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ENTITLED Mitochondrial Superoxide Dismutase: Construction of a Mutant Lacking the Presequence

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MITOCHONDRIAL SUPEROXIDE DISMUTASE: CONSTRUCTION OF A MUTANT LACKING THE PRESEQUENCE

BY

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Introduction

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen: $O_2^{-}\cdot + O_2^{-}\cdot \longrightarrow H_2O_2 + O_2$. In 1968, McCord and Fridovich isolated a copper and zinc-containing SOD (CuZnSOD) from erythrocytes. They followed the reduction of ferricytochrome c by superoxide anion which was generated through the catalytic action of xanthine oxidase upon its substrate, xanthine (1). By the same criteria, manganese-containing SOD (MnSOD) was isolated from Escherichia coli B in 1970 (2). Fridovich proposed that SOD protected cells against the deleterious effects of the superoxide radical and expected SOD to exist in all aerobic cells.

In 1973, two types of SOD were found in chicken liver cells and were shown to have organelle specificity (3). The mitochondrial MnSOD was similar to the MnSOD isolated from bacteria, but was different from the cytosolic CuZnSOD; the cytosolic CuZnSOD was similar to the CuZnSOD isolated from other eukaryotes. The authors, Weisiger and Fridovich, pointed out that this finding supported that mitochondrion evolved from aerobic prokaryotes that entered endocellular symbiosis with protoeukaryotes. The two enzymes were significantly different in molecular properties. CuZnSOD had a molecular weight of 31,000, 2 subunits of

equal size, and 1 atom of Cu and 1 of Zn per subunit (3). MnSOD had a molecular weight of 80,000, 4 subunits of equal size, and 2.3 atoms Mn per molecule (3). It was noted that CuZnSOD was inhibited by cyanide but not by chloroform-ethanol mixtures, while MnSOD was inhibited by the former but not by the latter. However, since the SOD catalytic activity was similar for the CuZnSOD and MnSOD, Fridovich posed the question, "Why cannot mitochondrion make do with CuZnSOD?"

It was known that CuZnSOD was encoded in the nucleus. Fridovich then sought the site of synthesis and the intramitochondrial localization of mitochondrial SOD (4). The MnSOD was deduced to be nuclear-encoded because a petite mutant of Saccharomyces cerevisiae that lacked mitochondrial DNA did contain MnSOD. The MnSOD activity was localized to the matrix fraction of chicken liver mitochondrion. By 1975, CuZnSOD and MnSOD were isolated and characterized from yeast (5), and, as had been shown for chicken liver cells, cytosolic SOD and mitochondrial matrix SOD were separate entities.

In 1982, Autor showed that in S. cerevisiae the nuclear-encoded MnSOD was synthesized in the cytoplasm as a higher molecular weight precursor (6). Recall (7) that the majority of proteins residing in the mitochondria are nuclear-encoded, synthesized in precursor form in the

cytoplasm, and targetted to the mitochondria. Prior to intramitochondrial localization of the mature protein, the precursor may be modified and its presequence amino-terminal amino acids processed by specific mitochondrial proteases.

The nuclear gene coding for MnSOD of yeast mitochondria was isolated, characterized and sequenced by van Loon *et al.* (8,9). The nuclectide sequence confirmed the predicted amino acid sequence; the initial 27 amino acids constitute the presequence. The van Loon group then used gene disruption to inactivate the MnSOD gene in the yeast nucleus (10). This mutant was found to be sensitive to oxygen; this supported Fridovich's idea that superoxide dismutases participate in the cellular defense against oxygen toxicity.

Fridovich's group has detected an inactive, but activatable, form of MnSOD (pro-MnSOD) in anaerobically-grown *E. coli* (11). Pro-MnSOD was shown to be MnSOD with some metal other than manganese at the active site. Since the addition of the electron sink NO_3^- to cells growing anaerobically resulted in increased accumulation of pro-MnSOD, Fridovich's experiments support the hypothesis that MnSOD biosynthesis in *E. coli* is under redox control. In addition, Scott *et al.* have published that SOD actually increases the lethality of radiation under aerobic, but not

anaerobic, conditions; in these studies, E. coli having low, normal, and high SOD activity were exposed to ionizing radiation (12).

