The 3′ Untranslated Region of Manganese Superoxide Dismutase RNA Contains a Translational Enhancer Element†

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ABSTRACT: A redox-sensitive protein that binds to the 3′ untranslated region (UTR) of manganese superoxide dismutase (MnSOD) RNA has been described previously [Fazzone, H., Wangner, A., and Clerch, L. B. (1993) *J. Clin. Invest. 92*, 1278−1281; Chung, D. J., and Clerch, L. B. (1997) *Am. J. Physiol. 16*, L714−L719]. In the present study, cross-competition gel retardation and RNase H assays were used to identify a 41-base region located 111 bases downstream of the stop codon as the 3′ UTR cis element involved in protein binding. The base sequence of this region is ∼75% conserved among the 3′ UTRs of rat, mouse, cow, and human MnSOD mRNAs at approximately the same distance downstream of the stop codon. The role of this protein-binding region in RNA translation was assessed in an in vitro rabbit reticulocyte lysate system. Translation of MnSOD RNA from which the 3′ UTR element was deleted decreased 60% compared with translation of MnSOD RNA containing the 3′ UTR cis element. In the presence of a specific competitor oligoribonucleotide that inhibits MnSOD RNA protein-binding activity, translation of MnSOD RNA containing the 3′ UTR was decreased by 65%. Thus, both the cis element and RNA protein-binding activity were required for more efficient translation of the MnSOD. An analysis of ribosomal profiles suggests the MnSOD RNA-binding protein participates in the formation of the translation initiation complex. When MnSOD RNA-binding activity was inhibited, initiation complex formation was decreased by 50%. From the data obtained in this study, we propose that the 3′ UTR cis element of MnSOD through its interaction with MnSOD RNA-binding protein may function as a translational enhancer.

Manganese superoxide dismutase (MnSOD)[1] catalyzes the conversion of superoxide radical to hydrogen peroxide. It is a critical enzyme in the ability of the cell to withstand oxidant stress. The importance of MnSOD is due, in part, to its intracellular location in mitochondria, a major site of free radical generation during oxidative phosphorylation even under normoxic conditions (*1*, *2*). Furthermore, during oxidant stress, for example during hyperoxia, the production of reactive oxygen species in the mitochondria is increased (*3*, *4*). Because mitochondria are the organelles responsible for maintaining cellular energy in the form of ATP, protection of the mitochondria from free radical toxicity is paramount in preventing cell damage and death under periods of oxidant stress. Thus, MnSOD is likely to play a significant role in the process of aging and in major illnesses in which free radical injury is implicated in pathogenesis including cancer, myocardial infarction, bronchopulmonary dysplasia, ischemia-reperfusion injury, and stroke.

In several animal models of oxidant stress, MnSOD is regulated, at least in part, at the level of mRNA stability or mRNA translation (*5*−*14*). Consequently, an understanding of the post-transcriptional mechanisms responsible for the regulation of MnSOD gene expression is important in devising strategies for up-regulating the enzyme during periods of free radical assault. A large and rapidly growing body of literature indicates that cytosolic RNA-binding proteins can function as trans-acting factors regulating post-transcriptional gene expression. Therefore, one approach to understanding the control of MnSOD expression was to look for proteins that specifically bind to MnSOD RNA. When lung extract was examined for the presence of a protein that binds to the 3′ UTR of MnSOD RNA, a redox-sensitive protein was found that bound to the 3′ UTR of MnSOD mRNA at a 216-base cis element located 111 bases downstream of the rat MnSOD RNA stop codon (*15*, *16*).

The current study was designed to delimit the cis element involved in protein binding and to investigate the function of the MnSOD RNA-binding protein and 3′ UTR cis element. Gel retardation and competition experiments were used to identify the region of protein binding, and a cell-free system of rabbit reticulocyte lysate was used to examine the role of

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[1] Abbreviations: MnSOD, manganese superoxide dismutase; UTR, untranslated region; S130, lung cytoplasmic extract; RRL, rabbit reticulocyte lysate; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; SEM, standard error of the mean; LPS, lipopolysaccharide; PABP, poly(A)-binding protein.

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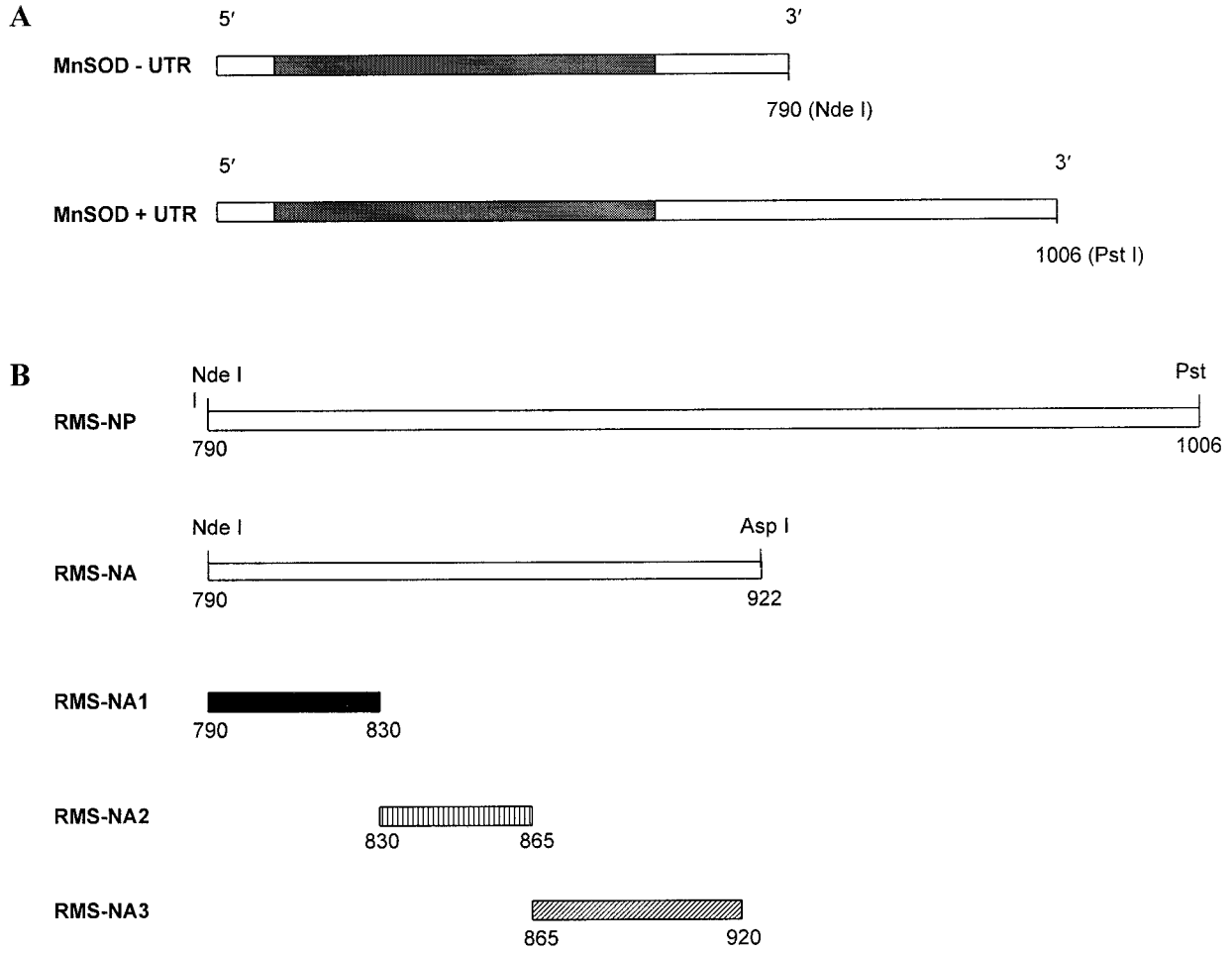

