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Heavy metal-mediated activation of the rat Cu/Zn superoxide dismutase gene via a metal-responsive element

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Abstract The Cu/Zn superoxide dismutase (SOD1) catalyzes the dismutation of superoxide radicals produced in the course of biological oxidations. When placed under the control of the rat *SOD1* gene promoter and transfected into human HepG2 hepatoma cells, the activity of a chloramphenicol acetyltransferase reporter gene was found to increase three- to four-fold in the presence of heavy metals (cadmium, zinc and copper). Functional analysis of mutant derivatives of the *SOD1* gene promoter and the use of a heterologous promoter system confirmed that the induction of the *SOD1* gene by metal ions requires a metal-responsive element (MRE) located between positions −273 and −267 (GCGCGCA). It was also shown by gel mobility shift assays that an MRE binding protein is induced by the exposure of the human liver cell line HepG2 to heavy metals. These results suggest that the MRE participates in the induction of the *SOD1* gene by heavy metals.

Key words *SOD1* gene · Metal-responsive element · Induction · Heavy metals

Introduction

Cu/Zn superoxide dismutase (SOD1) catalyzes the dismutation of superoxide radicals (O_2^-) to molecular oxygen and hydrogen peroxide (Fridovich 1975). The production and/or removal of superoxides has been observed to play a significant role in a variety of critical homeostatic mechanisms both at the cellular and the organismic levels. Production of superoxide following treatment with metal ions has been observed at the cellular and the organismic levels (Stohs and Bagchi 1995). Since biological macromolecules are prime targets for the damaging effects of the abundant oxygen radicals, it is assumed that these superoxides must be eliminated by SOD1. Therefore, the regulatory mechanisms that control the expression of the *SOD1* gene are of great interest (Kim et al. 1996). It has also been reported that SOD1 can act to prevent oncogenesis and tumor promotion (McCord et al. 1971); and protect against reperfusion damage to ischemic tissue (Fridovich 1983). A recent report suggested that overexpression of SOD1 and catalase could increase average lifespan in the fly *Drosophila melanogaster* (Orr and Sohal 1994). In yeast, the expression of SOD1 is regulated by copper ions at the level of transcription (Carri et al. 1991). Lutropin, Cu^{2+}, and reactive oxygen seem to induce SOD1 in rat (Laloraya et al. 1988; Nicotera et al. 1989; Percival and Harris 1991).

Metals are the common inducer of metallothionein (MT) genes, and all MT promoters from *Drosophila* to humans contain multiple copies of a semiconserved sequence that is responsible for induction by metals, called a metal-responsive element (MRE) (Culotta and Hamer 1989). MREs contain a 12- to 15-bp sequence consisting of a highly conserved heptanucleotide core, TGC(A/G)CNC, and a less conserved flanking sequence. Sequence analysis of transcription factor-binding sites has revealed an MRE sequence located between positions −273 and −267 in the 5′-flanking sequence of the *SOD1* gene from rat (Kim et al. 1993). The effect of this MRE sequence on the transcription of *SOD1* was therefore investigated. By transient transfection, mutagenesis, and mobility shift assays, it is shown here that the MRE in the proximal 5′-flanking sequence of *SOD1* is required for induction of the gene by heavy metals.

Materials and methods

Plasmid constructions

The 1.7-kb *Bam*HI-*Sma*I fragment (nucleotides −1633 to +85) of the rat *SOD1* gene (Kim et al. 1993) was inserted into the vector

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pBLCAT3 (Luckow and Schütz 1987). Unidirectional 5′ deletions were produced by cleavage of the 5′ region of the *SOD1* promoter with *Sph*I and *Bam*HI, followed by subsequent treatment with exonuclease III. Deletions were constructed that extend from position −1633 to −305 and to −257. These plasmids were designated pRSP-305 and pRSP-257, respectively. The endpoints of the deletions were confirmed by DNA sequencing with a Sequenase kit (US Biochemicals). For the construction of pMREtk, three copies of a double-stranded oligonucleotide that includes the MRE sequence between positions −273 and −267 of the *SOD1* promoter (SOD-MRE) were cloned into the *Bam*HI site of pBLCAT2Δ, which is derived from pBLCAT2 (Luckow and Schütz 1987). The plasmid pBLCAT2Δ contains a minimal promoter (−80 to +51) derived from the herpes simplex virus thymidine kinase (*tk*) gene. The plasmid pmMREtk is a mutant version of pMREtk with three copies of a mutated version of the MRE site (mMRE). To form SOD-MRE, the oligonucleotides 5′-GATCCTTCCAGGCGCGCACGCAGG-3′ and 3′-GAAGGTCCGCGCGTGCGTCCCTAG-5′ were annealed, giving a 4-bp overhang at each end. SOD-mMRE was obtained by annealing the sequences 5′-GATCCTTCCAGTCTCTACCGCAGG-3′ and 3′-GAAGGTCAGAGATGGCGTCCCTAG-5′. The insertion of the MRE consensus and mutant sequences was confirmed by DNA sequencing.

