The Journal of Microbiology, December 1997, p.309-314
Vol. 35, No. 4

Molecular Cloning and Characterization of Mn-Superoxide Dismutase Gene from *Candida* sp.

Yun Mi Hong, Yong-Suk Nam[1], and Soon-Yong Choi*

Department of Microbiology, HanNam University, Taejon 306-791, Korea
[1]Department of Legal Medicine, College of Medicine, Korea University, Seoul 136-701, Korea

(Received September 1, 1997 / Accepted November 4, 1997)

The manganese-containing superoxide dismutase (MnSOD) is a major component of the cellular defence mechanisms against the toxic effects of the superoxide radical. Within the framework of studies on oxidative stress-responsible enzymes in the *Candida* sp., the gene encoding the MnSOD was isolated and examined in this study. A specific primer was designed based on conserved regions of MnSOD sequences from other organisms, and was used to isolate the gene by PCR on reverse-transcribed *Candida* poly(A$^+$) RNA. The PCR product was used to screen a *Candida* genomic lambda library and the nucleotide sequence of positive clone was determined. The deduced primary sequence encodes a 25 kDa protein which has the conserved residues for enzyme activity and metal binding. The 28 N-terminal amino acids encoded by the *Candida* cDNA comprise a putative mitochondrial transit peptide. Potential regulatory elements were identified in the 5' flanking sequences. Northern blot analysis showed that the transcription of the MnSOD gene is induced 5-to 10-fold in response to mercury, cadmium ions and hydrogen peroxide.

Key words: *Candida*, stress, MnSOD, gene expression

The enzyme superoxide dismutase (SOD), a class of metalloprotein, protects living organisms from the damaging effects of the negatively-charged superoxide radical by catalysing the reaction $2O_2^{\cdot}+2H^+ \rightarrow H_2O_2+O_2$ (15). Its presence in all aerobic organisms examined has led to suggestions that they play a critical role in protecting cells against oxidative stress. The prime culprits which mediate oxygen toxicity are thought to be superoxide radicals and hydrogen peroxide, which can react to form the hydroxy radical ($OH^{\cdot}$), the most powerful oxidant known. The importance of SOD has been verified by the isolation of *Escherichia coli* (4) and yeast (25) mutants which are deficient in SOD enzymes. These mutants are all known to be hypersensitive to the oxidative stress conditions.

Three classes of SOD, with distinctive distributions characterized by metal requirements, are known to exist in aerobic organisms (15): copper/zinc, manganese, and iron forms. Eukaryotic cells contain two types of SOD: a copper and zinc-containing enzyme (Cu, ZnSOD) which is found in the cytosol and probably protects the cell from the detrimental effects of environmental oxygen and a manganese-containing enzyme (MnSOD) found in the mitochondrion which provides additional protection from respiration-derived superoxide radicals, a process much in evidence in these organelles (3). Although cytosolic Cu,ZnSOD and MnSOD catalyse the same reaction, the MnSOD is structually distinct from the CuZnSODs and each is encoded by a different gene. The MnSOD is encoded in the nucleus, synthesized in the cytosol and imported post-translationally into the mitochondrial matrix, where 90% of the cell's oxygen is consumed. Due to the lack of histones and inefficient DNA repair in mitochondria (19), mitochondrial DNA appears to be an important target of reactive oxygen and accumulates extensive oxidative damage, which is implicated in aging and in a number of degenerative conditions (18). Interestingly, the MnSOD is inducible by environmental oxidative stress (7). Here we report the structure of a gene for manganese SOD from the yeast *Candida* sp. HN95 and the deduced amino acid sequence of the protein. Possible regulatory sites present in the 5' flanking region and characteristic features of the primary structure are discussed.

* To whom correspondence should be addressed.

Materials and Methods

Culture media and growth condition

E. coli strains used for DNA manipulation were

grown in Luria broth supplemented with ampicillin (50 μg/ml) or on plates containing the same medium plus 1.8% agar. Plates were incubated at 37°C. *Candida* sp. strain HN95, isolated from heavy metal-contaminated soil in the Taejon Industrial Area, was grown in complete YPD medium at 30°C.

DNA and RNA techniques

Plasmid DNA was prepared from *E. coli* using the Wizard method (Promega). *Candida* chromosomal DNA was extracted by the method of Hoffman and Winston (12). Standard recombinant DNA techniques were performed essentially as described by Sambrook *et al.* (20).