FIGURE 1: Diagram of MnSOD RNA probes. The designated name is given on the left, and the 5′ to 3′ orientation is shown. The numbering of bases is from rat full-length MnSOD cDNA accession number Y00497. (A) RNAs used in stability and translation studies in RRL. The MnSOD coding region is depicted by the shaded bar. MnSOD-UTR contains the entire coding region plus 104 bases of 3′ UTR, thus terminating before the protein-binding sequence. MnSOD+UTR contains the entire coding region plus 327 bases of 3′ UTR, thus including the protein-binding element. (B) RNAs used in protein binding and competition studies. Both RMS-NP (216 bases) and RMS-NA (132 bases) begin at a *Nde*I restriction endonuclease site located 111 nucleotides downstream of the stop codon. RMS-NA1, RMS-NA2, and RMS-NA3 are oligoribonucleotide RNA-binding probes corresponding approximately to each consecutive third of RMS-NA.

the cis element and RNA-binding protein in MnSOD translation.

EXPERIMENTAL PROCEDURES

Animals. Specific pathogen-free Sprague Dawley rats were obtained from Taconic Farms, Germantown, NY, and were maintained in the animal care facility at Georgetown University Medical Center. Rats were killed by exsanguination after anesthesia with intraperitoneal injection of pentobarbital sodium (~200 mg/kg). Lungs were perfused with ice-cold 0.15 M NaCl, excised, and frozen in liquid nitrogen until use.

Preparation of Cell Extracts. Rat lung was homogenized in a 25 mM Tris buffer, pH 7.4, containing 0.1 mM EDTA, 1% Triton x-100, 40 mM KCl, 0.2 units/mL aprotinin, 10 μg/mL leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 27000*g* for 15 min at 4 °C. The supernatant fluid was subjected to centrifugation at 130000*g* for 2.5 h to obtain cytoplasmic extract (S130) for use in RNA-binding assays.

Chemical Assays. Protein concentrations were measured using the Coomassie Plus assay (Pierce Chemical Co.) with bovine serum albumin as a standard. RNA was quantitated using absorbance at 260 nm and an extinction coefficient of 0.025 $(\mu g/mL)^{-1}$ cm^{-1}.

Preparation of MnSOD 3′ UTR RNA Probes for Protein-Binding Studies. The MnSOD RNA probes were derived from a rat liver MnSOD cDNA-1 provided by Dr. Ye-Shih Ho, Wayne State University, Detroit, MI (*17*). For protein-binding experiments, a 216-base fragment located 111 bases downstream of the stop codon was cloned into pGEM-5Zf-(+); the resultant recombinant plasmid is designated RMS-12-Cl-16. RMS-12-Cl-16 was made linear with *Nco*I, and a labeled sense-strand transcript was prepared using the SP6 riboprobe system (Promega) in the presence of ^{32}P-CTP. Following electrophoresis in a 4% nondenaturing polyacrylamide gel, the transcript was eluted from the gel using 0.5 M ammonium acetate, 1% SDS, and 1 mM EDTA, precipitated by ethanol, and resuspended in 20 μL of H_2O. This transcript is designated RMS-NP (Figure 1). To delimit the region of binding, RMS-12-Cl-16 was made linear with *Asp*I to generate a 132-base probe designated RMS-NA (Figure 1B). For competition experiments, unlabeled cRNA transcripts were generated using the MEGAscript SP6 kit from Ambion for the synthesis of large amounts of RNA.

Preparation of Oligoribonucleotide Probes for Competition Studies. Oligoribonucleotide probes were synthesized from templates containing a T7 polymerase promoter sequence. These probes were designated RMS-NA1, RMS-NA2, and RMS-NA3, respectively (Figure 1B). The template for RMS-NA1 was 5′CATTTAATTAAGAAATTATCATAACTGTATGCTTACACATATATAGTGAGTCGTATTA3′. The template for RMS-NA2 was 5′AGTATGTACTGTTCTCAAACAGTTGCCTAACAATACTATAGTGAGTCGTATTA3′. The template for RMS-NA3 was 5′GTCCAAGCAATTCAAGCCTCTAATGAAAATGTTCAATCAAGAGCAGCTCACACCAATATAGTGAGTCGTATTA3′. The T7 promoter is underlined. The templates were incubated with a T7 primer (5′TAATACGACTCACTATA3′) for 3 min at 65 °C and then transcribed using either the Ribomax T7 system (Promega) or the T7 MEGAshortscript system (Ambion).

RNA Gel Retardation Assay. The binding of protein to RNA probes was assessed by means of a gel retardation assay as previously described (*15*, *16*). In some experiments, binding activity detected on the autoradiograph was measured using a scanning densitometer (Image Quant version 3.2; Molecular Dynamics, Inc.).

