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Multiple mRNA species generated by alternate polyadenylation from the rat manganese superoxide dismutase gene

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ABSTRACT

The mitochondrial enzyme, manganese superoxide dismutase (MnSOD) is an integral component of the cell's defense against superoxide-mediated cellular damage. We have isolated and characterized four cDNA clones and the structural gene for rat MnSOD. Northern analyses using MnSOD cDNA probes detected at least five mRNAs in all tissues and cell types examined. Southern and Northern analysis using a 3′ non-coding sequence probe, common to all the cDNAs, showed hybridization only to genomic restriction fragments that correspond to our genomic clone and the five MnSOD mRNAs. These data demonstrate that all of the rat MnSOD transcripts are derived from a single functional gene. Primer extension data indicate that transcription initiation is clustered within a few bases. Northern analysis using intron probes demonstrates that all five transcripts are fully processed. Northern analysis using cDNA and genomic probes from sequences progressively 3′ to the end of the coding sequence indicates that size heterogeneity in the MnSOD transcripts results from variations in the length of the 3′ non-coding sequence. From this data and the location of potential polyadenylation signals near the expected sites of transcript termination, we conclude that the existence of multiple MnSOD mRNA species originate as the result of alternate polyadenylation.

INTRODUCTION

Exposure of organisms to elevated concentrations of oxygen results in tissue and cellular damage (1). Many of the damaging effects of elevated O_2 concentrations have been attributed to the formation of reactive oxygen species such as superoxide ion (O_2-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH\cdot$) (2). Fridovich has expanded this hypothesis into a superoxide theory of oxygen toxicity which proposes that the formation of superoxide radicals *in vivo* plays a major role in oxygen toxicity (3). The primary cellular defense against the superoxide ion are the superoxide dismutases (SODs)[1], metalloenzymes which dismutate, and therefore scavenge, the superoxide free radical (O_2-) (4). Three forms of SOD, classified by their metal content, are known to exist: iron (FeSOD), copper and zinc (Cu/ZnSOD), and manganese (MnSOD). Prokaryotes generally contain an FeSOD and a MnSOD, though a few species of bacteria contain a Cu/ZnSOD as well (5). Eukaryotes contain a cytosolic Cu/ZnSOD (6), an extracellular Cu/ZnSOD (7), and a MnSOD which is specifically targeted to the mitochondrial matrix (8,9). The localization of the MnSOD exclusively within the mitochondria suggests an important role for this antioxidant enzyme in this organelle. The mitochondrial electron transport system is responsible for a significant proportion of O_2- production in mammalian cells (10), and it has been suggested that the mitochondrial MnSOD serves to protect against free radical damage to this organelle (9).

We have reported that bacterial endotoxin (LPS) selectively induces MnSOD protein, but not Cu/ZnSOD, in human monocytes (11) and bovine pulmonary endothelial cells (12). We have also demonstrated induction of steady-state MnSOD mRNA levels by LPS, interleukin-1 (IL-1) and tumor necrosis factor (TNF) in cultured rat lung epithelial and endothelial cells (13,14). The rapid activation of MnSOD in response to these three inflammatory mediators strongly supports an involvement of MnSOD in the acute inflammatory response. Further evidence for this role comes from our observation that IL-6, the primary cytokine responsible for acute phase gene induction in liver (15), stimulates MnSOD steady-state mRNA levels in isolated rat hepatocytes (16).

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Previously, two cDNA clones for rat MnSOD (17), as well as a corresponding genomic clone were isolated (18). Ho et al. (18) have proposed that the rat genome contains two copies of the MnSOD gene, which both may contribute to the expression of multiple species of MnSOD mRNA. However, several other mechanisms could account for the origin of these messages, including: utilization of multiple sites of transcription initiation, differential splicing of the mRNA, or use of different sites of polyadenylation at the 3′ end of the mRNA. Here, we report the isolation and characterization of three additional cDNA clones exhibiting heterogeneity in the size of the 3′ nontranslated termini. A combination of Northern and Southern analysis revealed that all detectable mRNA transcripts are derived from a single function gene with one copy per rat genome, and that message size heterogeneity results from an alternative polyadenylation mechanism.

