoter of the rat and human HO-1 genes. (**B–E**) Electrophoretic mobility-shift assays. (**B**) Two DNA–protein complexes are formed when radiolabeled MT-1ARE encoding the composite E-box/ARE is incubated for 10 min on ice with nuclear extracts (3 μg) from cells treated with 10 μM heme–hemopexin for 1 hr. Competitive inhibition was carried out with either the YaARE or the AdMLP oligonucleotide at the concentrations indicated. The magnesium concentration was 1 m*M*. (**C**) Two DNA–protein complexes are resolved when radioactive AdMLP oligonucleotide is incubated with the same nuclear extracts (1 m*M* $MgCl_2$), and specific competition with the YaARE and MT-1ARE probes are shown. (**D**) Nuclear extracts were first treated either with diamide or heated at 100°C as indicated before incubation with one of the following radiolabeled MT-1ARE, AdMLP, or Ya ARE probes. (**E**) Supershift assays are shown using antibodies to USF1 or to USF2 and the DNA–protein complexes formed with either the MT-1ARE or AdMLP radiolabeled probes in the presence of 1 m*M* Mg Mg to observe the shifted complexes (arrowhead). FP indicates that the sample electrophoresed in that lane contained only the radioactive probe and buffer.

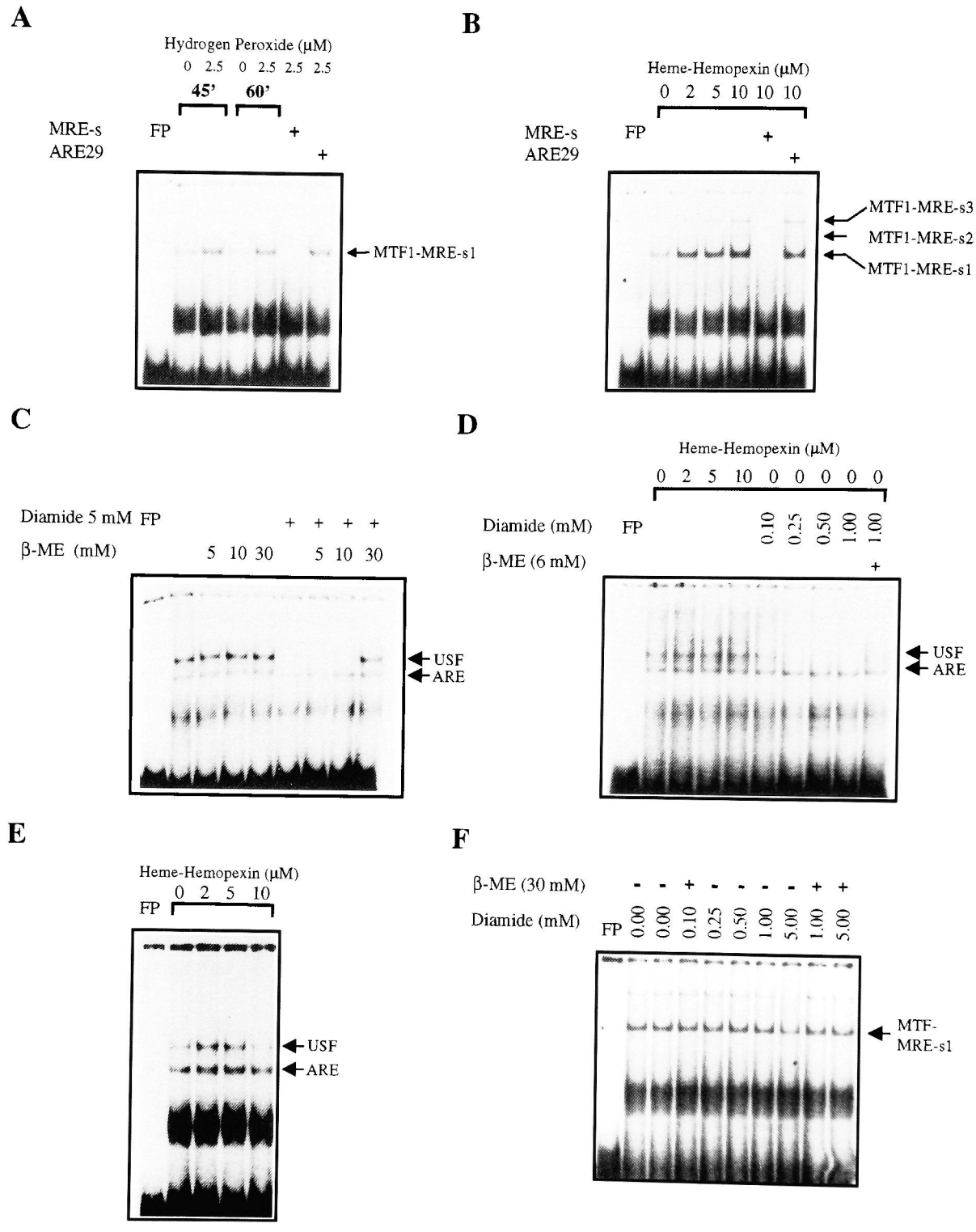