Total RNA from untreated and stress-induced cells was prepared using the method as described by Ausubel *et al.* (1). For Northern analysis, 15 μg of total RNA was electrophoresed through a 1.0% agarose gel containing 1.5% formaldehyde and transferred to a Zeta-probe nylon membrane (Bio-Rad). The *Bam*HI-digested 0.8 kb PCR DNA fragment including the MnSOD gene was radiolabeled by *in vitro* transcription with [α-^{32}P]UTP and T7 RNA polymerase and was used as a hybridization probe. Sequencing was performed by the dideoxy chain termination method (21) using synthetic oligonucleotides, T7 DNA polymerase and single stranded DNA as template. DNA analysis was performed using the PCGene software program. Database searches were done via the Blast program (National Center for Biotechnology Information).

Identification of *Candida* PCR fragment encoding the MnSOD

Total RNA isolated from *Candida* was reverse-transcribed into cDNA by M-MLV reverse transcriptase using Oligo d (T_{17}) as a primer. A specific 5'-primer (MnSOD1: 5'-CAAACTTACGTCAACGG-3') was designed based on two highly conserved regions of the Mn-SOD amino acid sequences from *Saccharomyces* and mouse. An oligod(T) primer (ADPT$_{17}$: 5'-GAGATCTGCTCTAGATCGATTTTTTTTTTT-TTTTTT-3') was used as a 3'-end primer. A PCR reaction with the MnSOD1 and ADPT$_{17}$ primers was performed using either *Candida* genomic DNA or oligo d(T)-pri- med *Candida* cDNA as a template. The PCR conditions were 35 cycles at 95°C for 1 min, 45°C for 1 min and 72°C for 1 min. The amplified PCR products were analysed by agarose gel electrophoresis, purified and subcloned in the pGEM-T vector (Promega).

Completion of the *Candida* MnSOD cDNA using 5' RACE

Primer extension using *Candida* mRNA was performed to obtain the full-length cDNA sequence. Purified *Candida* mRNA was used as the template in 5'-Rapid Amplification of cDNA Ends (RACE; Clontech) as described in the manufacturer's manual. MnSOD4 primer (5'-GTCAGCCTTGACGTTCTGGTA-3') was used to prime the synthesis of cDNA, after which the degradation of template RNA and purification with glass milk followed. The cDNA was tailed with AmpliFINDER anchor (5'-CACGAATTCAC-TATCGATTCTGGAACCTTCAGAG-3'). The anchored cDNA was then amplified by PCR with the MnSOD4 primer and the anchor primer (5'-CTGGTTCG-GCCCACCTCTGAAGGTTCCAGAATCGATAG-3'). The PCR product was ligated directly into the pGEM-T plasmid vector, and the nucleotide sequence was determined.

Identification of lambda ZAP genomic DNA clone

A *Candida* sp. HN-95 lambda ZAP (Stratagene) genomic DNA library was screened with the [^{32}P]-labelled *in vitro* transcript of the 0.8 kb PCR product of MnSOD using T7 RNA polymerase. Prehybridization and hybridization were performed at 68°C. Three positive clones were identified and two of them were selected for further analysis. Nested deletions were generated using the exonuclease III/Mungbean nuclease procedure (11).

Nucleotide sequence accession number

The *Candida* sp. HN95 MnSOD gene sequence has been deposited in the EMBL directory as accession no. Y11598

Results and Discussion

Identification of PCR fragment encoding the MnSOD

A comparison of the amino acid sequences of the MnSOD from yeast (14), human (26) and mouse revealed several regions which are highly conserved between their evolutionarily distant species. Based on the MnSOD cDNA sequences available in the database, an upstream primer of MnSOD1 corresponding to the amino acid strech QTYVNG (posi- tion 60 in the *Candida* sp. amino acids sequence in Fig. 2) was designed and used to amplify the MnSOD gene of *Candida* sp. by PCR. A 800 bp PCR fragment was identified following reverse transcription PCR (RT-PCR) with *Candida* sp. mRNA. The nucleotide sequence was determined and its partially deduced peptide sequence was found to have similarities with yeast and human MnSODs.

