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ISOLATION AND NUCLEOTIDE SEQUENCE OF RAT Cu/Zn SUPEROXIDE DISMUTASE cDNA CLONES

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Superoxide dismutase (SOD: EC 1.15.1.1) catalyzes the dismutation of oxygen radicals and is thought to protect cells against free radical damage. We have isolated and sequenced cDNA clones of the rat Cu/Zn SOD, and have used these clones to map the rat genomic sequences coding for this enzyme. Rat Cu/Zn SOD is coded for by a single copy gene which is transcribed into an mRNA species of approximately 800 bases. Comparison of the nucleotide sequences of rat and human SOD cDNAs shows that they are homologous over 83% of the coding sequences and that in the 3'-untranslated region the extent of homology drops to 66%. The predicted rat SOD amino acid sequence is very similar to that of other eukaryotic SODs, showing 70% homology with the SODs of other mammals. Sequence conservation is particularly high in domains believed to be of functional importance.

KEY WORDS: cDNA libraries, nucleotide sequencing, mRNA.

INTRODUCTION

Oxygen radicals generated in aerobic organisms during respiration can cause generalized cell damage through the oxidation of target macromolecules, viz. DNA, proteins, and lipids. This damage has been linked to irreversible biological consequences, such as chromosomal aberrations, carcinogenesis and senescence.[1,2] The scavanging action of superoxide dismutase (SOD)[3] protects the cells against the damaging effects of the superoxide radical[2,4,5] by catalyzing the redox of superoxide anion into peroxide and molecular oxygen. Eukaryotic organisms have two distinct forms of SOD, a mitochondrial Mn-containing enzyme and a cytosolic Cu/Zn-containing enzyme.[6] The amino acid sequence of the human Cu/Zn SOD is established[7,8] and cDNA clones of the gene have been isolated and sequenced[9,10] and used to characterize the expression of the human Cu/Zn SOD gene.[11]

In studies on aging, a correlation has been found between a species maximum lifespan potential and SOD tissue levels,[1] strongly suggesting that SOD may play an important role in slowing down the senescence process. This observation has lent support to the free radical theory of aging.[12] In agreement with this theory, the specific activity of Cu/Zn SOD has been found to decrease with age in mouse and rat tissues.[13,14]

To study the regulation of SOD gene expression during aging in rat, we have isolated Cu/Zn SOD recombinant DNA clones from an expression λgt_{11} library of rat

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chondrosarcoma cDNA. In this paper we present the nucleotide sequence of the entire coding region of rat Cu/Zn SOD plus 62 nucleotides from the 3′-untranslated mRNA. Comparison to human SOD cDNA shows that the two coding regions are over 80% homologous. The predicted amino acid sequence of the rat enzyme is very similar to other known mammalian Cu/Zn SODs. The gene is shown to be present as a single copy in the rat genome, with no evidence of the presence of pseudogenes. It produces a single mRNA species of about 800 bases.

MATERIALS AND METHODS

Construction and Screening of λgt_{11} Libraries

Recombinant cDNA libraries of rat mRNA were constructed essentially as described[15,16] using bacteriophage λgt_{11} as a vector. Two different libraries were screened for SOD-1 cDNA clones. One was constructed using mRNA from the Swarm rat chondrosarcoma cell line and was a gift from Drs. Pilar Fernandez and Yoshihiko Yamada. The other was constructed from mRNA of the rat insulinoma cell line RINm5F.[17] Briefly, RNA was extracted from RINm5F cells by the guanidium thiocyanate method,[18] pelleted through CsCl and poly A containing mRNA was isolated by one cycle of oligo dT-cellulose chromatography. Double-stranded cDNA was prepared with reverse transcriptase and the Klenow fragment of *E. coli* DNA polymerase I, using the conditions provided by the manufacturers (Bethesda Research Laboratories). Following hairpin digestion with S1 nuclease and methylation of possible EcoRI sites, EcoRI linkers were ligated to the ends of the cDNA. The preparation was digested with EcoRI and DNA molecules larger than 200 bp were selected by electrophoresis in 1.4% agarose gels. The DNA was ligated to the dephosphorylated arms of λgt_{11} (Promega Biotech.) and packaged into viable phage particles using commercially available packaging extracts (Vector Cloning Systems). Approximately 2×10^7 recombinant clones were obtained per microgram of cDNA.

