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Human Cu/Zn superoxide dismutase gene: molecular characterization of its two mRNA species

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ABSTRACT

Two cytoplasmic superoxide dismutase (SOD-1) mRNAs of about 0.7 and 0.9 kilobases (Kb.) were previously found in a variety of human cells. The two SOD-1 mRNAs are transcribed from the same gene and the major 0.7 Kb. species is approximately four times more abundant than the minor 0.9 Kb. mRNA. These two mRNAs differ in the length of their 3'-untranslated region and both have multiple 5'-ends. The longer transcript contains 222 additional nucleotides beyond the 3'-polyadenylated terminus of the short mRNA. S_1 nuclease mapping and sequence analysis showed that these extra 222 nucleotides are specified by sequences contiguous to those shared by the two SOD-1 mRNAs. The 5'-termini of the two SOD-1 mRNAs were identified and mapped by both primer extension and S_1 mapping. The majority of SOD-1 mRNA molecules (90-95%) have a 5'-start site located 23 base pairs (b.p.) downstream of the hexanucleotide -TATAAA-. The rest of the SOD-1 mRNA molecules have 5'-termini 30, 50 and 65 b.p. upstream from the major start region.

INTRODUCTION

Eukaryotic cells possess the enzyme cytoplasmic superoxide dismutase (SOD; EC 1.15.1.1.) which catalyzes the oxidation-reduction reaction $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ and thus provides a defense against oxygen toxicity (for reviews see 1,2,3). The human Cu/Zn SOD-1 is a dimeric protein (M-32,000) composed of identical noncovalently linked subunits (4) of known amino acid sequence (5, 6). The gene locus for human SOD-1 was localized at the distal portion of the long-arm of chromosome 21 in the segment 21q22 (7,8). This chromosomal region represents less than 0.1% of the human genome. Nevertheless, whenever it is present in triplicate it causes all the clinical symptoms of Down's syndrome (D.S.)(9-12). Although the relationship of trisomy 21 to Down's syndrome has been known for 25 years (13) there is no effective treatment and very little is known about the way in which the additional chromosomal segment (21q22) causes the disease. It is generally assumed that the extra 21q22 segment codes for normal products and that the abnormalities found in D.S. are produced by an excess of some of those products. Indeed, D.S.

patients show an increase of about 50% in SOD-1 activity, due to higher levels of SOD-1 protein (14,15,16,17). Moreover, it was suggested that the overproduction of SOD-1 may be involved in some of the clinical symptoms of Down's syndrome (18). Therefore characterization of the mRNA transcripts made from the SOD-1 gene and quantitative measurements of its expression in cells from Down's syndrome patients should contribute to our understanding of this genetic disease. A cDNA clone of the human SOD-1 was previously constructed (19) and used to identify the SOD-1 mRNAs. When poly(A)-containing RNA from various cells was analyzed it was found that SOD-1 cDNA probes hybridized to two species of mRNA differing in size by ~200 nucleotides (20). Here we report that the two SOD-1 mRNAs differ in the lengths of their 3'-untranslated region and that both of them possess multiple 5' termini. The 5' and 3' termini were mapped on the genome and a DNA probe for rapid and sensitive determination of the SOD-1 mRNAs by S_1 assay was defined.

MATERIALS AND METHODS

Preparation of poly(A)-containing RNA and blot hybridization

RNA from SV80 cells - a continuous line of Simian virus 40-transformed human fibroblasts (21) and FS11 - human foreskin diploid fibroblasts (22) was prepared as previously described (23). RNA from human placenta was prepared by a modification of the procedure described by Soreq et al., (24). Blotting of RNA from formaldehyde gels into nitrocellulose filters was described before (20). Blots were hybridized with nick-translated [^{32}P]-labeled probes ($2\text{-}4\times10^8$ cpm/μg), with [^{32}P]-end labeled DNA probes, or with single-stranded uniformly [^{32}P]-labeled probes prepared in M13 according to Hu & Messing (25).

DNA Sequence Analysis

The nucleotide sequence was determined by using both the chemical method (26) and the M13 phage dideoxynucleotide technique (27,28). The resulting fragments were resolved on 0.2 mm thin 6%, 8% and 15% acrylamide/8M urea gels.

Determination of SOD-1 mRNAs Length Differences by Ribonuclease H

Sixty μg of poly(A)$^+$ RNA from SV80 cells were coprecipitated with 120 ng of the 170 b.p. DNA fragment and 20 μg of carrier tRNA. The DNA was a HinfI-HinfI fragment of the SOD-1 cDNA clone pS61-10 (19). The precipitate was suspended in 30 μl of 40 mM pipes (pH 6.5) 400 mM NaCl, 1 mM EDTA 80% formamide, denatured 10 min at 70°C and annealed for 2.5 hrs at 47°C. After hybridization 10 μg of oligo(dT)(PL. Biochem) were added and the mixture was precipitated with ethanol. The dried precipitate was suspended in 200 μl of 20 mM Tris-HCl (pH7.5) 10 mM $MgCl_2$, 100 mM KCl, 0.1 mM DTT, 0.1 mM EDTA, 7 U

E.coli ribonuclease-H (BRL) and incubated at 37°C for 40 min. Ten µg/ml DNase (RNase free) were then added and incubation proceeded at 37°C for 30 min. The mixture was extracted with phenol-chloroform and precipitated by ethanol. RNase-H resistant RNA fragments were divided into two samples fractionated on 1.5% agarose formaldehyde gel and transferred to nitrocellulose filters.

