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Isolation and Characterization of the *sod2*$^+$ Gene Encoding a Putative Mitochondrial Manganese Superoxide Dismutase in *Schizosaccharomyces pombe*

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The fission yeast *Schizosaccharomyces pombe* contains two distinct superoxide dismutase (SOD) activities, one in the cytosol encoded by the *sod1*$^+$ gene and the other in mitochondria. The *sod2*$^+$ gene encoding putative mitochondrial manganese superoxide dismutase (MnSOD) was isolated from the *S. pombe* genomic library using a PCR fragment as the probe. The nucleotide sequence of the *sod2*$^+$ gene and its flanking region (4051 bp *Hin*dIII fragment) was determined. An intron of 123 nt in size was predicted and confirmed by sequencing the cDNA following reverse transcription PCR. The predicted Sod2p consists of 218 amino acid residues with a molecular mass of 24,346 Da. The deduced amino acid sequence showed a high degree of homology with other MnSODs, especially in the metal binding residues at the active site and their relative positions. The transcriptional start site was mapped by primer extension at 231 nt upstream from the ATG codon. A putative TATA box (TATAAAA) was located 58 nt upstream from the transcriptional start site and putative polyadenylation sites were located at 1000, 1062, and 1074 nt downstream from the ATG start codon.

Key words **:** manganese superoxide dismutase, mitochondria, *sod2*$^+$, *Schizosaccharomyces pombe*

Reactive oxygen species (ROS) are produced as a byproduct of aerobic metabolism. These include superoxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen, known to damage various cellular macromolecules, such as DNA, proteins, and lipids. Mitochondria constitute the prime source of ROS since its electron transport system consumes more than 90% of the oxygen utilized by the cell (15). As a defense against oxidative injury, cells have evolved a multilayered interdependent antioxidant system that includes enzymatic and nonenzymatic components (5). Individual antioxidant enzymes are located in specific subcellular sites and reveal distinct substrate specificity. Among these, manganese superoxide dismutase (MnSOD) has been the subject of particular interest because it is located in the mitochondria and represents the first line of defense against superoxide radicals produced as byproducts of oxidative phosphorylation (2).

Superoxide dismutases (SOD, EC1.15.1.1) are metalloproteins that dismutate two molecules of O_2^- to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). SODs are classified into four groups depending on their metal cofactors; MnSOD (containing manganese) (6), CuZnSOD (containing copper and zinc) (10), FeSOD (containing iron) (17) and NiSOD (containing nickel) (7, 18). Prokaryotes contain MnSOD and FeSOD that are similar in their amino acid sequences and structure (1). NiSOD has been found in some Gram-positive actinomycetes. In eukaryotes, a cytosolic CuZnSOD, a glycosylated extracellular CuZnSOD, and a mitochondrial MnSOD have been found (3).

Schizosaccharomyces pombe is an ascomycetous yeast that divides by binary fission. This yeast shares many fundamental cellular properties with higher multicellular eukaryotes (19). Conservation of the stress-activated cell signaling pathway between *S. pombe* and mammalian cells suggest that *S. pombe* can serve as a good model system to study stress response (16). *S. pombe* exhibits an adaptive response to oxidants like hydrogen peroxide and superoxide-generators by inducing such antioxidant enzymes as catalase and SODs (8, 9). Here we report on the isolation and characterization of the *sod2*$^+$ gene encoding a putative mitochondrial MnSOD in *S. pombe*.