Cell culture and CAT assay

Human HepG2 hepatoma cells were grown as described previously (Kim et al. 1997). Cells were grown (to 30–50% confluence) on 60-mm plastic culture dishes for 24 h prior to transfection. Then 3.0 pmol of the reporter plasmid, containing the upstream region of the rat *SOD1* gene fused to the structural gene for chloramphenicol acetyltransferase (CAT), was transfected into the cells by the calcium phosphate-DNA coprecipitation method (Chen and Okayama 1988). Five micrograms of pRSVβ-gal plasmid (Edlund et al. 1985) was also introduced in all experiments in order to be able to correct for variations in transfection efficiency. The CAT assay was performed as described previously (Kim et al. 1997). The conversion of chloramphenicol to the acetylated form was followed by radiolabeling, and the relative CAT activities were calculated from the degree of conversion. The results given are averages of three independent experiments.

Treatment of cells with metals and preparation of nuclear extracts

Metal ions were added to the culture medium 36 h after transfection, and the cells were incubated for an additional 22 h. By monitoring cell death, the cytotoxic effects of the various metal ions were evaluated for over a concentration range of 1–500 μM. Nuclear extracts were prepared by a modification of the procedure of Andrews and Faller (1991).

Mobility shift assay

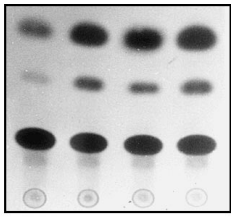
The synthetic oligonucleotides used to construct the MRE site were purchased from the Genosys. The double-stranded oligonucleotide was labeled with [γ-^{32}P]ATP and polynucleotide kinase (Sambrook et al. 1989). Aliquots (10 μg) of nuclear extract from each sample were incubated with labeled oligonucleotide for 20 min at 20° C in a 15 μl reaction containing 10 mM HEPES, 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol, and 2 μg of poly[dI-dC]. The mixtures were then fractionated by electrophoresis on 6% polyacrylamide gels in 0.5× TBE (44 mM TRIS, 44 mM boric acid, and 1 mM EDTA). After electrophoresis, the gels were dried and exposed to X-ray film. In competition assays, an excess of cold probe or competitor DNA was added to the binding reaction.

Results and discussion

Effect of heavy metals on the induction of the *SOD1* promoter

Production of superoxide has been observed following treatment with metal ions at the cellular and the organismic levels (Stohs and Bagchi 1995). It is assumed that these superoxides can be eliminated by the action of SOD1. In this study, the activation of the *SOD1* gene by metals was analyzed. HepG2 cells were transfected with plasmids containing the rat *SOD1* upstream region fused to the bacterial CAT gene. Recombinant plasmids containing the rat *SOD1* upstream region were derived from pRSP-1633. Deletions derivatives were constructed in the which region between −1633 and −305 or −257 was removed. The resulting plasmids were designated pRSP-305 and pRSP-257, respectively. These constructs were transiently expressed in HepG2 cells. At 36 h after transfection, metal ions were added to the transfected cells at the appropriate concentration. After a further 22 h, the CAT activity of the transfected cells was determined. To determine their effects on expression of the *SOD1* gene, cells transfected with pRSP-305 were treated with various doses of $CdCl_2$ (5 μM), $ZnCl_2$ (50 μM) and $CuSO_4$ (300 μM) (Fig. 1). The *SOD1* gene was induced about three- to four-fold by Cd, Zn, and Cu ions, as judged by the CAT activity in treated as compared to untreated cells.

When the transfected cells were treated with 300 μM $CuSO_4$, a three- to four-fold induction of CAT activity was observed with pRSP-1633 and pRSP-305. But pRSP-257 did not respond to the metal ion (Fig. 2). The plasmids pRSP-1633 and pRSP-305 contain the metal-responsive element (MRE) but pRSP-257 does not (Fig. 2A). These results therefore suggest that the *SOD1* gene is indeed inducible by heavy metals.