Meanwhile, the role and toxicity of oxygen radicals such as superoxide has been well studied. Cellular responses to oxygen and oxy-radicals are reviewed elsewhere (13). Recently, oxygen radicals have been shown to damage DNA and to increase cellular oxidative stress (14).

The goal of this project is to construct a mutant of the yeast MnSOD gene that lacks the presequence in order to test whether the mislocalized mitochondrial enzyme can compliment a mutant lacking the CuZnSOD activity. This will be done by cloning the mutant gene into a yeast expression vector. The $CuZnSOD^-$ mutant requires lysine and cysteine when grown in air but does not require supplementation when grown in nitrogen. Furthermore, Zamosc et al. have eliminated this oxygen-dependent auxotrophy by transformation of the mutant with a yeast expression vector carrying the yeast CuZnSOD gene (15).

The proposed construct would be used to address the question posed by Fridovich sixteen years ago, "Why are both MnSOD and CuZnSOD necessary if their enzymatic functions are virtually identical?" (3). In addition, the construct would be used to study the import of the MnSOD protein into the mitochondria in vitro and in vivo. The

presequence of the MnSOD gene can faithfully direct a passenger protein into the matrix of mitochondria *in vitro* (16), but the mechanism of import has yet to be defined *in vivo*.

Experimental Procedures

Materials -- DNA modification enzymes were from Bethesda Research Laboratories, New England Biolabs, and IBI. The pGem3zf plasmids were from Promega. Oligonucleotides were ordered from the University of Illinois DNA Synthesis Lab. Other chemicals were reagent grade from standard commercial sources.

Subcloning into pGem3z(+) -- The MnSOD gene obtained from A.P.G.M. van Loon was previously placed in vector pGem3z(-) by J. Rhodes (17).

In order to place the presequence region in sufficient proximity to the vector's SP6 promoter region for the purpose of future sequencing, the first 362 nucleotides of the 5' flanking region of the MnSOD gene were removed. This was accomplished by digestion of pGem3z(-)SOD (17) with restriction enzyme AvaII. The ends were modified with DNA Polymerase I Klenow fragment to fill-in the sticky ends, and the linearized plasmid was digested with the restriction enzyme BamHI. The 1705 nucleotide

fragment containing the MnSOD gene was isolated by size by 1% agarose gel electrophoresis; the appropriate band was purified by the Geneclean procedure. The pGem3z(+) vector was digested with BamHI and Hinc, and the 1705 nucleotide insert (+362 to +2067) were ligated with T4 DNA ligase to produce the pGem3z(+)SOD plasmid of 4890 nucleotides.

Oligonucleotide-directed Mutagenesis of the MnSOD Gene -- The Kunkel procedure (18) involves (a) phosphorylation of oligonucleotides for subsequent ligation, (b) preparation of uracil-containing DNA templates, (c) annealing, extension, and ligation of template to oligonucleotides, and (d) transformation and isolation of mutants.

The oligonucleotides were designed to hybridize to 11 to 13 bases immediatedly flanking either side of the 81 nucleotide presequence (Figure 1). The oligonucleotides were purified by 15% polyacrylamide gel purification and elution prior to phosphorylation. Phosphorylation of oligonucleotides was performed as described previously (18). A 30 ul solution containing 200 pmol oligonucleotide, 100 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 10 mM dithiothreitol, and 0.33 mM ATP was treated with 4.5 units of T4 polynucleotide kinase. After 45 minutes at 37°C, the reaction was terminated by heating at 65°C for 10 minutes.