S_1 Mapping

S_1 mapping was carried out by the method of Berk & Sharp (29) as modified by Weaver & Weissmann (30). Restriction fragments were labeled at their 5'-dephosphorylated end by polynucleotide kinase and [γ-^{32}]-ATP (>5000 Ci/mmol) or at their 3' end using avian myeloblastosis reverse transcriptase (Life Science Inc.) and the relevant [α-^{32}P]-deoxynucleotide (>3000 Ci/mmole).Labeled fragments were isolated by polyacrylamide gel electrophoresis and strand separated as described (26). Twenty to 50 ng ($2\text{-}5\times10^4$ Cherenkov cpm) of single-stranded DNA fragment were coprecipitated with 5-20 µg poly(A)$^+$ RNA and 30 µg tRNA, dissolved in 80% formamide, 40 mM Pipes (pH6.5), 400 mM NaCl 1 mM EDTA heated to 70°C for 10 min and annealed 3 hrs at 42°C. Hybridization was terminated with S_1 nuclease buffer containing 0.1 M Na acetate (pH4.8), 0.1 mM $ZnSO_4$ and 1000 U of S_1 nuclease (Boehringer) and incubated at 37°C or 42°C for 1 hr. Reaction was stopped by the addition of 50 µl 4 M NH_4-acetate and 100 mM EDTA. Samples were extracted with phenol-chloroform, precipitated with ethanol, washed twice with 70% ethanol and analysed on 6% and 8% urea/acrylamide gels.

Primer Extension

Restriction fragments were prepared, labeled at their 5' ends with [γ-^{32}P]-ATP and polynucleotide kinase and strand separated. cDNA was synthesized by reverse transcriptase as described by Ghosh et al., (31). 100 ng ($\sim$5 pmole) of purified primer ($\sim3\times10^6$ cpm/µg) were mixed with 100 µg poly(A)$^+$ RNA. The mixture was extracted once with phenol, twice with ether and precipitated with ethanol. The pellet was dissolved in 90 µl of 90% formamide and following incubation for 10 min at 57°C 10 µl of 100 mM Pipes (pH6.5) 4 M NaCl and 10 mM EDTA were added and samples were incubated at 42°C for 3 hrs. Hybridization was terminated by the addition of 2 ml 0.5 M KCl, 10 mM Tris-HCl (pH7.4) and DNA-RNA hybrids were purified by oligo(dT)-cellulose chromatography. Material eluted from the column was precipitated with ethanol and dissolved in 200 µl of 50 mM Tris-HCl (pH8.3) 10 mM $MgCl_2$, 75 mM KCl, 10 mM DTT and 0.5 mM of each dATP, dGTP, dTTP and dCTP. 100 U of reverse trancriptase were added and the reaction was incubated at 37°C for 3 hrs. After a further 1 hr incubation in 0.2 N NaOH the reaction was neutralized with 1 N

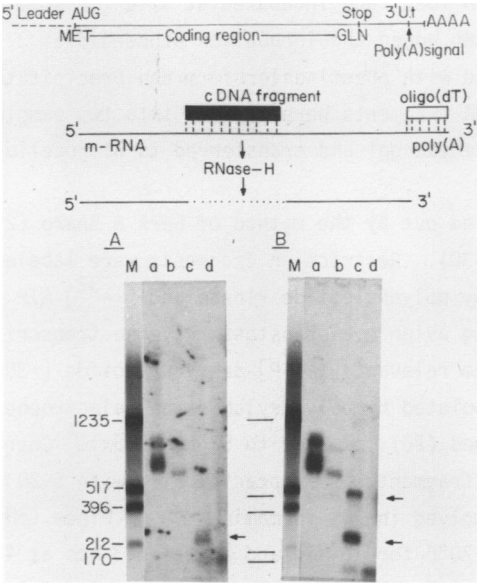

Figure 1. Determination of length differences of SOD-1 mRNAs by RNase-H. Poly(A)$^+$ RNA from SV80 cells was hybridized with the 170 b.p. HinfI-HinfI fragment derived from the middle region of the cDNA clone, treated by RNase-H and analysed as described in Materials and Methods. Hybridization were conducted with [^{32}P]-labeled cDNA subclones: A: 5'-specific probe. B: 3'-specific probe. (M) ^{32}P-end labeled DNA fragments of known size denatured in 1M glyoxal and 50% DMSO 1 hr 50°C prior to electrophoresis. (a) Control, 5 µg poly(A)$^+$ RNA untreated. (b) 15 µg poly(A)$^+$ RNA treated with RNase-H in the presence of oligo(dT). (c) 30 µg of poly(A)$^+$ RNA hybridized to 30 ng cDNA fragment and treated with RNase-H in the presence of oligo(dT). (d) 60 ng of the 170 b.p. HinfI-HinfI cDNA fragment treated by RNase-H in the presence of oligo(dT).

HCl extracted with phenol, precipitated with ethanol and analyzed on 6% or 8% acrylamide gels.

RESULTS

The Two Size Classes of Human SOD-1 mRNAs Differ in the Length of Their 3'-untranslated Regions

In our previous studies of SOD-1 mRNA expression in various human cells two species of mRNA, differing in size by ~200 nucleotides, were detected. The two RNAs were found in poly(A)-containing polysomal RNA and were translated *in vitro* into the same SOD-1 polypeptide (20). We therefore concluded that they are both functional messengers which differ in their untranslated region. To determine whether the extra segment is localized at the 5' or 3' end of the

molecule we have used the procedure of RNase H digestion previously applied to mouse α-amylase mRNA (32).

A 170 b.p. HinfI-HinfI fragment from the middle part of the cDNA coding region was hybridized to poly(A)$^+$ RNA from SV80 cells (Figure 1). The hybrids were treated with ribonuclease-H which specifically digests the RNA moiety within DNA-RNA hybrids (33,34). The RNase H treatment was performed in the presence of oligo(dT) so that the poly(A)-tails were also digested. The resulting 3' and 5' RNA fragments were separated by gel electrophoresis, transferred to nitrocellulose and identified by hybridization. As probes, we have used 3' and 5' subclones of the SOD-1 cDNA which share no homology with the internal DNA fragment. The lengths of the polyadenylated SOD-1 mRNAs were determined as 740 nucleotides for the small and more abundant mRNA and 950 nucleotides for the larger species (Figure 1, A and B, lane a). Upon deadenylation by RNase H treatment the RNA bands moved faster and became sharper (A and B, lane b) indicating that the broad bands were due to size heterogeneity of the poly(A)-tails. Following treatment of the DNA-RNA hybrids with RNase H, one RNA fragment of 250 nucleotides was detected with the 5' subclone (Figure 1A, lane c) and two RNA bands of 270 and 510 nucleotides were detected with the 3' subclone (Figure 1B, lane c). Control reactions from which poly(A)$^+$ RNA or internal cDNA fragments were omitted are shown in Figure 1,A and B, lanes b & d). Denatured DNA fragments were used as size markers to enable sizing of RNA fragments within the ±50 nucleotides accuracy limits of agarose gels. The 150 nucleotides band present in Figure 1, A and B, lane c, as well as in the control lane d, resulted from pBR322 fragment contaminating the internal cDNA fragment and therefore reacted with both the 5' and 3' subclones. The presence of a single 5' and two 3' RNA fragments indicated that the size difference between the SOD-1 mRNAs resides at the 3' terminus. The lower and more intense 3' band of 270 nucleotides originated most likely from the small mRNA, whereas, the 510 nucleotide band was derived from the longer 0.9 Kb. species of SOD-1 message.