The libraries were screened for the presence of rat Cu/Zn SOD-1 cDNA clones by hybridization with various restriction endonuclease fragments of the human SOD cDNA plasmid pUC61-10[9], kindly provided by Dr. Yoram Groner. Positive clones were plaque-purified, tested by restriction enzyme analyses for insert size, and subcloned in bacteriophage M13 for sequencing.

DNA Sequencing

The DNA inserts of selected λgt_{11} clones were subcloned into the mp18 and mp19 derivatives of bacteriophage M13 by standard techniques.[19] Initial M13 clones were prepared using the EcoRI sites of both vector and insert DNA. Derivatives of these subclones, containing shorter inserts were constructed using restriction enzyme sites determined during sequencing of the larger inserts. Nucleotide sequences were determined by the dideoxy chain termination method,[20] using [^{35}S]dATP as the labeled precursor, and the sequencing kit available from International Biotechnologies, Inc.

Restriction Enzyme Cleavage, Agarose Gel Electrophoresis and Southern and Northern Blots

DNA preparations were digested with the appropriate restriction enzymes in T4 DNA polymerase salts.[21] High molecular weight genomic DNA from adult Fisher rat liver

or from tissue culture RINm5F cells was prepared as described.[22] DNA fragments were separated by agarose gel electrophoresis and blotted onto nitrocellulose paper.[23] Polyadenylated mRNA species were separated in formaldehyde agarose gels and transferred to nitrocellulose.[24]

Labeling of Hybridization Probes

Short DNA fragments (25–150 bp) used to screen the libraries were end-labeled using the terminal nucleotide exchange activity of T4 DNA polymerase.[25] Thus, Hae III fragments having 5′-dG residues were labeled with [^{32}P] dGTP (3000 Ci/m mole) and 2 units of T4 DNA polymerase at a molar ratio of 1:2 (DNA:dGTP). This resulted in specific activities of $1 \times 10^6 - 3 \times 10^6$ cpm/pmole of DNA or $1 \times 10^7 - 3 \times 10^7$ cpm/μg of a 150 bp fragment, sufficient for several screenings. Larger DNA fragments, up to 600 bp in length, were labeled by limited digestion and resynthesis with T4 DNA polymerase. Briefly, 100–500 ng of plasmid DNA containing the insert to be labeled were linearized at a site near the insert. Polymerase was added in the absence of dNTPs for 20–30 min at 37°C to allow for exonucleolytic digestion of several hundred nucleotides, after which the reaction mixture was supplemented with 250 μM dATP and dTTP and 1 μM [^{32}P]dGTP and [^{32}P]dCTP. After 15 minutes at 37°C, the reaction was chased with 250 μM of unlabeled dCTP and dGTP and continued for an additional 15 minutes. The DNA was digested with a second restriction enzyme that would cut the insert from the vector, and the fragments produced were separated in low melting point agarose gels. Agarose slices were cut from the gel and portions melted at 100°C for 10 minutes as needed for hybridization. With this method, specific activities of 2×10^8 to 5×10^8 cpm/μg were routinely obtained. Hybridizations of plaque lifts and Southern blots were done in 5 X SET (1 X SET = 0.15 M NaCl, 0.03 M Tris-HCl pH 8.0, 2 mM EDTA), 2 X Denhardt's solution (0.04% BSA, 0.04% polyvinylpyrrolidone, 0.04% Ficoll), 01% SDS and 25–50 μg/ml *E. coli* DNA, for 12–18 hours at 65–70°C, with the exception of screenings with a 25-mer oligonucleotide, that were done at 30°C. For Northern blots the above buffer also contained 50% deionized formamide and 50 mM sodium phosphate buffer, pH 6.8, and hybridization was carried out at 45°C. Blots were washed in 0.1 X SET, 0.1% SDS at 50–60°C. Filters hybridized with the 25-mer oligonucleotide were washed in 2 X SET and autoradiographed several times after washes at increasing stringencies, beginning at 5°C above the hybridization temperature. After exposure, filters were washed again at 5°C above the prior wash and reexposed. This process was repeated until all non-specific hybridization was eliminated. Autoradiography was on Kodak XAR-5 film, with intensifying screens when needed.

