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Cloning, Expression, and Characterization of Two Manganese Superoxide Dismutases from *Caenorhabditis elegans**

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Two genes encoding manganese superoxide dismutase (*sod-2* and *sod-3*) have been identified in the nematode *Caenorhabditis elegans*. Each gene is composed of five exons, and intron positions are identical; however, intron sizes and sequences are not the same. The predicted protein sequences are 86.3% homologous (91.8% conservative), and the cDNAs are only 75.2% homologous. Both deduced protein sequences contain the expected N-terminal mitochondrial transit peptides. Reverse transcriptase polymerase chain reaction analysis shows that both genes are expressed under normal growth conditions and that their RNA transcripts are *trans*-spliced to the SL-1 leader sequence. The latter result together with Northern blot analysis indicate that both genes have mono-cistronic transcripts. The *sod-3* gene was mapped to chromosome X, and the location of *sod-2* was confirmed to be chromosome I. Polymerase chain reaction was used to amplify the cDNA regions encoding the predicted mature manganese superoxide dismutase proteins and each was cloned and expressed to high levels in *Escherichia coli* cells deficient in cytosolic superoxide dismutases. Both proteins were shown to be active in *E. coli*, providing similar protection against methyl viologen-induced oxidative stress. The expressed enzymes, which were not inhibited by hydrogen peroxide or cyanide, are dimeric, show quite different electrophoretic mobilities and isoelectric points, but exhibit comparable specific activities.

A fraction of the molecular oxygen generated in respiring cells is subjected to sequential univalent reduction to superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals. Unless effectively removed, these highly reactive oxygen species cause damage to DNA (1, 2), proteins, and lipids (3–7). To protect their cellular components, organisms have evolved a series of chemical and enzymatic mechanisms that remove these detrimental oxygen species (8). In the first line of defense is the family of superoxide dismutases (SOD,[1] EC. 1.15.1.1), which scavenge superoxide anions and catalyze their dismutation into hydrogen peroxide and oxygen (9) (Eq. 1).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{(Eq. 1)}$$

Four genetically distinct forms of SOD occur that differ in their active site prosthetic metal ion and/or cellular localization. Eukaryotic cells possess a cytosolic copper/zinc SOD (Cu/ZnSOD) (10), a glycosylated extracellular copper/zinc SOD (11), and a mitochondrial manganese SOD (MnSOD) (12). Most prokaryotes generally have both MnSOD and iron SOD (FeSOD) that are similar in their amino acid sequence and structure (13).

Mitochondrial MnSOD is encoded by a nuclear gene and is produced as a precursor protein targeted to the mitochondrial matrix during which it is processed into the mature form by cleavage of the transit peptide (14). The expression of MnSOD is usually inducible in both prokaryotic and eukaryotic cells, its levels increasing under conditions of elevated oxidative stress in the immediate environment (15–19). A superoxide response regulon has been identified in *Escherichia coli* (20). The level of MnSOD encoded by the *sodA* gene in this organism is regulated both transcriptionally (17) and post-translationally (18, 21), and involves the participation of at least six global transcriptional regulators (22).

Free radicals have been directly and indirectly implicated in the aging process (23). A transgenic strain of *Drosophila melanogaster* overexpressing both SOD and catalase exhibited a life-extension of up to one-third that of wild-type (24). Long-lived genetic variants of the metazoan *Caenorhabditis elegans*, *age-1* (25) and *daf-2* (26), provide evidence for a positive correlation between cellular levels of SOD and extended lifespan (27–30). Furthermore, Ishii *et al.* (31) isolated a short-lived methyl viologen-sensitive mutant *mev-1* which exhibited hypersensitivity to both oxygen and methyl viologen and half the cytosolic SOD activity of wild type. Much evidence has been provided supporting the theory that longevity is determined, at least in part, by polygenes mediating stress-related responses (28, 32, 33). These stress-response genes are in turn believed to be under regulatory control. The *age-1* gene product of *C. elegans* has recently been characterized as a homologue of the mammalian phosphatidylinositol-3-OH kinase which suggests a role for inositol signaling in the oxidative stress response and, in particular, a free radical protection pathway (28, 34). The gene product of another mutant *rad-8* (35), which is hypersensitive to ultraviolet radiation, oxygen and methyl viologen, may also, similar to *age-1*, be a component of the nematode's antioxidant defense system.

To understand the free radical protection pathway in *C. elegans,* we have characterized the manganese superoxide dismutases of this organism. The gene sequence for an Mn-SOD (*sod-3*) was determined in our laboratory (36), and the gene of a second MnSOD (*sod*-2) has now been described (37), its cDNA sequence already having been established (EMBL entry CELMNSOD).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X85790.

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[1] The abbreviations used are: SOD, superoxide dismutase; FeSOD and MnSOD, iron- and manganese-containing SOD, respectively; Cu/ZnSOD, copper and zinc-containing SOD; bp, base pair(s); IPTG, isopropyl-β-D-thiogalactopyranoside; NBT, nitro blue tetrazolium chloride; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; RT-PCR, reverse transcriptase-polymerase chain reaction.

We report the elucidation of the cDNA sequence of *sod-3*, characterization of the gene, and RNA transcript together with expression and characterization of the presumed mature protein of both MnSODs in *E. coli*.

EXPERIMENTAL PROCEDURES

Isolation of cDNA Clones and Sequencing—Standard methods (38) were used to screen approximately 2×10^6 plaques of a *C. elegans* mixed stage Uni-ZAP XR λ cDNA library (Stratagene, La Jolla CA) with a radiolabeled probe corresponding to exon 2 of *sod-3* (see below). The *sod-2* cDNA was isolated serendipitously while screening for *sod-3* using an oligonucleotide probe (CM3-A+) (Table I).

Phagemid (pBlueScript) DNA was prepared from λ clones using the *in vivo* excision protocol utilizing ExAssist helper bacteriophage as described by the manufacturer's instructions. Single strand DNA was prepared by superinfection of phagemid-containing cells utilizing helper bacteriophage VCS-M13. Four positive *sod-3* clones and two positive *sod-2* clones were sequenced by primer walking using both single-stranded and double-stranded DNA templates utilizing dye terminator cycle sequencing chemistry and *Thermus aquaticus* DNA polymerase in an ABI CATALYST 800 laboratory workstation and an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA).