Mapping the 3' Termini of SOD-1 mRNA Species

We previously identified, what now seems to be, the 3' terminus of the short SOD-1 mRNA by comparing the DNA sequences of the gene with that of the cDNA clone which contains a poly(A)-tail (20). A polyadenylation signal (ATTAAA) that plays a role in processing and poly(A) addition (35,36) was found 16 nucleotides upstream of that poly(A)-tail. To identify and map the sequences of the 3'-terminus of the long SOD-1 mRNA a HindIII-HindIII genomic fragment containing the 3' exon (V) and extending 450 nucleotides downstream from the

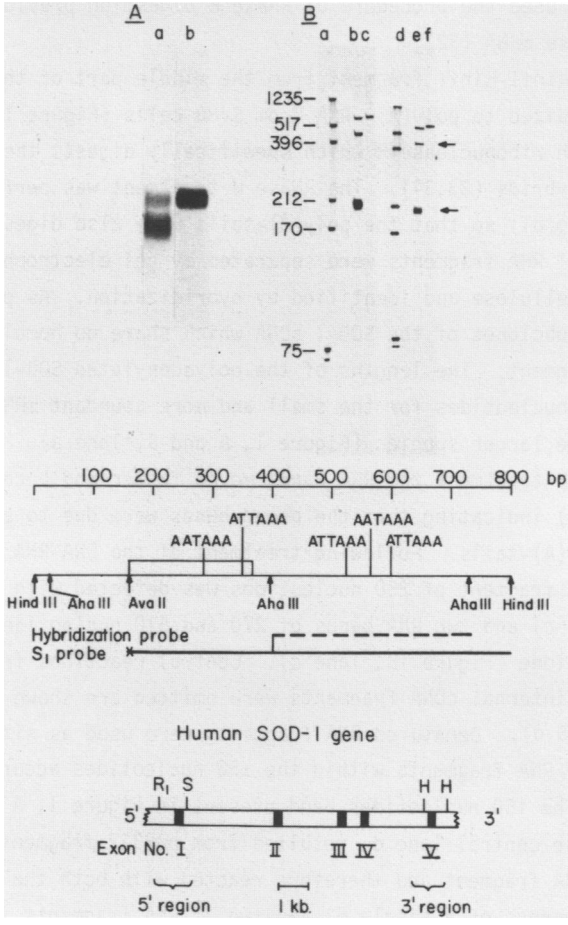

Figure 2. Mapping the 3' termini of SOD-1 mRNAs. A: Localization of the 3'-untranslated region of the 0.9 Kb SOD-1 mRNA by blot analysis. Human placenta poly(A)$^+$ RNA (10 µg) was size fractionated on 1.5% agarose formaldehyde gel transferred to nitrocellulose and probed with (a) [^{32}P]-labeled nick-translated HindIII-HindIII fragment, or (b) [α^{32}P]-dATP uniformly labeled AhaIII-AhaIII genomic fragment designated by the dashed line at the bottom. B: Examination of the 3'-termini of SOD-1 mRNAs by nuclease S1 digestion of mRNA/DNA hybrids. The 634 b.p. AvaII-HindIII fragment was 3'-end labeled, strand separated and hybridized to 10 µg of poly(A)$^+$ RNA from human placenta. (b) or SV80 cells (e). Nuclease S$_1$ treatment and analysis of the DNA protected fragments by polyacrylamide gels were described in Materials and Methods. (c) and (f) are control reactions with no RNA added. (a) and (d) are DNA size markers.

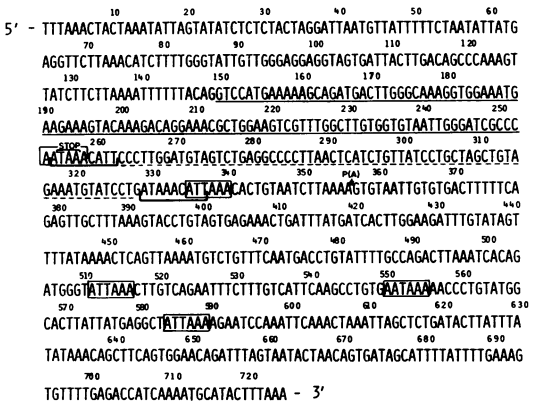

Figure 3. Nucleotide sequence of the 3'-region of SOD-1 gene. Underlined is the sequence of exon V coding the 3' part of the SOD-1 protein. The dashed line indicates the 67 b.p. region flanked by two underlined 9 b.p. direct repeats (-ATAAACATT-). The five possible polyadenylation signals are boxed and the polyadenylation site of the 0.7 Kb. SOD-1 mRNA is designated by an arrow.

poly(A) addition site of the short mRNA was subcloned in M13 (Figure 2). This probe, as well as the shorter AhaIII-AhaIII fragment of 390 nucleotides derived from it, hybridized to both mRNAs(Figure 2, Aa). On the other hand, the downstream AhaIII-AhaIII fragment (marked in Figure 2 by the dashed line) hybridized only to the longer species (Figure 2 Ab). The 3' termini of the 0.7 Kb. and 0.9 Kb. SOD-1 mRNAs were mapped on the DNA by S_1 nuclease protection experiment (29;30). A 636 b.p. AvaII-HindIII fragment which spans the 3' exon sequences of the small mRNA and extends 432 nucleotides further downstream was labeled at the 3' end of the AvaII site (Figure 2). The two strands of the labeled DNA were separated and the 3'-labeled strand was hybridized with poly(A)$^+$ RNA from human placenta and SV80 cells. The hybrids were digested with S_1 nuclease and the products separated on acrylamide sequencing gels (Figure 2B). Two protected fragments of 203 and 425 b.p., corresponding to the two SOD-1 mRNAs were resolved. Identical DNA fragments were protected by the two preparations of poly(A)$^+$ RNA (Figure 2B, lanes b & e). The size of the small more abundant 204 b.p. fragment agreed well with the measured distance between the AvaII site and the end of the small SOD-1 mRNA identified in the cDNA clone. The larger SOD-1 mRNA species protected a fragment of 425 b.p. long which extends 222 nucleotides further downstream. From these results it was also evident that the DNA fragment used for hybridization and S_1 mapping was colinear with both species of SOD-1 mRNA, and that the extra 222 nucleotides were specified by sequences contiguous to those shared by the two mRNAs.

