

Translation of a Testis-Specific Cu/Zn Superoxide Dismutase (SOD-1) mRNA Is Regulated by a 65-Kilodalton Protein Which Binds to Its 5' Untranslated Region

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Mouse testes contain two distinct superoxide dismutase (SOD-1) transcripts which differ by 114 nucleotides in their 5' untranslated regions (UTRs) (W. Gu, C. Morales, and N. B. Hecht, *J. Biol. Chem.* 270:236–243, 1995). The shorter SOD-1 mRNA, a somatic type SOD-1 mRNA ($S_{\text{SOD-1}}$), is ubiquitously expressed in all somatic tissues as well as in testes. The larger SOD-1 mRNA, a testis-specific SOD-1 mRNA ($T_{\text{SOD-1}}$), derived from an alternative upstream start site, is transcribed solely in postmeiotic germ cells and is translationally regulated during spermiogenesis. Since the two mRNAs have identical nucleotides except that $T_{\text{SOD-1}}$ has an additional sequence at its 5' terminus, we have proposed that the extra 5' UTR sequence may be involved in the translational control of the $T_{\text{SOD-1}}$ mRNA during spermiogenesis. Here we show that, when assayed in a cell-free system, $T_{\text{SOD-1}}$ is translated only slightly less efficiently than $S_{\text{SOD-1}}$. RNA gel retardation and UV cross-linking assays reveal that a testicular cytoplasmic protein (Cu/Zn superoxide dismutase RNA-binding protein [SOD-RBP]) of about 65 kDa specifically binds to the extended 5' UTR of $T_{\text{SOD-1}}$. After purification of SOD-RBP by RNA affinity chromatography, we demonstrate that SOD-RBP can repress the *in vitro* translation of $T_{\text{SOD-1}}$ mRNA but not $S_{\text{SOD-1}}$ mRNA or cotranslated luciferase mRNA. We conclude that SOD-RBP serves as a repressor in the translation of $T_{\text{SOD-1}}$ mRNA during spermiogenesis and thereby fine-tunes the level of Cu/Zn superoxide dismutase produced in maturing germ cells.

Translation of mRNAs is often regulated by specific cytoplasmic proteins that interact with regulatory elements within their 5' or 3' untranslated regions (UTRs). The iron-responsive-element-binding protein, one of the best-characterized RNA-binding proteins, regulates cellular iron metabolism by binding to sequence elements in the 5' UTR of ferritin mRNA and the 3' UTR of transferrin receptor mRNA (30, 31, 34, 38, 67). Other RNA-binding proteins such as a cytoplasmic polyadenylation element-binding protein mediate mRNA poly(A) elongation during *Xenopus* oocyte maturation (25, 52), a 70-kDa poly(A)-binding protein binds to poly(A) tails and helps stabilize mRNAs (9, 60), and *Xenopus* p54 and p56 (47–49) or their mouse homologs p48 and p52 (42, 64) function as masking proteins by binding to stored mRNAs in an apparent sequence- or structure-independent manner.

Translational regulation of stored mRNAs plays an important role in programmed early development. During oogenesis in *Xenopus laevis*, as much as 90% of the total poly(A) RNA is sequestered from translation by repressor (masking) proteins that bind to the mRNAs and inhibit their translation (14, 35, 58). Similarly, during spermatogenesis, nearly 70% of mouse testicular mRNAs are subject to translational control, leading to their germ cell stage-specific expression (19, 20, 28, 29).

The mechanisms regulating translation of two mouse sperm nuclear proteins, protamines 1 and 2, whose mRNAs are stored during spermatogenesis, have begun to be understood. Braun et al. (10), using transgenic mice, have demonstrated that the temporal expression of protamine 1 protein is regulated solely by its 3' UTR. Moreover, a specific cytoplasmic phosphoprotein that binds to a conserved sequence within the 3' UTR of the protamines and represses its translation in a cell-free system has been identified (41). This protein is also

present in the brain, where it binds mRNAs to microtubules and facilitates their transport (26).

Cu/Zn superoxide dismutase (SOD-1, EC 1.15.1.1) is an essential enzyme that catalyzes the reaction $2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$, thus providing a means for protection of cells against damage from oxygen radicals. Studies have revealed that mutations in the human SOD-1 gene are associated with Lou Gehrig's disease, familial amyotrophic lateral sclerosis (15, 59). In *Saccharomyces cerevisiae*, SOD-1 plays a vital role in protecting cells from oxidative stress, since a number of oxygen-related growth defects are seen when the gene is deleted (11, 45). In the mammalian testis, SOD-1 is believed to be essential to maintain the viability of spermatozoa (2–4).

In most mammalian somatic cells, SOD-1 is expressed primarily from one mRNA of about 0.70 kb (8, 44). However, two size classes of SOD-1 mRNAs, which differ by 200 nucleotides in their 3' UTRs, have been detected in certain human somatic tissues (61, 62). Recently, it has been shown that the longer somatic SOD-1 mRNA produces three times more enzyme when transfected into COS 1 and human 293 cells than the shorter SOD-1 mRNA (36). Rat and mouse testes also contain multiple SOD-1 mRNAs (24b, 33a). In the mouse testis, two SOD-1 transcripts, which differ by 114 nucleotides in their 5' UTRs, have been characterized (24b). The shorter SOD-1 mRNA, the somatic-type SOD-1 ($S_{\text{SOD-1}}$), is ubiquitously expressed in somatic tissues and in testes. The longer SOD-1 mRNA, a testis-specific SOD-1 ($T_{\text{SOD-1}}$) mRNA derived from a more upstream transcriptional start site, is solely transcribed in male postmeiotic germ cells. In contrast to $S_{\text{SOD-1}}$, $T_{\text{SOD-1}}$ is translationally regulated during spermiogenesis (24b). Here we demonstrate that the two testicular SOD-1 mRNAs show only slight differences in translation ability when assayed in a cell-free system, suggesting the existence of a regulatory factor that interacts with the 5' UTR of $T_{\text{SOD-1}}$ and modulates its translation during spermatogenesis. On the basis of gel retardation

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and UV cross-linking assays, we have identified a testicular cytoplasmic protein of about 65 kDa (Cu/Zn superoxide dismutase RNA-binding protein [SOD-RBP]) that binds to the novel 114 nucleotides in the extended 5' UTR of T_{SOD-1} mRNA. After purification of SOD-RBP by RNA affinity chromatography, we demonstrate that the addition of SOD-RBP to a cell-free translation system represses the translation of T_{SOD-1} mRNA but not S_{SOD-1} mRNA or cotranslated luciferase mRNA.