Computer Software

Computer analyses and assembly of nucleotide sequences utilized programs written by Staden[26,27] and Brutlag *et al.*[28] Optimal alignments of protein sequences were obtained using PRTALN[29] and SEQA.[30]

RESULTS

Isolation and Nucleotide Sequence of Rat Cu/Zn SOD-1 cDNA Clones

To select SOD-1 cDNA clones from a λgt_{11} library of rat chondrosarcoma mRNA,

we initially used the insert of the human Cu/Zn SOD-1 cDNA clone pUC61-10 as a hybridization probe. Approximately 1% of the clones screened showed positive hybridization, a number that seemed unusually large if it were to correspond to the actual abundance of Cu/Zn SOD-1 mRNA in the total mRNA population. We suspected that either the presence of oligo-dC and oligo-dG tails at the ends of the cDNA, or cross-hybridization with related sequences, or both, were responsible for the large number of clones detected. Forty randomly-chosen positive clones were plaque-purified, their inserts mapped with several restriction enzymes and hybridized in Southern blots to the human cDNA probe. None of the clones isolated showed mapping and hybridization data consistent with the presence of a true rat Cu/Zn SOD-1 DNA insert.

As an alternative approach, we selected as probes regions of the human SOD cDNA more likely to be conserved among species. First, three regions of amino acid sequence conservation were determined by comparison of the bovine,[31] equine[32] and human[7,8,10] enzymes using the PRTALN algorithm.[29] These regions are located at the carboxyl- and amino-termini and in the interior of the polypeptide moiety (data not shown). Second, restriction enzyme fragments of the human cDNA clone corresponding to these regions of sequence conservation were generated by Hae III digestion. The three fragments prepared were: a 25 bp-long fragment (position 45 to 70) from the 5′ end; an internal 64 bp fragment (from residue 254 to 318); and a 146 bp-long fragment from the 3′ end (from residue 376 to 522). These fragments are highlighted in *white letters on black background* in the human sequence shown in Figure 2.

The rat chondrosarcoma library was re-screened with these three fragments as probes, and the results were quite different from those obtained with the full length human cDNA. Only 0.01% of the clones; hybridized with the 3′ end probe. Of a total of 26 positives, 13 were also positive using the internal 64 bp fragment as a probe. Of these, only one hybridized to the 25 bp 5′ end probe.

The DNA insert in one representative of each of the three groups of clones was excised with EcoRI and subcloned into the mp18 and mp19 derivatives of bacteriophage M13 for sequence determination. λgtRSOD7 contained sequences arising from the 3′ end which were subcloned into M13 as mp18RSOD7; λgtRSOD16 extended to the middle of the mRNA (M13 subclone mp18RSOD16); and λgtRSOD31 contained cDNA sequences up to the 5′ end of the mRNA (subclone mp18RSOD31). Each original M13 clone, and derivatives therefrom generated by shortening of the insert with appropriate restriction enzyumes, were sequenced at least four times in both directions. The complete nucleotide sequence was assembled using Staden's algorithms for random sequencing[26,27] and the contiguity relationships defined by the derivation of each clone.

A single open reading frame 456 nucleotides long was found, coding for a polypeptide of 152 amino acid residues. An additional 62 nucleotides extended beyond the termination codon, ending with 9 A-residues immediately before the EcoRI linker (Fig. 1). Surprisingly, mp18RSOD31, the clone with the longest insert, extending two nucleotides beyond the initiation codon, did not contain 3′ poly A sequences, but ended at an EcoRI restriction site at position 460. This EcoRI site had been spuriously created by linker addition, since it did not appear in the two other independently isolated overlapping clones. We have now reconstructed a complete 539 bp cDNA clone in plasmid pGEM2 (TM, Promega Biotech.) using the unique BstNI site at position 257, common to the inserts in mp18RSOD31 and mp18RSOD16.