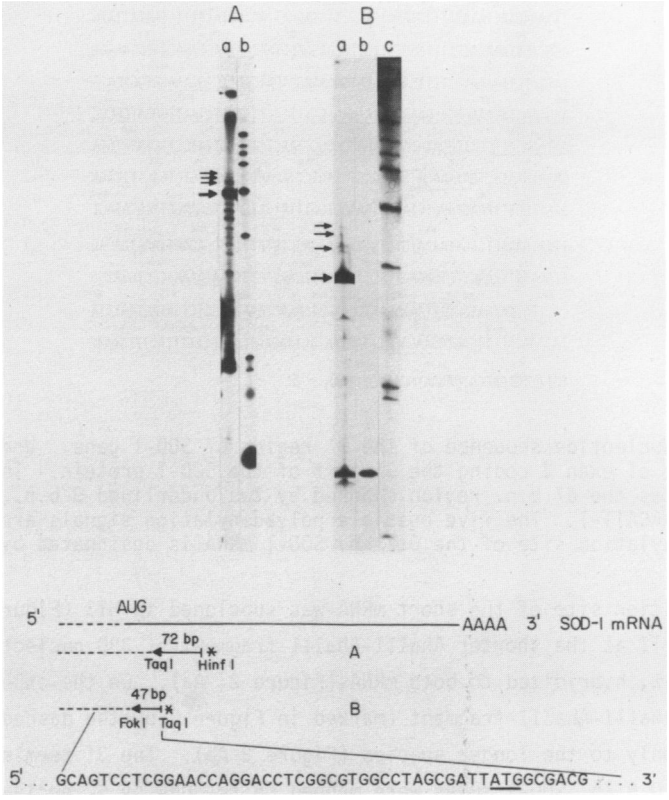

Figure 4. Primer extension analysis of SOD-1 mRNA. Single-stranded [^{32}P]-labeled probes (A) and (B) were hybridized to poly(A)$^+$ RNA, extended with reverse transcriptase and analyzed as described in Materials and Methods. (A) The 72 b.p. HinfI-TaqI fragment was used as primer and one fifth of the extension reaction was analyzed (a). Size markers depicted in (b) are ^{32}P-end labeled HinfI digest of pBR322. (B) The 47 b.p. TaqI-FokI fragment was used as primer. The main extension product of 140 nucleotides (a) was removed from the gel and sequenced. Part of this sequence is shown at the bottom as the mRNA sense. The position of the primer is depicted in (b). (c) are size markers as above.

The nucleotide sequence of the 3' region of the SOD-1 gene including the 3' exon and further downstream sequences, is shown in Figure 3. Inspection of this sequence revealed five polyadenylation sites. The first hexanucleotide (AATAAA) is fused to the translatable region. It contains the two A residues of the last codon (CAA=GLN) of SOD-1 protein and the TAA stop codon. The second signal (ATTAAA) is the one involved in the formation of the 0.7 Kb. mRNA species. Out of the three downstream poly(A) signals at positions 511,

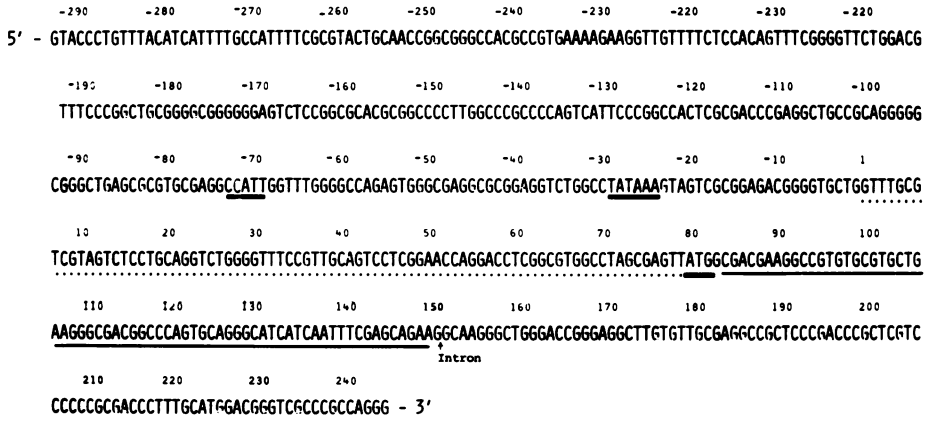

Figure 5. DNA sequence of the 5'-region of SOD-1 gene. This sequence was independently determined by the Maxam-Gilbert sequencing procedure and by the use of dideoxynucleotide chain termination procedure. The coding region of the first exon starting with the marked ATG is underlined with a solid line. The 5'-non coding region is underlined with a dotted line. The -TATAAA- sequence and the -70 (CCATT) are underlined with heavy lines.

550, and 584 (Figure 3) the middle one would generate a transcript which is 220 b.p. longer than the small SOD-1 mRNA, in good agreement with the S_1 mapping experiment (Figure 2).

We therefore concluded that the AATAAA hexanucleotide at position 550 was used as a processing signal in formation of the 0.9 Kb. SOD-1 mRNA.