The pGem3z(+) is a phagemid, that is, a plasmid carrying the intragenic region of bacteriophage f1 that allows the packaging of one strand of the double-stranded vector as single-stranded (ss) DNA into a viral particle by a helper phage; the helper phage supplies the genes necessary for assembly, packaging, and secretion of ssDNA viral particles (19). Uracil-containing templates were prepared using E. coli CJ236 (dut^-ung^-) carrying the pGem3z(+)SOD plasmid and infecting with the helper phage IR1. The single stranded phagemid DNA was isolated by PEG-NaCl precipitation and phenol-chloroform extraction. The CJ236 strain was maintained in LB media supplemented with uridine (0.25 ug/ml) and chloramphenicol (10 ug/ml) to maintain the f-pili.

Phosphorylated oligonucleotides were annealed to the templates, extended with Klenow fragment and deoxynucleotide triphosphates, and ligated. The annealing reaction contained 10 ul total volume of 2 pmol oligonucleotide, 40 pmol template DNA, 20 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, and 50 mM NaCl. This solution was heated to 70°C and cooled to 30°C over a 40-minute period. The extension and ligation reaction was initiated by addition of 0.4 mM each dNTP, 0.75 mM ATP, 17.5 mM Tris-HCl, pH 7.4, 3.8 mM $MgCl_2$, 21.5 mM DTT, 1 unit T4 DNA ligase, 1 unit DNA

polymerase I Klenow fragment. The reaction mixture was kept on ice 5 minutes, transferred to 25°C for 5 minutes, shifted to 37°C for 90 minutes, and stopped by freezing. Restriction enzyme digest was used for initial screening of the transformed E. coli NM522 colonies.

Results

Subcloning into pGem3z(+) -- Colonies of transformants were "mini-prepped" (20) and DNA was digested with BamHI, Kpn, and EcoRI-HindIII. A subclone that produced the appropriate restriction map (Figure 2) was found.

Oligonucleotide-directed Mutagenesis of the MnSOD Gene -- The preparation of uracil-containing DNA templates created more problems than expected. Agarose gel analysis of the single-stranded DNA indicated that on the occasions that single-stranded DNA was isolated, it was ss helper phage DNA and not the desired ss phagemid DNA.

The ratio of single-stranded DNA from helper phage to that of the phagemid varies depending on the helper phag and the bacterial host strain used, the multiplicity of infection, and the phagemid and insert employed. The above were varied and analyzed on agarose gels. When the E. coli strain NM522 was used, the yield of helper phage to phagemid ss DNA was 2 to 1 and easily visualized on the

gel; the yield from NM522 was compared side-by-side with that from CJ236 and the desired phagemid DNA band was confirmed. Apparently, the previous yields from CJ236 may have contained the desired phagemid as DNA, but in such small amounts that were not seen on ethidium bromide-stained agarose gel. (NM522 does not allow incorporation of uracil and thus could not be used as a replacement for CJ236.)

The first attempt at construction af a signal sequence deficient mutant was not successful, as judged by the fact that recombinant colonies did not exceed the background colonies. As previously described (18), unsuccessful experiments are often the result of incomplete synthesis of the complementary strand or absence of streng selection for the mutant strand over the uracil-containing template strand.

In the case of incomplete synthesis, hybridisation conditions may need to be altered to assist base-pairing between the oligonucleotide and the template and to optimize the activity of the DNA polymerase. If the low percentage of mutants is due to weak selection for the mutants, possibilities such as oligonucleotide displacement during the *in vitro* DNA synthesis and the participation of the *E. coli* mismatch correction repair synthesis must be entertained.

Discussion

The aim of this thesis was to construct a mutant of the MnSOD gene that lacks the 81 nucleotides that encode the presequence. The 5' flanking region was successfully shortened, and the gene was placed into reverse orientation for future sequencing to cofirm the presequence deletion. This vector will be more useful for *in vitro* import experiments because removal of 362 5' nucleotides will make a shorter SP6-promoted transcript. Transcripts containing fewer 5' nucleotides are translated more efficiently in *in vitro* systems (J. Kaput, personal communication). The oligonucleotide-directed mutagenesis of the MnSOD gene is in progress, as the initial attempt at isolation of the desired mutant was not successful.