Isolation of Total RNA—C. elegans Bristol strain N2, (a kind gift of J. Hodgkin, MRC, Cambridge, UK) was maintained on NGM agar seeded with *E. coli* strain OX326A (Δ*sod* AΔ*sod* B, provided by H. Steinman, Albert Einstein College of Medicine, New York) and grown at 20 °C in a liquid culture of Basal S medium (0.1 M NaCl, 50 mM potassium phosphate, pH 6.0, 5 μg/ml cholesterol, 10 mM potassium citrate, 3 mM $CaCl_2$, 3 mM $MgSO_4$, 50 μM EDTA, 25 μM $FeSO_4$, 10 μM $MnCl_2$, 10 μM $ZnSO_4$, and 1 μM $CuSO_4$), also in the presence of OX326A cells (39). After 4 days mixed stage nematodes were harvested and purified from bacterial debris by sucrose flotation (40). The pelleted nematodes were resuspended in M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 85 mM NaCl, 1 mM $MgCl_2$) and frozen instantly by dropping into liquid nitrogen.

Total RNA was isolated by first grinding 1 g of mixed stage nematodes with pestle and mortar under liquid nitrogen, homogenizing the powder with a Polytron probe after dissolution in guanidinium thiocyanate, and then phenol-extracted using the Stratagene RNA Isolation kit. All solutions and glassware were treated with diethyl pyrocarbonate prior to use (38).

Hybridization Probes—The probe for *sod-2* was generated by PCR using the cDNA clone, CEL9, as template (this study) and oligonucleotides CM2-P5 and CM2-P3 as primers (Table I). The resulting 594-bp product (which corresponds to nucleotides 207–1132, Fig. 1) was purified from an agarose gel by the glass binding method. *sod-3* was detected using a 133-bp DNA fragment prepared by an *Sfu*I and *Bgl*II digest of the *sod-3* genomic clone 17MNCE (36) (this probe covers nucleotides 501–634, Fig. 1). Each probe (200 ng) was radiolabeled with [α^{32}P]ATP (100 μCi) by nick translation and purified via NucTrap columns (Stratagene).

Southern Blot Analysis—Genomic DNA was isolated by grinding 1-g aliquot of worm with a pestle and mortar submerged in liquid nitrogen and incubating the powdered tissue in 30 ml of lysis solution (100 mM EDTA, 0.5% SDS, 50 μg/ml proteinase K, 1% β-mercaptoethanol) for 3 h at 50 °C (40). The cell lysate was phenol-extracted in the cold and ethanol-precipitated. The recovered DNA was digested with restriction endonucleases and resolved on a 0.7% agarose gel. After denaturation and neutralization, the DNA was capillary transferred onto Hybond N nylon membrane (Amersham Corp.) (41). The blot was prehybridized overnight at 65 °C in a solution containing 0.5 M $NaHPO_4$, 1 mM EDTA, pH 8.0, 1% BSA, 7% SDS, 100 μg/ml denatured herring sperm DNA and hybridized in the same buffer containing α-^{32}P-labeled heat-denatured DNA probe. After an overnight hybridization at 65 °C, the filters were washed three times for 10 min at room temperature in low stringency wash (0.5% BSA, 1 mM EDTA, pH 8.0, 40 mM $NaHPO_4$, 5% SDS) and once for 10 min at 65 °C in high stringency wash solution (1 mM EDTA, 40 mM $NaHPO_4$, 1% SDS) (42). This was followed by autoradiography over several days.

Northern Blot Analysis—Total RNA was size-fractionated on a 1% agarose gel containing 2.2 M formaldehyde and 1 × MOPS running buffer (0.4 M MOPS, 0.1 M sodium acetate, 0.01 M EDTA). RNA (50 μg in a volume of 11 μl) was mixed with 39 μl of denaturing solution (500 μl of deionized formamide, 162 μl of 12.3 M formaldehyde, 100 μl of MOPS buffer) (MOPS buffer is 0.2 M MOPS, 0.5 M sodium acetate, 0.01 M EDTA) and incubated at 55 °C for 15 min (42). The samples were electrophoresed at 5 V/cm in 1 × MOPS running buffer. Hybridization probes and conditions were as described above.

Physical Mapping of the Gene—Two polytene filters (kind gift of A. Coulson, MRC, Cambridge, UK) were screened for *sod-2* and *sod-3*. Hybridization was performed overnight at 65 °C in a solution comprised of 6 × SCP (0.6 M NaCl, 1.8 M Na_2HPO_4, 6 mM EDTA, pH 6.2), 3% (v/v) Sarkosyl NL30, and 10% (w/v) dextran sulfate containing heat-denatured hybridization probes and herring sperm DNA as described above. Filters were washed four times in 0.5 × SCP, 0.5% SDS at 50 °C. Autoradiography was over several days.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)—RT-PCR was carried out using Perkin-Elmer rTth RT-PCR kit according to the manufacturer's instructions except for the following details: 50 ng of *sod-2* or *sod-3* downstream primers (CM2-NC3 or CM3-P3, respectively) (Table I) were annealed to 250 ng of total RNA and extended in manganese buffer at 50 °C for 30 min. After the addition of 50 ng of upstream primers (CM2-P5 and CM3-P5 or SL-1 or SL-2) (Table I), chelating buffer, and $MgCl_2$, the reactions were held at 95 °C for 1 min and then cycled at 95 °C for 10 s and 50 °C for 1 min for 35 cycles in a Perkin-Elmer 9600 Thermal Cycler.

Primer Extension Analysis—Antisense 26-mer oligonucleotide CM3+9M (Table I) (corresponding to nucleotides 8–33, Fig. 1) was radiolabeled utilizing [γ-^{32}P]ATP at its 5′ terminus using T4 polynucleotide kinase (1 ng, approximately 10^5 cpm) and annealed to *C. elegans* total RNA (10 μg) by heating to 70 °C in the presence of 10 mM Tris, pH 8.3, 1 mM EDTA, and 30 mM KCl and slowly cooling to room temperature. The reaction mixture was transferred to 42 °C and extended using avian myeloblastosis virus reverse transcriptase (25 units, 1 h). Following 10-fold dilution, RNase (DNase-free) was added and the reaction mixture incubated at 37 °C for 15 min, phenol-extracted, and ethanol-precipitated. Reaction products were characterized by electrophoresis on a 7 M urea, 6% polyacrylamide gel and subsequent autoradiography.

Cloning and Expression of Sod-2 and Sod-3—PCR was performed on each cDNA clone to amplify only the presumed mature coding region following the protocol provided with the GeneAmp PCR reagent kit using AmpliTaq DNA polymerase (Perkin-Elmer). In addition to standard components, each 20-μl reaction contained 1 ng of template (double-stranded DNA phagemids CEL 9 and 8-1 for *sod-2* and *sod-3*, respectively), 100 ng of each primer (CM2-P5 and CM2-P3 for *sod-2*; CM3-P5 and CM3-P3 for *sod-3*, Table I), and 1.5 mM $MgCl_2$. Thermal cycling conditions were 94 °C for 30 s, 50 °C (for *sod-2*) or 60 °C (for *sod-3*) for 30 s, and 72 °C for 1 min, applied for 30 cycles after which the reactions were held at 4 °C until 1 unit of Klenow polymerase was added, and the reactions were incubated at 30 °C for 30 min. The reaction mixtures were purified using Microcon-100 centrifugal concentrators (Amicon), washed with TE buffer, and ligated to *Nco*I-digested, Klenow-treated pTrc99A (Pharmacia Biotech Inc.) in the presence of 8 units of T4 DNA ligase (Pharmacia FPLC-Pure). Positive clones in the correct orientation were designated pTrcsod-2 and pTrcsod-3 and fully sequenced as described above.