Isolation and analysis of the MnSOD gene and its nucleotide sequence

Using the [^{32}P]-labeled *in vitro* transcript of the 0.8 kb PCR product of MnSOD as a probe, two independent genomic clones were isolated from approx. 10^5 plaques of *Candida* sp. lambda genomic library. Both clones were characterized and found to be overlapping fragments containing the same nuclear gene. A restriction enzyme cleavage map of the 4.1 kb *Candida* MnSOD genomic DNA insert was constructed (Fig. 1). Southern analysis of *Candida* sp. genomic DNA digested with a restriction enzyme, *Hin*dIII or *Bam*HI, and probed with the *in vitro* transcript of the 0.8 kb PCR product is shown in Fig. 2. There is no internal cleavage site for any of the two restriction enzymes in the coding region. The restriction patterns obtained are consistent with the restriction map of the genomic DNA sequence and suggest the presence of one gene copy of MnSOD in the genome of *Candida* sp. HN95.

On the basis of the genomic sequence, PCR primers were designed and utilized for cloning the full-length cDNA corresponding to the isolated genomic clone. The cDNA clone isolated by PCR was sequenced and found to contain a single open reading frame of 227 amino acids corresponding to an estimated molecular mass of 25 kDa. The complete MnSOD nucleotide sequence including promoter and coding region and its derived amino acid sequence of MnSOD is shown in Fig 1.

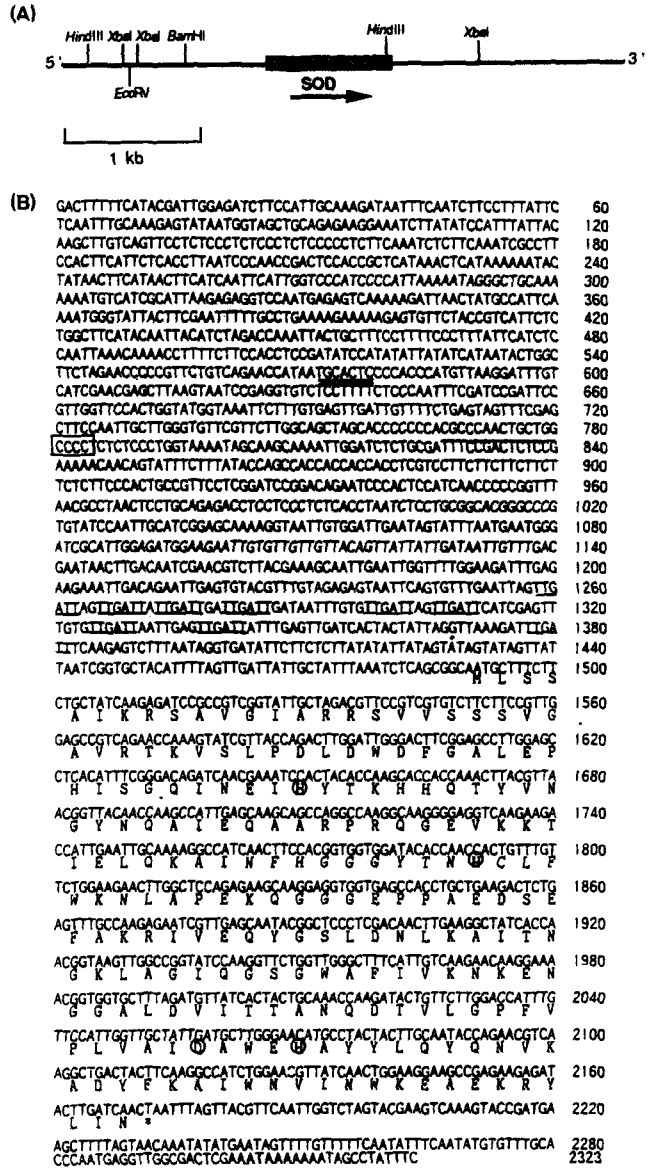

Fig. 1. Partial restriction map (A) and nucleotide sequence (B) of MnSOD gene of *Candida* sp. HN95 with upstream sequence and the deduced amino acid sequence. The transcription initiation site is indicated over the base (.) and the poly(A$^+$) signal is boldfaced. The repetitive nine hexanucleotides are underlined. Putative STRE sequence is boxed and Metal Responsive Element (MRE) site is double underlined. The nucleotides overlined represent UAS-like sequence. The conserved metal-binding residues are circled. Abbreviation: B, *Bam*HI; E, *Eco*RV; H, *Hin*dIII; X, *Xba*I.