Once the mutant is acquired, the gene will be placed into a yeast expression vector. This vector will be transformed into a yeast mutant that lacks CuZnSOD activity; the mutant yeast has been rescued from its oxygen-dependent auxotrophy by transformation with a yeast expression vector carrying the full-length yeast CuZnSOD gene (15). Since the yeast mutant does have MnSOD activity, it will be interesting to examine whether the MnSOD introduced into the cytoplasm will substitute for the cytoplamic CuZnSOD's function. This assumes that the MnSOD that lacks its presequence amino acid signal will indeed be

stably retained in the cytoplasm; thus, these experiments will also add to the understanding of how proteins are directed and imported into mitochondria.

The overall goal of the experiments with the yeast CuZnSOD mutant that is oxygen-sensitive is to uncover the metabolic pathways that are sensitive to oxygen and its reduction by-products. Supplementation studies using various growth conditions and introduction of the proposed "anti-oxidant" enzymes themselves may define the probable cellular targets.

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Finally, I do hope all will proceed with imagination and positive persistence so as to better understand the beguiling, at-times-elusive , little biological curiosities that may fascinate us.

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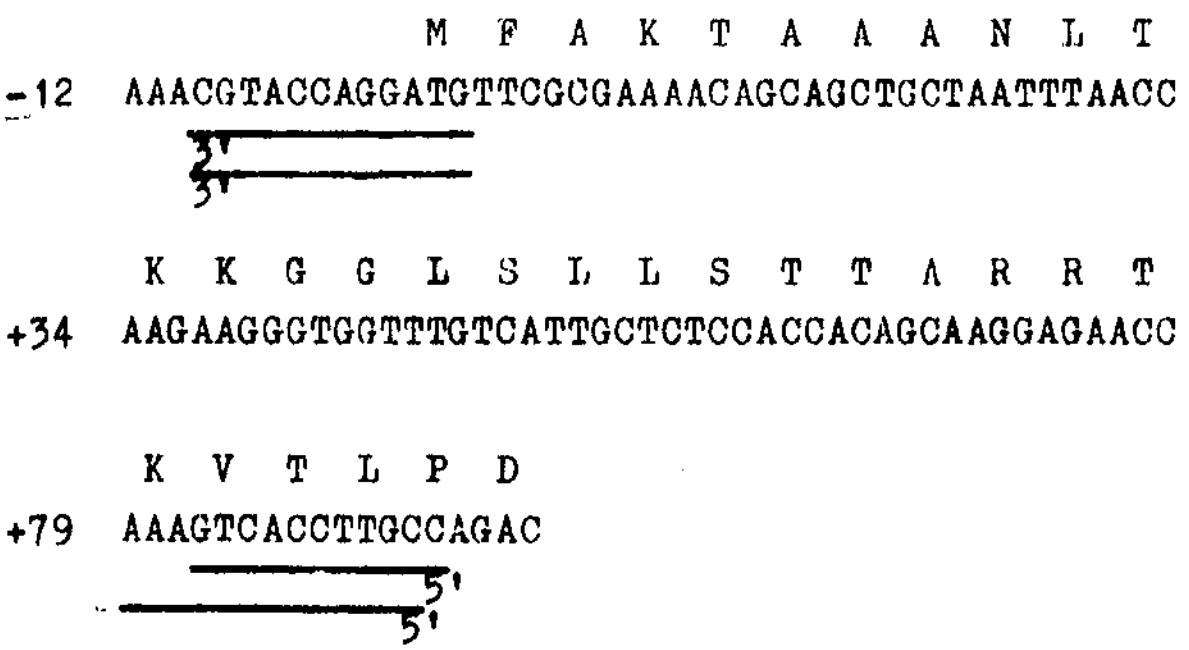

Figure 1. Nucleotide sequence and deduced protein of MnSOD and the 23mer and 25mer oligonucleotides designed to hybridize to this region. Sequence is numbered relative to the A (+1) in the translation-initiation ATG.