MATERIALS AND METHODS

Preparation of SOD-1 transcripts. For the in vitro translation assays, RNA was transcribed from pGEM 3Z constructs of cDNAs encoding mouse T_{SOD-1} (759 bp) and S_{SOD-1} (645 bp) as previously described (24b). The two SOD-1 cDNAs are identical except that T_{SOD-1} has an additional 114 nucleotides in its 5' UTR. For the gel retardation assays, the novel 114 nucleotides of the 5' UTR of the T_{SOD-1} cDNA (5'T_{SOD-1}) were PCR amplified and subcloned into pGEM 3Z by using the *EcoRI* and *AvaI* sites. Deletion constructs of this 114-nucleotide fragment were produced by digesting the 5'T_{SOD-1} subclone with the restriction enzyme *SacI*, *SylI*, or *StuI*, which cut at nucleotides 36, 63, and 84 within the 5'T_{SOD-1}, respectively. To subclone these deletion constructs, the restriction-digested fragments were incubated for 30 min at 30°C with Klenow enzyme (50 U/ml) and religated. With +1 designating the transcription start site of T_{SOD-1}, seven deletion constructs of the 5'T_{SOD-1} were generated by this procedure. Δ₁ contains nucleotides 1 to 84, Δ₂ contains nucleotides 1 to 64, Δ₃ contains nucleotides 1 to 36, Δ₄ contains nucleotides 36 to 84, Δ₅ contains nucleotides 84 to 114, Δ₆ contains nucleotides 64 to 114, and Δ₇ contains nucleotides 36 to 114 (see Fig. 4A).

For the RNA affinity column, the novel 114 nucleotides of the T_{SOD-1} 5' UTR were PCR amplified and subcloned into the *HindIII* and *AvaI* sites of the pSP64 poly(A) vector (Promega), creating pSP64-5'T_{SOD-1}. After pSP64-5'T_{SOD-1} was linearized with *EcoRI*, SP6 RNA polymerase was used to transcribe the vector to synthesize RNA transcripts which contain the 114 nucleotides of the 5' UTR of T_{SOD-1} and 30 nucleotides of poly(A).

Cell-free translation of S_{SOD-1} and T_{SOD-1} mRNAs. Capped SOD-1 mRNAs were transcribed in vitro with the Message Machine kit (Ambion) and used according to the supplier's instructions. To facilitate quantitation of synthesized RNAs, a trace amount of [³H]UTP was included in the transcription reactions. Constructs of T_{SOD-1} and S_{SOD-1} in pGEM 3Z were linearized at the *AvaI* site and transcribed with T7 RNA polymerase, and the RNAs were resolved in 6% urea-polyacrylamide gels. The RNA bands were excised, eluted in a solution of 2 M ammonium acetate and 1% sodium dodecyl sulfate (SDS), and precipitated with 70% ethanol. The RNAs were dissolved in RNase-free water and used immediately for in vitro translation or stored at -80°C.

In vitro translations were carried out in rabbit reticulocyte lysates (Promega). Equimolar amounts of in vitro-transcribed T_{SOD-1} or S_{SOD-1} mRNA were used as templates for protein synthesis in a 25-μl reaction mixture containing 17.5 μl of reticulocyte lysate, 20 μCi of [³⁵S]methionine (Amersham), and 10 U of RNasin (Promega). After a 60-min incubation at 30°C, the synthesized protein products were analyzed by electrophoresis in an SDS-12.5% polyacrylamide gel (SDS-PAGE) and visualized by autoradiography. In some assays, an aliquot of RNA affinity column-purified SOD-RBP was preincubated with the SOD-1 transcripts for 5 min at 25°C in RNA-binding buffer before the mRNAs were added to the reticulocyte lysates. For some cotranslations, an aliquot of RNA affinity column-purified SOD-RBP was added to SOD-1 and control luciferase transcripts (Promega). Aliquots of the cotranslated transcripts were then deproteinized with phenol-chloroform and retranscribed in rabbit reticulocyte lysates.

To verify that the protein synthesized in vitro was SOD-1, immunoprecipitation was performed. An aliquot (5 μl) of the reaction mixture was incubated with 1 μl of rabbit antiserum against mouse SOD-1 protein (kindly provided by L.-Y. Chang of Duke University, Durham, N.C.) in 20 μl of phosphate-buffered saline for 1 h at 4°C and then with 2 μl of protein A-agarose beads (Sigma) for 1 h at 4°C. After centrifugation at 8,000 × g for 5 min, the supernatant and pellet were separated and analyzed by SDS-12.5% PAGE. Rabbit preimmune serum was utilized as a negative control.

Gel retardation binding assays. ³²P-labeled RNA probes were transcribed in vitro from 1 μg of an *AvaI*-linearized pGEM 3Z construct of 5'T_{SOD-1} by using a Riboprobe Gemini system (Promega). Transcriptions were carried out with T7 RNA polymerase (Promega) and 60 μCi of [³²P]CTP (Amersham Corp.). After incubation overnight at 37°C, the synthesized probes were resolved in 6% urea-polyacrylamide gels and the gel slice containing the predicted size of RNA was removed. The RNA was eluted in 500 μl of buffer containing 2 M ammonium acetate, 1% SDS, and 1 mM EDTA and precipitated with 70% ethanol. When large amounts of unlabeled RNAs were needed, a MEGAscript kit (Ambion) was used and trace amounts of [³²P]CTP were added to allow detection and quantitation of the synthesized RNAs.