RNase H Experiments. Antisense oligodeoxyribonucleotides complementary to bases 793−831 (RMS-NA1), 834−866 (RMS-NA2), and 868−903 (RMS-NA3) were synthesized by the Georgetown University Core Facility using an Applied Biosystems DNA synthesizer. The sequences of the oligonucleotides were as follows: RMS-NA1, 5′CATTTAATTAAGAAATTATCATAACTGTATGCTTACACA3′; RMS-NA2, 5′AGTATGTACTGTTCTCAAACAGTTGCCTAACAA3′; and RMS-NA3, 5′TCTAATGAAAATGTTCAATCAAGAGCAGCTCACACC3′. The antisense oligodeoxyribonucleotides (150 ng and 15 μg) were annealed to ^{32}P-labeled RMS-NA sense-strand transcript in the gel retardation binding buffer for 15 min. RNase H (0.75 units, Promega) was added, and the resulting mixture was incubated for 15 min at 25 °C. Lung protein extract (20 μg) was then added, and a gel retardation assay was performed (*15*, *16*). Briefly, the lung extract was incubated with the probe for 20 min at 25 °C in binding buffer containing 5% glycerol, 40 mM KCl, 3 mM $MgCl_2$, 10 mM Hepes, pH 7.6. T1 RNase (1 unit) was then added and incubation continued for 10 min. The reaction mixture was subjected to electrophoresis in a low cross-linked 4% polyacrylamide gel (acrylamide-bis-acrylamide 60:1) containing 89 mM Tris-borate (pH 8.0) and 2 mM EDTA. The probe remaining after the RNase H digestion that was protected against the RNase T1 by protein binding was visualized on an autoradiograph of the gel.

In vitro Synthesis of MnSOD RNA Transcripts for RNA Translation Studies. The *Eco*RI insert from a full-length, rat liver MnSOD cDNA-1 provided by Dr. Ye-Shih Ho, Wayne State University, Detroit, MI (*17*), was cloned into pGem-7Z (Promega). Dideoxy sequencing of the insert was done to determine the orientation of the cDNA in the recombinant plasmid designated RMS-7-2. RMS-7-2 was made linear with *Pst*I and transcribed with T7 RNA polymerase to generate MnSOD RNA full-length sense-strand transcript containing 327 bases of 3′ UTR (thus containing the region of protein binding); this RNA is designated MnSOD+UTR (Figure 1A). RMS-7-2 was made linear with *Nde*I and transcribed with T7 RNA polymerase to generate MnSOD RNA full-length transcript containing 104 bases of 3′ UTR (thus terminating before the protein-binding site); this RNA is designated MnSOD-UTR (Figure 1A). The 5′ cap was added to both RNAs by including m7(5′)ppp(5′)G cap analogue in the transcription reaction. Synthesis of large amounts of RNA was done using the Ribomax T7 system (Promega) in the presence of 3 mM m7(5′)ppp(5′)G cap analogue, and the product was recovered by alcohol precipitation.

Rabbit Reticulocyte Lysate (RRL) Translation Experiments. For translation, 2 μg of nonradiolabeled capped MnSOD+UTR or MnSOD-UTR was incubated with 17.5 μL of RRL in the presence of L-[3,4,5 ^{3}H(N)] leucine for 2 h at 30° C. The translation reactions were performed under protein synthesizing conditions specified by Promega. Luciferase RNA (Promega) was used as a control. The reaction was terminated by incubation for 5 min at 30 °C in the presence of RNase A (0.2 μg/mL). The incorporation of the labeled amino acid into MnSOD was assessed by electrophoresis on a 10% polyacrylamide gel in the presence of SDS. At the end of electrophoresis, the gel was fixed in 2-propanol-acetic acid-H_2O (25:10:65), treated with Amplify (Amersham), dried under vacuum, and exposed to preflashed Hyperfilm MP. The band on the resultant fluorograph was quantitated by laser densitometry (Image Quant version 3.2; Molecular Dynamics, Inc.), and the values were calculated as relative densitometry units normalized to the measurement of translation of MnSOD+UTR in the absence of competitor oligoribonucleotides. The apparent molecular mass of the observed band was estimated by calibration against ^{14}C-labeled protein standards (Amersham).

Translation Initiation Assays. RRL extract (Promega) was incubated with or without RMS-NA1, RMS-NA2, or RMS-NA3 RNA in the presence of RNasin (20 units) for 30 min at 25 °C. Incubation was continued for 3 min at 30 °C after addition of 20 μM complete amino acid mix, 0.5 mM cycloheximide (Sigma) or 0.5 mM anisomycin (Sigma), and, where indicated, 2 mM GMP-PNP (Boehringer Mannheim). ^{32}P-labeled MnSOD+UTR was added and incubated for 10 min at 30 °C. Reactions were stopped by adding 10 volumes of ice-cold buffer A (25 mM Hepes, pH 7.0, 50 mM KCl, 2 mM MgAc) and placing on ice for 5 min. Translation initiation complexes were then resolved on 10-mL linear 5%-25% sucrose gradients in buffer A (25 mM Hepes, pH 7.0, 50 mM KCl, 2 mM MgAc), by centrifugation at 175000*g* for 110 min at 4 °C. Fractions (500 μL) were collected from the bottom of the gradient, and the RNA was recovered by adding an equal volume of ice-cold 10% TCA, followed by filtration through GF/C filters. The filters were washed with 5% TCA-95% ethanol, and the radioactivity in each sample was determined by scintillation counting of the filter dissolved in optifluor-o (Packard). The sum of the ^{32}P disintegrations per minute was determined for each gradient, and the data are given as the percentage of total radioactivity present in each fraction.

Statistical Analysis. For densitometry data, the values for individual animals were averaged per experimental group, and the standard error of the group mean was calculated. The significance of the difference between two groups was obtained using an unpaired *t*-test analysis (*18*).