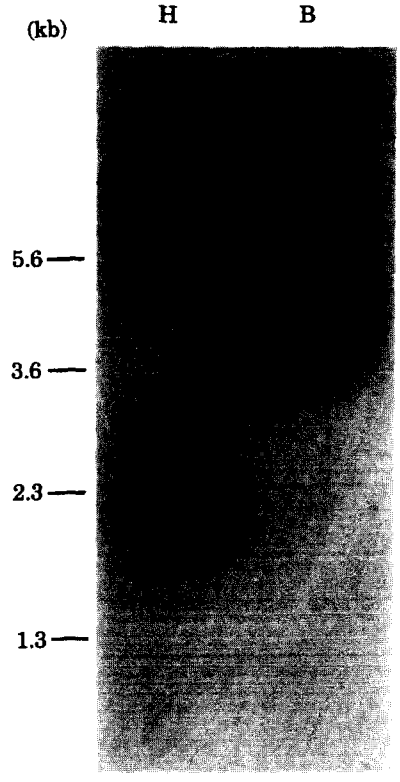

Fig. 2. Southern hybridization analysis of *Candida* sp. genomic DNA. Chromosomal DNA was digested with the enzymes as indicated, electrophresed in a 1.0% agarose gel and blotted to a nylon membrane. The blot was hybridized to the *in vitro* transcript of the 0.8 kb PCR product. The restriction enzymes used were *Bam*HI (B) and *Hin*dIII (H).

The upstream sequence was examined for potential regulatory elements (Fig. 1). Notably, no "TATA box" and "CAAT box" were found in the 5' flanking region. A GC-rich region with 65% G-C content is located between positions -1188 and -1348. These features are common to the promoters of many other housekeeping genes as well as the human, bovine, and rat MnSOD gene (26). Interestingly, nine hexanucleotide (TTGATT) sequences, are interspersed between positions -176 and -57 upstream of the transcription start site. Whether these sequences are involved in regulation has yet to be determined. In the upstream of the *Candida* MnSOD gene coding region a number of transcription factor binding DNA elements were found, but many of Them match loosely with their respective consensus sequence (6). The sequence CCCCT, a potential stress response element (STRE)(13), is located between positions -644 and -648 5' upstream. One copy of the consensus metal regulatory element (TGCA/GCNC) (5) that is responsible for the induction by metals is found between positions -791 and -797. The 5' flanking region also possesses an 'UAS(Upstream activation site of CYC 1)'-like sequence, located at about -601 from the transcription initiation site. Heme, a prosthetic group in the cytochromes and some oxygen-binding proteins, regulates transcription of the CYC1 gene which encodes iso-1-cytochrome c via an upstream activation site (9). The synthesis of MnSOD is also known to be regulated by O_2 or heme at the level of transcription (17). It has been reported that the regulation of the biosynthesis of MnSOD resembles that of several other yeast mitochondrial protein, and also the cytosolic catalase T (14). Although sequences similar to the CYC1-UAS is present upstream of *Candida* MnSOD, their functional importance has to be assesed. In addition, despite the high homology of the *Candida* MnSOD coding region to that of the *S. cerevisiae*, rat, or human MnSODs, the promoter sequences of the genes show a virtual lack of similarity. At the 3' end, a polyadenylation signal sequence, AATAAA, was found at position 876 downstream of the transcription start site.

Analysis of the deduced amino acid sequence of the *Candida* MnSOD

The determined nucleotide sequence of the SOD gene and predicted amino acid sequence of the protein are presented in Fig. 1. The open reading frame encodes a 227 amino acid with an estimated molecular mass of 25 kDa. The *Candida* MnSOD sequence appears to have a 28 aa-long putative N-terminal mitochondrial leader peptide which may target the preprotein to the intermembrane mitochondrial space (14). Although comparison of the mitochondrial targeting signal peptides of MnSODs from several species show no true homologies, transit peptides are generally rich in positively charged and hydroxylated amino acids and lack acidic amino acids (24). The putative transit peptide of the *Candida* MnSOD possesses all of these characteristics. It lacks acidic residues, containing one lysine and four arginine residues dispersed among hydrophobic amino acids and the hydoxylated amino acid serine and threonine. One of the positively charged arginine residues lies in position 27 of the transit peptide, which is invariable in all eukaryotic MnSODs (Fig. 2). It is believed that this unique feature of the transient peptide present in the *Candida* MnSOD might be related to its subcellular location.