Mapping the 5' Termini of SOD-1 mRNA

Two methods were used to locate and map the 5' sequences of SOD-1 mRNA; primer extension (31) and S_1 mapping (29;30). For DNA extension experiments, poly(A)-containing RNA from human placenta was used as template with two different DNA fragments from the 5' region of SOD-1 cDNA clone as primers. When the 72 b.p. TaqI-HinfI fragment labeled at the HinfI site was extended it yielded one major product of 210 b.p. and three minor larger bands of 240, 260 and 275 b.p. (Figure 4A). The lower bands observed in this experiment were probably due to premature termination of the reverse transcriptase. The 47 b.p. TaqI-FokI primer gave a 140 b.p. product and again a few minor higher bands of 170, 190, 205 b.p. (Figure 4B). The two primer extension experiments gave consistent results indicating that the majority of SOD-1 mRNAs have 5'-termini about 80 nucleotides upstream from the ATG codon which specify the initiator methionine of the SOD-1 polypeptide (Figure 5). The results also suggested that a minor fraction of SOD-1 messenger has 5'-untran-

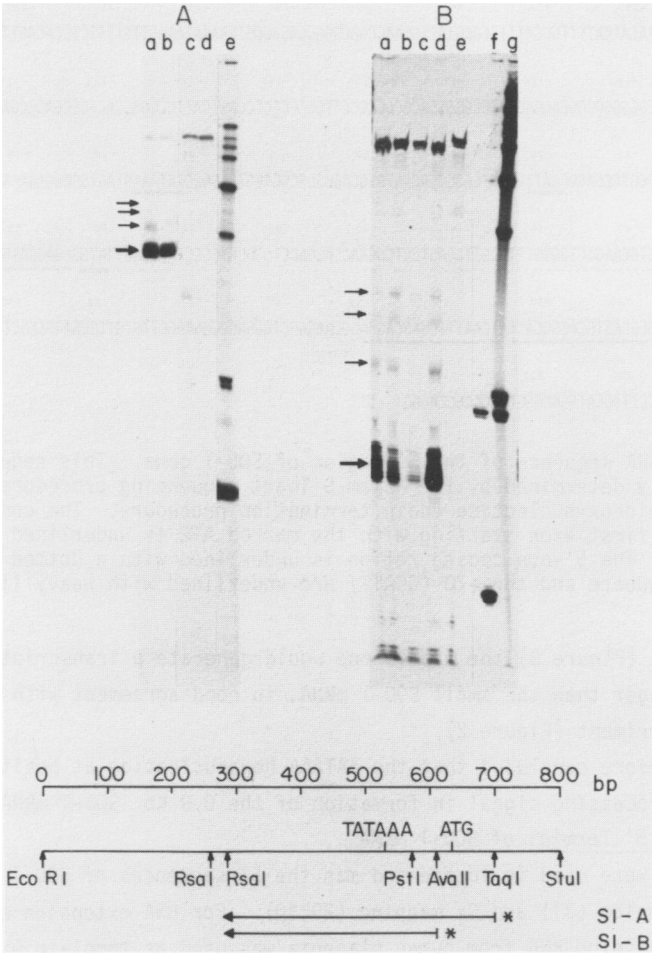

Figure 6. S_1 mapping of the 5'-end of SOD-1 mRNAs from different sources. S_1 mapping was performed as described in Materials and Methods. The fragments used as probes and their labeling sites (denoted by asterisks) are presented at the bottom. (A) The 405 b.p. TaqI-RsaI probe was hybridized to poly(A)$^+$ RNA (20 µg) from human placenta. Hybrids were digested by S_1 nuclease for 1 hr at (a) 37°C and (b) at 42°C. (c and d) control reactions with tRNA instead of poly(A)$^+$ RNA. (e) ^{32}P-end labeled markers as in Fig. 4. (B) The 356 b.p. AvaII-RsaI probe was hybridized to (a) poly(A)$^+$ RNA (12 µg) from FS11 cells. (b and c) poly(A)$^+$ RNA from SV80 cells size fractionated on a sucrose gradient; 120 µg poly(A)$^+$ RNA were sedimented through 15-30% SDS sucrose gradient and fractions containing SOD-1 RNA were analyzed by agarose gel and blot hybridization. Fractions containing the 0.7 Kb. species were used in (b), whereas in (c) the 0.9 Kb. mRNA was hybridized to probe B. (d) poly(A)$^+$ RNA (10 µg) from human placenta. Hybrids were digested by S_1 nuclease for 1 hr at 42°C. (e) control lane with tRNA added instead of poly(A)$^+$ RNA. (f and g) size markers.

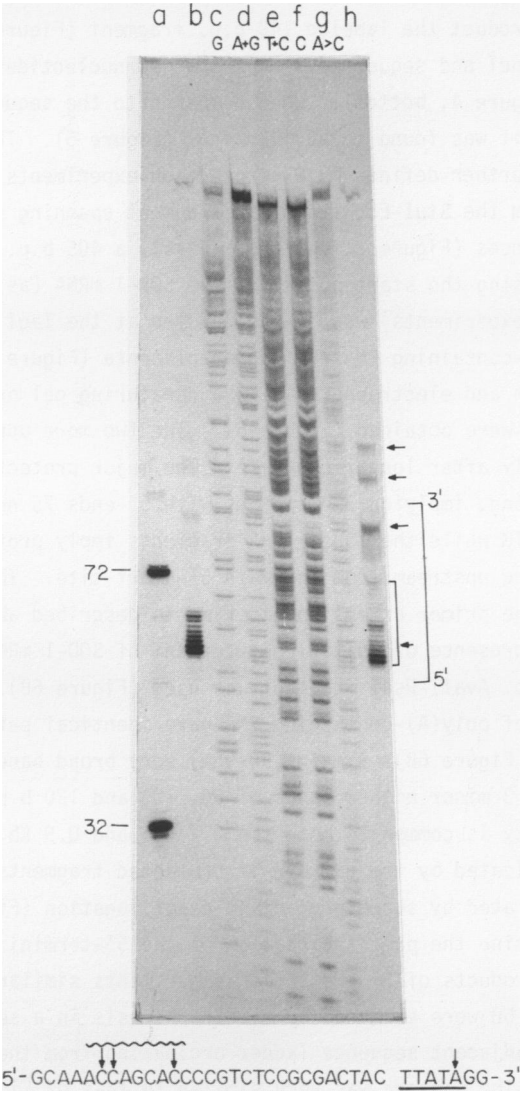

Figure 7. Localization of the 5'-termini of SOD-1 mRNAs by analysis on sequencing gel. The 356 b.p. AVaII-RsaI fragment was 5'-end labeled, strand separated and the coding strand was sequenced using the chemical cleavage method (lanes c-g). The same probe was used for S_1 mapping of poly(A)$^+$ RNA (6 µg) from human placenta (b) or SV80 cells (h). Hybrids were digested by S_1 nuclease for 1 hr at 42°C. (a) Size markers. The main initiation region is marked by the waved lines. The three upstream secondary sites are marked by thick arrows. The nucleodie sequence of the non coding strand containing the main start site is presented at the bottom.