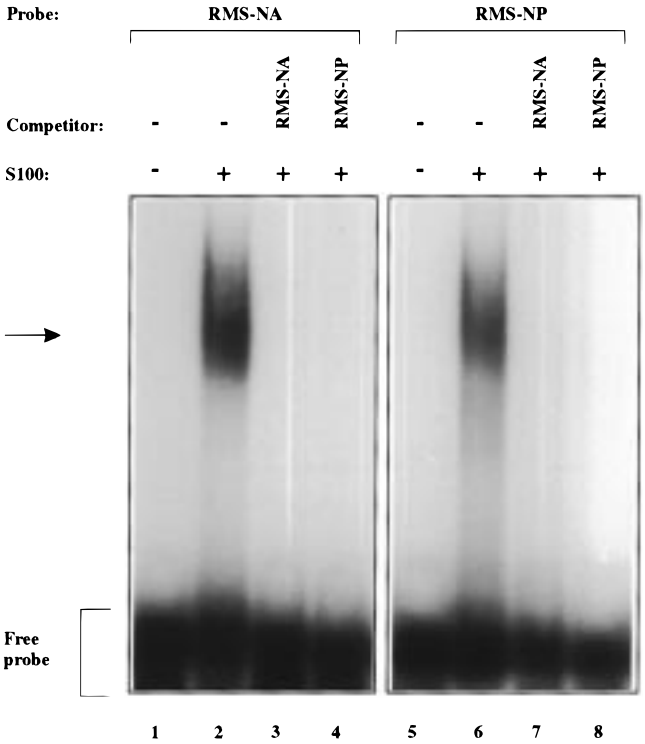

FIGURE 2: Cross-competition RNA-binding assays. Gel retardation assays show that an RNA-protein complex was formed (arrow) between ^{32}P-labeled RMS-NA probe and 20 μg of S130 lung protein extract (lane 2). Complex formation was effectively eliminated by competition with unlabeled RMS-NA (115 pmol) and RMS-NP (70 pmol), lanes 3 and 4, respectively. In a cross-competition study, RNA-protein complex formation between 20 μg of S130 and ^{32}P-labeled RMS-NP probe (lane 6) was blocked by RMS-NA (115 pmol) and RMS-NP (70 pmol) competition, lanes 7 and 8, respectively. Lanes 1 and 5 are controls that show that no complex was formed in the absence of S130. The migration of free probe is indicated by the brackets.

RESULTS

Mapping the Protein-Binding cis Element in the 3′ UTR of MnSOD RNA. Earlier studies indicated that the 3′ UTR of MnSOD RNA bound a redox-sensitive protein at a region that was 450 bases long (*15*). Subsequently, a 216-base element, RMS-NP, was shown to be involved in binding to protein (*16*). Gel retardation and cross-competition studies now show that protein binding takes place on a 132-base cis element located between *Nde*I and *Asp*I restriction endonuclease sites; this region is designated RMS-NA. Figure 2 shows the results of a cross-competition gel retardation assay. An RNA-protein complex was formed using either RMS-NP or RMS-NA (lanes 3 and 6). Autoradiographic evidence of complex formation was abolished when either unlabeled RMS-NP or RMS-NA was used in a competition assay (lanes 3, 4, 7, and 8). These data indicate that RMS-NA contains the protein-binding region of the 3′ UTR. RMS-NA is located 111 bases downstream of the stop codon, is present in the 3′ UTR of all rat MnSOD mRNA species resulting from alternate polyadenylation (*19*), and contains the first polyadenylation signal.

Analysis of sequence information available in GenBank using the FASTA program of the Genetics Computer Group revealed significant homology between the rat cis element, RMS-NA, and regions in the 3′ UTR of mouse, cow, and human MnSOD RNAs at approximately the same distance downstream of the stop codon (Table 1, Figure 3). Together with the fact that MnSOD RNA-binding protein activity was present in cells of all species tested including rat, baboon, human, mouse, monkey, and hamster (data not shown), the finding of a 3′ region with ~75% conserved sequence among 4 species (Table 1, Figure 3) suggests that our studies with the rat MnSOD 3′ UTR may elucidate mechanisms of post-transcriptional regulation that apply to many species.

Table 1. Homology between MnSOD 3′ UTRs Compared to Rat cis Element, RMS-NA[a]

species (accession no.)	% identity	distance from stop codon
cow (L22092)	73.1% in 108 b overlap	138 b
human (X15132)	74.7% in 79 b overlap	131 b
mouse (X04972)	71.4% in 56 b overlap	118 b

[a] "b" stands for the number of bases.

Gel retardation competition studies were performed using oligoribonucleotides corresponding approximately to each third of RMS-NA. The results of competition between ^{32}P-labeled RMS-NA and nonlabeled sense-strand RMS-NA1, RMS-NA2, and RMS-NA3 oligoribonucleotides indicate that specific binding occurs in the region mapped to RMS-NA1. A dose-titration competition experiment showed that RMS-NA1 competed with RMS-NA for protein binding whereas RNA-protein complex formation was not inhibited by RMS-NA2 or RMS-NA3 (Figure 4).

RNase H experiments further substantiated that RMS-NA1 is the cis element involved in binding to protein. Specific antisense oligodeoxyribonucleotides were synthesized for RMS-NA1, RMS-NA2, and RMS-NA3 and were incubated with ^{32}P-labeled sense-strand RMS-NA. After treatment with RNase H to degrade the RNA in the RNA-DNA duplexes, only RMS-NA1 antisense prevented MnSOD RNA-binding protein activity (Figure 5). These data indicate that specific protein binding takes place on the 41-base element designated RMS-NA1. The sequence of this region is as follows: 5′AUGUGUAAGCAUACAGUUAUGAUAAUUUCUUAA-UUAAAUG3′.

The Role of the 3′ UTR in Translation of MnSOD RNA in Rabbit Reticulocyte Lysate. Before using RRL to examine the role of the MnSOD RNA 3′ UTR in translation, we determined that RRL contains MnSOD RNA-binding protein activity and that the RNA-binding activity in RRL detected in a gel retardation assay could be specifically inhibited by unlabeled RMS-NA1, but not by RMS-NA3 (Figure 6).