FIG. 2. Mobility-shift assays of MTF-1–MRE-s complexes in nuclear extracts from cells incubated with heme–hemopexin or H_2O_2 and sensitivity to oxidation of USF, ARE-, and MRE-binding proteins and the effects on DNA binding. Nuclear extracts, isolated as usual from cells incubated with H_2O_2 in the presence of 5% FBS (**A**) or with 2–10 μM heme–hemopexin (**B**) at the concentrations and times indicates, were incubated with the radiolabeled MRE-s oligonucleotide as described in Materials and Methods. Up to three specific MTF-1–MRE-s complexes, identified by competitive inhibition, are apparent using nuclear extracts from cells incubated with hemopexin complexes. One, constitutively expressed, is here designated MTF-1–MRE-s1 and the two additional DNA–protein species that migrate more slowly through the native gel are designated s-2 and s-3. FP indicates the lane containing only free probe. Antibodies to MTF-1 currently available are not suitable for supershift studies and were used for immunoblotting of nuclear extracts. Mobility-shift assays of DNA protein complexes formed using nuclear extracts isolated from mouse Hepa (**C, E,** and **F**) or human retinal pigment epithelial cells (**D**) incubated with 2–10 μM heme–hemopexin, as indicated, for 1 hr. The nuclear proteins were oxidized chemically by incubation for 10 min on ice with diamide followed by reduction for 10 min with up to 30 mM reducing agent at the concentrations indicated before addition of either the radiolabeled MT-1ARE or MRE oligonucleotide and a further incubation for 10 min before electrophoresis.