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                                                30                                  60
GAATTCCGCG ATG AAG GCC GTG TGC GTG CTG AAG GGC GAC GGT CCG GTG CAG GGC GTC ATT
           Met Lys Ala Val Cys Val Leu Lys Gly Asp Gly Pro Val Gln Gly Val Ile

                                                90                                 120
CAC TTC GAG CAG AAG GCA AGC GGT GAA CCA GTT GTG GTG TCA GGA CAG ATT ACA GGA TTA
His Phe Glu Gln Lys Ala Ser Gly Glu Pro Val Val Val Ser Gly Gln Ile Thr Gly Leu

                                               150                                 180
ACT GAA GGC GAG CAT GGG TTC CAT GTC CAT CAA TAT GGG GAC AAT ACA CAA GGC TGT ACC
Thr Glu Gly Glu His Gly Phe His Val His Gln Tyr Gly Asp Asn Thr Gln Gly Cys Thr

                                               210                                 240
ACT GCA GGA CCT CAT TTT AAT CCT CAC TCT AAG AAA CAT GGC GGT CCA GCG GAT GAA GAG
Thr Ala Gly Pro His Phe Asn Pro His Ser Lys Lys His Gly Gly Pro Ala Asp Glu Glu

                                               270                                 300
AGG CAT GTT GGA GAC CTG GGC AAT GTG GCT GCT GGA AAG GAC GGT GTG GCC AAT GTG TCC
Arg His Val Gly Asp Leu Gly Asn Val Ala Ala Gly Lys Asp Gly Val Ala Asn Val Ser

                                               330                                 360
ATT GAA GAT CGT GTG ATC TCA CTC TCA GGA GAG CAT TCC ATC ATT GGC CGT ACT ATG GTG
Ile Glu Asp Arg Val Ile Ser Leu Ser Gly Glu His Ser Ile Ile Gly Arg Thr Met Val

                                               390                                 420
GTC CAC GAG AAA CAA GAT GAC TTG GGC AAA GGT GGA AAT GAA GAA AGT ACA AAG ACT GGA
Val His Glu Lys Gln Asp Asp Leu Gly Lys Gly Gly Asn Glu Glu Ser Thr Lys Thr Gly

                                               450                                 483
AAT GCT GGA AGC CGC TTG GCT TGT GGT GTG ATT GGG ATT GCC CAA TAA ACGTTCCCTATGTGG
Asn Ala Gly Ser Arg Leu Ala Cys Gly Val Ile Gly Ile Ala Gln

                                               522
GTCTGAGTCTCAGACGGCATCTGCTGCCTGCTAAACTGTAAAAAAAAACGGAATTC
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FIGURE 1 Assembled nucleotide sequence of rat Cu/Zn SOD cDNA and predicted amino acid sequence of the translated protein. Residue 1 is the first residue of the 5′ EcoRI linker. Translation starts at the ATG triplet (residues 11–13) and continues uninterrupted until the stop signal at residues 467–469. The 3′ EcoRI linker is also shown. The three original M13 clones contain the following parts of the sequence: mp18RSOD31, from residue 1 to 460; mp18RSOD16, from residue 190 to 485; mp18RSOD7 from residue 465 to 539.

Alignment of the rat and human Cu/Zn SOD cDNAs revealed a high degree of sequence conservation between the two species (Fig. 2). Homology extends over 83.3% of the coding sequences, although this value drops to 66.2% in the 3′-untranslated region, the most rapidly divergent in gene families.

Comparison with Other Known Cu/Zn SOD Amino Acid Sequences

In addition to man, cow, and horse, the primary structure of Cu/Zn SOD is known in four other eukaryotes: pig,[33] swordfish,[34] yeast,[35,36] and *D. melanogaster*.[37] Homology among the previously known sequences is very high, being highest among the mammals.[33,37] The rat sequence is no exception, sharing 115 of 154 residues (74%) with the other mammals (Fig. 3). The major difference that we find from this conservation pattern occurs at the amino terminus with an apparent deletion of Ala-Thr or Ala-Leu in the rat sequence. Minor differences are found at residue 51, which has Phe in all the other eukaryotes and Tyr in the rat; and at Thr-89 and Asp-91 in the other animals, which are replaced by Ala and Gly, respectively, in the rat.