E. coli OX326A cells transformed with pTrcsod-2 or pTrcsod-3, were grown in 2TY medium (16 g/liter tryptone, 10 g/liter yeast extract, and 5 g/liter NaCl), supplemented with 50 μM $MnSO_4$ to an A_{600} of 0.4 when IPTG was added to a final concentration of 1.0 mM. Growth was continued for 6 h when the cells were harvested by centrifugation, resuspended in KP buffer (50 mM potassium phosphate, 1 mM EDTA, pH 7.8), and lysed by passage though a French pressure cell at 16,000 p.s.i. Protein concentration was measured by the method of Bradford (43), SDS-15% polyacrylamide gel electrophoresis was carried out after reduction with dithiothreitol according to the method of Laemmli (44) as described (38), and 8% native PAGE gels were stained for superoxide dismutase using nitro blue tetrazolium (45).

Characterization of MnSOD-2 and MnSOD-3—Superoxide dismutase assays were carried out essentially as described by McCord and Fridovich (9) and Ysebaert-Vanneste and Vanneste (46) using cytochrome *c* as detector and xanthine-xanthine oxidase as superoxide generator.

Molecular weights of MnSOD-2 and MnSOD-3 were estimated by loading 250 μl of each sample onto a Superose 12 (HR 10/30, 10 × 300 mm) gel permeation FPLC column (LKB-Pharmacia) and eluted with 50 mM phosphate buffer, pH 7.2, and 150 mM NaCl at a flow rate of 0.4 ml/min in 1-ml fractions. The column was previously calibrated with gel filtration calibration molecular weight markers (Sigma; blue dextran, M_r 2,000,000; bovine lung aprotinin M_r 6,500; horse heart cytochrome *c*, M_r 12,400; bovine erythrocyte carbonic anhydrase, M_r 29,000; bovine serum albumin, M_r 66,000). The collected fractions were analyzed on an 8% native PAGE gel stained for SOD activity using the NBT method (45).

Isoelectric points of the two MnSODs were determined by focusing

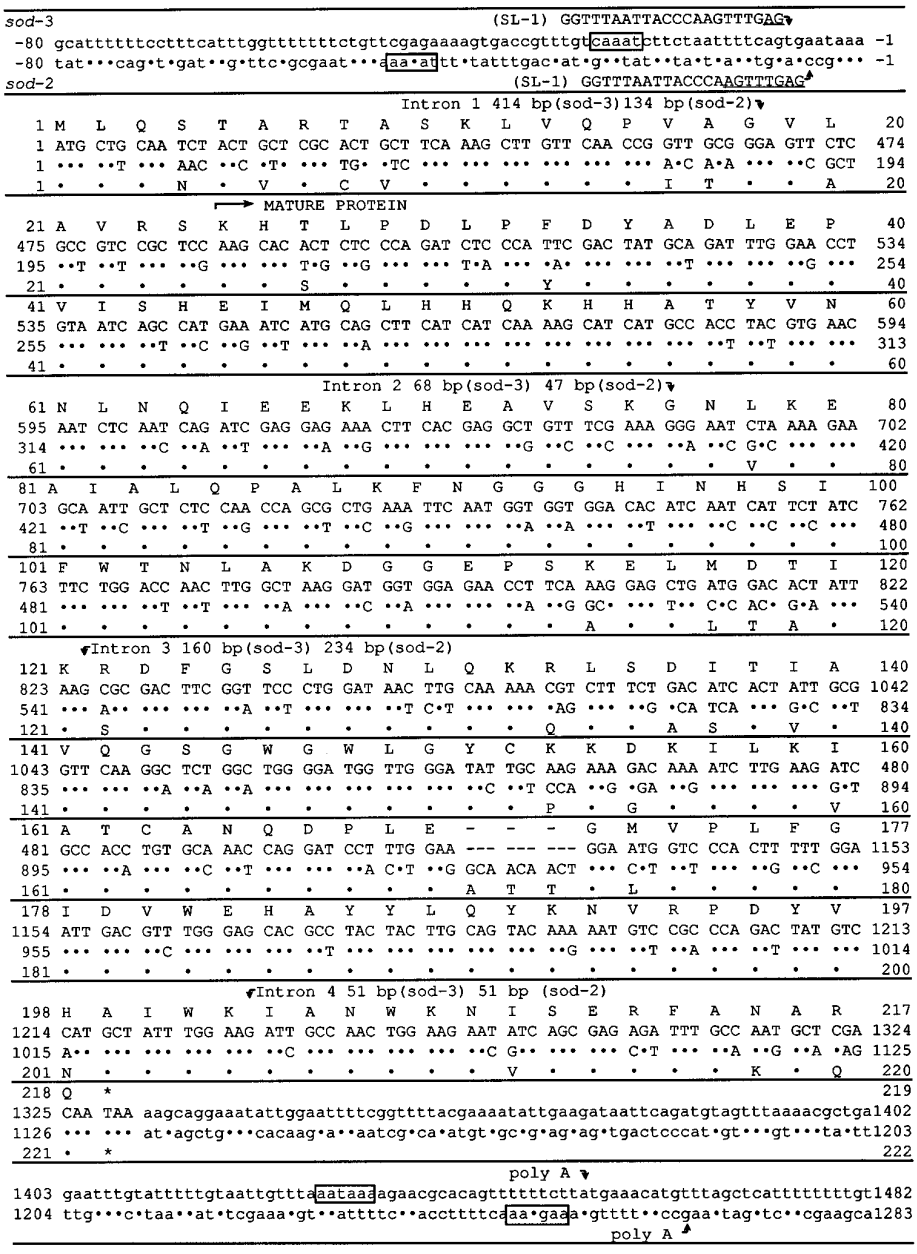

FIG. 1. **Sequence comparison of the two MnSOD genes, cDNAs, and proteins of *C. elegans*.** The *sod-3* gene sequence is presented with the encoded amino acid sequence above. The *sod-2* gene sequence is presented below the *sod-3* gene sequence with the encoded amino acids below this. Identity between the sequences is indicated by a *dot*, and differences are shown. The predicted N terminus for mature MnSOD proteins is indicated by an *arrow above* the *sod-3* amino acid sequence (Lys-25). Numbering (beginning at the coding region and including introns) is given for the gene and amino acid sequences, the positions and sizes of the four introns in each gene being indicated by *curved arrows* (intron sequences not shown). Putative CAAT boxes and poly(A) signals are boxed at the 5′ and 3′ ends of the genes, respectively. Actual poly(A) attachment sites are indicated by a *curved arrow* to the last nucleotide of the gene sequence before the poly(A) tract (as determined by cDNA sequencing). Similarly, the SL-1 *trans*-spliced leader sequences are shown adjacent to the gene sequences with *curved arrows* indicating the first nucleotide of the gene found in the *trans*-spliced RNA (as determined by sequence analysis of RT-PCR products). The beginning of sequenced cDNAs is indicated by *underlined bases* within the SL-1 leaders. Noncoding DNA sequences (5′ and 3′) for *sod-2* were taken from EMBL data base entry CELSOD2.