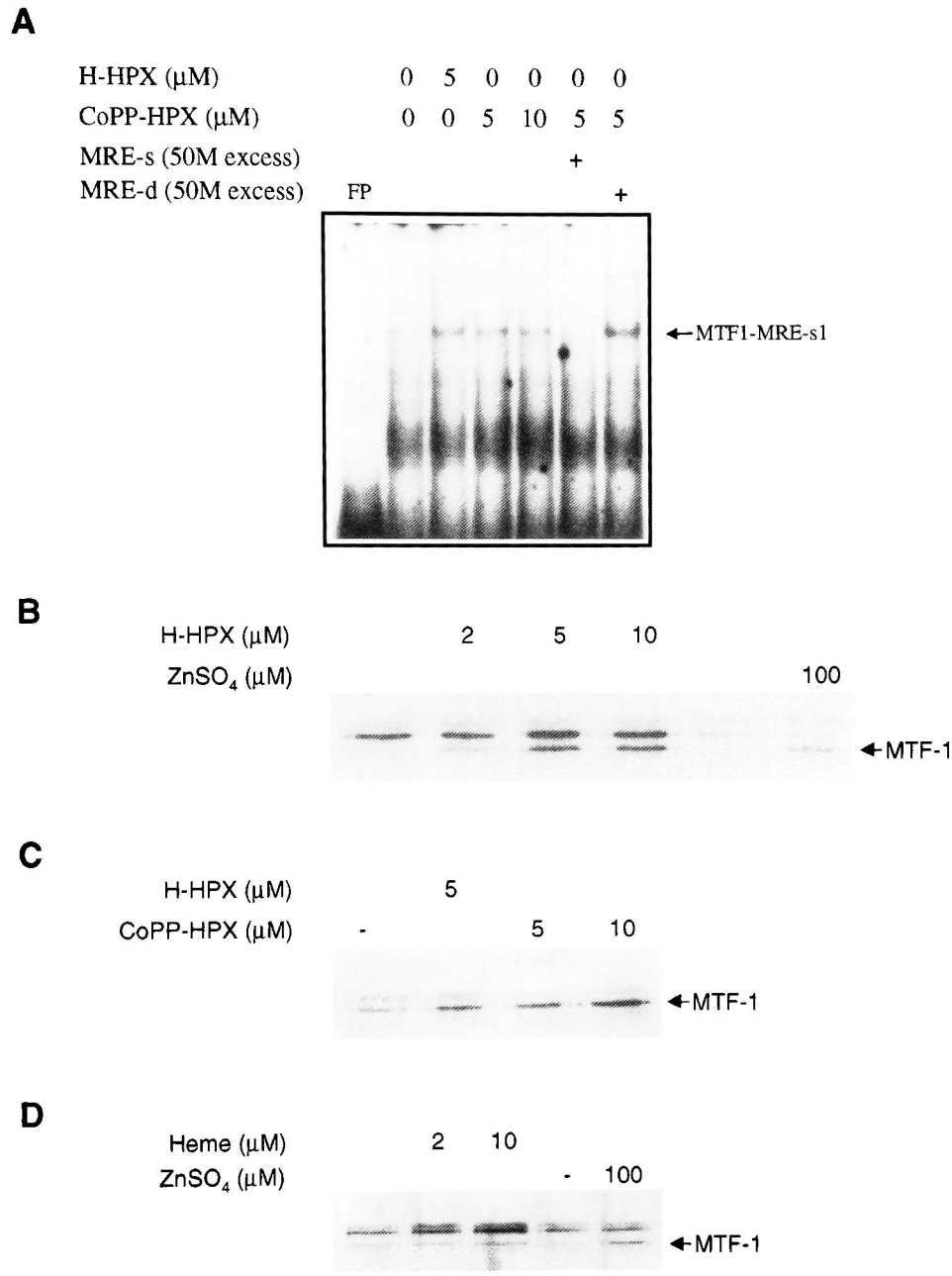

FIG. 3. MTF-1–MRE-s complexes in nuclear extracts from cells incubated with heme– or CoPP–hemopexin together with the effect of heme-hemopexin, CoPP–hemopexin, and free heme on the nuclear translocation of MTF-1: comparison with Zn. (**A**) Results of a mobility-shift assay using nuclear extracts from cells incubated with either 5 or 10 μM heme- or CoPP–hemopexin for 1 hr before addition of the radiolabeled MRE-s oligonucleotide as usual followed by electrophoresis. Using Western immunoblotting, the levels of MTF-1 in nuclear extracts (3 μg per lane) isolated as usual following incubation of cells with 2–10 μM heme-hemopexin or 100 μM $ZnCl_2$ (**B**), 5 or 10 μM CoPP–hemopexin (**C**), 2–10 μM free heme or 100 μM $ZnCl_2$ (**D**) for 1 hr were determined. Control cell were incubated with PBS (**A** and **C**) or with DMSO (**D**).

BCDS, we have proposed that redox-active processes participate in cellular responses to heme–hemopexin and heme uptake. Using the effect of heme-hemopexin on known oxidation sensitive transcription factors, USF 1 and 2, MTF-1, and ARE-binding proteins required for MT-1 transcription and of NF-κB, we investigated the relative roles of oxidation, receptor occupancy, and copper on the DNA binding of these transcription factors to their cognate sites as well as effects on nuclear translocation of MTF-1 and NF-κB.

Both the USF and ARE families of transcription factors have been implicated in MT-1 and