To test the hypothesis that the 3′ UTR plays a role in MnSOD translation, capped MnSOD RNAs with and without the 3′ UTR protein-binding element were translated in vitro. Synthesis of MnSOD was then assessed by SDS-polyacrylamide gel electrophoresis. The RRL was treated with micrococcal nuclease to degrade endogenous mRNA while leaving the ribosomal RNA intact, thus ensuring complete synthesis of proteins with minimal background. The 22-kDa band detected on the fluorograph was verified as MnSOD by Western analysis (data not shown). Figure 7A is a representative fluorograph showing that MnSOD translation is diminished in the reaction containing MnSOD-UTR compared to the reaction mixture containing MnSOD+UTR. There was no translation in the absence of exogenous RNA. Analysis of nine separate experiments by scanning densito-

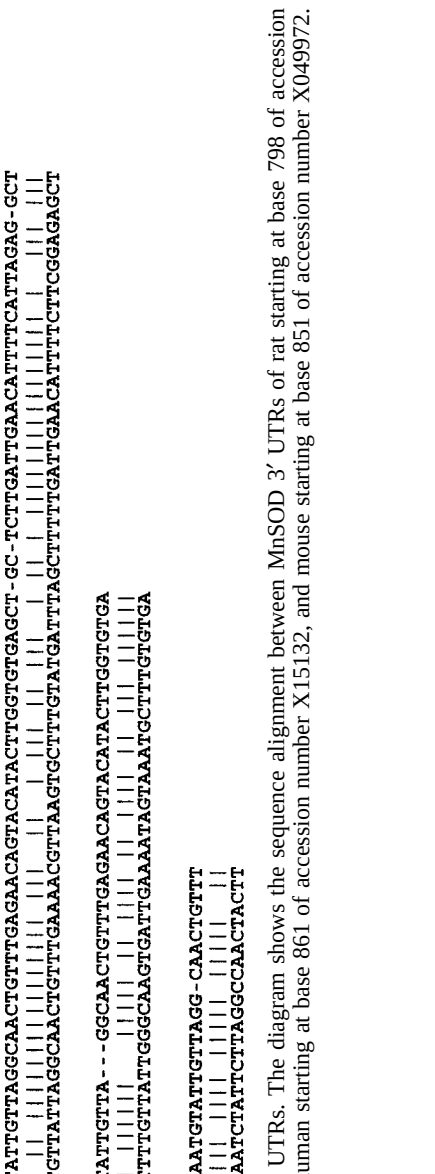

FIGURE 3: Sequence conservation among rat, cow, human, and mouse 3′ UTRs. The diagram shows the sequence alignment between MnSOD 3′ UTRs of rat starting at base 798 of accession number Y00497, cow starting at base 835 of accession number L22092, human starting at base 861 of accession number X15132, and mouse starting at base 851 of accession number X049972.

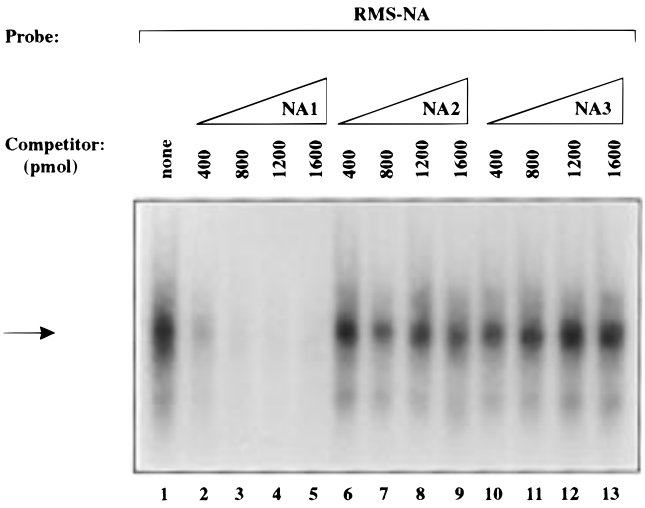

FIGURE 4: Mapping the cis element: competition RNA-binding assays. Gel retardation assay shows complex formation (arrow) between ^{32}P-labeled RMA-NA and 15 μg of S130 lung extract in the absence of competitor RNA (lane 1). In the dose-titration competition study, protein binding to RMS-NA is competed more efficiently by RMS-NA1 than by RNA from either region RMS-NA2 or region RMS-NA3. The amount of competitor in picomoles is given at the top of the autoradiograph.

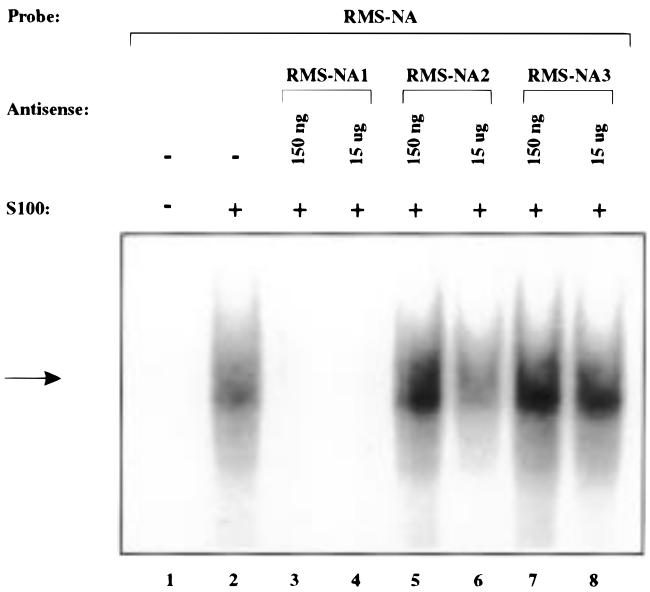

FIGURE 5: Mapping the cis element: RNase H assays. The gel retardation assay shows that the complex (arrow) between ^{32}P-labeled RMS-NA probe and 20 μg of S130 lung extract protein (lane 2) was eliminated when antisense oligodeoxyribonucleotide RMS-NA1 (lanes 3 and 4) was prehybridized to the probe and treated with RNase H before incubation with S130. Complex formation was not eliminated when ^{32}P-labeled RMS-NA was prehybridized with either antisense oligodeoxyribonucleotide RMS-NA2 (lanes 5 and 6) or RMS-NA3 (lanes 7 and 8) and treated with RNase H before incubation with S130. The amount of antisense oligodeoxyribonucleotide used is indicated at the top of the figure.

metry indicated that translation in the presence of the 3′ cis element was 60% greater than when the protein-binding region was absent. Translation in the absence of the 3′ UTR was only 0.40 ± 0.04 ($p < 0.05$, $n = 9$) relative to translation of MnSOD+UTR.