4 μl of each protein sample on an ampholine PAGplate gel, pH range 5.5–8.5 (T = 5, C = 3%) (Pharmacia Biotech). The samples were loaded in duplicate alongside 5 μl of Bio-Rad isoelectric focusing standards and electrophoresed at 1.5 watts/cm for 2.5 h at 10 °C. When the electrophoresis was complete, the pH gradient was measured with a surface electrode and then the PAGplate divided into two: one-half was stained with Coomassie Brilliant Blue as recommended by the manufacturer and the second half was stained for SOD by the NBT method (45).

RESULTS

Structure and Comparison of the Genes for Sod-2 and Sod-3—Four *sod-3* cDNA clones were isolated from a lambda phage library by hybridization to a 133-bp fragment prepared from an *Bgl*II/*Sfu*I double digest of the *sod-3* genomic clone 17MNCE previously described (36) (see "Experimental Procedures"). Each fully sequenced cDNA clone (813 bp total, Fig. 1) aligned to the gene sequence of *sod-3* (36) (Fig. 1). Initially, two *sod-2* cDNA clones had been isolated by hydridization to an oligonucleotide probe (CM3-A+) (Table I) while screening for the *sod-3* cDNA. The sequence of these cDNA clones (807 bp total, Fig. 1) matched the previously determined *sod-2* cDNA sequence (GenBank accession number D12984, 814 bp). Intron positions within the *sod-2* gene were determined by direct sequencing of a PCR product obtained from *C. elegans* genomic DNA using specific primers for *sod-2* (CM2-P5 and CM2-NC3, Table I). Both MnSOD genes contain four introns each located between the same nucleotide positions (Fig. 1 and Table II), and each intron is flanked by 5′ donor and 3′ acceptor splice sites which conform to the known *C. elegans* consensus sequences (Table

TABLE I
Name and sequence of oligonucleotides used in this study

Oligonucleotide	Sequence
SL-1	GGTTTAATTACCCAAGTTTGAG
SL-2	GTTTTAACCCAGTTACTCAAG
CM2-P5	AAGCACTCGCTGCCAGATTTAC
CM2-P3	TTATTGCTGTGCCCTTTGCAAAAAC
CM2-NC3	CGGTGAAAACTTTTCATTTGA
CM3-P5	AAGCACACTCTCCCAGATCTC
CM3-P3	TTATTGTCGAGCATTGGCAAATC
CM3+9M	CTTTGAAGCAGTGCGAGCAGTAGATT
CM3-A+	GATTTGGAACCTGTAATCAGC
PK-PRO2	TGTGTGGAATTGTGAGC
PK-TERM	GGCTGAAAATCTTCTCT

TABLE II
Sequences of exon/intron junctions

Sequences are presented of exon (uppercase) and intron (lowercase) junctions within the MnSOD genes of *C. elegans*. Sizes of intron (bp) are also given.

Intron	Gene	Sequence of exon/intron junctions
1	*sod-2*	..CACAGG/gttagt..134 bp..ttttag/AGTCGC..
	sod-3	..TGCGGG/gtaagt..414 bp..tttcag/AGTTCT..
2	*sod-2*	..CTCCAA/gtaatt.. 47 bp..ttgcag/GAAACG..
	sod-3	..CGAAAG/gttttt.. 48 bp..atatag/GGAATC..
3	*sod-2*	..ATTAAG/gtagtt..234 bp..ttcaag/AGCGAC..
	sod-3	..ATTAAG/gtaaaa..160 bp..tttcag/CGCGAC..
4	*sod-2*	..TGGAAG/gtagtt.. 51 bp..tttcag/ATCGCC..
	sod-3	..TGGAAG/gtgatt.. 51 bp..tttcag/ATTGCC..

II) (47). Introns 1 and 3 were of significantly different sizes (Table II), and no homology was found between the intron sequences of each gene other than polynucleotide tracts.[2] The sequences of the 5′- and 3′-untranslated regions of the *sod-2* gene were obtained from the GenBank data base (accession number D85499). No significant homology was found between the 5′-untranslated region sequences of *sod-2* and *sod-3* (using GenBank CELC08A9), and poly(A) splice sites within the 3′-untranslated region were identified by comparison to the corresponding cDNA clones (Fig. 1). Putative CAAT signals are also shown (Fig. 1).

The predicted protein sequences are 86.3% homologous (91.8% conservative), and the cDNA sequences are 75.2% homologous. No significant difference was found in codon usage between the two genes. It has been suggested that a bias in synonymous codon usage may be useful in identifying highly expressed or lowly expressed genes (48). Analysis of relative synonymous codon usage could not unambiguously classify either *sod-2* or *sod-3* in this way[2] suggesting that expression levels may be the same for each. These similarities suggest that the two genes are the result of a recent gene duplication event.

The amino acid sequences deduced from each cDNA were analyzed using the Prosite data base which identified each as MnSOD with the consensus pattern D*X*WEH(STA)(FY) (residue Asp-179 to Tyr-185, Fig. 1) (49) although this pattern cannot discriminate between MnSOD and FeSOD sequences. Each protein sequence was also aligned with other MnSOD and FeSOD sequences[2] which allowed identification of the metal-binding residues as His-50, His-98, Asp-182, and His-186 (*sod-2*) and His-50, His-98, Asp-179, and His-183 (*sod-3*). There is a three-amino acid deletion in the *sod-3* protein sequence in comparison with *sod-2* between positions Glu-170 and Gly-174 (*sod-2* numbering, Fig. 1). Furthermore, the protein sequences conform to the signature of Parker and Blake (49) that defines an SOD as an MnSOD (Gly, Gly, Phe, Gln, and Asp, residue positions 93, 94, 101, 166, and 167 respectively, Fig. 1). Further analysis of the deduced protein sequences confirms that both MnSODs possess a putative mitochondrial transit peptide comparable to those present in MnSODs from other sources (Table III). It has been shown that mitochondrial transit peptides in the yeast *Saccharomyces cerevisiae* are capable of forming amphiphilic helices that contain positively charged residues (50) as are all the MnSOD transit peptides presented in Table III, including *C. elegans* MnSOD-2 and MnSOD-3. All transit peptides of mitochondrially targeted MnSODs possess positively charged residues at the beginning of the mature peptide and within the transit peptide penultimate to the cleavage site (Table III). This property was used to identify the predicted cleavage site

[2] T. Hunter, W. H. Bannister, and G. J. Hunter, unpublished results.