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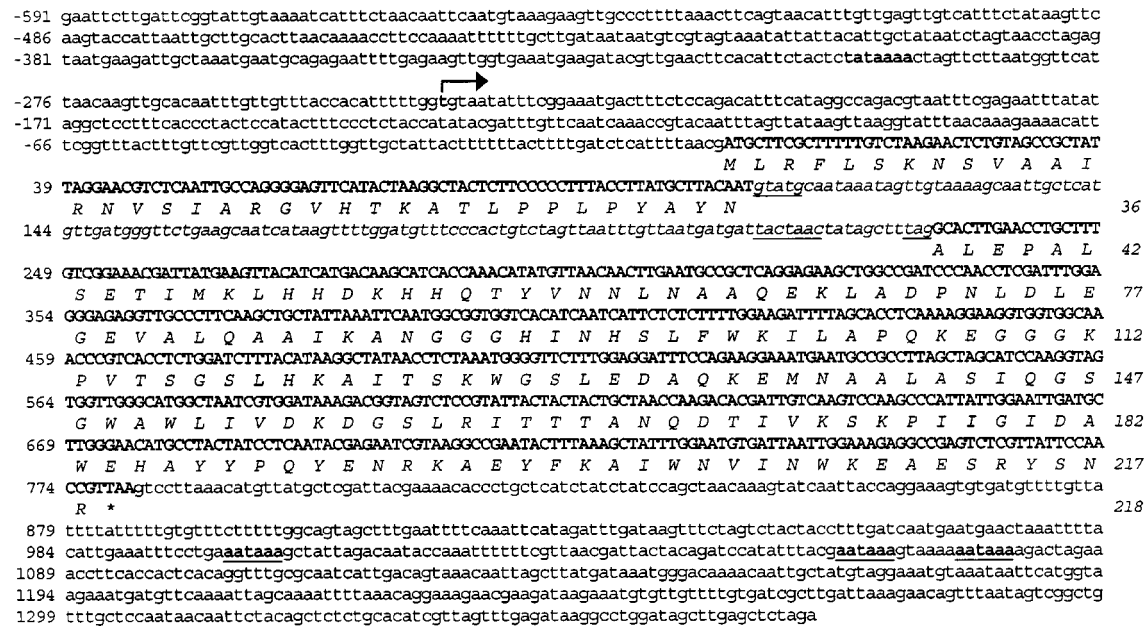

Fig. 1. Nucleotide sequence of the *sod2*$^+$ gene. The nucleotide sequence of 1965 bp *Eco*RI/*Xba*I fragment containing the *sod2*$^+$ gene is shown with its deduced amino acid sequence. The transcription start site is indicated with an arrow, the putative TATA box in bold face, and the intron sequences in italics. The conserved regions in the intron junctions as well as the branch site in *S. pombe* are underlined. Three putative polyadenylation sites are underlined. The nucleotide sequence of the *sod2*$^+$ gene and its flanking region (*Hin*dIII fragment of 4051 bp in length) has been deposited in the GENBANK database under accession number AF069292.

Materials and Methods

Strains and culture media

S. pombe strains 972 (*h*$^-$) and ED665 (*h*$^-$, *ade6-M210 leu1-32 ura4-D18*) were used in this study. For routine cultures, YES [0.5% (w/v) yeast extract, 3% (w/v) dextrose, and 100 mg/l supplements] and YPD [1% (w/v) yeast extract, 2% (w/v) Bacto-peptone, and 2% (w/v) dextrose] media were used. EMM with appropriate supplements (11) was used to isolate the *sod2*$^+$ gene-disrupted cells.

Isolation of the sod2$^+$ *gene*

Two degenerate oligonucleotide primers corresponding to the conserved residues of MnSOD in *S. cerevisiae* were used to amplify homologous DNA from *S. pombe* by PCR; MSN2 for residues 55 to 61 (KHHQTYV; 5'-AARCAYCAYCARACNTAYGT-3') and MSC1 for residues 196 to 202 (WEHAYYL; 5'-ARRTARTANGCRTGYTCCCA-3'), where R=A+G, Y=C+T, N=A+C+G+T. The amplified DNA fragment of 411 bp was cloned and sequenced. It was used as a probe to isolate the entire *sod2*$^+$ gene. *S. pombe* genomic DNA, prepared as described by Moreno *et al.* (11), was completely digested with *Sal*I, from which fragments in the range of 5.5~8.0 kb were obtained by elution from agarose gel electrophoresis. The DNA fraction hybridizing to the PCR product was ligated to *Sal*I-cut pTZ18R plasmid DNA to construct a sublibrary, and transformed into *E. coli* DH5α cells. About 200 transformants were screened by colony hybridization (14), and two positive clones containing a common 6.1 kb *Sal*I fragment were selected.

Reverse transcription (RT)-PCR

To verify the predicted intron junctions, primers MS5N1 (5'-CGATTTGTTCAATCAAACCGTAC-3') and MS5C2 (5'-AACTATTGTTTGGTGATGCTTGTC-3') were synthesized. The 5' ends of the MS5N1 and MS5C2 primers correspond to nucleotide positions -129 and +300, respectively, relative to the translation start codon (Fig. 1). The reverse transcription (RT) mixture contained 1 × RT buffer (Poscochem, Korea), 1 mM dNTP, 25 pmol of MS5C2 primer, 2.5 mM of $MgCl_2$, 40 U of RNasin (Promega) and 7 μg of *S. pombe* RNA in total 20 μl of reaction. The mixture was incubated at 65°C for 5 min and then at 37°C for 10 min, followed by addition of 10 units of AMV RTase (Poscochem) and further incubation for 1 h at 37°C. To amplify the cDNA, 50 pmol of each primer were added to the reaction mixture adjusted to a final 1 × PCR buffer (Poscochem) containing 1.5 mM $MgCl_2$ in 100 μl total volume. The resulting PCR product was checked on an agarose gel and the intron junction was verified by sequencing.