To test whether the interaction between the cis element and the binding protein was required for the increased

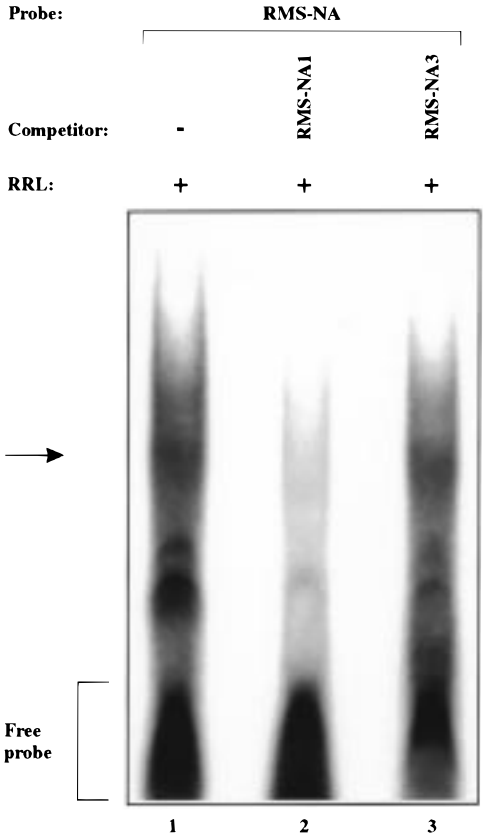

FIGURE 6: RNA-binding assay in RRL. The gel retardation assay shows complex formation (arrow) between ^{32}P-labeled RMS-NA and 17.5 μL of RRL (same amount used in in vitro translation assays). Binding protein activity was inhibited by unlabeled competitor RNA and by RMS-NA1 (740 pmol) (lane 2), but not by RMS-NA3 RNA (1080 pmol) (lane 3).

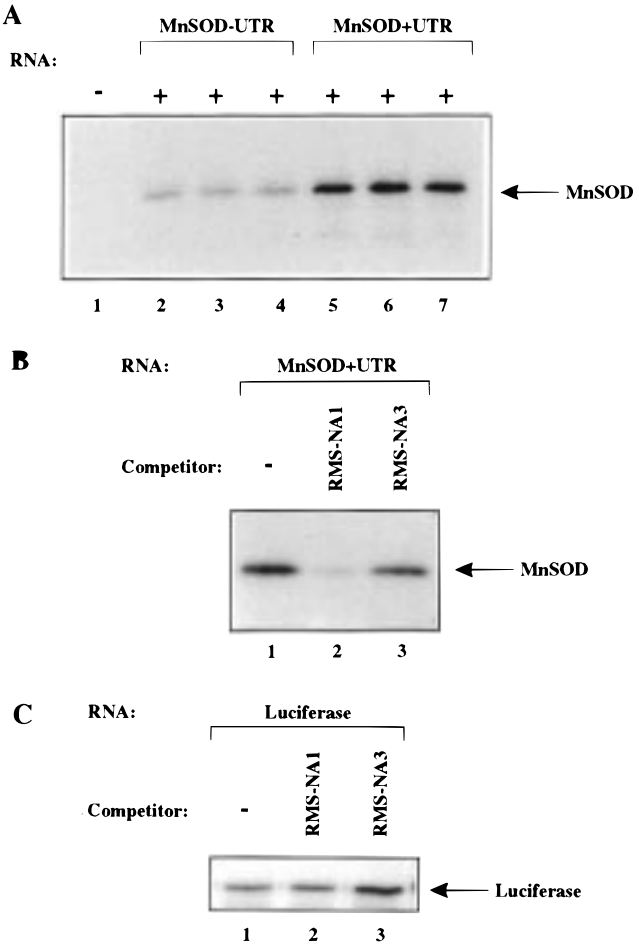

FIGURE 7: Translation studies in RRL. (A) Fluorograph of translation products in RRL when no exogenous RNA (lane 1), MnSOD-UTR RNA (lanes 2−4), or MnSOD+UTR RNA (lanes 5−7) was added to the translation reaction. The arrow points to the 22-kDa monomer of MnSOD that is revealed by fluorography of the SDS-polyacrylamide gel. (B) Fluorograph of the translation of MnSOD (arrow) synthesized when MnSOD+UTR RNA was added to the translation reaction alone (lane 1), in the presence of the specific inhibitor RMS-NA1 (lane 2), or in the presence of noncompetitive RMS-NA3 (lane 3). (C) Fluorograph of the translation products in RRL when control luciferase RNA was added to the translation reaction alone (lane 1), in the presence of RMS-NA1 RNA (lane 2), or in the presence of RMS-NA3 RNA (lane 3). The arrow points to the 61-kDa monomer of luciferase revealed by fluorography of the SDS-polyacrylamide gel.

translational efficiency, RMS-NA1 was added to the RRL to limit the availability of unbound MnSOD RNA-binding protein. Figure 7B shows that, when binding protein activity was specifically competitively inhibited by preincubation with RMS-NA1, translation of capped full-length MnSOD+UTR was diminished significantly. In four separate trials, translation in the presence of the specific competitor, RMS-NA1, was only 0.35 ± 0.10 ($p < 0.05$) relative to translation in control reactions. These results indicate that RMS-NA1 was sequestering a protein component required for translation. Thus, when MnSOD RNA-binding activity is inhibited, translation decreased 65%. The specificity of the effect is indicated by the finding that the noncompetitive oligoribonuceotide, RMS-NA3, had no significant effect on MnSOD translation (Figure 7B). In addition, neither RMS-NA1 nor RMS-NA3 diminished the translation of luciferase RNA that was used as a control (Figure 7C). The data from the competition experiments indicate that the effect of the cis element is specific and operates through a trans-acting protein factor. It is possible the effect of the 3′ UTR on translation was due to an alteration in RNA degradation. However, in the translation studies, a large amount of transcript (2 μg) was added to RRL, and ^{32}P-labeled RNA was added as a tracer; under these circumstances there was no detectable difference in the amount of RNA remaining at the end of the 2-h incubation (data not shown). Therefore, the difference in translation between the RNA containing the cis element and the RNA from which the protein-binding element was deleted was not due to alterations in stability under the conditions of the translation studies. Collectively, the data from the translation experiments indicate that the 3′ cis element of MnSOD RNA together with RNA-protein-binding activity is required for efficient translation of MnSOD; in the absence of the 3′ cis element or in the presence of a specific competitor RNA that blocks MnSOD RNA-protein binding, translation is significantly diminished.