Comparative coding sequence alignments of *Candida* MnSOD with *Saccharomyces* MnSOD, human MnSOD and rat MnSOD revealed amino acid homologies of 75, 52 and 54%, respectively (Fig. 3). Notably, mature MnSOD showed homologies of 80, 60 and 61%, respectively. The mitochondrial targeting signal peptide, anchored by a conserved Lys-29, displayed the lowest level of sequence identity. The potential N-glycosylation site at His-100 is present in the MnSOD of *Saccharomyces* as well. The N-ter-

```
                                  ↓
SODCAN   MLSSAIKRSAVGIARRSVVSS-SVGAVRTKVSLPDLDWDFGALEPHISGQ   49
SODSCE   MFA---KTAAANLTKKGGLSLLSTTARRTKVTLPDIKWDFGALEPYISGQ   47
SODHUM   MLSRAVCGTSRQLA-----PALGYLGSRQKHSLPDLPYDYGALEPHINAQ   45
SODRAT   MLCRAACSTGRRLG-----PVAGAAGSRHKHSLPDLPYDYGALEPHINAQ   45
         *.       .. ..     .  .  . * * .***. .*.*****.*..*

SODCAN   INEIHYTKHHQTYVNGYNQAIEQ--------AARPQGEVKKTIELQKAI   91
SODSCE   INELHYTKHHQTYVNGFNTAVDQFQELSDLLAKEPSPANARKMIAIQQNI   97
SODHUM   IMQLHHSKHHAAYVNNLNVTEEKYQEA---LAK----GDVTAQIALQPAL   88
SODRAT   IMQLHHSKHHAAYVNNLNATEEKYHEA---LAK----GDVTTQVALQPAL   88
         * ..*..***..***. * . ..       *     ...   ...* ..

SODCAN   NFHGGGYTNHCLFWKNLAPEKQGGGEPPAEDSEFAKRIVEQYGSLDNLKA  141
SODSCE   KFHGGGFTNHCLFWENLAPESQGGGEPPT--GALAKAIDEQFGSLDELIK  145
SODHUM   KFNGGGHINHSIFWTNLSPN--GGGEP---KGELLEAIKRDFGSFDKFKE  133
SODRAT   KFNGGGHINHTIFWTNLSPK--GGGEP---KGELLEAIKRDFGSFEKFKE  133
         .*.*** .** .**.**.*.  *****    ... . *  ..**....

SODCAN   ITNGKLAGIQGSGWAFIVKNKENGGALDVITTANQDTVLGP--FVPLVAI  189
SODSCE   LTNTKLAGVQGSGWAFIVKNLSNGGKLDVVQTYNQDTVTGP--IVPLVAI  193
SODHUM   KLTAASVGVQGSGWGWLGFNKERG-HLQIAACPNQDPLQGTTGLIPLLGI  182
SODRAT   KLTAMSVGVQGSGWGWLGFNKEQG-RLQIAACSNQDPLQGTTGLIPLLGI  182
           ..  .*.*****...  * ..*  *..     ***.. *.  ..**..*

SODCAN   DAWEHAYYLQYQNVKADYFKAIWNVINWKEAEKRYLIN--   227
SODSCE   DAWEHAYYLQYQNKKADYFKAIWNVVNWKEASRRFDAGKI   233
SODHUM   DVWEHAYYLQYKNVRPDYLKAIWNVINWENVTERYMACKK   222
SODRAT   DVWEHAYYLQYKNVRPDYLKAIWNVINWENVTERYTACKK   222
         *.*********.* ..**.******.**.... *.
```

Fig. 3. Comparison of deduced MnSOD amino acid sequences. The sources of the MnSODs described on the left are SODCAN (*Candida* sp. HN95), SODSCE (*Saccharomyces cerevisiae*), SODHUM (human) and SODRAT (rat). Gaps are introduced to facilitate optimal alignment of sequences. Asterisks indicate the perfectly conserved amino acid; dots denote the well conserved sequences. The beginning of the mature protein is shown by the arrow.