A comparison of the primary sequence of rat SOD with all other known Cu/Zn SOD enzymes is shown in Figure 3. To optimize congruence for sequence com-

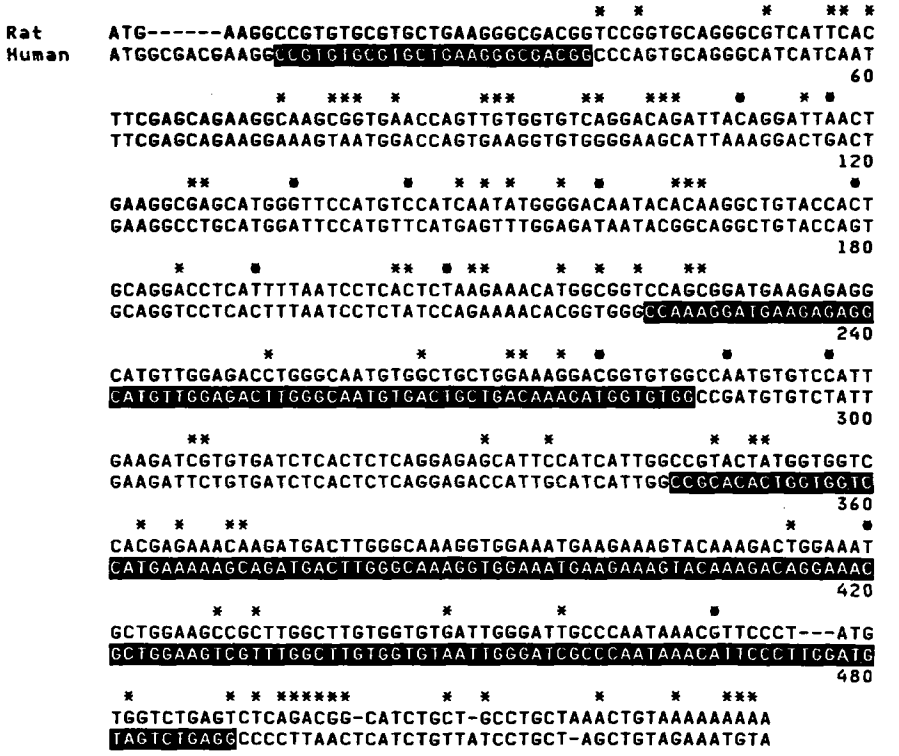

FIGURE 2 Alignment of the nucleotide sequences of human and rat SOD cDNA. The EcoRI linkers at the 5′ and 3′ ends of the rat sequence are not shown. Residue 1 is the first nucleotide in the ATG triplet. Shown in *white letters in black boxes* are the Hae III fragments of the human cDNA used as probes. *Asterisks* denote mismatches in the sequences; gaps introduced to optimize the match are indicated by *hyphens*. The human sequence is taken from ref. 10. The alignment was obtained with the SEQ algorithm.[28]

parisons, gaps were introduced at position 38 in all animals, at positions 18 and 19 in *Drosophila*, at positions 24 and 25 in cow and pig, and at position 103 in the swordfish. Furthermore, the rat sequence was shifted two positions to the right, to account for the two-amino acid deletion; this shift was not compensated by additional gaps anywhere else in the sequence. Figure 3 visually emphasizes the high degree of sequence homology found among the eight known eukaryotic enzymes.

Mapping and Expression of the Rat Cu/Zn SOD Gene

Southern blot hybridizations of restriction endonuclease fragments of adult Fisher rat liver DNA are shown in Figure 4A. The probe for these experiments was the complete 539 bp cDNA clone pG2RSOD reconstructed in plasmid pGEM2. The combination of single and double digestions with five restriction enzymes was sufficient to construct the approximate restriction map shown in Figure 4B. The Cu/Zn SOD gene is present in single copy within the rat genome, with no evidence for the presence of pseudogenes, as discrete single fragments are found for several of the restriction enzymes used. As expected from the presence of a PstI site in the cDNA, this enzyme cuts the