RNA-protein binding complexes were analyzed at room temperature as previously described (40). Briefly, 4 × 10⁴ cpm of radiolabeled RNA probes (approximately 0.2 ng) was incubated with testicular protein extracts (20 to 40 μg)

for 20 min at room temperature in 20 μl of binding solution containing 20 mM HEPES (*N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5), 3 mM MgCl₂, 40 mM KCl, 2 mM dithiothreitol, and 5% glycerol. In some cases, protein extracts from adult mouse somatic tissues were utilized. Unbound RNA was removed by a 10-min incubation with 1 U of RNase T₁ (Gibco BRL), and nonspecific RNA-protein interactions were minimized by incubation with heparin (5 mg/ml) for an additional 10 min. RNA-protein complexes were resolved in 4% polyacrylamide native gels and visualized by autoradiography. The specificity of RNA-protein binding was established by competition assays in which unlabeled RNA competitors were incubated for 5 min with the reaction components before the labeled RNA was added.

UV cross-linking of RNA-protein complexes. UV cross-linked RNA-protein complexes were analyzed by gel retardation assays followed by SDS-PAGE. After RNA-protein complexes were formed as described above, the complexes were irradiated on ice in a UV Stratalinker (Stratagene) with 254-nm, 8-W UV bulbs for 10 min. The UV cross-linked samples were then digested with RNase T₁ for 10 min (1 U per reaction) and then incubated for 10 min with heparin (5 mg/ml) at room temperature. The ³²P-labeled RNA-protein complexes were resolved in 4% native polyacrylamide gels and localized by autoradiography. The radiolabeled bands, corresponding to the RNA-protein complexes, were cut from the gel, soaked in SDS buffer (125 mM Tris [pH 6.8], 0.1% SDS, 1 mM EDTA, and 10 U of RNase T₁) for 20 min, electrophoresed by SDS-10% PAGE, and detected by autoradiography.

Affinity purification of SOD-RBP, the T_{SOD-1} 5' UTR-binding protein. A modification of the methods of Neupert et al. (50) and Hake and Richter (25) was used. Polyadenylated RNA transcripts were generated in vitro with the SP6 MEGAscript kit (Ambion) from *EcoRI*-linearized pSP64-5'T_{SOD-1}. The synthesized RNA transcripts contain the 114 novel nucleotides of the 5' UTR of T_{SOD-1} and a poly(A) tail of 30 nucleotides. To maximize RNA yield, the reaction mixture was incubated overnight at 37°C and RNA was quantitated by incorporating a trace amount of [³²P]CTP into the transcripts. One milliliter of poly(U)-agarose beads (type 6; Pharmacia) was suspended in RNA binding buffer (25 mM HEPES [pH 7.5], 100 mM KCl) and packed into a 15-ml column. About 400 μg of the in vitro-transcribed polyadenylated 5'T_{SOD-1} (in 2 ml of RNA binding buffer) was added to the column at 4°C and recycled five times. The efficiency of RNA binding to the poly(U)-agarose beads was determined by monitoring ³²P-labeled RNA. With this procedure, we found that about 95% of the poly(A)-RNA was bound to the poly(U)-agarose after five rounds of binding and less than 1% of bound RNA was released from the poly(U)-agarose beads in the subsequent wash and salt elution steps. The poly(A)-5'T_{SOD-1}-bound poly(U)-agarose beads were equilibrated with the testicular extract buffer, and 25 ml of testicular cytoplasmic extract (10 to 15 mg/ml) was mixed with the affinity beads at room temperature and incubated for 1 h with gentle shaking to maximize protein binding. The extract also contained RNasin (Promega) at a final concentration of 60 U/ml (to inhibit endogenous RNase). To minimize nonspecific binding, heparin and yeast tRNA at final concentrations of 5 mg/ml and 40 μg/ml, respectively, were added. The beads were then pelleted by centrifugation at 1,000 rpm for 5 min, resuspended in 20 ml of washing buffer A (20 mM HEPES [pH 7.5], 40 mM KCl, 5 mg of heparin per ml, 50 μg of yeast tRNA per ml), and repacked into the column. The column was extensively washed with 20 ml of washing buffer B (20 mM HEPES [pH 7.5], 40 mM KCl, and 0.5% Nonidet P-40), 20 ml of washing buffer C [20 mM HEPES (pH 7.5), 40 mM KCl, and 40 μg of poly(A · C · U) per ml], and 20 ml of washing buffer D (20 mM HEPES [pH 7.5] and 100 mM KCl). Bound proteins were step eluted with 2 ml of 0.5, 1, and 2 M KCl in 20 mM HEPES, pH 7.5. The eluted protein fractions were dialyzed against buffer containing 10 mM HEPES (pH 7.5), 40 mM KCl, 3 mM Mg₂Cl₂, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride at 4°C before being analyzed by SDS-PAGE, gel shift, and Northwestern blot assays.

The proteins of the RNA affinity-purified fractions were visualized by electrophoresis by SDS-10% PAGE followed by silver staining as described by Wray et al. (66). Gel retardation assays were performed as described above, and Northwestern blots were performed by using a modification of the procedure of Houman et al. (33). Aliquots of protein samples were resolved by SDS-10% PAGE and transferred to Zeta membranes (Bio-Rad) by utilizing a Semi-Dry Trans-Blot SD (Bio-Rad; 20 V for 1 h at 4°C). The membranes were incubated in renaturation buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1% bovine serum albumin (Sigma) for 1 h at room temperature and then overnight at 4°C. SOD-RBP was detected by autoradiography after the membrane was incubated with a ³²P-labeled 114-nucleotide T_{SOD-1} RNA probe (2 × 10⁵ cpm/ml) in a binding solution containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5 μg of yeast tRNA per ml, and 5 μg of poly(A · C · U) per ml for 1 h and washed three times, 10 min each, in binding solution at room temperature.