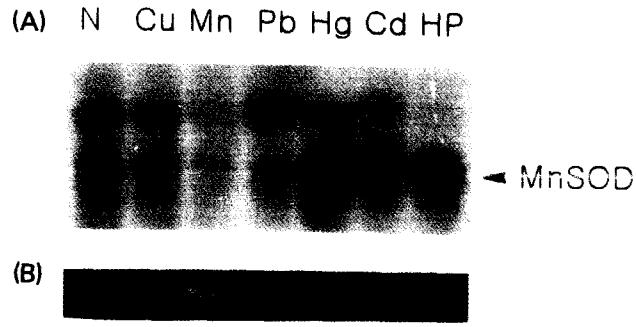

Fig. 4. Northern blot analysis of MnSOD mRNA in *Candida* cells. (A) Total RNAs (15 μg) isolated from cells treated for 3 h with Cu (500 mM $CuCl_2$), Mn (500 mM $MnCl_2$), Pb (500 mM $PbCl_2$), Hg (200 mM $HgCl_2$), Cd (200 mM $CdCl_2$), and HP (2 mM H_2O_2), respectively, were separated by electrophoresis, transferred to nylon membrane and hybridized with the *in vitro* RNA transcript of 0.8 kb PCR fragment. N denotes untreated *Candida* cells. (B) Ethidium bromide staining of 18S ribosomal RNAs was used to confirm e-

minal LPD_{32-34} region, which is completely conserved in eukaryotic MnSODs, is also present in the *Candida* sequence at position 32. In addition, the putative metal(Mn) binding ligands as determined in prokaryotes (22) are well conserved at His-54, His-101, Asp-190 and His-194 of the deduced amino acid sequence in *Candida*. Notably, the region surrounding the last two ligands is highly conserved, as these two sites are separated by only three amino acids. The positions of aspartate 190 and histidine 194 in *Candida* Mn enzyme coincide with those of residues 148 and 152, found as Fe ligands, in the bacterial FeSOD enzyme (23). This suggests that both SOD enzymes arise from common ancestral SOD enzymes.

Expression of the *Candida* MnSOD gene

To investigate the extent to which MnSOD mRNA is induced by various environmental stresses, *Candida* cells were incubated with copper, manganese, mercury, cadmium or hydrogen peroxide for 3 h. Total RNAs were isolated and analyzed by northern hybridization. The result (Fig. 4) shows that nonessential heavy metal (mercury or cadmium) treatments results in a 5-to 10-fold induction of mRNA over control levels. The transcription of Cu, ZnSOD is known to be partially inducible by copper via the copper-dependent *trans*-activator *ACE1* gene product (8). It is possible that some kinds of regulatory proteins can be employed for mercury-and cadmium-sensing at the MnSOD promoter. It is interesting to note that hydrogen peroxide, the final product of the SOD reaction, also induced the expression of MnSOD mRNA. Several investigations have proposed (2, 10, 15) that O_2^- may directly or indirectly regulate superoxide dismutase biosynthesis under conditions of oxidative stress. However, since the experimental procedures used to create oxidative stress will cause a variety of cellular changes in addition to an increase in O_2^- flux, it can not be dismissed that MnSOD may not be induced by O_2^- itself but rather is affected by one of the other cellular changes consequent to oxidative stress produced by toxic metals or hydrogen peroxide treatment.

Our results represent a basic description of the isolation and expression of the *Candida* MnSOD gene and determination of its nucleotide sequence. It could now be possible to utilize the gene to examine the role of the *Candida* MnSOD enzyme in oxygen or heavy metal tolerance. In addition, we are dissecting the putative *cis*-acting elements upstream of the SOD gene responsible for expression induced by environmental stresses.

Acknowledgments

This study was supported by the '95 Domestic Apparatus Research Fund of KOSEF, and by the '97 HanNam University Research Fund to S.Y. Choi.