TABLE III
Alignment of manganese superoxide dismutase mitochondrial transit peptides

Selected MnSOD mitochondrial transit peptide sequences are presented with the first 10 amino acids of the mature MnSOD protein. Positively charged amino acid residues are presented in bold type, and an asterisk above the sequences indicates the conserved lysine and arginine residues at the beginning of the mature protein and penultimate to the maturation cleavage site respectively.

Organism/gene	Signal sequence	Mature start
	*	*
Human[a]	MLS**R**A---VCGTS**R**QLA-PALGYLGS**R**Q	**K**HSLPDLPYD
Mouse[b]	MLC**R**A---ACSTG**RR**LG-PVAVAAGS**R**H	**K**HSLPDLPYD
Drosophila[c]	MFVA---**R**KISPNC**K**PG--------V**R**G	**K**HTLP**K**LPYD
Yeast[d]	MFA**K**TAAA--NLT**KK**GGLSLLSTTA**RR**T	**K**VTLPDL**K**WD
Onchocerca[e]	MNLIIGIAG**R**LLVG**K**N----YCLNTQ**R**L	**K**HVLPDLPYD
MnSOD-2[f]	MLQN----TV**R**CVS**K**LVQPITGVAAV**R**S	**K**HSLPDLPYD
MnSOD-3[g]	MLQS----TA**R**TAS**K**LVQPVAGVLAV**R**S	**K**HTLPDLPFD

[a–g] Sequences were obtained from the following Swiss-Prot entries: [a] SODM HUMAN; [b] SODM MOUSE; [c] SODM DROME; [d] SODM YEAST; [e] SODM ONCVO; [f] SODM CAEEL; and [g] SODN CAEEL.

of both *sod-2* and *sod-3* precursor MnSODs (Table III).

Physical Mapping of the sod-2 and sod-3 Genes—Physical mapping using polytene filters confirmed the position of *sod-2* on chromosome I (YAC positives Y37F9 and Y38C11, Fig. 2*A, upper panel, closed arrow*). The *sod-3* gene was similarly mapped to chromosome X (YAC positives Y52F1, Y46C2, and Y46B6, Fig. 2*A, lower panel, closed arrow*). Although the two polytene filters were screened with probes specific for each gene (see "Experimental Procedures") and no cross-hybridization was observed between the two MnSODs, each probe did, however, hybridize to a second identical YAC clone (Fig. 2*A, open arrows*), identified as Y45D2 mapping to chromosome IV. This may be indicative of a further MnSOD gene in *C. elegans*. Southern blot analysis of each gene using several restriction endonucleases (see "Experimental Protocols" and Fig. 2*B*) also suggests the presence of another gene. When a *sod-3*-specific probe was utilized, only single DNA fragments were observed by Southern analysis (Fig. 2*B, lower panel*). These correspond to the expected sizes determined from the known sequence (GenBank CELC08A9) (19274, 9937, 6182, 6262, 7930, and 2747 bp, respectively, as shown in Fig. 2*B*). When a *sod-2*-specific probe was used, more restriction fragments were visualized than expected (Fig. 2*B, upper panel*). Although digestion of genomic DNA with *Bam*HI or *Hin*dIII was expected to yield two hybridizing fragments (1806 bp and >482 bp with *Bam*HI; 305 bp and >1000 bp with *Hin*dIII), three can clearly be seen (Fig. 2*B*). Furthermore, all other restriction digests would be expected to yield only one hybridizing fragment, but two fragments are clearly visible in the lanes containing DNA digested with *Sal*I, *Eco*RI, *Xba*I, and *Pst*I (Fig. 2*B, upper panel*). The unexpected additional bands observed by Southern hybridization are smaller in size than that observed with uncut DNA (*U*, Fig. 2*B*) and are therefore not due to failure of the restriction enzymes to digest the DNA. A third gene for MnSOD may therefore be present in *C. elegans* as suggested by both the physical mapping and Southern blot analysis. Different hybridization solutions were used to screen the polytene filters and the Southern blots although the probes used were identical (see "Experimental Procedures"). This may explain why a second signal was observed with probes specific for *sod-2* and *sod-3* in the polytene filters (Fig. 2*A*), but only a second signal was observed for *sod-2* by Southern blot analysis (Fig. 2*B, upper panel*).

In Vivo Expression of Sod-2 and Sod-3 in C. elegans—Northern blot analysis revealed single transcripts for *sod-2* and *sod-3* of approximately the same size (800 nucleotide) (Fig. 2*C*) indi-