slated regions longer by 30, 50, and 65 nucleotides. To verify the identity of the extended product the labeled 140 b.p. fragment (Figure 4B) was recovered from the gel and sequenced. More than 40 nucleotides upstream of the ATG were read (Figure 4, bottom). When compared to the sequence of the genomic fragment it was found to be identical (Figure 5). The 5'-ends of the SOD-1 mRNA were further defined by S_1 protection experiments. Two probes were prepared from the StuI-EcoRI genomic fragment spanning exon #I and the 5'-flanking sequences (Figure 2, bottom). First, a 405 b.p. TaqI-RsaI fragment encompassing the start points of the SOD-1 mRNA (as deduced from the primer extension experiments) was 5'-end labeled at the TaqI site and hybridized with poly(A)-containing RNA from human placenta (Figure 6). After S_1 nuclease digestion and electrophoresis in a denaturing gel one major band and three minor bands were obtained (Figure 6). The two more upper ones in Fig.6A become visible only after longer exposure. The major protected band was 138 nucleotides long, implying RNA species with 5'-ends 75 nucleotides upstream from the ATG while the longer DNA fragments imply protection by RNA species originating upstream from the main 5'-start site. These results were consistent with the primer extension experiments described above. To further substantiate the presence of multiple 5'-termini of SOD-1 mRNAs a second S_1 probe, the 356 b.p. AvaII-RsaI fragment was used (Figure 6B). Three different preparations of poly(A)-containing RNA gave identical patterns of protected fragments (Figure 6B): a main (90-95%) very broad band with a size of about 57 b.p. and 3 minor higher bands of 86, 105 and 120 b.p. The 5'-terminal heterogeneity is common to both the 0.7 Kb. and 0.9 Kb. species of SOD-1 mRNA as indicated by the pattern of protected fragments obtained with mRNA species separated by sucrose gradient centrifugation (Figure 6B, lanes b & c). To determine the precise location of the 5'-termini of the SOD-1 transcripts the products of S_1 protection experiments similar to those described in Figure 6B were examined by electrophoresis in a sequencing gel and compared with an adjacent sequence ladder orginating from the same DNA fragment (Figure 7). The pattern was very similar to that of Figure 6; three larger bands of lower intensity and a smaller major fragment now migrating as a group composed of 10-15 nucleotides (Figure 7, lanes b & h). Within the protected fragment (waved line in Figure 7) the 3 prominent bands corresponded to G residues at position 1, -2 and -7 of the SOD-1 gene (see Figure 5). The match was done by moving the mRNA start points 1½ nucleotides relative to the sequencing ladder to account for the migration diference of products generated by the chemical sequencing method (26). This is because the mobility of the

3'-OH-terminated S_1 fragment is slightly slower than that of their 3'-P-terminated counterparts produced by chemical cleavage (37). From the length of the other three RNA-protected DNA fragments the additional less abundant 5'-ends of the SOD-1 mRNAs were localized at positions -30, -50, and 65 on the genomic DNA (Figure 5). The determination of the start sites of the SOD-1 mRNA by S_1 nuclease mapping with two different probes (Figures 6 & 7) is in excellent agreement with the determination of the 5'-ends established by extending two different primers with reverse transcriptase (Figure 4).

DISCUSSION

Two species of SOD-1 mRNA

We previously reported that two functional SOD-1 mRNA species that share a common translated region, but differ in size by about 200 nucleotides, are present in human cells (19;20). Several lines of evidence support the conclusion that the two SOD-1 mRNAs are transcribed from the same gene. First, hybridization experiments with defined restriction fragments of SOD-1 cDNA clone showed that both mRNAs contain sequences of the entire coding region plus the 95 nucleotides representing the 3'-untranslated region of the small SOD-1 mRNA. Second, primer extension and S_1-mapping experiments with genomic DNA probes from the 5' and 3'-ends of the SOD-1 gene indicated that the DNA sequences encoding the large transcripts are contiguous to those shared by the two mRNA species. Thirdly, only one region of human DNA with nucleotide sequences identical to the SOD-1 cDNA was detected (Levanon et al., in preparation). The RNase H experiment clearly indicated that sequences at the 3'-untranslated region account for the differences in size between the SOD-1 mRNAs.

The 3'-ends of SOD-1 mRNAs

The 3'-termini of both SOD-1 mRNAs were mapped on a single genomic fragment which covers the 3' region of the small mRNA and extends further downstream 450 b.p. Accurate sizing of the two protected fragments showed that the length of the shorter one was 203 nucleotides. This value is in very good agreement with the distance of 204 nucleotides between the labeled AvaII site and the polyadenylated 3'-end of the small SOD-1 mRNA represented in the cDNA clone (Figure 3). The larger SOD-1 mRNA protected a DNA fragment extending 222 nucleotides further downstream. Examination of the DNA sequence at the 3' region of the SOD-1 gene revealed two groups of processing/polyadenylation signals; the first group contains two such signals 72 b.p. apart. The first hexanucleotide - a classical AATAAA is fused to the coding