MATERIALS AND METHODS

cDNA Library Screening

A lambda gt11 rat liver cDNA library (Clonetech Laboratories, Inc., Palo Alto, CA) was screened for the expression of β-galactosidase-MnSOD fusion protein with polyclonal antisera raised against rat MnSOD (19−21). Four positive plaques were identified using immunoscreening and/or by hybridization with one of the positive rat MnSOD cDNAs, which had been radiolabeled by the random primer extension method (22). These cDNAs were inserted into pUC19 and M13 for characterization by restriction mapping and sequencing (21,23).

Genomic Library Screening

A lambda Charon 4A rat genomic library (kindly provided by Dr. Thomas Sargent) was screened for MnSOD DNA sequence using a cDNA probe (21). The library was constructed from Sprague-Dawley rat genomic DNA that had been partially cleaved with EcoRI. Phage were plated on *E. coli* Y1088 and screened with a radiolabeled MnSOD cDNA probe (22). A single positive phage plaque was isolated, the genomic insert was excised with EcoRI and inserted into the EcoRI site of pUC19.

To characterize the structure of the MnSOD genomic clone, all exon containing restriction fragments were identified by Southern blot analysis with cDNA probes or oligonucleotides complementary to cDNA sequence. These fragments were ligated into the SmaI site of M13 mp18 and the sequence determined by the method of dideoxynucleotide chain termination (23,24). The sequences were analyzed with the aid of the Beckman Microgenie program.

RNA Isolation and Northern Analysis

Total RNA was isolated from rat tissues by the acid guanidinium thiocyanate-phenol-chloroform extraction method as described previously (25) with modifications (13). Twenty μg of total cellular RNA was denatured in 25 μl and fractionated by size on a 1% agarose/formaldehyde gel (21). The RNA was electrotransferred to a noncharged nylon membrane (Genescreen, DuPont-New England Nuclear, Boston, MA) and covalently cross-linked to the membrane with UV light (26). The membranes were hybridized with a radiolabeled rat MnSOD cDNA probe (22) and washed in 0.04 M sodium phosphate and 0.1% SDS at 65°C and then subjected to autoradiography using an intensifying screen at −85°C (26).

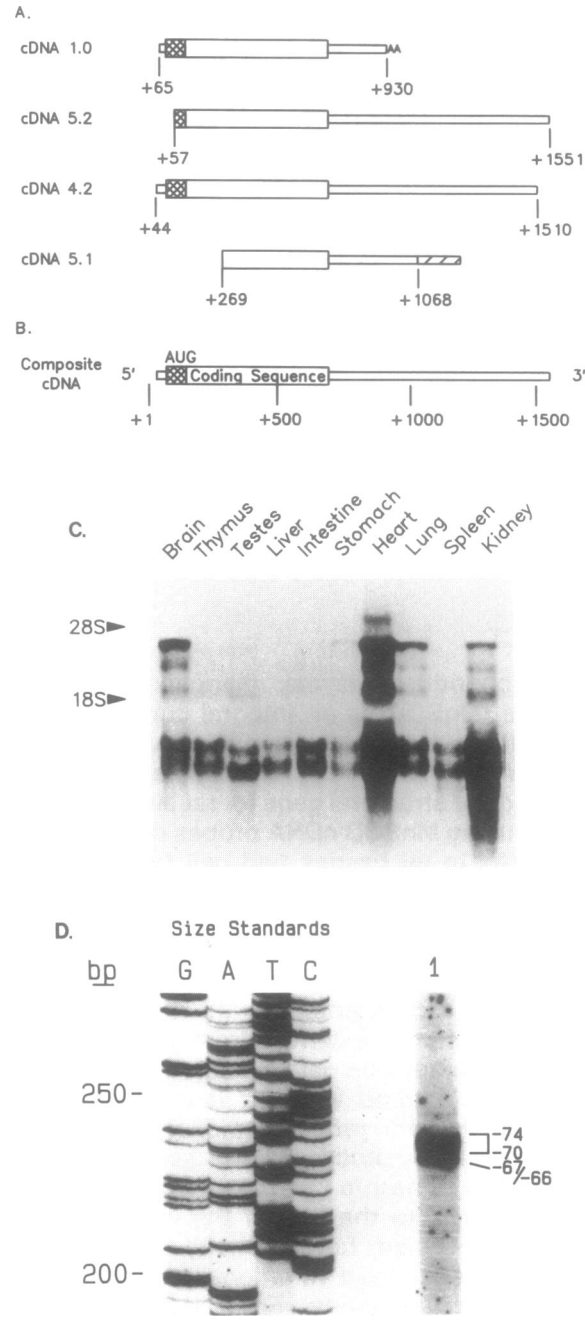
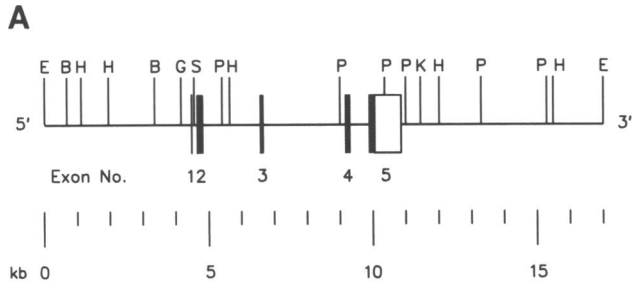