Primer extension analysis

Primer extension analysis was performed according to Grimm *et al.* (4) with a slight modification as follows. An anti-sense oligonucleotide SD2prim2 (5'-AAAGGAGCCTATATAAATTCTCGAAATT-3') was radioactively labeled

with [γ-^{32}P]ATP by T4 polynucleotide kinase. Total RNA (150 μg) denatured with DMSO in the presence of the radioactive primer was precipitated with ethanol. The pellet was dissolved in 30 μl of RT buffer (5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, and 50 mM Tris-HCl, pH 8.3) and heated at 55°C for 30 min. This was slowly cooled to 22°C and precipitated with an equal volume of isopropanol. The pellet was resuspended in 30 μl of RT buffer containing 2 mM dNTP. The cDNA was synthesized by adding 200 units of MMLV RTase (Promega) and incubated at 37°C for 2 h. RNA was hydrolyzed in 0.3 N NaOH at 56°C for 10 min and the reaction was neutralized by adding Tris-HCl, pH 8.0, to 60 mM, and HCl to 22 mM. The mixture was extracted with phenol/chloroform once and precipitated with ethanol. The cDNA was run on a 6% polyacrylamide sequencing gel containing 7 M urea.

Results and Discussion

Isolation of the sod2$^+$ *gene*

From the known amino acid sequences of MnSODs of other organisms, several highly conserved regions were identified. The primer pair corresponding to the residues 55-61 (KHHQTYV) and 196-202 (WEHAYYL) of the *SOD2* gene of *S. cerevisiae* amplified a single species of PCR product from the chromosomal DNA of *S. pombe* strain 972. The deduced amino acid sequence of the amplified product revealed high homology with that of MnSODs from *S. cerevisiae* and humans. Using this PCR product as a probe, the genomic library was screened and two positive clones containing a common 6.1 kb *Sal*I fragment were selected. Genomic Southern analysis revealed that there is only one copy of the MnSOD gene in *S. pombe* (data not shown). The nucleotide sequence of the *sod2*$^+$ gene and its flanking region in the 4051 bp *Hin*dIII fragment was determined and deposited in the database under the accession number AF069292. The nucleotide and deduced amino acid sequences of *Eco*RI/*Xba*I fragment containing the *sod2*$^+$ gene are presented in Fig. 1.

Characterization of the sod2$^+$ *gene*

From the nucleotide sequence, we predict that the *sod2*$^+$ gene contains an intron of 123 nt near the N terminus of the open reading frame. This intron has the canonical 5' donor (GTATG), branch site (TACTAAC), and the 3' acceptor (TAG) sequences for the splicing known in *S. pombe* (12, Fig. 1). The presence of the intron was verified by RT-PCR and sequencing of the cDNA (Fig. 2). The reverse transcription of *sod2*$^+$ mRNA followed by PCR generated the 306 bp fragment whereas the genomic DNA produced the PCR fragment of 429 bp. This suggests that there is indeed an intron of 123 bp (Fig. 2A). The intron junction was sequenced, verifying the 5' and 3' splice sites (Fig. 2B).