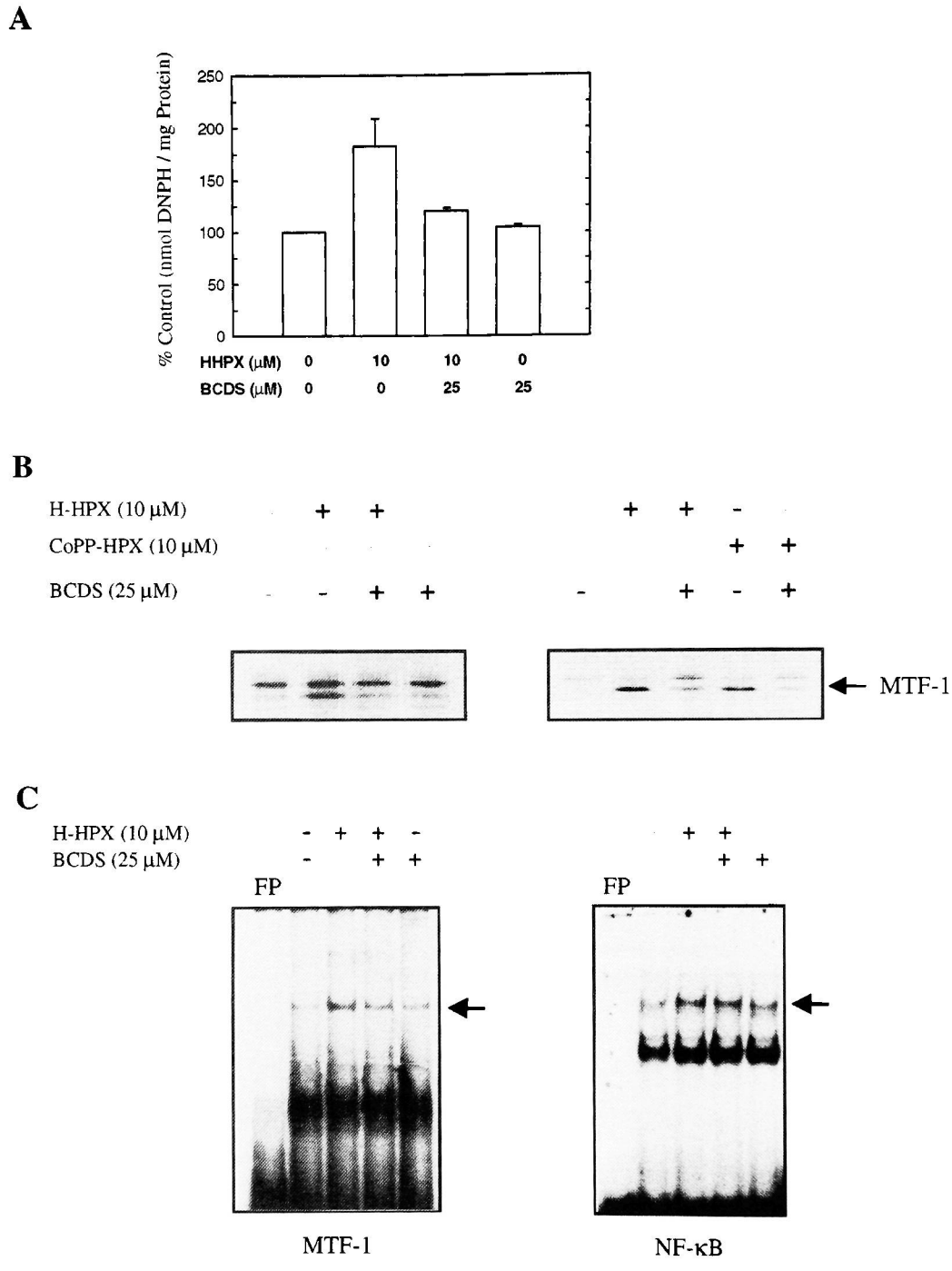

FIG. 4. Effects of the Cu(I) chelator bathocuproine sulfonate on the carbonyl production by heme–hemopexin and on the nuclear translocation and functional DNA binding of MTF-1 and NF-κB. The effect of incubating Hepa cells in the absence and presence of 25 μM BCDS with 10 μM heme–hemopexin for 1 hr on the protein carbonyl content, determined as described in Materials and Methods, and on the nuclear levels of MTF-1 induced by heme– and CoPP–hemopexin complexes as determined by Western immunoblotting are shown in **A** and **B**, respectively. (**C**) Data from mobility-shift assays of the amount of specific MTF-1– and NF-κB–DNA complexes (indicated by the arrows) in nuclear extracts from cells incubated with heme–hemopexin for 1 hr in the absence or presence of BCDS

HO-1 transcription and, together with MTF-1, in cellular responses to oxidative stress. The ubiquitous USF family is involved in basal expression of MT-1 (Carthew *et al.*, 1987), in the response of MT genes to several inducers (Dalton *et al.*, 1996), and, interestingly, also in the regulation of rat HO-1 (Maeshima *et al.*, 1996). AREs were first identified in the promoters of