Determination of partial protein sequence. Protein fractions containing SOD-RBP eluted from the RNA affinity column were concentrated with Centricon-30 filters (Amicon), and aliquots were electrophoresed by SDS-10% PAGE after dialysis. Proteins in the gel were transferred to a polyvinylidene difluoride membrane by using a Semi-Dry Trans-Blot SD at 20 V for 5 min at 4°C. Guide strips at both sides of the membrane were used to detect the protein by Northwestern blotting as described above. The protein band, corresponding to the RNA-binding protein detected by Northwestern blotting, was visualized by Ponceau S

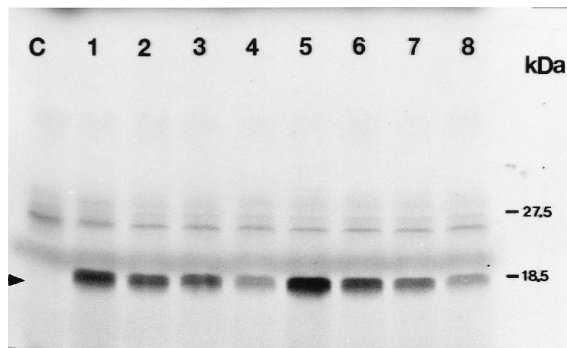


FIG. 1. Cell-free translation of T_{SOD-1} and S_{SOD-1} mRNAs. In vitro-synthesized T_{SOD-1} and S_{SOD-1} RNAs were translated in a rabbit reticulocyte lysate. SOD-1 protein synthesis was detected by [35 S]methionine incorporation. Arrowhead, synthesized SOD-1 protein with its expected size of 16 kDa. Lane C, a control translation reaction without an RNA template; lanes 1 to 4, T_{SOD-1} RNA templates of 4, 2, 1, and 0.5 pmol, respectively; lanes 5 to 8, S_{SOD-1} RNA templates of 4, 2, 1, and 0.5 pmol, respectively.

staining, cut out, and microsequenced by the Department of Microchemistry at Harvard University.

RESULTS

When translated "in vitro," T_{SOD-1} mRNA translates slightly less efficiently than S_{SOD-1} mRNA. To compare the translational efficiencies of T_{SOD-1} and S_{SOD-1} mRNAs, cell-free translation experiments were performed with T_{SOD-1} and S_{SOD-1} mRNAs. At template concentrations of 4, 2, 1, and 0.5 pmol, the in vitro translational efficiency of S_{SOD-1} mRNA was modestly higher than that of T_{SOD-1} mRNA, with differences of about 1.7-, 1.3-, 1.1-, and 1.2-fold, respectively, by densitometric analysis (Fig. 1; compare lane 5 with lane 1 and lane 6 with lane 2, etc.). This reproducible small difference in translational efficiency of the two SOD-1 mRNAs was also observed when 8 pmol of RNA template was used (data not shown). Both mRNAs encode a protein of about 16 kDa.

To confirm that the protein synthesized in the reticulocyte lysates is SOD-1, the translation product was immunoprecipitated with an antiserum specific to mouse SOD-1 (13). The synthesized protein was precipitated with the antiserum to SOD-1 but not with a control rabbit preimmune serum, establishing that the protein synthesized in the cell-free extract is SOD-1 (data not shown). Since the additional 5' UTR of the T_{SOD-1} mRNA appears to only slightly repress the in vitro translation of T_{SOD-1} whereas in vivo over 90% of the T_{SOD-1} is nonpolysomal (24b), this suggests that other factors such as protein binding to the 5' UTR of the T_{SOD-1} mRNA could modulate its translation in vivo.

A protein in testis extracts specifically binds to the 5' UTR of T_{SOD-1} . To identify proteins that bind to the 5' UTR of T_{SOD-1} mRNA, radiolabeled RNA transcripts were synthesized from the 5' T_{SOD-1} plasmid and used for gel retardation assays. When the RNA probe was incubated with testicular cytoplasmic extracts and then with RNase T_1 and heparin, a distinct RNA protein complex was detected (Fig. 2, lane 1). To determine the specificity of the RNA-protein complex, competition assays with increasing amounts of unlabeled specific or nonspecific RNA competitors were performed. Preincubation of testicular extract with a 2 \times molar excess of an unlabeled 5' T_{SOD-1} transcript reduced the amount of the RNA-protein complex seen (Fig. 2, lane 2), and a 20 \times molar excess or more completely prevented our ability to detect radiolabeled RNA-protein complexes (Fig. 2, lanes 3 and 4). A nonspecific com-

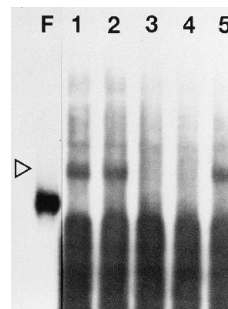


FIG. 2. The 5' UTR of T_{SOD-1} forms a specific RNA-protein complex with a protein in the testicular cytosol. 32 P-labeled transcripts encoding the 114 nucleotides of the 5' UTR of T_{SOD-1} were incubated with testicular extract (40 μ g) and then with RNase T_1 (50 U/ml) and heparin (5 mg/ml) at room temperature. RNA-protein complexes were resolved in 4% polyacrylamide native gels. Arrowhead, RNA-protein complex. Lane F, 32 P-labeled transcript alone; lane 1, 32 P-labeled transcript incubated with testicular extract; lanes 2 to 4, 32 P-labeled transcript incubated with testicular extract in the presence of 2 \times , 20 \times , and 200 \times molar amounts of unlabeled 5' UTR of T_{SOD-1} , respectively; lane 5, 32 P-labeled transcript incubated with testicular extract in the presence of a 200 \times molar amount of a nonspecific unlabeled 3' UTR of mouse protamine 2 RNA (transcript c).

petitor such as transcript c of the 3' UTR of mouse protamine 2 (41) did not inhibit complex formation up to a 200 \times molar excess (Fig. 2, lane 5). This indicates that a testicular protein specifically recognized a region of the 5' UTR of T_{SOD-1} .