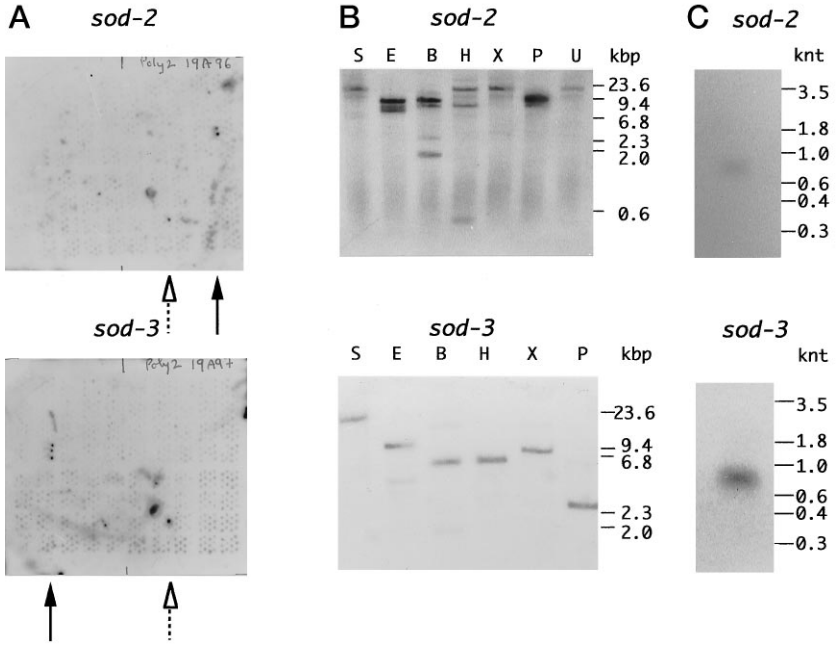

FIG. 2. **Chromosome mapping and Southern and Northern blot analysis of *sod-2* and *sod-3*.** *A*, polytene filters containing YAC clones in a gridded array and spanning most of the *C. elegans* genome were probed with ^{32}P-labeled DNA fragments obtained from either *sod-2* or *sod-3* clones (see "Experimental Procedures"). The *bold arrows* in each figure show the gene-specific hybridization to clones Y37F9 and Y38C11 (chromosome I) in the case of *sod-2* (*upper*) and to Y52F1, Y46C2 and Y46B6 (chromosome X) in the case of *sod-3* (*lower*). An additional clone was observed to cross-hybridize to each SOD fragment (*open arrow*) corresponding to Y45D2 (chromosome IV). *B*, genomic DNA was digested with the restriction endonuclease indicated at the *top* of each lane (*S*, *Sal*I; *E*, *Eco*RI; *B*, *Bam*HI; *H*, *Hin*dIII; *X*, *Xba*I; *P*, *Pst*I; *U*, uncut) and then separated on a 0.7% agarose gel. A fragment of the *sod-2* (*upper*) or *sod-3* (*lower*) gene was labeled with ^{32}P and used to probe a blot of the genomic digests. Size markers (lambda *Hin*dIII in kilobase pairs (*kbp*)) are indicated to the *right*. *C*, total RNA (50 μg) from *C. elegans* mixed-stage culture was separated on a 1.0% agarose-formaldehyde gel and hybridized to a ^{32}P-labeled DNA fragment isolated from the *sod-2* (*upper*) or *sod-3* (*lower*) gene. RNA size markers (*knt*) are shown to the *right*.

cating that only two MnSOD genes are actually expressed *in vivo* in *C. elegans*. The intensity of the respective signals for *sod-2* and *sod-3* may not be indicative of the quantitative level of expression of each transcript in *C. elegans*. The specific activities of each probe were not measured quantitatively, although the larger *sod-2* probe might have been expected to yield the more intense signal. In two separate experiments, Northern blot results were identical.

Expression and *trans*-splicing of the two genes was further investigated by RT-PCR. Using primers specific to the coding regions of each SOD cDNA (Table I), RT-PCR produced amplicons of the expected sizes (728 bp for *sod-2* and 585 bp for *sod-3*, Fig. 3). Replacement of the upstream (5′) primer with either SL-1- or SL-2-specific primers (Table I) yielded products only with SL-1 in each case (Fig. 3). RT-PCR with spliced leader upstream primers has been reported to give multiple products. We observed two bands only with *sod-3* (Fig. 3*B, lane 5*). This is probably due to the downstream (3′) primer as two bands were also observed with the sequence-specific upstream primer (Fig. 3*B, lane 4*). This result indicates that both *sod-2* and *sod-3* are *trans*-spliced to SL-1 and not SL-2 leader sequences. RT-PCR products for *sod-2* (Fig. 3, *lanes 1* and *2*) and for *sod-3* (Fig. 3, *lanes 4* and *5, lower bands*) were purified from the gel, cloned into pUC 18, and sequenced. The results indicated that the SL-1 leader sequence was *trans*-spliced to the consensus splice acceptor site TTTCAG at position −8 for *sod-3* (Fig. 1) and to acceptor site TTGGAG at position −6 for *sod-2* (Fig. 1). The sequence of the *trans*-spliced RT-PCR products also confirmed that the cDNAs of each SOD contained the *trans*-spliced SL-1 sequences at their 5′ end (Fig. 1).

Primer extension analysis using total *C. elegans* RNA and a *sod-3*-specific primer (CM3+9 M, Table I) gave a product of 63 nucleotides (Fig. 4). This is the expected product size of the *trans*-spliced *sod-3* RNA product. As the splicing reaction is very rapid, primer extension could not be used to determine the transcription start site but merely confirms SL-1 *trans*-spliced product. We therefore made no attempt to analyze the *sod-2* transcript by primer extension.

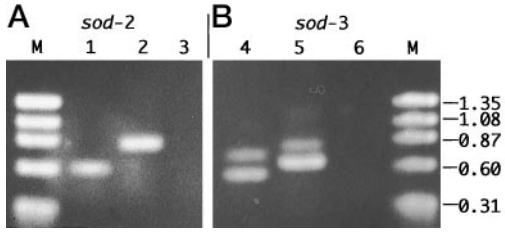

FIG. 3. **Reverse-transcriptase polymerase chain reaction (RT-PCR) of *sod-2* and *sod-3*.** Total RNA (250 ng) was hybridized to the downstream oligonucleotides CM2-NC3 (*sod-2*, *lanes 1–3*) or CM3-P3 (*sod-3*, *lanes 4–6*) and reverse-transcribed. PCR was then carried out after the addition of upstream oligonucleotides CM2-P5 (*lane 1*), SL-1 (*lanes 2* and *5*), SL-2 (*lanes 3* and *6*), or CM3-P5 (*lane 4*). PCR products were run on 1% agarose gels and stained with ethidium bromide. DNA size markers (ϕOX174, *Hae*III) were run in lanes labeled *M* and sizes (kilobase) are indicated to the *right*.

Expression of Sod-2 and Sod-3 in E. coli—The coding regions for the predicted mature MnSOD proteins (Fig. 1 and Table III) were amplified by PCR using the primer pairs CM2-P5 and CM2-P3 (*sod-2*) or CM3-P5 and CM3-P3 (*sod-3*) (Table I) and the respective cDNA clone as template. Each product was subcloned into the *E. coli* expression vector pTrc99A and fully sequenced before transformation into *E. coli* OX326A cells that lack the cytosolic FeSOD and MnSOD. Control of the level of expression of each gene from the *trc* promoter encoded by the vector was demonstrated by addition of different amounts of the inducer IPTG to cultures followed by SDS-PAGE analysis (Fig. 5). Interestingly, initial experiments performed using the amplicon generated by PCR using primers CM2-P5 and CM2-

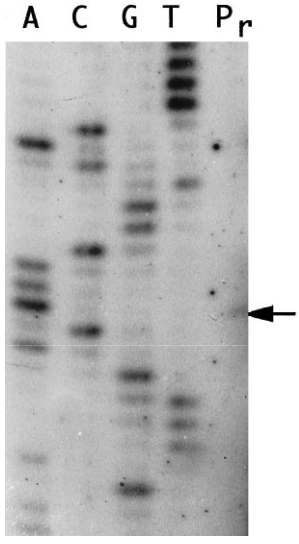

FIG. 4. **Primer extension product for *sod-3*.** Oligonucleotide CM3+9M, complementary to the coding strand and radiolabeled with ^{32}P, was annealed to total RNA (10 μg) isolated from a *C. elegans* mixed-stage culture. After extension with reverse transcriptase the products were denatured and electrophoresed on a 6% polyacrylamide sequencing gel (*lane* P_r). A DNA sequence ladder was generated using the same labeled oligonucleotide primer and *sod-3* genomic clone 17MNCE as template (*lanes A, C, G*, and *T*). Autoradiography was over 14 days. The *arrow* to the *right* of the figure indicates the position of the primer extension product (*lane* P_r).