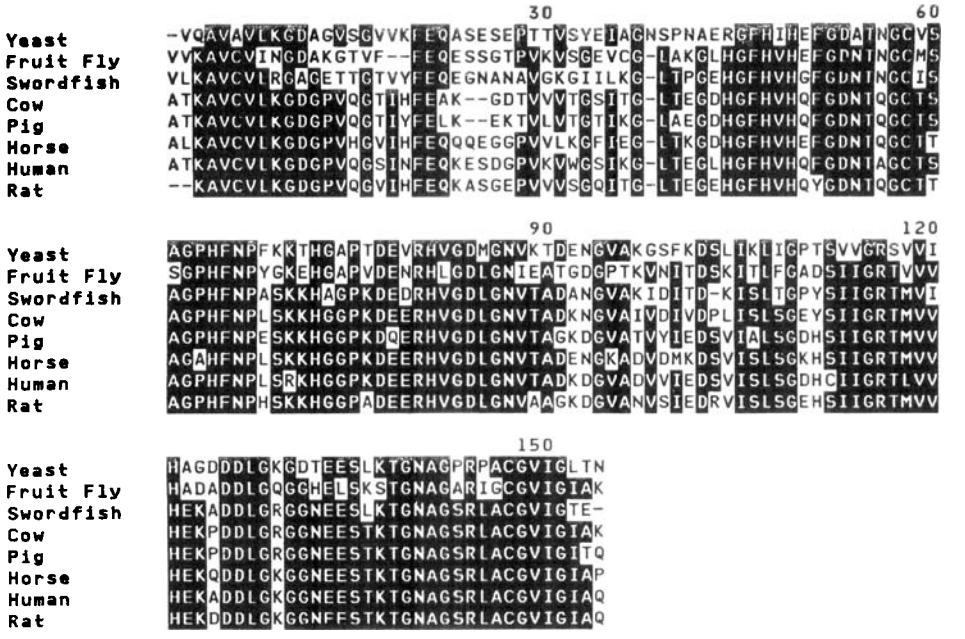

FIGURE 3 Comparison of the amino acid sequence of rat Cu/Zn SOD with all other known eukaryotic Cu/Zn enzymes (see text for references). The *hyphen* denotes gaps introduced to maximize sequence homology. Amino acids shared by five or more of the eight sequences are indicated by *white letters on black background.*

SOD gene producing three fragments, two of which hybridize strongly to the probe and a third that hybridizes weakly. Similarly, two EcoRI fragments hybridize with the probe, although no EcoRI sites are found in the cDNA. We would predict that at least one of the PstI sites, and the EcoRI, BamHI and SacI are internal to the gene and present in one or more introns. A maximum size of 4–5 kb can be calculated from this data for the entire rat Cu/Zn SOD gene.

Hybridization of Northern blots of poly A containing RINm5F mRNA separated in formaldehyde-agarose gels revealed that the SOD-1 mRNA is a single species of ca. 800 bases, by comparison with 28S and 18S rRNA size markers (Fig. 4C). The broad band distribution of the hybridizing mRNA may be indicative of a size variability of $\pm$ 100 bases, but no evidence for two distinct species of SOD-1 mRNA, as is the case in human cells[10,11] was found in rat cells, even after long autoradiographic exposures, or when large amounts (7 μg) of poly A mRNA were loaded on the gels.

DISCUSSION

We have assembled the DNA sequence of the complete rat Cu/Zn SOD coding region with an additional 62 bp from the 3′ untranslated end. The identity of this sequence was established from its homology with the nucleotide sequence of the human cDNA and from the comparison of the predicted amino acid sequence with the sequences of the other known eukaryotic enzymes.