A 65-kDa testicular protein UV cross-links to the 5' UTR of T_{SOD-1} . To begin to characterize the protein component that binds to the 5' UTR of the T_{SOD-1} mRNA, we have used UV cross-linking to covalently link the protein to a radiolabeled RNA probe. RNA-protein complexes of 32 P-labeled 5' T_{SOD-1} RNA and testicular extracts were cross-linked with UV irradiation, and the complex was resolved by native gel electrophoresis followed by SDS-PAGE. RNA-protein complexes were formed when the RNA probe was incubated with testicular extracts in the absence of competitor RNA (Fig. 3A, lanes 1 and 2) or when nonspecific RNA competitor was present (Fig. 3A, lane 4) but not when specific unlabeled RNA competitor was added (Fig. 3A, lane 3). We detected a protein-RNA complex with an estimated molecular mass of about 65 kDa with the samples previously UV cross-linked in the absence of additional RNA or presence of 100 \times molar excess amounts of unlabeled nonspecific RNA transcripts (Fig. 3B, lanes 2 and 4, respectively). Only unbound RNA was detected by SDS-PAGE with the non-cross-linked control sample (Fig. 3B, lane 1), and no RNA-protein complex was detected when a 100-fold molar excess of an unlabeled specific RNA transcript was added before cross-linking (Fig. 3B, lane 3). We conclude that a protein of about 65 kDa, SOD-RBP, binds to the 5' UTR of T_{SOD-1} .

The full length of 5' T_{SOD-1} is needed for optimal protein binding. To determine which sequence elements within the 114 nucleotides of 5' T_{SOD-1} are required for protein binding, radiolabeled transcripts from seven deletion constructs of the 5' UTR of T_{SOD-1} were used in gel retardation binding assays (Fig. 4). In contrast to the large amount of RNA-protein complex formed with the full-length 5' T_{SOD-1} (Fig. 4B, lane C), we observed greatly reduced levels of protein binding with deletion transcripts that contain the middle region (nucleotides 36 to 84) of the 5' UTR RNA sequence (Fig. 4B, lanes Δ_1 , Δ_4 , and Δ_7). No RNA-protein complex of the same electrophoretic mobility as that seen with the full-length 5' T_{SOD-1} was observed with the other deletion transcripts (Fig. 4B, lanes Δ_2 , Δ_3 , Δ_5 , and Δ_6), although low levels of a more slowly migrating

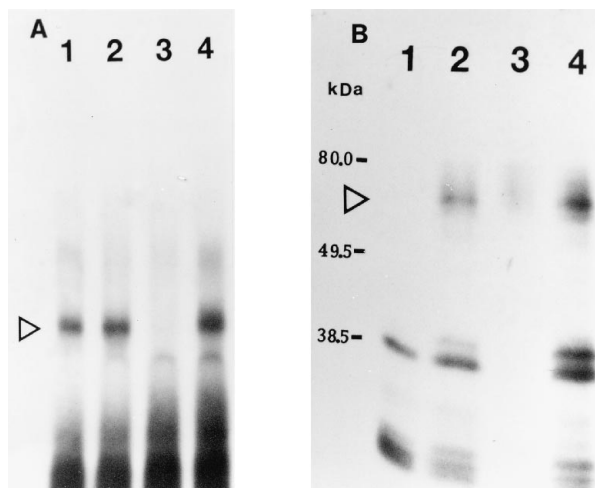


FIG. 3. UV cross-linking of the 5' UTR of T_{SOD-1} to a testicular protein. ^{32}P -labeled transcripts were incubated with testicular extracts and treated with RNase T_1 and heparin as described in the legend to Fig. 2. RNA-protein complexes were identified and isolated from a 4% polyacrylamide native gel, soaked in SDS buffer, and resolved by SDS-10% PAGE. (A) Gel retardation assay. Arrowhead, RNA-protein complexes. Lane 1, radiolabeled transcript incubated with testicular extract without UV cross-linking; lane 2, radiolabeled transcript incubated with testicular extract and subjected to UV cross-linking; lane 3, radiolabeled transcript incubated with testicular extract in the presence of 100 \times excess unlabeled 5' UTR of T_{SOD-1} transcripts added before UV cross-linking; lane 4, transcript incubated with testicular extract in the presence of 100 \times excess nonspecific RNA (3' UTR of mouse protamine 2) added before UV cross-linking. (B) SDS-PAGE analysis of the RNA-protein complexes from panel A. Arrowhead, UV cross-linked RNA-protein complex. The additional faster-migrating bands in lanes 1, 2, and 4 are RNase T_1 -digested RNA probes which are not cross-linked with protein.

RNA-protein complex were seen. This suggests that nucleotides 36 to 84 within the 5' UTR of T_{SOD-1} are essential but not sufficient for maximal formation of the RNA-protein complexes, since the binding activity is substantially lower with this shortened sequence than with the full-length 5' UTR probe (Fig. 4B, lane C).

Distribution of SOD-RBP in mouse somatic tissues. To determine how widely expressed SOD-RBP is in mouse somatic tissues, gel retardation assays were performed with protein extracts from seven different mouse tissues. Equal aliquots of protein from each tissue extract were incubated with ^{32}P -labeled RNA transcripts and digested with RNase T_1 , and the RNA-protein complexes were resolved by electrophoresis in native gels (Fig. 5). Among the seven different mouse tissues, the distinct RNA-protein complex is observed only with the testis extract (Fig. 5, lane T). A small amount of a more slowly migrating RNA-protein complex is also seen with the brain extract (Fig. 5, lane B). These data indicate that, in agreement with the restriction of expression of the longer SOD-1 mRNA to the testis (24b), SOD-RBP, the protein that binds to this mRNA, was not detectable in the extracts from mouse heart, spleen, lung, brain, liver, and kidney.