Figure 1. The cDNAs and mRNA transcripts for MnSOD. (A) The size and structures of the isolated cDNA clones. The coding regions for the mature polypeptide are represented by the large open boxes, the coding region for the putative mitochondrial targeting signal is indicated by the cross hatched boxes. 5′ and 3′ non-coding sequences are represented by smaller open boxes. Clone 1.0 contains a 48 bp Poly (A) tail represented by the 'AA' at its 3′ terminus. All the sequences from the cDNA clones are identical, except for a 157 base pair divergent sequence at the 3′ end of clone 5.1 (represented by a striped box). Nucleotides are numbered with +1 being the start of transcription (74 base pairs 5′ to the first AUG). (B) The composite structure of the rat MnSOD cDNAs based on sequences common to all the cDNA clones. (C) Northern analysis of total cellular RNA isolated from adult rat tissues using cDNA 1.0 as a probe. The positions of the 18S and 28S ribosomal RNAs are shown to the left. (D) A synthetic oligonucleotide (45 nucleotides) complementary to nucleotide positions +120 to +164 of the 1st exon of the MnSOD gene was used for primer extension analysis (see 'Materials and Methods'). As a negative control, primer extension was performed using yeast tRNA as a substrate, and no reaction products were detected (data not shown). The size standards were prepared by dideoxy sequencing of a known recombinant M13 clone.

Primer Extension

Total cellular RNA from heart tissue was used as the template for primer extension analysis (21). The RNA (50 μg) was annealed to 2 pmoles of a [^{32}P]-radiolabeled oligonucleotide (complementary to nucleotides +120 to +164) in a buffer containing 0.4 M NaCl, 40 mM Pipes (pH 6.4), and 1 mM EDTA at 65°C for 2 h. The samples were precipitated with ethanol, dried, and resuspended in 60 μl of a buffer containing 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 1 mM each of the deoxynucleoside triphosphates, 50 μg/ml actinomycin-D, and 400 units of Moloney Murine Leukemia Virus Reverse Transcriptase (BRL), and incubated at 42°C for 2 h. The reaction was terminated by incubation in 0.6 M NaOH/50 mM EDTA for 1 h at 65°C, neutralized, and precipitated with ethanol. The sample was resuspended in a formamide dye mix and subjected to electrophoresis on a 6% polyacrylamide/7 M urea denaturing gel. Size standards were generated by dideoxy sequencing of a known M13 recombinant clone.