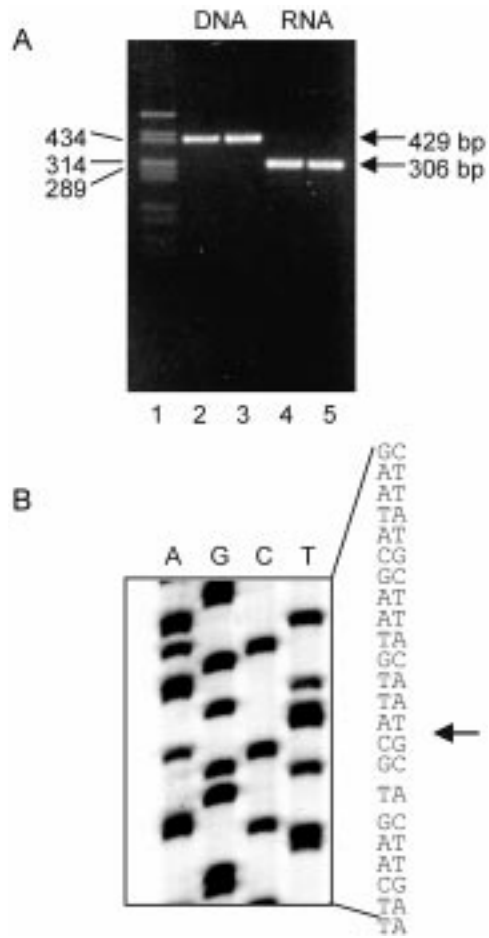

Fig. 2. Characterization of an intron by RT-PCR. (A) Total RNA was reverse transcribed followed by PCR. Genomic DNA was also amplified as a comparison. PCR amplified 429 bp fragment from the genomic DNA (lanes 2, 3) and 306 bp fragment from RNA (lanes 4, 5), indicating the presence of a 123 nt-long intron. (B**)** The nucleotide sequence near the intron junction of the *sod2*$^+$ cDNA. The intron junction where two exons were ligated is marked with an arrow.

The predicted Sod2p consists of 218 amino acid residues with a molecular mass of 24,346 Da. Comparison of the amino acid sequence with those from known MnSODs revealed a high degree of conservation (Fig. 3). It showed about 75% and 71% homology with those of humans and *S. cerevisiae*, respectively, and the metal binding residues at the active site and their relative positions were very well conserved. Computer-based analysis of the primary amino acid sequence suggested that Sod2p could be a mitochondrial protein where the 22nd valine becomes the N-terminal amino acid after targeting into mitochondria. The N-terminal 21 amino acids of the predicted Sod2p exhibited a putative signal sequence for mitochondrial transport, being a sequence of 20-30 amino acids with 3-5 lysine or arginine residues, each separated by 2-5 hydrophobic residues (13). Protein identification and analysis tools calculated that the molecular mass of MnSOD is 21,909 Da with pI 7.28 after processing.

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S. pombe        ----------MLRFLSKN--SVAAIRNVSIARGVHTKATLPPLPYAYNALEPALSETIMKLHHDKHHQTYVNNLNAAQEKLADP
Maize           MALRTLASKKVLSFPFGG--AGRPLAAAASARG-VTTVTLPDLSYDFGALEPAISGEIMRLHHQKHHATYVANYNKALEQLETA
Human           ----------MLSRAVCG--TSRQLAPALGYLGSRQKHSLPDLPYDYGALEPHINAQIMQLHHSKHHAAYVNNLNVTEEKYQEA
Mouse           ----------MLCRAACS--TGRRLGPVAVAAGSRHKHSLPDLPYDYGALEPHINAQIMQLHHSKHHAAYVNNLNATEEKYHEA
S. cerevisiae   ----------MFAKTAAANLTKKGGLSLLSTTARRTKVTLPDLKWDFGALEPYISGQINELHYTKHHQTYVNGFNTAVDQFQEL
E. coli         -----------------------------------MSYTLPSLPYAYDALEPHFDKQTMEIHHTKHHQTYVNNANAALESLPEF
                                                   :** * : : **** :      :*: *** :**   * : :

S. pombe        N---------LDLEGEVALQAAIKANGGGHINHSLFWKILAPQKEGGGKPVTSGSLHKAITSKWGSLEDAQKEMNAALASIQGS
Maize           VS-------KGDASAVVQLQAAIKFNGGGHVNHSIFWKNLKPISEGGGEPP-HGKLGWAIDEDFGSFEALVKKMNAEGAALQGS
Human           LA-------KGDVTAQIALQPALKFNGGGHINHSIFWTNLSPN--GGGEP--KGELLEAIKRDFGSFDKFKEKLTAASVGVQGS
Mouse           LA-------KGDVTTQVALQPALKFNGGGHINHTIFWTNLSPK--GGGEP--KGELLEAIKRDFGSFEKFKEKLTAMSVGVQGS
S. cerevisiae   SDLLAKEPSPANARKMIAIQQNIKFHGGGFTNHCLFWENLAPESQGGGEPP-TGALAKAIDEQFGSLDELIKLTNTKLAGVQGS
E. coli         ANLPVEELITKLDQLPADKKTVLRNNAGGHANHSLFWKGLKKG------TTLQGDLKAAIERDFGSVDNFKAEFEKAAASRFGS
                          :  :: : **  ** :**  *             * *  **   :** :              **