Purification of SOD-RBP. To allow us to functionally test how protein interactions within the 5' UTR of T_{SOD-1} could regulate translation, it was necessary to purify SOD-RBP. To accomplish this, we passed testicular extracts over an RNA affinity chromatography column. After extensive washes, protein fractions were eluted with increasing salt concentrations and analyzed by SDS-PAGE and silver staining (Fig. 6). Although the starting crude protein extract was heterogeneous (Fig. 6, lane TE), after a series of washes a greatly reduced number of proteins was retained on the RNA affinity column

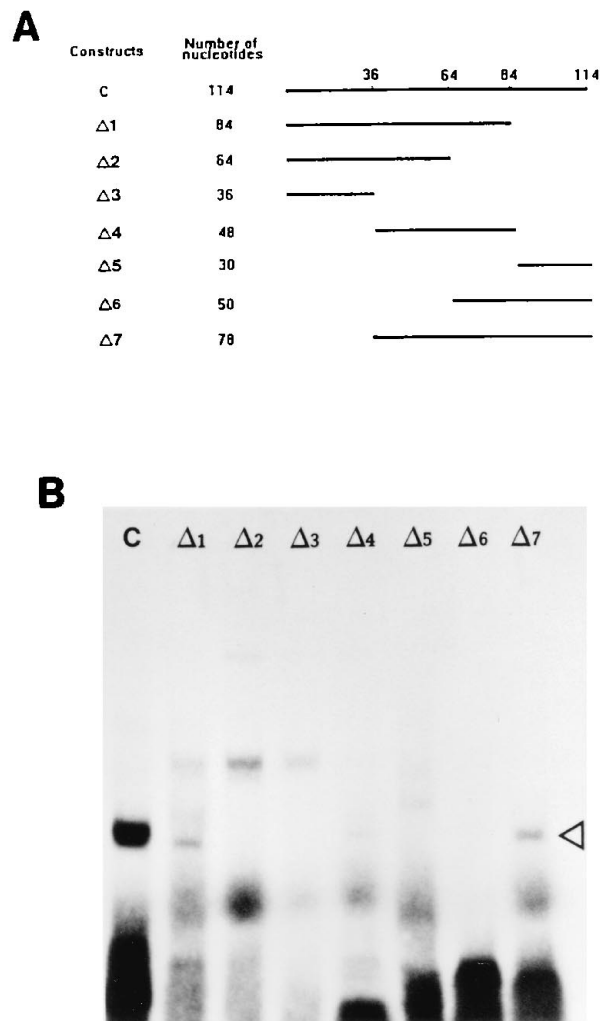


FIG. 4. Gel retardation assays with deletion constructs of the 5' UTR of T_{SOD-1} . (A) Diagram of RNA constructs prepared from the 5' UTR of T_{SOD-1} . (B) ^{32}P -labeled transcripts were incubated with 40 μ g of testicular extract, digested with RNase T_1 , and incubated with heparin. The samples were resolved in 4% native polyacrylamide gels and visualized by autoradiography. Arrowhead, RNA-protein complex. Lane C, full-length 5' UTR of T_{SOD-1} ; lanes $\Delta 1$ to $\Delta 7$, RNA-protein complexes formed with the deletion transcripts diagrammed in panel A.

(Fig. 6, lanes E_1 to E_3). The fraction eluted with 2 M KCl from the RNA affinity column contained a distinct protein band of about 65 kDa (Fig. 6, lane E_3), an estimated molecular mass in close agreement with that of the RNA-protein complex detected by UV cross-linking (Fig. 3B).

To establish where active SOD-RBP eluted from the RNA-affinity column, gel retardation assays were performed on aliquots of protein from each purification stage with a radiolabeled 5' T_{SOD-1} transcript (Fig. 7). In a comparison of the RNA-protein complexes detected with the crude extract and aliquots of the washes and salt-eluted fractions, the 2 M KCl-eluted fraction (E_3) showed good enrichment in RNA binding activity (Fig. 7, lane E_3). A densitometric scan revealed that about 70 to 80% of the original SOD-RBP activity was recovered in the E_3 fraction. No specific RNA-protein complexes were detected in aliquots of the wash fractions or the 0.5 M KCl fraction (Fig. 7, lanes W_1 to W_4 and E_1), and small amounts of binding activity were seen in the flowthrough and



FIG. 5. Tissue distribution of SOD-RBP. ^{32}P -labeled transcripts of the 5' UTR of $T_{\text{SOD-1}}$ were incubated with protein aliquots (40 μg) of mouse extracts from heart (lane H), spleen (lane S), lung (lane Lu), brain (lane B), liver (lane Li), kidney (lane K), and testis (lane T), digested with RNase T_1 , and incubated with heparin. The RNA-protein complexes were resolved in a 4% native polyacrylamide gel. Arrow, RNA-protein complex.

1.0 M KCl elution fractions (Fig. 7, lanes FT and E_2). This indicates that RNA affinity chromatography was an effective means to purify SOD-RBP and maintain its RNA binding activity.

To demonstrate which protein detected in the E_3 fraction by silver staining of the SDS gel was SOD-RBP, we used Northwestern blots. Aliquots of protein from each fraction of the RNA affinity column were resolved by SDS-10% PAGE, transferred to Zeta membranes, incubated overnight in renaturing solution, and probed with ^{32}P -labeled 5' $T_{\text{SOD-1}}$ (Fig. 8). A protein of about 65 kDa was detected in the crude testicular extract and the 2.0 M KCl fraction (Fig. 8, lanes TE and E_3) but not in the flowthrough, washes, and other salt elution fractions. This establishes that the 65-kDa protein in the E_3 fraction was the protein which binds to the 5' UTR of SOD-1 mRNA.