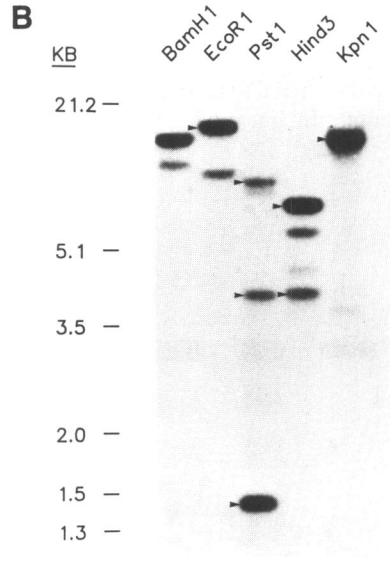


Figure 2. Comparison of the isolated rat MnSOD gene to a rat genomic Southern. (A) The size and restriction map of the genomic fragment containing the rat MnSOD gene. Five exons, identified from cDNA sequence, are represented by solid boxes. The open box at the end of exon 5 corresponds to untranslated sequence contained in cDNA clone 5.2. (B) Southern blot analysis of a rat genomic DNA using cDNA 1.0 as a probe. Arrowheads identify genomic DNA fragments whose hybridization to the cDNA probe correlates to the restriction map of the genomic clone. Position of size markers are shown to the left. E, EcoRI; B, BamHI; H, HindIII; G, BglI; S, SmaI; P, PstI; K, KpnI.

Isolation of Genomic DNA and Southern Blotting

Genomic DNA was isolated from adult male Sprague-Dawley rats utilizing standard techniques (21). For Southern analysis (21), 10 μg of genomic DNA was digested with a variety of restriction endonucleases and size-fractionated on a 1% agarose gel. The DNA was transferred to a nylon membrane and covalently crosslinked by UV light. Hybridization with radiolabeled probes are as described for Northern analysis.

RESULTS

The size and orientation of each cDNA clone are depicted above a diagram of a composite MnSOD cDNA (Figure 1A and 1B). The cDNAs all share identical protein-coding sequences, but possess differences in the size and sequence of their 3′ nontranslated regions. The 3′ end of clone 1.0 contains 190 bp of untranslated sequence ending with a poly $(A)^+$ tail of 48 bp. The 3′ end of clone 4.2 contains 756 bp of 3′ untranslated sequence, while lacking a poly $(A)^+$ tail. The 3′ terminus of clone 5.2 was identical to that of 4.2, except for an additional 70 bp. Clone 5.1 shared sequence identity with clones 4.2 and 5.2 until position +994, where the next 151 bp diverged completely with all other MnSOD cDNA sequences. Ho and Crapo (17) have reported the sequence of two MnSOD cDNA clones, one similar to our clone 5.1, and the other containing sequences contiguous to the sequences in our larger cDNAs. However, neither of the cDNA clones identified by Ho and Crapo (17) contained a poly $(A)^+$ tail.

With cDNA probes available, we determined whether the basal levels of MnSOD mRNA showed tissue specific expression in adult rat tissues by Northern analysis of total cellular RNA.

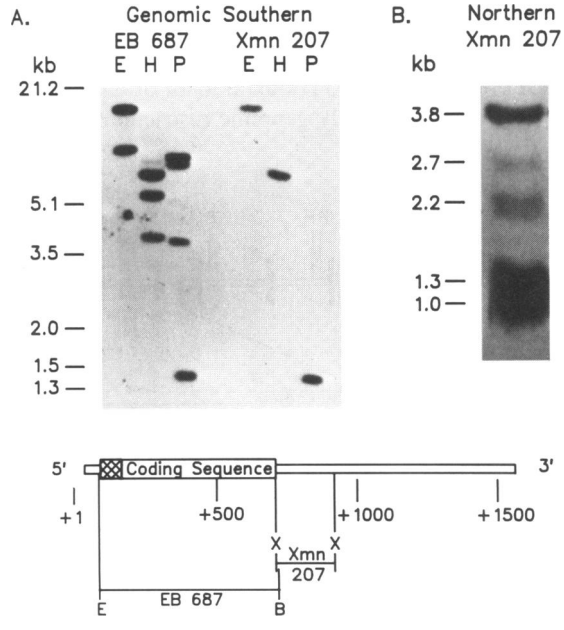


Figure 3. Comparison of Southern and Northern analysis using a non-coding sequence cDNA probe. (A) Southern analysis of rat genomic DNA using a coding sequence probe, EB 687, and a 3′ non-coding sequence probe, Xmn 207. Size markers are shown to the left. (B) Northern analysis of total cellular mRNA using the Xmn 207 probe. The sizes of each MnSOD mRNA transcripts are shown to the left. The location of the cDNA probes sequences are shown below the composite cDNA. E, EcoRI; X, XmnI.

Hybridization revealed the same five MnSOD mRNA species, with the quantity and ratio of the five transcripts varying between the tissues tested (Figure 1C).