Conc. of metal (μM)	-	Cd 5	Zn 50	Cu 300
Relative CAT activity	**1.0**	**2.5**	**3.1**	**3.8**

Fig. 1 Effect of metals on expression of the *SOD1* gene. Plasmid pRSP-305 containing an SOD1-CAT reporter gene, was transfected into HepG2 cells. The cells were treated with cadmium, zinc, or copper for 22 h and CAT activity was assayed, as described in the Materials and methods. CAT activity is expressed relative to that observed in untreated cells

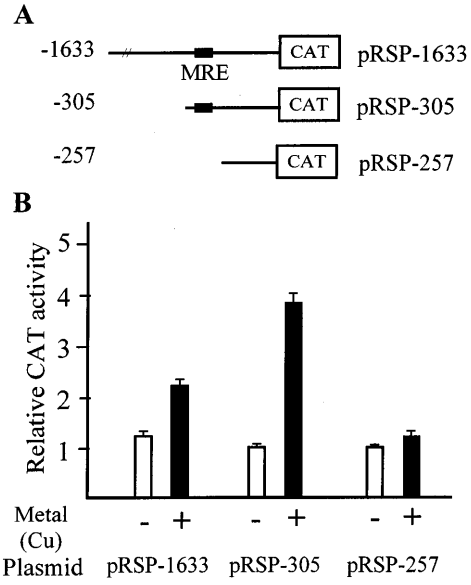


Fig. 2A, B Effects of various promoter constructs on the induction of the *SOD1* gene by metals. **A** Schematic diagram of the *SOD1* promoter attached to the CAT reporter gene. The plasmid names indicate the positions of the deletions in the SOD1 5′-flanking sequence. The plasmids pRSP-1633 and pRSP-305 contain the MRE site, but pRSP-257 does not. **B** Activation of the *SOD1* promoter by copper. The transfected cells were treated with Cu^{2+} (300 μM) for 22 h. The values are the means of three experiments. Relative CAT activities with (+) and without (–) copper ion are indicated by the *solid* and *open bars*, respectively

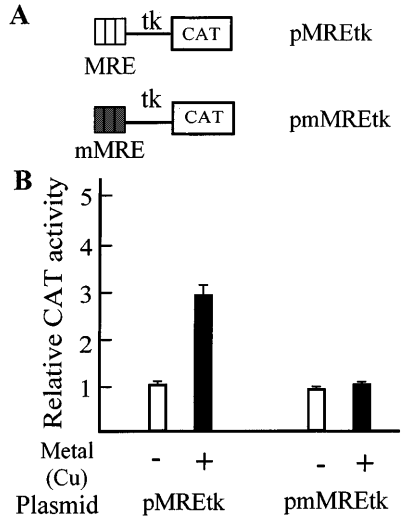


Fig. 3A, B Functional analysis of the MRE using a heterologous promoter system. **A** Schematic diagram of a synthetic MRE (pMREtk) and its mutated derivative (pmMREtk) attached to a heterologous promoter (*tk*) which was fused to the CAT gene. **B** Effect of metals on the synthetic MRE and its mutant derivative in the heterologous promoter. Relative CAT activities with (+) and without (–) Cu^{2+} (300 μM) are indicated by the *solid* and *open bars*, respectively. The values are the means of three experiments

Transcriptional activation by copper is mediated by the MRE

We demonstrated above that the *SOD1* promoter is inducible by heavy metals. An MRE sequence is located between positions −273 and −267 (GCGCGCA) in the 5′-flanking sequence of the *SOD1* gene. The role of MREs in regulating the expression of the metallothionein genes has been well established (Stuart et al. 1985; Karin et al. 1987; Culotta and Hamer 1989). We therefore wished to confirm that the activation of the *SOD1* promoter by metals is mediated by the MRE. Cells were therefore transfected with the plasmid pMREtk, which carries three copies of a double-stranded oligonucleotide that includes the *SOD1* MRE linked to a herpes simplex virus-thymidine kinase promoter (*tk*) fused to the CAT gene (Fig. 3A). CAT activity was induced about three-fold by copper in cells transfected with pMREtk. But CAT activity was not induced by copper when pmMREtk, containing mutated MRE sites, was transfected (Fig. 3B) into the HepG2 cells. The mutation within the MRE also abolished the ability to bind the MRE binding protein (see below; data not shown). This confirms that the MRE of the *SOD1* gene is responsive to copper. These results indicate that the MRE sequence in the *SOD1* gene promoter is indeed responsive to metals and mediates a strong induction response, as in the case of the MT gene of mouse. In yeast, copper ions act in two ways: they induce *SOD1* gene expression via the protein ACE1 and also serve to activate the SOD1 proenzyme (Carri et al. 1991; Gralla et al. 1991). Thus, copper appears to have a dual function in the regulation of SOD1. One involves transcriptional activation of the *SOD1* gene through the MRE; the other post-transcriptional activation of its product the proenzyme.