On the basis of the electrophoretic mobility of the 65-kDa protein in the silver-stained SDS-polyacrylamide gel (Fig. 6) and the identical-size protein detected by Northwestern blot-

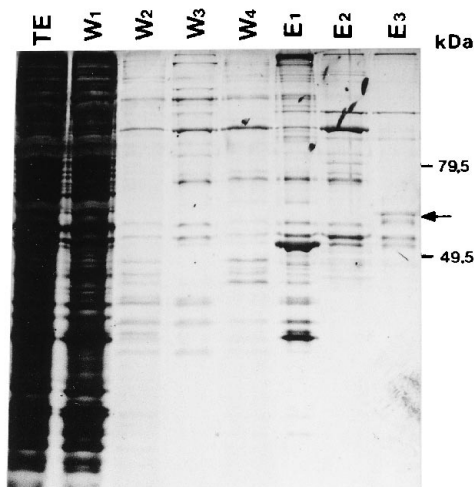


FIG. 6. SDS-PAGE analysis of the protein fractions of the RNA affinity column. Aliquots of protein in each purification step were resolved by SDS-10% PAGE and silver stained. Lane TE, 40 μg of the starting testicular extract. All other samples contain 30- μl aliquots of protein fractions. W_1 to W_4 , the four washing steps; E_1 to E_3 , 0.5, 1.0, and 2.0 M KCl step elution fractions, respectively. Arrow, RNA-binding protein.

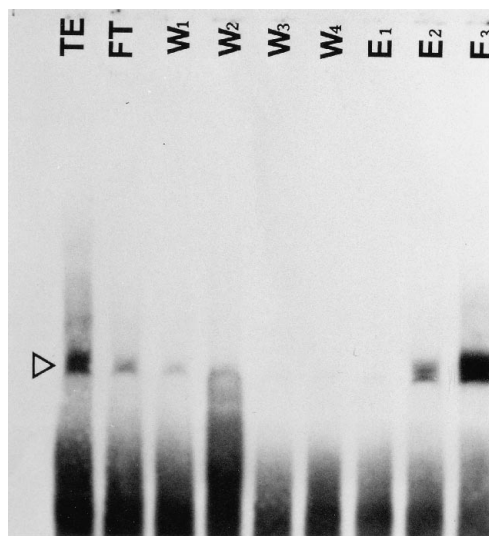


FIG. 7. Gel retardation assay of the protein fractions of the RNA affinity column. Aliquots (5 μl) of the fractions in Fig. 6 were assayed for RNA-protein complex formation by the gel retardation assay. Each fraction was incubated with a ^{32}P -labeled transcript of the 5' UTR of $T_{\text{SOD-1}}$, and the complexes formed were resolved in 4% native polyacrylamide gels. TE, starting crude extract; FT, flowthrough; W_1 to W_4 , washing fractions; E_1 to E_3 , 0.5, 1.0, and 2.0 M KCl step elution fractions, respectively.

ting (Fig. 8) and by UV cross-linking (Fig. 3), we conclude that the 65-kDa protein isolated by RNA affinity chromatography is SOD-RBP. The additional proteins present in the E_3 fraction may specifically or nonspecifically bind to SOD-RBP or to other sequences in the polyadenylated 5' $T_{\text{SOD-1}}$ RNA.

Amino acid sequence of partial SOD-RBP. Protein in the E_3 fraction of the RNA affinity column (Fig. 6, lane E_3) was concentrated with a Centricon-30 filter, electrophoresed by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. The 65-kDa protein band that comigrated with the band detected by ^{32}P -labeled 5' UTR of $T_{\text{SOD-1}}$ was visualized by Ponceau S staining and excised for microsequencing. Two

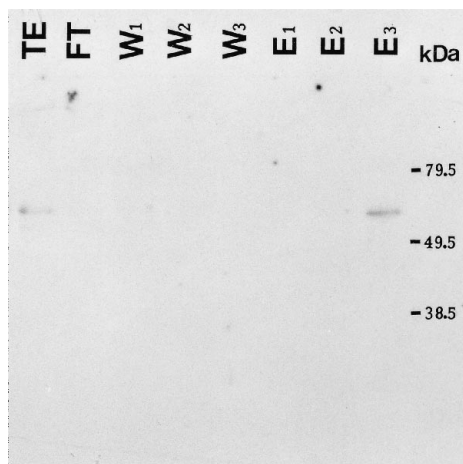


FIG. 8. Northwestern blot analysis of the protein fractions of the RNA affinity column. Aliquots (30 μl) of proteins from each purification step were resolved by SDS-10% PAGE and transferred to Zeta membranes. The membranes were renatured and probed with a ^{32}P -labeled transcript of the 5' UTR of $T_{\text{SOD-1}}$. TE, starting material (10 μg); FT, flowthrough fraction; W_1 to W_3 , washing-step fractions; E_1 to E_3 , step elution fractions.