Based on the sequence of our four MnSOD cDNAs, we were able to extend the previously published sequence at the 5′ end by 19 additional bases (17). To verify if the 5′ untranslated sequence of our longest cDNA begins at the transcriptional start site of the gene, we utilized a ^{32}P-labeled oligonucleotide complementary to nucleotides +120 to +164 (relative to the AUG start codon) for primer extension analysis. Total cellular RNA from heart tissue was used as the template, because the steady state levels of MnSOD mRNA is highest in this tissue (Figure 1C). Five major primer extension products were observed with lengths corresponding to transcriptional initiation sites between positions −70 to −74 (Figure 1D) yielding an additional 44 bp compared to the 5′ end of cDNA clone 4.2. We also observed minor extension products ending at positions −67, and −66.

The structure and restriction map of the isolated genomic clone is depicted in Figure 2A. To determine the copy number of the MnSOD gene in the rat genome, Southern analysis of rat liver DNA was performed using cDNA 1.0 as a probe. KpnI, BamHI, and EcoRI digested rat liver genomic DNA showed strongly hybridizing bands at 14.5, 15, and 17 kb respectfully (Figure 2B). The sizes of these restriction fragments were consistent with Southern analysis of the MnSOD genomic clone digested with the same enzymes (data not shown). However, additional weakly hybridizing bands were detected in each genomic digest that do not correlate with the restriction map of our genomic clone.

To determine whether the MnSOD transcripts originate from a single gene, cDNA probes were used for Southern and Northern analysis. The left side of Figure 3A illustrates a genomic Southern following hybridization with a cDNA probe containing only coding sequence (EB 687). As in Figure 2B both bands corresponding to our genomic clone and the additional weakly hybridizing fragments are detected. However, a 3′ non-coding sequence probe (Xmn 207) common to all of our cDNA clones shows hybridization to only genomic restriction fragments that correlate to our genomic clone. The weakly hybridizing genomic fragments were also not detected using probes derived either from intron 2 or a restriction fragment encompassing genomic sequence from 280 bp 5′ to first codon and extending 30 bp into exon 2 of MnSOD gene (data not shown). Like the coding sequence probe (Figure 4, lane 1) all five MnSOD transcripts hybridized to the Xmn 207 fragment using Northern analysis (Figure 3B).

Additional mechanisms that may lead to the generation of the five MnSOD mRNA transcripts include: 1) multiple transcriptional initiation sites; 2) alternative or incomplete splicing; or 3) differential polyadenylation. Primer extension analysis indicates that transcriptional initiation is clustered between 74 to 66 base pairs 5′ to the start of translation (Figure 1D). Northern analysis using probes derived from the three largest introns (introns 2, 3, and 4) showed no hybridization to any of the MnSOD transcripts (data not shown).

To test the hypothesis that the larger transcripts were produced by variation in the length of their 3′ non-coding sequence, separate lanes of total cellular RNA were hybridized to probes derived from the 3′ non-coding ends of the cDNA and genomic clones by Northern analysis (Figure 4A). As mentioned previously, the Xmn 207 probe, which begins 747 bp 3′ to the start of transcription, hybridized to all five transcripts (Figure 3B). Probe 2 begins 853 bp 3′ to the start of transcription, and failed to hybridize to the 1.0 kb message (Figure 4B, lane 2). Probe 3, which begins 954 bp 3′ to the transcription start, did not hybridization with either the 1.0 or 1.3 kb messages (Figure 4B, lane 3). Probe 4 begins approximately 2.2 kb 3′ to the transcription start site and hybridized only to the 2.7 and 3.8 kb transcripts (Figure 4B, lanes 4). Finally, by using probe 5, which begins approximately 3.5 kb 3′ to the transcription start site, only the 3.8 kb mRNA was detected (Figure 4B, lane 5).