the glutathione *S*-transferase (Nguyen and Pickett, 1992) and quinone reductase (Favreau and Pickett, 1995) genes, whose protein products provide protection against oxidative damage from redox cycling chemicals. HO-1 is also induced by electrophiles via an ARE consensus sequence (which overlaps with two phorbol ester-responsive TRE sites) in the SX2 enhancer (Prestera *et al.*, 1995). MTF-1 binding to MREs is increased by oxidative stress from H_2O_2 and tBHQ (Dalton *et al.*, 1996; Sung *et al.*, 2000), and footprints are detected over the E-box/ARE region and the MREs (Dalton *et al.*, 1996). The increased oxidation from heme–hemopexin is not reflected in decreased binding of the extremely oxidation-sensitive USF to the MT-1 E-box/ARE. The MRE- and ARE-binding proteins are far more resistant to chemical oxidation than USF 1 and 2, consistent with their proposed regulation of gene expression in response to oxidative stress. However, as discussed below, the activation of MTF-1 by CoPP–hemopexin and lack of effect of heme show that the primary stimulus comes from the hemopexin receptor, rather than oxidation *per se*. A two-fold increase in ARE binding to the E-box/ARE by heme–hemopexin is consistent with a two-fold activation in transient transfection assays of the fusion gene, $ARE_2MT\beta$-Geo, which contained two copies of the 10-nucleotide ARE element linked to the basal MT promoter (Ren and Smith, 1995).

When heme is bound by hemopexin, it no longer catalyzes lipid peroxidation (Gutteridge and Smith, 1988), yet the protein carbonyl production detected when heme is transported into cells is indicative of metal-catalyzed oxidation involving hydroxyl radicals leading to the oxidation of proteins (Levine *et al.*, 1990, 1994). Addition of free heme to cultured cells causes a similar level of protein carbonyl production, and the effect of free heme, like that of heme–hemopexin, is rapid but transient and is inhibited by BCDS (J.D. Eskew and A. Smith, unpublished observations). The carbonyls are proposed to be derived from the participation and activation of electron transport processes at the plasma membrane, and, furthermore, by analogy with iron uptake in yeast, reduction of the heme-iron is followed by oxidation via a copper oxidase (Smith, 1999). Incomplete reduction by the oxidase would be a source of reduced oxygen species like superoxide, from which H_2O_2 is derived by dismutation. Hydroxyl radicals are then generated from H_2O_2 in the presence of redox-active metals like iron and copper at the cell surface leading to the carbonyl production. The transient protein oxidation in response to heme-hemopexin is prevented by the Cu(I)-chelator, BCDS, which does not penetrate cells, and, therefore, the redox cycling of copper takes place at the cell surface. One of the site(s) of BCDS action is presumed to be the oxidase homologous to yeast Fep3.

Other data also point to separation of the responses to oxidation and signaling cascades activated by hemopexin receptor occupancy in the events leading to the induction of MT-1 mRNA, including the nuclear translocation of MTF-1. BCDS prevented the nuclear translocation and increased DNA binding of MTF-1 induced by heme–hemopexin but not the DNA binding of USF or ARE binding proteins. BCDS also inhibits the increase of protein carbonyl formation by heme–hemopexin. CoPP–hemopexin does not increase the cell oxidation state, consistent with the lower redox activity of cobalt compared with iron, but nevertheless this complex increases MTF-1 nuclear concentration and MTF-1 binding in EMSAs; thus, the surface oxidation is not the principal stimulus for MTF-1 activation. Rather, the hemopexin receptor initiates the process with an additional role for Cu(I) in the signaling cascade for MT transcription because BCDS prevents the induction of MT-1 mRNA by CoPP–hemopexin (Sung *et al.*, 1999). The pathways activated by heme-hemopexin leading to the nuclear translocation of MTF-1 and NF-κB, both considered to be oxidation-responsive transcription factors because they respond to H_2O_2, are distinguished by BCDS. BCDS decreased JNK activation by heme-hemopexin but not by CoPP–hemopexin. This copper chelator not only distinguishes effects of surface signals, in part via the hemopexin receptor, on MTF-1 and NF-κB but also on JNK activation.

The importance of receptor-mediated events in MTF-1 and NF-κB activation is further reinforced by the fact that heme, which causes oxidation, is a poor inducer of MT-1 mRNA (Ren and Smith, 1995) and of both MTF-1 and NF-κB in mouse hepatoma cells. Whether MTF-1 is a substrate for a kinase is not known, although

there are putative phosphorylation sites in the deduced amino acid sequence of MTF-1 (Radtke *et al.*, 1995).