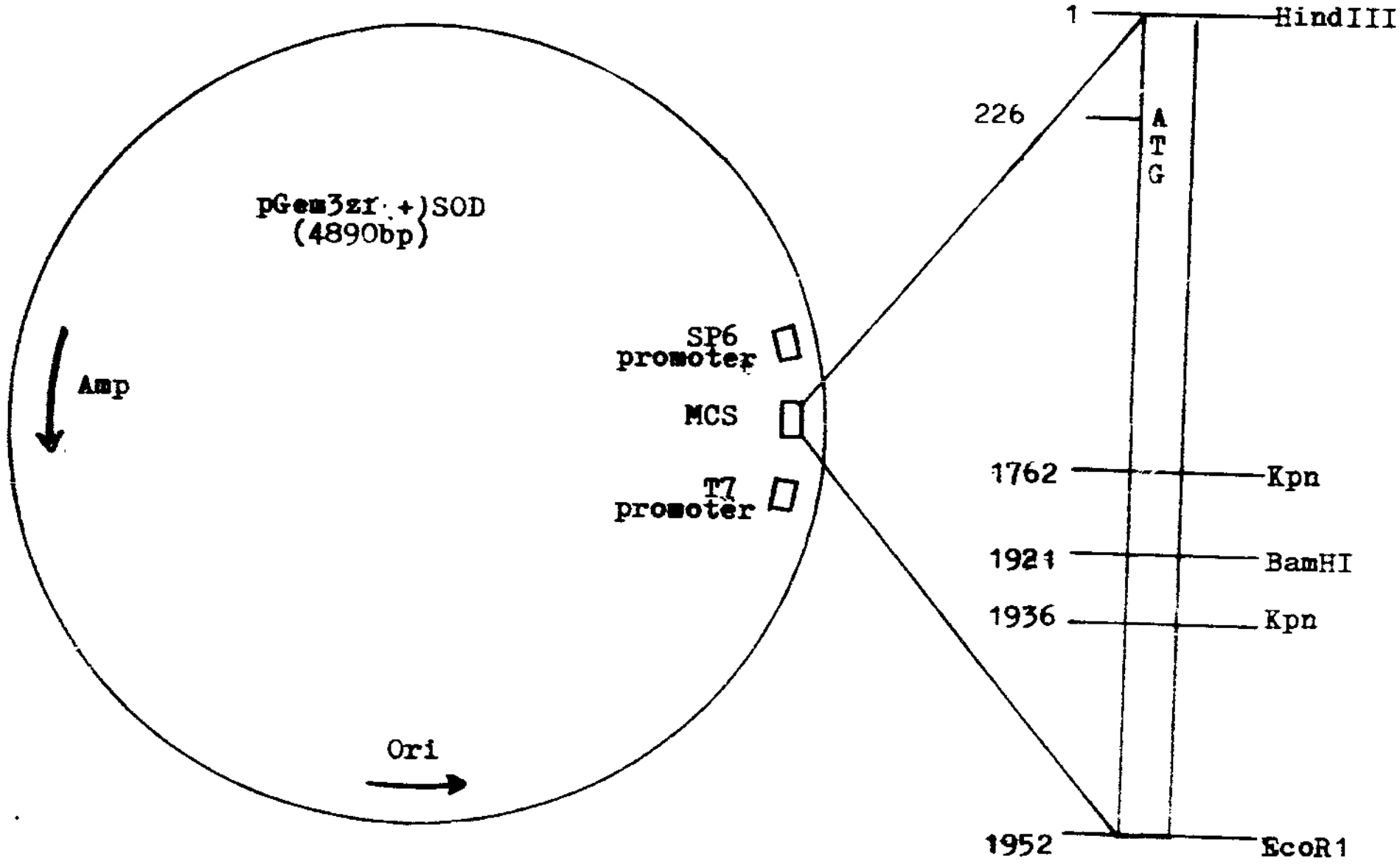

Figure 2. The pGem3zf(+) plasmid carrying the MnSOD gene. The 1705 nucleotide insert is 226 to 1931.