S. pombe        GWAWLIVDKD--GSLRITTTANQD-TIVKS-------KPIIGIDAWEHAYYPQYENRKAEYFKAIWNVINWKEAESRYSNR--
Maize           GWVWLALDKEA-KKVSVETTANQD-PLVTKGAS---LVPLLGIDVWEHAYYLQYKNVRPDYLNNIWKVMNWKYAGEVYENVLA
Human           GWGWLGFNKER-GHLQIAACPNQD-PLQGT-TG---LIPLLGIDVWEHAYYLQYKNVRPDYLKAIWNVINWENVTERYMACKK
Mouse           GWGWLGFNKEQ-GRLQIAACSNQD-PLQGT-TG---LIPLLGIDVWEHAYYLQYKNVRPDYLKAIWNVINWENVTERYTACKK
S. cerevisiae   GWAFIVKNLSNGGKLDVVQTYNQD-TVTGP------LVPLVAIDAWEHAYYLQYQNKKADYFKAIWNVVNWKEASRRFDAGKI
E. coli         GWAWLVLKGD---KLAVVSTANQDSPLMGEAISGASGFPIMGLDVWEHAYYLKFQNRRPDYIKEFWNVVNWDEAAARFAAKK-
                ** ::         : :     ***  :            *:: :* ****** :::* : :*:: :*:*:**      :
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Fig. 3. Comparison of deduced amino acid sequence of the *sod2*$^+$ gene with other MnSODs. The deduced amino acid sequence of *sod2*$^+$ gene was compared with MnSOD genes from *E. coli* (X03951), *S. cerevisiae* (X02156), humans (X15132), mice (X04972), and maize (M33119). Sequences were aligned using the Clustal W program. Identical and similar matches are indicated by asterisks(*) and colons(:), respectively. Positions of the metal binding ligands are presented in bold. Amino acids of *S. cerevisiae* MnSOD employed to design MSN2 and MSC1 PCR primers are underlined.

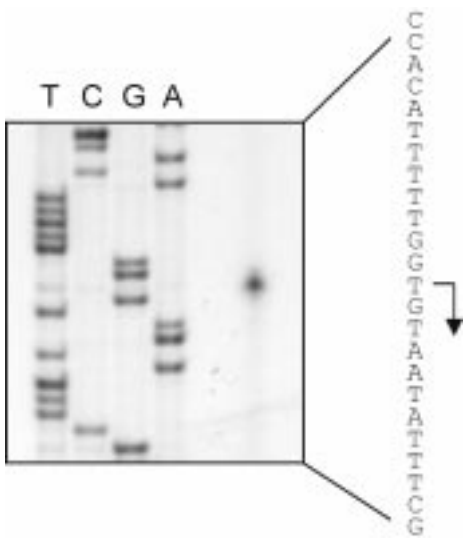

Fig. 4. Determination of the transcription start site by primer extension analysis. An oligonucleotide primer SD2prim2, complementary to the coding strand, was extended by reverse transcription. The same primer was used for sequencing. The transcriptional start point was localized on the T residue at 231 nt upstream from the initiation codon.

Transcription start site of the sod2$^+$ *gene*

To determine the transcriptional start site of the *sod2*$^+$ gene, primer extension analysis was performed. The primer whose 5' end corresponds to the 164th residue upstream from the ATG start codon was extended by reverse transcription. The length of the extended cDNA indicated that the transcription initiated at the T residue 231 nt upstream from the start codon (Fig. 4). This position coincided with the one predicted from the S1 nuclease protection analysis (data not shown). A putative TATA box (TATAAAA) was located 58 nt upstream from the transcriptional start site (Fig. 1). Putative polyadenylation sites were located at 1000, 1062, and 1074 nt downstream from the ATG start codon. The length of the mature *sod2*$^+$ transcript is thus predicted to be about 1.1 kb.

Sod2p as a mitochondrial MnSOD in S. pombe

The finding that the deduced amino acid sequence exhibits high homology with those of other MnSODs, especially in the metal binding residues of the active site, strongly suggests that the Sod2p is indeed an Mn-containing SOD. The presence of the mitochondrial target sequence at the N-terminus of Sod2p predicts that it is localized in mitochondria. Even though these predictions are very likely, it is still necessary to purify the protein to determine its metal content, as well as the N-terminal amino acid sequence. The subcellular localization of Sod2p needs to be verified experimentally as well.

Based on these findings, further investigation of the role and regulation of *sod2*$^+$ in the growth and stress response of *S. pombe* will provide valuable insights to the role of mitochondrial MnSOD in eucaryotic cells.

Acknowledgments

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