The rapid and extensive nuclear translocation of MTF-1 by both heme–hemopexin and CoPP–hemopexin shows for the first time a response of this transcription factor to a receptor and a nonmetal inducer. Heme alone does not cause a similar effect. The increased DNA binding of MTF-1 detected by EMSAs may reflect an increase in transcriptional activity, but this remains to be shown. These data contrast with the lack of activation of a fusion gene containing five copies of MREd, MREβGeo, by heme–hemopexin in transient transfection assays (Ren and Smith, 1995). One explanation is that chromatin structure influences the transcriptional activation via MREs of the MT-1 gene at least by the hemopexin system. Both Zn (Fig. 3) and pyrrolidine dithiocarbamate (PDC) activate MTF-1 (see Fig. 3 and Palmiter, 1994), and the activity of MREβGeo is induced by Zn (Westin and Schaffner, 1988; Y. Ren and A. Smith, unpublished observations) and by PDC in the Hepa cells (Ren and Smith, 1995). In addition, PDC is a far less effective inducer of MREβGeo than Zn. MTF-1 is detected by Southwestern analysis (Radtke *et al.*, 1993) and thus does not require a protein partner to bind to the MRE.

Several observations point to multiple protein–protein interactions, likely to involve MTF-1, at the MT-1 proximal promoter both constitutively and in response to hemopexin and other inducers. Dithiocarbamate and PDC augment the induction by heme–hemopexin of MT-1 mRNA levels and also of reporter gene activity in transient transfections via the ARE by both MRE-dependent and -independent mechanisms (Ren and Smith, 1995). The original proposal that release in the cytosol for nuclear translocation of constutively active MTF-1 Zn-sensitive inhibitor by Zn(II) uptake via PDC (Palmiter, 1994) provided a basis for the stimulation by PDC of the effects of hemopexin on a fusion gene construct, $-153(-67)$ MTβ-Geo comprised of MREc, MREd, and the E-box/ARE. Overall, these published data implied an interaction between proteins bound to E-box/ARE and MREc and/or MREd in response to cell-surface events caused by hemopexin binding to its receptor and heme uptake. Constitutive binding over the E-box/ARE region as well as occupancy of MREd (Dalton *et al.*, 1996) has been shown by footprinting studies *in vivo* with genomic DNA, which correlates with the EMSAs shown here and in Dalton *et al.* (1996), as well as with the low level of MTF-1 binding in EMSAs and in immunoblots of nuclear extracts from control cells. Extensive nuclear protein binding over the MREs and a region downstream of the ARE occurred in response to tBHT, Zn, or H_2O_2 with some temporal changes in intensity that were correlated to the time course of changes in MT-1 mRNA levels by these inducers (Dalton *et al.*, 1996).

Perspectives

Study of the hemopexin system has revealed links between heme, iron, copper, and zinc metabolism as well as several roles for copper in the activation of signaling cascades, nuclear translocation of the transcription factor MTF-1, and HO-1 and MT-1 gene regulation. Furthermore, the activation by heme–hemopexin of JNK, for which extensive cross-talk is established, provides a basis whereby such a variety of stimuli at the cell surface induce MT-1, and potentially also HO-1, expression. However, in the case of ARE-like sequence regulation, c-Jun and family members do not act on TRE-like and ARE regulatory regions as transcriptional activators, as do Nrf 1 and 2 for ARE-mediated activation of the human quinone reductase gene, but as negative regulators, *e.g.*, c-Fos and Fra-1 (Venugopal and Jaiswal, 1996). The rapid activation of JNK by hemopexin suggests that c-Jun acts indirectly by sequestering protein partners. Thus, the hemopexin receptor exerts specific and coordinate effects on individual transcription factors that are further modified when heme transport takes place.

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ABBREVIATIONS

AP-1, Activator protein; ARE, anti-oxidant response element; β-MSH, β-mercaptoethanol; CoPP, cobalt-protoporphyrin IX; BCA, bicinchoninic acid; BCDS, bathocuproinedisulfonate; DMSO, dimethylsulfoxide; DNPH, dinitrophenylhydrazine; DTT, dithiothreitol; EGF, epidermal growth factor; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; heme, iron protoporphyrin IX; HO-1, heme oxygenase; HPX, hemopexin; H_2O_2, hydrogen peroxide; IFκB, inhibitory factor κB; LSDMEM, low serum DMEM (contains 0.5% FBS); MT, metallothionein, MTF-1, metal-responsive transcription factor; MRE, metal responsive element; NF-κB, Nuclear factor-κB; NIK, NF-κB inducing kinase; PBS, phosphate buffered saline; ROS, reactive oxygen species; TPA, 12-O-tetradecanoylphorbol-13-acetate; TRE, TPA response element; t-BHQ, *tert*-butylhydroxyquinone; TNF, tumor necrosis factor; USF, upstream stimulating factor.

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