DISCUSSION

We report the isolation and characterization of four independent MnSOD cDNA clones with heterogeneity in the size of their 3′ untranslated termini. In line with this observation, Northern

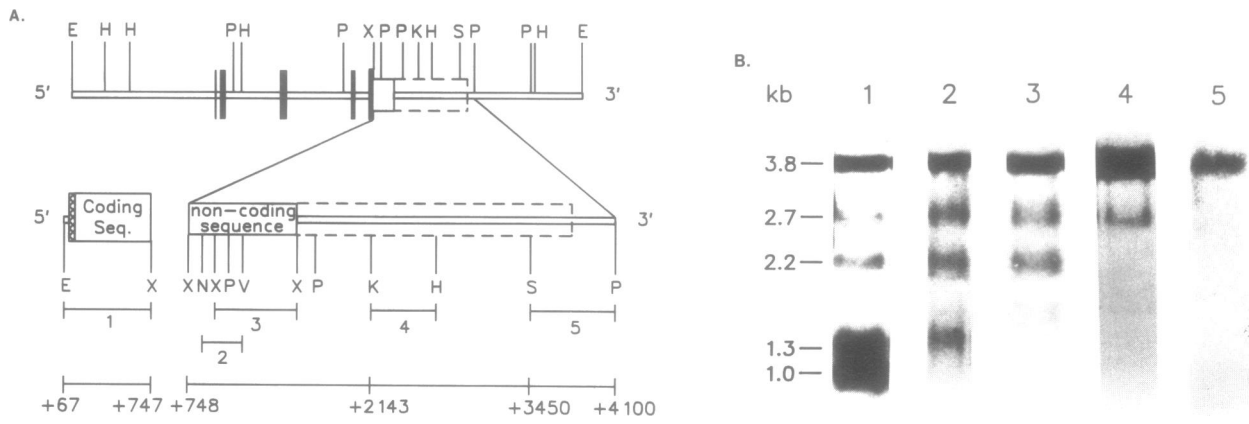


Figure 4. Northern analysis using 3′ non-coding sequence probes. (A) The genomic clone is illustrated as in figure 2, except the non-coding sequence at the end of exon 5 is represented by an open box of intermediate size. An extension of exon 5 representing sequences contained in the larger mRNA transcripts is represented by a box with broken edges. Below, a region of 3′ non-coding genomic sequence is expanded next to a stretch of cDNA coding sequence (probe 1) to show the location of probes 1 to 5 relative to a large mRNA transcript. +1 on this scale is the site of transcription initiation. Probes 1, 2, and 3 are derived from cDNA sequence, while probes 4 and 5 are derived from sequence contained in the genomic clone. (B) Hybridization of probes 1 to 5 to total cellular RNA in Northern analysis. The probe number is listed above each lane. The size of each MnSOD transcript is shown to the left. E, EcoRI; H, HindIII; P, PstI; X, XmnI; K, KpnI; S, StyI; N, NdeI; V, EcoRV.

analysis with a MnSOD cDNA hybridized to five distinct mRNA species (Figure 1C). We also isolated a MnSOD genomic clone which contains all the sequences within these cDNAs, except for the divergent fragment at the end of cDNA 5.1. This 151 bp of divergent sequence does not hybridize to any of transcripts by Northern analysis (data not shown) and we believe that this fragment results from a cloning artifact during library construction.

We postulate that the multiple transcripts could arise from at least four possible mechanisms: 1) transcription from more than one MnSOD gene; 2) multiple sites of transcriptional initiation; 3) incomplete splicing; or 4) alternative polyadenylation.

The presence of additional functional MnSOD genes was ruled out by Southern and Northern analyses using a 3′ non-coding sequence probe (Xmn 207). This probe is contained in all the isolated cDNAs and in our genomic clone as part of exon 5. The results in Figure 3A show that the extra bands seen during genomic Southern analysis with a coding sequence probe (EB 687) do not hybridize with the Xmn 207 probe. This indicates that the Xmn 207 sequence is unique to the region of the rat genome depicted by our genomic clone. This sequence is also contained in all five MnSOD mRNA transcripts visualized by Northern analysis (Figure 3B). This data and extensive genomic Southern analysis unequivocally demonstrates that all MnSOD mRNA transcripts detectable by Northern analysis originate from a single functional gene depicted by our genomic clone (Figure 2A), and that there is a single copy of this locus in the rat genome. This contrasts the speculation by Ho et al. (18) that the rat genome contains multiple functional MnSOD genes.

Our primer extension data indicates that MnSOD transcription initiates at essentially one site 74 to 70 bp 5′ to the start of translation (Figure 1D). Thus, major variations in MnSOD message size can not be explained by differences in the size of the 5′ end.