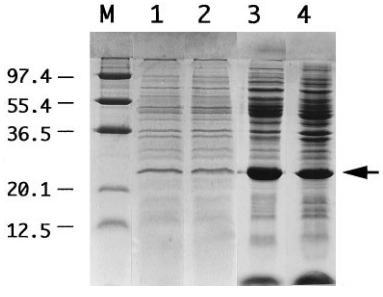

FIG. 5. **SDS-PAGE of *MnSOD-2* and *MnSOD-3* proteins expressed in *E. coli*.** *C. elegans* MnSODs were induced from expression clones pTrcsod-2 (*sod-2* gene, 10 μg, *lanes 1* and *3*) or pTrcsod-3 (*sod-3* gene, 10 μg, *lanes 2* and *4*) in SOD-deficient *E. coli* OX326A cells with 0.1 mM IPTG (*lanes 1* and *2*) or 1.0 mM IPTG (*lanes 3* and *4*) and electrophoresed through a 15% polyacrylamide gel. The *arrow* on the *right* indicates the position of the expressed SOD protein bands. Molecular mass size markers were run in the *lane labeled M* and sizes (kDa) are indicated on the *left*.

NC3 (Table I) to express MnSOD-2 in *E. coli* always exhibited lower expression levels with equivalent IPTG concentrations than MnSOD-3. Evidently this is due to the increased size of the RNA transcript at the 3′ end generated by expression from this amplicon. At 1.0 mM IPTG expression of each MnSOD, as exhibited by a pronounced band at approximately 22,200 Da, corresponded to approximately 32% of the total protein as determined by laser densitometry[2] (Fig. 5, *lanes 3* and *4*). When OX326A cells harboring either expression plasmid were placed under induced oxidative stress by the addition of 250 μM methyl viologen to exponentially growing cultures, induction with 0.1 mM IPTG (to levels exhibited in Fig. 5 *lanes 1* and *2*) was sufficient to allow the cells to recover to approximately half the growth rate exhibited without methyl viologen. Increasing the amount of IPTG to cultures under these conditions (to 1.0 mM) restored the growth rates to almost the same level as exhibited without methyl viologen[2] (or without IPTG in the absence of methyl viologen). These results indicate that each MnSOD from *C. elegans* can protect *E. coli* cells *in vivo* from the

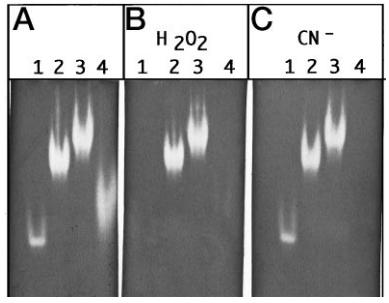

FIG. 6. **Native PAGE of SOD proteins stained for SOD activity.** SOD proteins were electrophoresed through 8% polyacrylamide gels and stained for SOD activity. SOD inhibitors (none, *A*; hydrogen peroxide, 5 mM, *B*; or potassium cyanide, 10 mM, *C*) were added during incubation in NBT. Sample lanes correspond to *E. coli* FeSOD (Sigma, 5 μg, *lane 1*), *C. elegans sod-2* expressed from pTrcsod-2 in *E. coli* OX326A (10 μg, *lane 2*), *C. elegans sod-3* expressed from pTrcsod-3 in *E. coli* OX326A (10 μg, *lane 3*), and bovine Cu/ZnSOD (Sigma, 5 μg, *lane 4*) in each panel. *Lanes 2* and *3* contained the same samples as *lanes 3* and *4*, Fig. 5.

detrimental effects of the intracellularly superoxide-producing herbicide methyl viologen. As each MnSOD was obviously active *in vivo,* we proceeded to further characterize each protein.

Characterization of Mature MnSOD-2 and MnSOD-3—The observed protein expression products shown in Fig. 5 (*lanes 1–4*) are in excellent agreement with the calculated molecular masses of the predicted mature proteins MnSOD-2 (21,986 Da, 192 amino acids) and MnSOD-3 (22,197 Da, 194 amino acids).

The quaternary structure of each protein was determined by gel filtration. The measured molecular masses of 44,961 and 47,279 Da for MnSOD-2 and MnSOD-3, respectively, indicate that when expressed in *E. coli* each protein is an active dimer.

The migration patterns of each of the MnSODs on native 8% PAGE was shown to be capable of discriminating between the two enzymes. MnSOD-2 had a retardation factor (R_F) of 0.365 and MnSOD-3 of 0.250 (Fig. 6, *lanes 2* and *3*, respectively). Furthermore, the insensitivity of each MnSOD toward the SOD inhibitors hydrogen peroxide and potassium cyanide was as expected for MnSODs (Fig. 6, *panels A–C, lanes 2* and *3*) (7). The former is known to inhibit all FeSODs and Cu/ZnSODs (Fig. 6, *panels A* and *B, lanes 1* and *4*, respectively) and the latter only Cu/ZnSODs (Fig. 6, *panels A* and *C, lane 4*).

Isoelectric focusing proved conclusively that each MnSOD protein was inherently different. MnSOD-2 exhibited a pI of 6.5 and MnSOD-3 a pI of 7.3 (Fig. 7, *lanes 2* and *1*, respectively). Two other minor SOD activity bands were also observed for each MnSOD as was a minor band for purified MnSOD from *E. coli*. Multiple bands in isoelectric focusing gels are commonplace for SODs and have been observed in *C. elegans* (51). Possible causes include protein conformers, aggregates, proteolysis, and interactions with the gel buffer constituents. No definitive study has been made of this apparently anomalous behavior.

Spectrophotometric assay of SOD activity revealed that the *C. elegans* MnSODs were not significantly different in terms of their specific activities. In crude extracts from *E. coli* expressing the *C. elegans* SODs, MnSOD-2 was determined to have a specific activity of 2516 units/mg protein and MnSOD-3 2731 units/mg protein.