Effect of MnSOD RNA-Binding Protein Activity on Translation Initiation Complexes. To determine the step in translation at which the loss of MnSOD RNA-binding protein activity was modulating translational efficiency, translation initiation assays were performed in which RRL and ^{32}P-labeled MnSOD+UTR were incubated with and without competitor RNA. The polysomal profile distribution of MnSOD RNA was then assessed by isolating the RNA and measuring the radioactivity recovered after fractionation on

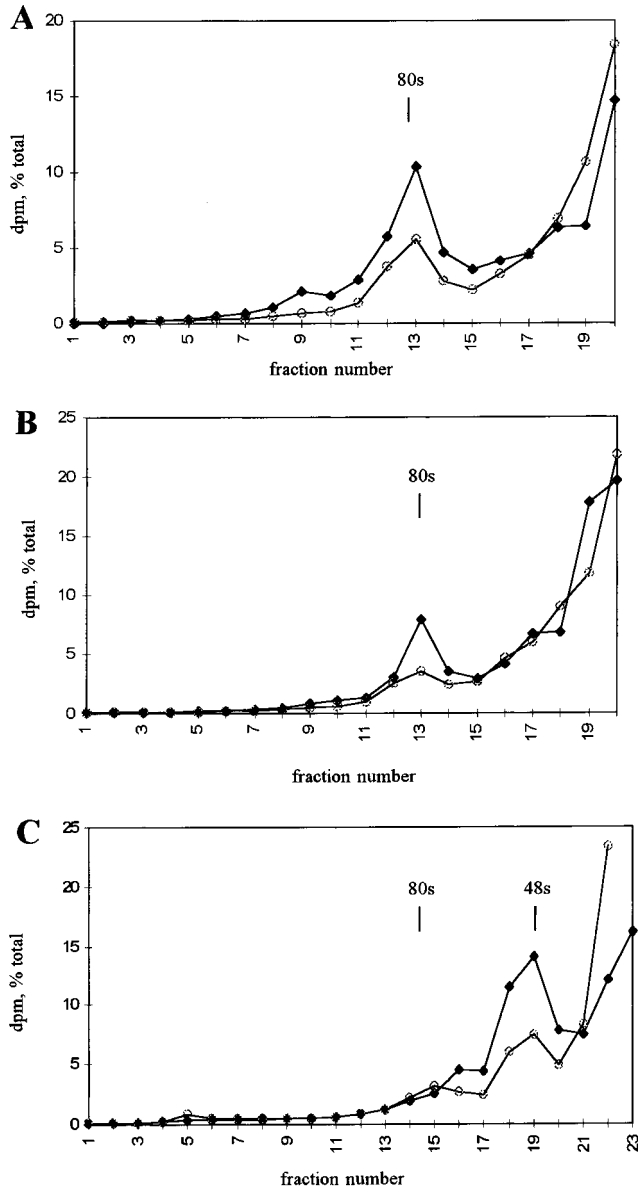

FIGURE 8: Sucrose gradient profiles of translation initiation assays. Equal amounts of ^{32}P-labeled 5′ capped MnSOD+UTR RNA were mixed and incubated in RRL in the absence (filled diamonds) or in the presence of competitor RMS-NA1 RNA to inhibit the activity of the binding protein (open circles). After fractionation on linear 5%−25% sucrose gradients, fractions were collected from the bottom of the gradient and absorbance at 254 nm was monitored. The positions of 80s and 48s particles are indicated. The labeled RNA in fractions is expressed as a percentage of the total counts recovered and is plotted against the fraction number. (A) The assay was done in the presence of 0.5 mM cycloheximide. (B) The assay was done in the presence of 0.5 mM anisomycin. (C) The assay was done in the presence of 0.5 mM cycloheximide and 2 mM GMP-PNP.

a 5%−25% linear sucrose gradient. To resolve the pattern of complexes that sediment at 80s or more slowly, the translation initiation assays were performed in the presence of 0.5 mM cycloheximide, an inhibitor of elongation that traps 80s ribosomes at the AUG initiator codon to prevent runoff. Figure 8A shows that MnSOD+UTR was located in two peaks, one major peak at the location of the 80s ribosome and another very small peak migrating slightly faster than 80s. When binding protein was made unavailable by incubating RRL in the presence of the MnSOD-NA1 competitor, there was ∼50% less MnSOD+UTR at the location of 80s and a loss of the smaller second peak. The position of labeled MnSOD+UTR shifted from the location of 80s to fractions at the top of the gradient that cosediment with the small ribosomal subunit (Figure 8A). Thus, it appears that the translation repression observed following inhibition of RNA-binding protein activity was associated with a reduction in 80s complex formation. These experiments were repeated nine times, and on each gradient the same pattern shift in the presence of the MnSOD-NA1 competitor was observed; that is, when protein binding to the ^{32}P-labeled RNA was inhibited, there was a reduction in the fraction of MnSOD RNA bound to the 80s ribosome. In addition to the ∼50% decrease in the percentage of total MnSOD+UTR associated with 80s, the second smaller peak was eliminated. The exact nature of this second peak is unclear. Although 0.5 mM cylcoheximide completely blocked incorporation of ^{3}H-labeled leucine into MnSOD (data not shown), the small peak may represent partial elongation. When anisomycin was used in lieu of cycloheximide to block elongation, the small peak with faster mobility was not observed (Figure 8B). As was the case in the presence of cycloheximide, the peak at 80s was diminished in the gradient from RRL in which the binding protein activity was inhibited (Figure 8B). There was no decrease in the formation of the initiation complex when the experiments were carried out in the presence of RMS-NA2 or RMS-NA3 (Figure 9), indicating that the effect of RMS-NA1 is specific and that, when MnSOD-RNA binding protein activity is inhibited, translation initiation is reduced to 50% of the control value. When the translation initiation assays contained GMP-PNP, a nonhydrolyzable GTP analogue that prevents joining of the 40s and 60s ribosomal subunits, the sucrose gradient profile detected the 48s preinitiation complexes stalled at the initiation codon (Figure 8C). Figure 8C is representative of three separate experiments and shows that as a percentage of total radioactivity recovered, significantly less MnSOD RNA is present in preinitiation complexes when the RNA-binding protein activity is blocked by competition with RMS-NA1. Thus, it appears that the RNA-binding protein plays a role as a positive regulator of translational efficiency and that it may exert its effect at the level of preinitiation complex formation.