region (#255 in Figure 3). Although the latter is the more proximal signal it is not utilized. The poly(A)-tail of the small SOD-1 mRNA is attached 95 nucleotides further downstream at position 356. This point is 16 nucleotides beyond the second less conventional ATTAAA hexanucleotide. It is interesting to note that the 67 b.p. fragment between the two polyadenylation signals is flanked by two 9 nucleotide direct repeats (-A-T-A-A-A-C-A-T-T). This may suggest that the 67 b.p. region is actually an insert which by integrating into this region created the second polyadenylation signal at position 335 (Figure 3). In any event, it is clear that in the case of the small SOD-1 mRNA the variant (ATTAAA) and the more downstream signal is utilized as a recognition site for cleavage and polyadenylation. A similar situation occurs with the larger species of SOD-1 mRNA. The second group of processing/polyadenylation signals contain three tandem hexanucleotides ATTAAA, AATAAA and ATTAAA at positions 511, 550 and 584, respectively. From those the middle one specifies the 3'-end of the longer SOD-1 transcript. Whether the two SOD-1 mRNAs are produced by selective cleavage of the same primary transcript or whether transcription termination of the small mRNA occurs before the second group of processing signals, is still an open question. A generation of multiple mRNAs from a single gene has been previously reported. It was first found in the late-Ad2 mRNAs (38;39) but since then many other cases where multiple transcripts are produced from a single gene have been reported (40-53). Although the phenomena is wide-spread in most of the cases the physiological significance is totally unclear, mainly because the biological function of the 3' and 5'-untranslated regions is unknown. In the case of human SOD-1 mRNAs, the ratio of the two species appears to be very similar in many different cells and tissues including monosomy-21 cells and cells from patients with Down's syndrome (20, and unpublished data).

The 5'-ends of SOD-1 mRNAs

The 5'-termini of SOD-1 mRNAs were mapped on the genomic DNA by a combination of primer extension and S_1 nuclease protection experiments. As judged by the relative intensity of the S_1-resistant bands, transcription of 90-95% of SOD-1 mRNA appears to start around position +1, whereas, the rest of the mRNA molecules had termini further upstream at positions -30, -50, and -65 of the genomic DNA (Figure 5). The length heterogeneity at the 5'-end was observed in both cytoplasmic and nuclear poly(A)-containing RNA and it appeared to be independent from the selection of the 3' polyadenylation site since both the 0.7 and 0.9 Kb. mRNA species had multiple 5'-termini. As discussed above, there is good evidence that all the SOD-1 transcripts are derived from the

same gene; therefore, the additional minor 5'-ends must result either from multiple transcription initiation sites or from cleavage of a longer precursor. Since the 5'-cap structures of SOD-1 mRNAs were not analysed we can not distinguish between these two possibilities. At 23 nucleotides upstream from the major 5'-end (designated #1, Figure 5) there is the hexanucleotide TATAAA ("Goldberg-Hogness" box) found in many eukaryotic type II genes 20-30 b.p. upstream from the mRNAs 5'-start (reviewed in 54). On the other hand, a similar TATA box sequence is not present at the DNA region upstream from the minor 5'-termini. This provides an additional example for a TATA-less transcription start (see below). Multiple 5'-ends were first observed in polyoma and SV40 late mRNAs (55;56;23;57), as well as in Ad-2 early mRNAs (58;59). It was also shown that the multiple 5'-caps represent initiation points of RNA polymerase II (60-63). More recently, heterogeneity at the 5'-end of cellular mRNA has also been reported (42;46;63-68). In all these cases (except mouse α-amylase,65) the biological significance of the 5'-terminal heterogeneity is not yet understood. It may be that generations of multiple 5'-terminal is an intrinsic feature of RNA polymerase II transcription mechanism. In this context we have previously suggested (23) that a repertoire of initiation points that generate transcripts differing only in length of the 5'-untranslated region might render the synthesis of mRNA less vulnerable to mutation in promoter sites.

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REFERENCES

1. Fridovich, I. (1975) Ann. Rev. Biochem. 44, 147-159.
2. Fridovich, I. (1978) Science 201, 875-880.
3. Fridovich, I. (1979) in: Advances in Inorganic Chemistry (Eichhorn, G.L. and Marzilli, L.G., Eds) (Elsevier/North Holland, New York), pp 67-90.
4. Briggs, R.G. and Fee, J.A. (1978) Biochim. Biophys. Acta 537, 86-99.
5. Jabusch, J.R., Farb, D.L., Kerschensteiner, D. and Deutsch, H.F. (1980) Biochemistry 19, 2310-2316.
6. Barra, D., Martini, F., Bannister, J.V., Schinina, M.E., Rotilio, W.H., Bannister, W.H. and Bossa, F. (1980) FEBS Lett. 120, 53-56.
7. Tan, Y.H., Tischfield, J. and Ruddle, F.H. (1973) J. Exp. Med. 137, 317-330.
8. Sinet, P.M., Allard, D. and Lejeune, J. (1974) C.R. Acad. Sci. 278, 3267-3270.
9. Niebuhr, E. (1974) Humangenetik 21, 99-101.