Introns 2, 3 and 4 contain 1667, 2473, and 551 base pairs respectively, and could contribute to the larger size mRNA species as unprocessed transcripts. However, probes derived from these introns did not hybridize to any of the MnSOD transcripts by Northern analysis, indicating that incomplete splicing does not generate the different sized transcripts.

The remaining possibility for the generation of multiple mRNA species is the alternative cleavage and polyadenylation of the precursor RNA. Recently, several cDNAs have been isolated for the human MnSOD which also show 3′ end size heterogeneity.

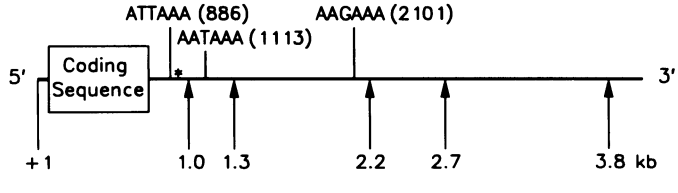


Figure 5. An Extended MnSOD mRNA transcript is depicted. The large open box represents coding sequence, the vertical bars represent non-coding sequence. +1 on this scale is located at the site of transcription initiation. The location of polyadenylation signals that would generate cleavage and polyadenylation sites near the 1.0, 1.3, and 2.2 kb transcripts' termini are listed above the vertical bars. The site of the 1.0 cDNA clone polyadenylation is designated by a '*'. Expected sites of message termination that would generate the five MnSOD transcripts detected by Northern analysis are listed below the arrows.

It has been postulated that the size heterogeneity results both from alternate polyadenylation and an intronic splicing event in the 3′ non-coding sequence (27).

All five rat MnSOD mRNA species are polyadenylated, based on the fact that they are present on Northern blots of poly(A)$^+$ selected mRNA (data not shown). Northern analysis using probes derived from sequences sequentially 3′ to the coding sequence of MnSOD showed a consecutive loss of hybridization from the smallest to the largest MnSOD transcripts. The loss of message hybridization to each successive probe corresponded to that expected, if the differences in the message sizes were the result of alternate polyadenylation.

Based on its inherent size we predict that cDNA clone 1.0 originates from the 1.0 kb mRNA transcript. This cDNA contains the first identifiable polyadenylation signal (ATTAAA) 40 residues upstream of its poly(A) sequence. This signal is the only common natural variant of the strict polyadenylation consensus sequence (AATAAA) (28). Experimental evidence comes from hybridization of this transcript with the Xmn 207 probe and the loss of hybridization with probe 2. Clone 1.0 contains 190 bp of untranslated sequence, and the Xmn 207 probe is almost exclusive to that region.

Further support of the alternative polyadenylation mechanism, stems from the identification of additional polyadenylation signals in the genomic sequence. The locations of these sites are depicted in Figure 5, as well as the predicted termination sites for each of the mRNAs. We were also able to identify numerous other potential polyadenylation signals, however those shown in the figure correlated best with the sizes of each mRNA transcript based on Northern analysis. The alternate signal sequences were defined from a comprehensive analysis of 269 eucaryotic cDNAs and were chosen based on their frequency in this cDNA population and experimental ability to promote cleavage and polyadenylation (29). Signals for the two largest messages could not be localized because available genomic sequence ends approximately 400 bp past the last putative polyadenylation signal at position 2101 relative to the start of transcription.

As illustrated by the tissue distribution in Figure 1C, the relative abundance of the different size transcripts varies from tissue to tissue. For example, the intensities from the two smallest transcripts are similar in all tissues except in the testes and liver where the smallest message is more abundant. This type of variation is also apparent when comparing the ratio of intensities for the three largest transcripts in the brain, lung and kidney. The differences in the intensity of each transcript signal could be attributed to tissue-specific selection of polyadenylation signals and the ability of each signal to promote cleavage. Alternatively, this variation in transcript intensity may result from tissue-specific differences in the regulation of the half-life of each mRNA (30).

Our experiments show that all five MnSOD transcripts detected by Northern analysis are derived from a single functional gene in the rat genome and that the size differences in the MnSOD transcripts are produced by alternate cleavage and polyadenylation.

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