DISCUSSION

In all aerobic organisms, SOD is required to combat the detrimental effects of the superoxide anion, itself a product of the reduction of molecular oxygen. In *C. elegans* protein extracts, only Cu/ZnSOD has been observed by activity stained native PAGE gels and has been partially characterized (51). Clones obtained from a cDNA library illustrate that the MnSODs are indeed both expressed even under normal oxidative

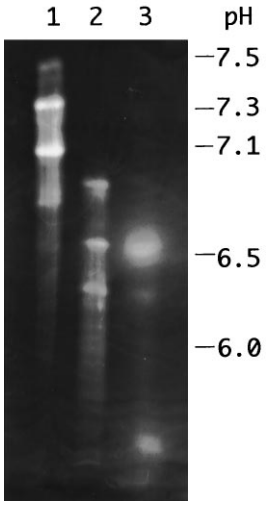

FIG. 7. **Isoelectric focusing of SOD proteins.** SOD proteins were electrophoresed through a precast 5% polyacrylamide gel containing ampholines to generate a gradient after 2.5 h electrofocusing of pH 5.5–8.5. After electrofocusing the gel was stained for SOD activity (see "Experimental Procedures"). Total cell protein from *E. coli* OX326A overexpressing the *C. elegans* MnSODs was loaded in *lane 1* (20 μg, SOD-3) and *lane 2* (20 μg, SOD-2). *Lane 3* contained MnSOD purified from *E. coli* (4 μg, Sigma). After electrofocusing, a pH surface electrode was used to record the pH at various intervals along the gel and confirmed the positions of marker proteins whose pI values are indicated to the *right* of the figure.

conditions. Furthermore, Northern blots have characterized the primary transcripts as mono-cistronic. RT-PCR has confirmed this by demonstrating that each is *trans*-spliced to an SL-1 leader sequence which is usually associated with monocistronic transcripts (52). Presumably, the levels of each protein are simply too low to be detected or definitively identified by the traditional techniques employed. Estimates of the proportion of MnSOD activity in crude extracts of wild-type strains range from one-fifth (53) to one- fiftieth (29) of the total spectrophometrically measured SOD activity. By characterization of the MnSOD proteins we hope to be able to study further their expression *in vivo* in *C. elegans.*

Although the presence of multiple genes appears to be a commonplace phenomenon in *C. elegans* (54, 55), multiple gene copies of MnSOD are rare in other organisms. An exception is *Zea mays* which has four distinct MnSOD genes (56). There is also experimental evidence of the existence of two MnSOD genes per haploid rat genome (57). Both the human and bovine MnSOD genes on the other hand are present as single copies but appear to encode multiple polyadenylation sites resulting in multiple RNA transcripts of widely different sizes (58, 59). Furthermore, analysis of the expression of murine MnSOD revealed two RNA transcripts resulting from multiple transcriptional start sites (60). This does not appear to be the case for either of the *C. elegans* MnSODs.

Although only one *C.elegans* Cu/ZnSOD has so far been cloned and characterized (30, 61), the nematode genome sequencing project has identified two other Cu/ZnSOD genes. These are presumably extracellular Cu/ZnSOD proteins as the deduced protein sequences contain N-terminal signal peptides (not present in other Cu/ZnSODs) for cellular export or membrane anchoring. It has not yet been determined whether these are expressed in the nematode, although we have affirmative results by RT-PCR for the expression of Cu/ZnSOD encoded by the gene F55H2.1.[2]

Prediction of the relative importance of each MnSOD is difficult as there do not appear to be significantly distinct differences between either gene or protein sequences. Eukaryotic MnSODs are mitochondrial, and both MnSOD-2 and MnSOD-3 contain the expected mitochondrial transit peptide sequences at their N termini (Table III). The measured specific activities of the two proteins are very similar as is their gross behavior toward the SOD inhibitors hydrogen peroxide and potassium cyanide (Fig. 6). Each appears to be dimeric in nature but may be distinguished by their net electrical charge. MnSOD-2 has a measured pI of 6.5 and MnSOD-3 of 7.1 (Fig. 7). Differences between the highly homologous FeSOD and MnSOD of *E. coli* have been shown to determine a bias in the protective roles played by each of these enzymes (62). The FeSOD enzyme has been shown to preferentially protect the protein components of the cell against oxidative damage, whereas the MnSOD has been shown to preferentially protect DNA. This has been attributed to an increased positive charge present on the surface of the MnSOD protein. Whether such physical differences observed between the MnSODs of *C. elegans* result in a similarly preferential role for each within the mitochondrion is worthy of further examination. Each of the MnSODs studied in this work protected SOD-deficient *E. coli* cells from the effects of chemically induced oxidative stress to the same degree, which is indicative of their similar specific activities (63).

The two MnSOD genes *sod-2* and *sod-3* have been mapped to chromosomes I and X, respectively (Fig. 2*A*). However, neither this result nor an analysis of possible preferential codon usage or bias indicated any possible differences between the genes which would demonstrate an increased expression of one compared with the other. It is known that proteins encoded on the X chromosome can exhibit a gene dosage effect on expression levels between males and hermaphrodites in *C. elegans* (64–66), but any such effect has yet to be elucidated for the two MnSODs. Further characterization of these genes *in vivo* in *C. elegans* may also be compounded by the possible existence of a third MnSOD gene, evidence for which we have presented (Fig. 2). So far efforts to obtain such a third MnSOD from a cDNA library by degenerate PCR have failed, in itself a result which may be indicative of the presence of an unexpressed pseudogene as suggested by Northern blot analysis (Fig. 2*C*).

SOD expression has been linked with longevity in several organisms (24, 25, 28). The mutant *C. elegans age-1*, which exhibits an increased life expectancy, has indeed been shown to increase the levels of expression of this antioxidant enzyme in adult tissue by 50-fold (25, 29, 67). However, characterization of precisely which SOD(s) has not yet been established. No apparent significant effect on levels of MnSOD activity has been observed (29) in this strain. In addition, Ishii *et al.* (31) isolated a mutant, *mev-1*, which is four times more sensitive to methyl viologen than the wild-type and exhibits half the activity of Cu/ZnSOD compared with wild-type. Under certain conditions such as starvation, *C. elegans* can form dauer larvae which enter a resting phase for long periods which may be followed by complete recovery. The SOD activity in this stage was shown to be 4-fold higher than in young adults (68). Protection against oxidants and the overexpression of SOD during dauer larvae formation is another interesting facet of this enzyme's role in antioxidant defense. Darr and Fridovich (69) demonstrated that young *C. elegans* adapt to oxidative stress by increasing their content of SOD, whereas older worms fail to do so. This, as suggested by the authors, could contribute to an age-related acceleration of senescence. However, other studies have failed to detect age-dependent alteration of SOD activity in wild-type strains (29, 30).

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