10. Hagemeijer, A. and Smit, E.M.E. (1977) Hum. Genet. 38, 15-23.
11. Williams, J.D., Summit, R.L., Martens, P.R. and Kimbrell, R.A. (1975) Am. J. Hum. Genet. 27, 478-485.
12. Sinet, P.M., Couturier, J., Dutrillaux, B., Poissonnier, M., Rauol, O., Rethore, M., Allard, D., Lejeune, J. and Jerome, H. (1976) Exp. Cell. Res. 97, 47-55.
13. Lejeune, J.M., Gautier, M. and Turpin, R. (1959) Compt.Rend.Acad.Sci. 248, 1721-1722.
14. Sinet, P. M., Allard, D., Lejeune, J. and Jerome, H. (1974) Compt.Rend. Acad.Sci. 278, 3267-3270.
15. Erikson, A.W., Frants, R.R. and Jonbgbloet, P.H. (1975) Abstr. Amer. J. Hum. Genet. 27, 33A.
16. Crosti, N., Serra, A., Rigo, A. and Viglino, P. (1976) Hum. Genet. 31, 197-203.
17. Feaster, W.W., Kwok, L.W. and Epstein, C.J. (1977) Amer. J. Hum. Genet. 29, 563-570.
18. Sinet, P.M. (1982) Annals of the New York Academy (Sinex F.M. and Merril, C.R. Eds) 396, 83-94.
19. Lieman-Hurwitz, J., Dafni, N., Lavie, V. and Groner, Y. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 2808-2811.
20. Sherman, L., Dafni, N., Lieman-Hurwitz, J. and Groner, Y. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 5465-5469.
21. Todaro, G.J., Green, H. and Swift, C. (1966) Science 153, 1252-1254.
22. Weissenbach, J., Zeevi, M., Landau, T. and Revel, M. (1979) Eur. J. Biochem. 98, 1-8.
23. Canaani, D., Kahana, C., Mukamel, A. and Groner, Y. (1979) Proc.Natl. Acad.Sci. U.S.A. 76, 3078-3082.
24. Soreq, H., Safran, A. and Zisling, R. (1982) Developmental Brain Research 3, 65-79.
25. Hu, N. and Messing, J. (1982) Gene 17, 271-277.
26. Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. 65, 499-560.
27. Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H. and Roe, B.A. (1980) J. Mol. Biol. 143, 161-178.
28. Heidecker, G., Messing, J. and Gronenborn, B. (1980) Gene 10, 69-73.
29. Berk, A.J. and Sharp, P.A. (1977) Cell 12, 721-732.
30. Weaver, R.F. and Weissman, C. (1979) Nucleic Acid Res. 7, 1175-1193.
31. Ghosh, P.K., Reddy, V.B., Pialak, M., Lebowitz, P. and Weissman, S.M. (1980) Methods Enzymol. 65, 580-595.
32. Hagenbuchle, O. and Schibler, U. (1981) Proc.Natl.Acad.Sci. U.S.A. 78, 2283-2286.
33. Jacquet, M., Groner, Y., Monroy, G. and Hurwitz, J. (1974) Proc.Natl. Acad.Sci. U.S.A. 71, 3045-3049.
34. Groner, Y., Monroy, G., Jacquet, M. and Hurwitz, J. (1975) Proc.Natl. Acad.Sci. U.S.A. 72, 194-199.
35. Proudfoot, N.J. (1982) Nature 298, 516-517.
36. Montell, C., Fischer, E.F., Caruthers, M.H. and Berk, A.J. (1983) Nature 305, 600-605.
37. Sollner-Webb, B. and Reeder, R.H. (1979) Cell 18, 485-499.
38. Nevins, J.R. and Darnell, J.E. (1978) Cell 15, 1477-1493.
39. Ziff, E. and Fraser, N. (1978) J. Virol. 25, 897-906.
40. Setzer, D.R., McGrogan, M., Nunberg, J.H. and Schimke, R.T. (1980) Cell 22, 361-370.
41. Hofer, E. and Darnell, J.E. (1981) Cell 23, 585-593.
42. Grez, M., Land, H., Giesecke, K., Schutz, G., Jung, A. and Sippel, A.E. (1981) Cell 25, 743-752.
43. Tosi, M., Young, R.A., Hagenbuchle, O. and Schibler, U. (1981) Nucleic Acid Res. 9, 2313-2322.

44. Cheng, H.L., Blattner, F.R., Fitzmaurice, L., Mushinski, J.F. and Tucker, P.W. (1982) Nature 296, 410-415.
45. Alt, F.W., Bothwell, A.L.M., Knapp, M., Siden, E., Mather, E., Koshland, M. and Baltimore, D. (1980) Cell 20, 293-301.
46. Gerlinger, P., Krust, A., LeMeur, M., Perrin, F., Cochet, M., Gannon,F., Dupret, D. and Chambon, P. (1982) J. Mol. Biol. 162, 345-364.
47. Lee, M.S.-S., Lewis, S.A., Wilde, C.D. and Cowan, N.J. (1983) Cell 33, 477-487.
48. Parnes, J.R., Robinson, R.R. and Seidman, J.G. (1983) Nature 302, 449-452.
49. Scarpulla, R.C. and Wu, R. (1983) Cell 32, 473-482.
50. Zehner, Z.E. and Paterson, B.M. (1983) Proc.Natl.Acad.Sci. U.S.A. 80, 911-915.
51. Wang, J.Y.J. and Baltimore, D. (1983) Mol. and Cell. Biol. 3, 773-779.
52. Aho, S., Tate, U. and Boedtker, H. (1983) Nucleic Acid Res. 11, 5443-5450.
53. Capetanaki, Y.G., Ngai, J., Flytzanis, C.N. and Lazarides, E. (1983) Cell 35, 411-420.
54. Breathnach, R. and Chambon, P. (1981) Ann. Rev. Biochem. 50, 349-383.
55. Flavell, A.J., Cowie, A., Legon, S. and Kamen, R. (1979) Cell 16, 357-371.
56. Ghosh, P.K., Reddy, V.B., Swinscoe, J., Lebowitz, P. and Weissman, S.M. (1978) J. Mol. Biol. 126, 813-846.
57. Haegeman, G., Van Heuverswyn, H., Gheysen, D. and Fiers, W. (1979). J. Virol. 31, 484-493.
58. Baker, C.C., Herisse, J., Courtois, G., Galibert, F. and Ziff, E. (1979) Cell 18, 569-580.
59. Hashimoto, S. and Green, M. (1979) Virology 94, 254-272.
60. Kahana, C., Gidoni, D., Canaani, D. and Groner, Y. (1981) J. Virol. 37, 7-16.
61. Gidoni, D., Kahana, C., Canaani, D. and Groner, Y. (1981) Proc.Natl. Acad. Sci. U.S.A. 78, 2174-2178.
62. Contreras, R. and Fiers, W. (1981) Nucleic Acids Res. 9, 215-236.
63. Hagenbuchle, O., Tosi, M., Schibler, U., Bovey, R., Wellauer, P.K. and Young, R.A. (1981) Nature 289, 643-646.
64. Malek, L.T., Eschenfeldt, W.H., Munns, T.W. and Rhoads, R.E. (1981) Nucleic Acid Res. 9, 1657-1673.
65. Young, R.A., Hagenbuchle, O. and Schibler, U. (1981) Cell 23, 451-458.
66. Faye, C., Leung, D.W., Tatchell, K., Hall, B.D. and Smith, N. (1981) Proc.Natl.Acad.Sci. U.S.A. 78, 2258-2262.
67. Lai, E.C., Roop, D., Tsai,M.-J., Woo, S.L.C. and O'Malley, B.W. (1982) Nucleic Acid Res. 10, 5553-5567.
68. Allan, M., Lanyon, W.G. and Paul, J. (1983) Cell 35, 187-197.