DISCUSSION

The aim of the experiments in this paper was to initiate a mechanistic approach to understanding the regulation of MnSOD gene expression at the post-transcriptional level. In experimental models of oxidant stress, the ability to withstand the damaging effects of hyperoxia appears to be regulated, at least in part, by the translational efficiency of MnSOD in the lung. Understanding the mechanism controlling MnSOD translational yield is important because an inability to defend against free radical damage to the mitochondria is responsible, in large part, for the pulmonary oxygen toxicity that results in mortality. In addition, because the potential for gene therapy in the lung is advancing quickly, it is important to understand ways in which the resultant increased concentration of RNA can be maximally utilized for protein synthesis (minimize blocks to translation and enhance translational efficiency).

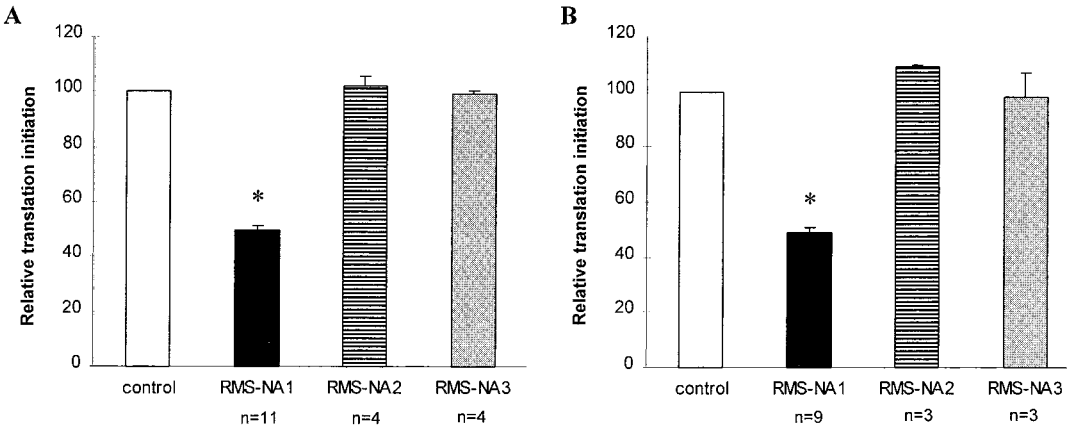

FIGURE 9: Translation initiation complex formation in the presence of competitive and noncompetitive oligoribonucleotides. The effect of RMS-NA1, RMS-NA2, and RMS-NA3 on formation of the 80s initiation complex was calculated compared to a control reaction in the absence of oligoribonucleotide set at 100%. Data are given as mean ± SEM. (A) The assay was done in the presence of 0.5 mM cycloheximide. (B) The assay was done in the presence of 0.5 mM anisomycin. The asterisk (*) indicates $p < 0.01$ compared to RMS-NA2 or RMS-NA3.

There is a rapidly growing number of reports in the literature describing the role of RNA-binding proteins in post-transcriptional regulation at the level of both RNA stability and translation. The results of this report have mapped the cis element of MnSOD RNA involved in protein binding to within 41 bases of the 3′ UTR. There is significant sequence homology at this region of the 3′ UTR among different species suggesting that, as MnSOD is found in the mitochondria of all aerobic eukaryotes, an RNA-binding protein may also be ubiquitous and act by a universal mechanism.

The value of controlling MnSOD expression at a post-transcriptional level is partly due to the very large amplification that occurs in protein synthesis. Typically more than 10^4 mol of protein is synthesized per mole of mRNA (*20*, *21*); thus, it is energetically favorable to increase protein synthesis by increasing the translational yield. Our data suggest that MnSOD translational efficiency is increased through the action of a protein that binds to the 3′ UTR of MnSOD RNA. The result of this RNA-binding activity is increased translation, and the RNA-binding protein may achieve its effect by enhancing the formation of the preinitiation complex. From a physiological point of view, these studies must be confirmed using an in vivo system. However, there is favorable correlative evidence supporting the concept that the MnSOD RNA-binding protein is a positive regulator of translational efficiency in vivo. When rats are treated with lipopolysaccharide (LPS), lung MnSOD activity is increased by ~40% (1.38-fold) 6 h after LPS treatment whereas MnSOD mRNA is elevated 20-fold, indicating that translational efficiency is markedly decreased by LPS treatment (*22*). This response is preceded by a fall, 1 h after LPS treatment, in the activity of MnSOD RNA-binding protein (*22*), suggesting that the lack of RNA-binding protein activity might be responsible, at least in part, for diminished translation of the markedly elevated MnSOD RNA.

The finding of a translational enhancer element in the 3′ UTR of MnSOD RNA supports the concept proposed by Sachs and Buratowski that transcription and translation may have evolved similar mechanisms to regulate gene expression (*23*). As transcriptional enhancer elements have been shown to exert their effect at long distances from the transcription site, so too translational enhancer elements can be located in regions other than the site of translation initiation. As suggested by Jacobson (*24*), translational enhancer elements are functionally equivalent to transcriptional enhancers, and in both cases the activity of the enhancer can be modulated by trans-acting factors. There are several examples in mammalian systems in which 3′ UTR elements and their binding proteins function as negative regulators, not enhancers, of translation. These include mouse protamine 2 mRNA (*25*, *26*), human interferon-β mRNA (*27*), erythroid 15-lipoxygenase mRNA (*28*, *29*), and the α_{2c}-adrenergic receptor mRNA (*30*). To our knowledge, apart from the report for MnSOD in this paper, there are only two examples of a 3′ UTR element and its binding protein acting as a positive regulator of translation. The first is the recent finding that microtubule-associated protein 1 light chain 3 (LC3) binds to the 3′ UTR of fibronectin mRNA and enhances fibronectin mRNA translation (*31*). The second example is the poly(A) tail and poly(A)-binding protein (PABP) that stimulate translation by interacting with the 5′ UTR in the formation of the preinitiation complex (*32*−*37*). One model to explain the translational effects of the poly(A) tail is the closed-loop model (*24*); in this paradigm, the poly(A) tail at the 3′ end of the RNA interacts with the 5′ region through the action of the bound PABP. The results of our study indicate that the interaction between the 3′ UTR of MnSOD RNA and the MnSOD RNA-binding protein may have a role in the initiation of protein synthesis at the level of preinitiation complex formation. Whether the MnSOD 3′ UTR cis element and binding protein interact with the 5′ UTR to provide a pseudo-circular structure that improves translational efficiency comparable to the closed-loop model for PABP bound to a poly(A) tail remains to be determined.

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