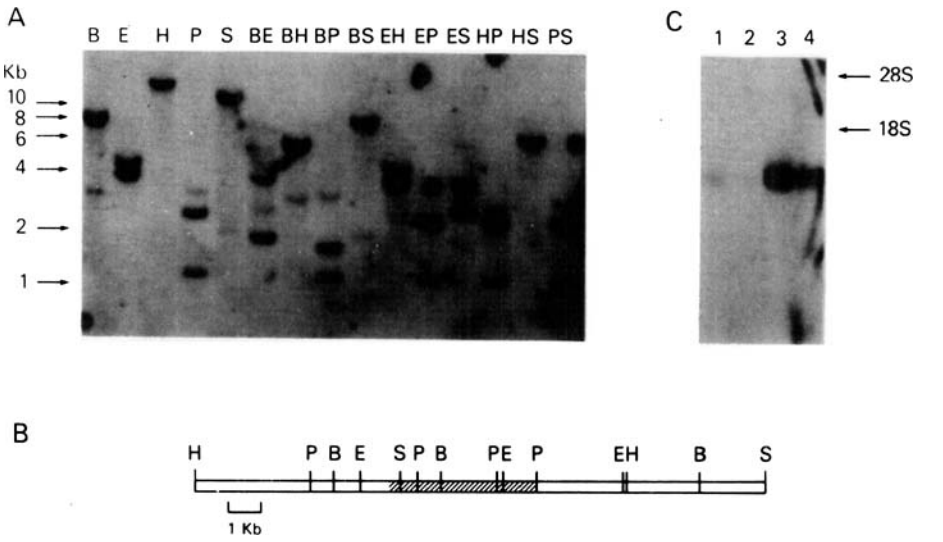

FIGURE 4 Southern and Northern blots and restriction map of the rat Cu/Zn SOD gene. (A) Ten micrograms of rat liver DNA were digested to completion with the restriction enzymes indicated at the top of each lane and electrophoresed in 1% agarose gels. After transfer to nitrocellulose, the blot was hybridized with ^{32}P-labeled SOD cDNA as described in the Methods section. Sizes of the fragments were determined by comparison with the 1 kb marker ladder (Bethesda Research Laboratories), indicated by the arrows. (B) The restriction map was established from the data in (A). The maximum size of the gene is indicated by the shaded area. The right-most end located between an EcoRI and a PstI site cannot be unambiguously placed. B = BamHI; E = EcoRI; H = HindIII; P = PstI; S = SacI. (C) 7 μg (lanes 1 and 3) and 2.5 μg (lanes 2 and 4) of RINm5F poly A mRNA were electrophoresed in 1.5% agarose containing 2.2 M formaldehyde and transferred to nitrocellulose. Hybridization to ^{32}P-SOD cDNA was as described in Methods. The position of 28S and 18S ribosomal RNA markers are indicated by arrows. Autoradiographic exposures were 8 hours (lanes 1 and 2) and 6 days (lanes 3 and 4).

The size of the cDNA is shorter than the size of the mRNA detected in Northern blots by approximately 300 bases. Assuming that the length of the 5′ untranslated region is 80 nucleotides, the same as in the human gene,[39] and that the poly A tail may extend over 100–150 residues, there is still a discrepancy of 50–100 nucleotides. A possible explanation for this discrepancy is that the clones that we isolated had been internally primed at the 9-mer poly A tract found at the 3′ end. This tract may have served as an internal binding site for the oligo dT primer as well as a block for synthesis primed at the true poly A tail. We are presently screening the cDNA library of RINm5F cells in search of longer cDNA clones. Interestingly, we find 10 to 15 times more SOD cDNA clones in this library than in the chondrosarcoma library, perhaps reflecting differences in SOD gene expression between the two cell lines.

Comparison of the primary structure of the eight eukaryotic proteins indicates that SOD is highly conserved. Single amino acid differences from a possible consensus for all mammals[37] found in rat enzyme may be due to experimental errors, although all the clones were sequenced several times and confirmatory data for the complementary strand was always obtained. One of these changes, His for Leu-68, alters the hydrophobicity of this region appreciably (data not shown); two others, Ala for Thr-89 and Gly for Asp-91, may affect the possible beta-sheet character of this domain. These

differences are not unique to the rat enzyme; for instance, the pig sequence has Glu for Leu-68 and Gly for Asp-91; the human enzyme has Arg for Lys-70 and Cys for Ser-112. Most likely these changes represent true interspecies differences, or possibly intraspecies allotypes, as found for the porcine enzyme.[33] On the other hand, all the regions of functional relevance are conserved, such as the metal-liganding segments between residues 49–81, Asp-84, the His residues, and most of the glycine residues, essential for the formation of the β-strand barrel structure characteristic of Cu/Zn SOD.[39,40]

The availability of Cu/Zn SOD cDNA clones from rat makes it possible to study the mechanisms involved in the regulation of SOD gene expression, and to test whether changes in expression of this gene occur during senescence. These studies may help elucidate the role that SOD plays in the aging process.

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