Identification of an MRE binding protein

Stuart et al. (1985) have shown that the MREs of the metallothionein I promoter are composed of the sequence TGCRCNC. To explore whether a nuclear protein(s) binds to the *SOD1* MRE, gel mobility shift assays using HepG2 nuclear extracts were performed with the synthetic MRE as a probe. Nuclear proteins prepared from cells not treated with copper ion showed a faint MRE-protein complex (Fig. 4A). This complex disappeared when unlabeled oligonucleotide was added to the reaction mixture in 50- or 100-fold excess, indicating the specificity of this protein-DNA interaction (Fig. 4A, lanes 3 and 4). The formation of a protein-DNA complex was not inhibited by addition of the mutated MRE oligonucleotide or non-specific competitors (Fig. 4A, lanes 5 and 6). When nuclear proteins prepared from copper-treated cells (24 h) were used, a much stronger band was seen at the same position as that observed with extracts from untreated cells (Fig. 4B, lane 3). This band could be competed by the cold MRE oligonucleotide, but not by the mutated MRE oligonucleotide or non-specific competitors (Fig. 4B, lanes 6 and 7). A protein-DNA complex of

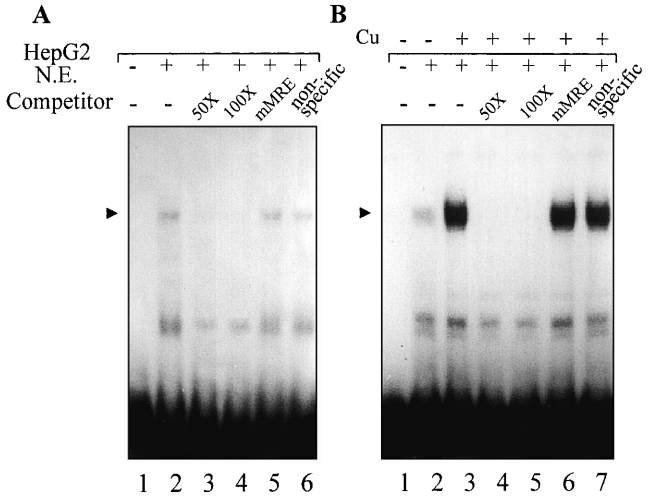


Fig. 4A, B Binding and induction of MRE-binding transcription factors. Gel mobility shift assays were performed using nuclear extracts (HepG2 N.E.) prepared from untreated HepG2 cells (**A**) and copper-treated HepG2 cells (**B**). When the HepG2 cells were treated with Cu^{2+} (**B**, lane 3), a strong DNA-protein complex appeared. This band could competed out by adding cold probe in excess. The location of the specific DNA-protein complex is indicated by the *arrowhead*

higher mobility was not efficiently competed out by the MRE fragment; this complex therefore appears to be non-specific. These results show that an MRE binding protein is induced by exposure of cells to heavy metals, and the induction of *SOD1* is mediated by the MRE in the promoter. Genomic footprinting of nuclei from induced and uninduced cells has indicated that a factor is bound to the MREs in the MT I gene only during induction, suggesting that metals allow a positively acting transcription factor to interact with the MREs (Anderson et al. 1987; Mueller et al. 1988). A clone encoding an MRE-binding protein (MTF-1) has been isolated from a cDNA expression library by using labeled MREs as a probe (Radtke et al. 1993). Thus, MTF-1 is a candidate transcription factor for the interaction with MRE, but it is not clear how it is regulated by metals. Recent studies have shown that metals undergo redox cycling, which results in the production of reactive oxygen species such as superoxide ion and hydroxyl radical. Therefore, it is very interesting that the MRE sequence contributes to the induction of the *SOD1* gene by heavy metals.

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