References

1. **Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology. New York: Green Publishing Associates/Wiley Interscience.
2. **Autor, A.P.** 1982. Biosynthesis of mitochondrial manganese superoxide dismutase in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **257**, 2713-2718
3. **Bannister, J.V., W.H. Bannister, and G. Rotilio.** 1987. Aspects of the structure, function, and applications of superoxide dismutase. *CRC Crit. Rev. Biochem.* **22**, 111-180
4. **Carlioz, A. and D. Touati.** 1986. Isolation of superoxide dismutase mutants in *Escherichia coli*: is superoxide dismutase necessary for aerobic life? *EMBO J.* **5**, 623-630
5. **Culotta, V.C. and D.H. Hamer.** 1989. Fine mapping of a mouse metallothionein gene metal response element. *Mol. Cell. Biol.* **9**, 1376-1380
6. **Faisst, S. and S. Meyer.** 1992. Compilation of vertebrate-encoded transcription factors. *Nucl. Acids Res.* **20**, 3-26
7. **Galiazzo, F. and R. Labbe-Bois.** 1993. Regulation of Cu, Zn-and Mn-superoxide dismutase transcription in *Saccharomyces cerevisiae*. *FEBS Lett.* **315**, 197-200
8. **Gralla, E.B., D.J. Thiele, P. Sila, and J.S. Valentine.** 1991. ACEi, a copper-dependent transcription factor, activates expression of the yeast copper, zinc superoxide dismutase gene. *Proc. Natl. Acad. Sci. USA* **88**, 8558-8562
9. **Guarente, L., B. Lalonde, P. Gifford, and E. Alani.** 1984. Distinctly regulated tandem upstream activation sites mediate catabolite repression of the CYC1 gene of *S. cerevisiae*. *Cell* **36**, 503-511
10. **Hassan, H.M. and I. Fridovich.** 1977. Regulation of

superoxide dismutase synthesis in *Escherichia coli*: glucose effect. *J. Bacteriol.* 132, 505-510
11. **Henikoff, S.** 1987. Unidirectional digestion with exonuclease III in DNA sequence analysis. *Methods Enzymol.* **155**, 156-165
12. **Hoffman, C.S. and F. Winston.** 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* **57**, 267-272
13. **Marchler, G., C. Schuller, G. Adam, and H. Ruis.** 1993. A *Saccharomyces cerevisiae* UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. *EMBO J.* **12**, 1997-2003
14. **Marres, C.A., A.P. Van Loon, P. Oudshoorn, H. Van Steeg, L.A. Grievell, and E.C. Slater.** 1985. Nucleotide sequence analysis of the nuclear gene coding for manganese superoxide dismutase of yeast mitochondria, a gene previously assumed to code for the Rieske iron-sulphur protein. *Eur. J. Biochem.* **147**, 153-161
15. **McCord, J.M. and I. Fridovich.** 1969. Superoxide dismutase. *J. Biol. Chem.* **244**, 6049-6055
16. **Moody, C.S. and H.M. Hassan.** 1984. Anaerobic biosynthesis of the manganese-containing superoxide dismutase in *Escherichia coli*. *J. Biol. Chem.* **259**, 12821-12825
17. **Pinkham, J.L., Z. Wang, and J. Alsina.** 1997. Heme regulates SOD2 transcription by activation and repression in *Saccharomyces cerevisiae*. *Curr. Genet.* **31**, 281-291
18. **Richter, C.** 1992. Reactive oxygen and DNA damage in mitochondria. *Mutat. Res.* **275**, 249-255
19. **Richter, C., J. Park, and B.N. Ames.** 1988. Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc. Natl. Acad. Sci. USA* **85**, 6465-6467
20. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York, New York.
21. **Sanger, F., S. Nicklen, and A.R. Coulson.** 1977. DNA sequencing with chain-termination inhibitor. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467
22. **Stallings, W.C., K.A. Pattridge, R.K. Strong, and M. L. Ludwig.** 1985. The structure of manganese superoxide dismutase from *Thermus thermophilus* HB8 at 2.4 A resolution. *J. Biol. Chem.* **260**, 16424-16432
23. **Stallings, W.C., T.B. Powers, K.A. Pattridge, J.A. Fee, and M.C. Ludwig.** 1983. Iron superoxide dismutase from *E. coli* at 3.1-A resolution: a structure unlike that of copper/zinc protein at both monomer and dimer levels. *Proc. Natl. Acad. Sci. USA* **80**, 3884-3888
24. **Stuart, R.A. and W. Neuport.** 1996. Topogenesis of inner membrane proteins of mitochondria. *Trends in Biochem. Sci.* **21**, 261-267
25. **Van Loon, A.P.G.M., B. Pesold-Hurt, and G. Schatz.** 1986. A yeast mutant lacking mitochondrial manganese-superoxide dismutase is hypersensitive to oxygen. *Proc. Natl. Acad. Sci. USA* **83**, 3820-3824
26. **Zang, N.** 1996. Characterization of the 5' flanking region of the human MnSOD gene. *Biochem. Biophys. Res. Comm.* **220**, 171-180