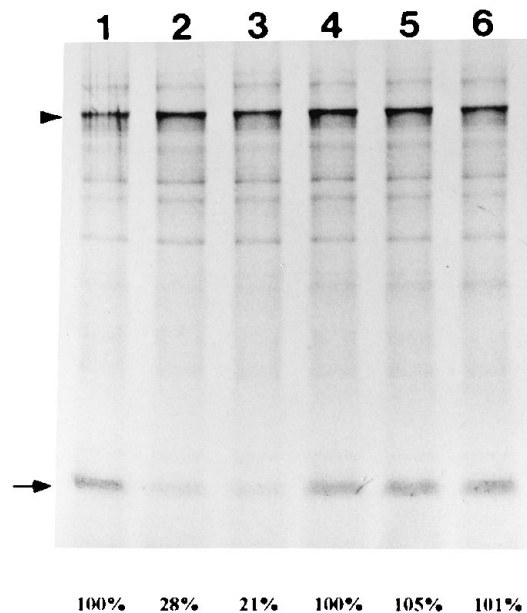


FIG. 9. Protein binding to the 5' UTR of the T_{SOD-1} mRNA represses its translation in vitro. About 4 pmol of T_{SOD-1} mRNA (lanes 1 to 3), S_{SOD-1} mRNA (lanes 4 to 6), or a control luciferase mRNA was cotranslated in rabbit reticulocyte lysates, and translation efficiencies were measured by incorporation of [35 S]methionine. Lanes 1 and 4, cotranslation of luciferase mRNA without added protein with T_{SOD-1} and S_{SOD-1} mRNAs, respectively; lanes 2 and 5, cotranslation of luciferase mRNA with 150 ng of the total E_3 protein fraction with T_{SOD-1} and S_{SOD-1} mRNAs, respectively; lanes 3 and 6, cotranslation of luciferase mRNA with 300 ng of the total E_3 protein fraction with T_{SOD-1} and S_{SOD-1} mRNAs, respectively. Arrowhead, in vitro-synthesized luciferase protein; arrow, in vitro-synthesized SOD-1 protein. Relative translational efficiencies of the T_{SOD-1} and S_{SOD-1} mRNAs are indicated at the bottom.

peptides of 9 and 20 amino acids of purified SOD-RBP with the sequences Phe-Thr-Pro-Gln-Glu-Leu-Asn-Ser-His and Glu-Gly-Ala-Pro-Ala-Gly-Gly-Asp-Gln-Gln-Ala-Ala-Ser-Gly-Pro-Ala-Ala-Gly-Thr-Pro were obtained following tryptic digestion. A FASTA search (54, 55) with the sequence of the 29 amino acids against the GENEEMBL database failed to identify any known protein in the database. However, one consecutive 6-amino-acid sequence, Ala-Pro-Ala-Gly-Gly-Asp, in the longer peptide exactly matches a sequence in a transcription factor that binds to an inverted CCAAT box (16).

SOD-RBP represses translation of T_{SOD-1} mRNA but not S_{SOD-1} mRNA in vitro. To demonstrate that the translational repression of T_{SOD-1} was due to the specific interaction of SOD-RBP with the 5' UTR of T_{SOD-1} , we performed cotranslation assays in rabbit reticulocyte lysates using T_{SOD-1} and S_{SOD-1} transcripts and a control luciferase mRNA. Compared with the translational efficiency of T_{SOD-1} mRNA in the absence of SOD-RBP (Fig. 9, lane 1), marked decreases of translational efficiencies to 28 and 21% of the control were observed when T_{SOD-1} transcripts were preincubated with 150 and 300 ng, respectively, of the SOD-RBP fraction (Fig. 9, lanes 2 and 3). No significant decrease was seen in the amount of the 61-kDa luciferase protein produced from the luciferase mRNA cotranslated with T_{SOD-1} (Fig. 9, lanes 1 to 3), suggesting that SOD-RBP binding was specific to the T_{SOD-1} mRNA. Moreover, translational inhibition was not observed in identical assays when S_{SOD-1} mRNAs were cotranslated with luciferase mRNAs in the presence of SOD-RBP (Fig. 9, lanes 4 to 6). The translational inhibition of T_{SOD-1} was not due to a selective RNA degradation activity present in the SOD-RBP frac-

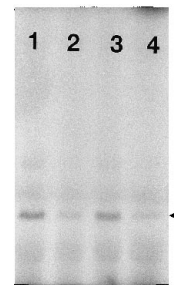


FIG. 10. Cell-free translation competition assays. T_{SOD-1} mRNA templates (2 pmol) were translated in rabbit reticulocyte lysates in the presence of 300 ng of the E_3 fraction after preincubation with 100 ng of the 5' UTR of T_{SOD-1} or 100 ng of nonspecific 3' UTR of mP2 RNA. The translation efficiencies were measured by incorporation of [35 S]methionine. Lane 1, translation of T_{SOD-1} mRNA; lane 2, translation of T_{SOD-1} mRNA in the presence of 300 ng of the E_3 fraction; lane 3, translation of T_{SOD-1} mRNA in the presence of 300 ng of the E_3 fraction after preincubation with 100 ng of an unlabeled T_{SOD-1} 5' UTR transcript; lane 4, translation of T_{SOD-1} mRNA in the presence of 300 ng of the E_3 fraction after preincubation with 100 ng of unlabeled nonspecific transcript c. Arrow, in vitro-synthesized SOD-1 protein.

tion since retranslation in vitro of reextracted aliquots of the mRNAs of Fig. 9 produced similar amounts of SOD-1 and luciferase (data not shown). This suggests that, when assayed in vitro, the enriched SOD-RBP present in the E_3 fraction specifically repressed the translation of T_{SOD-1} mRNA but not S_{SOD-1} mRNA.

To rule out the possibility that other proteins present in the E_3 fraction were the cause of the translational repression of T_{SOD-1} mRNA, we titrated SOD-RBP from the E_3 fraction by preincubating aliquots of the E_3 fraction with either unlabeled 5' UTR T_{SOD-1} transcripts or nonspecific protamine 2 3' UTR transcripts (Fig. 10). As in Fig. 9, addition of 300 ng of the E_3 fraction inhibited translation of T_{SOD-1} about 80% (Fig. 10, lane 2) compared with the control translation with no added protein (Fig. 10, lane 1). After preincubation of the E_3 fraction with 100 ng of the 5' T_{SOD-1} transcript, no inhibition of translation of T_{SOD-1} mRNA was seen (Fig. 10; compare lane 3 with lane 1). T_{SOD-1} translation was reduced when the added protein was preincubated with 100 ng of nonspecific RNA, transcript c (Fig. 10, lane 4). This indicates that SOD-RBP selectively binds to T_{SOD-1} and serves as a repressor of T_{SOD-1} mRNA translation in a cell-free system.

DISCUSSION

The secondary structure of the 5' UTR of many mRNAs plays a crucial role in regulating their translation (39). Stable secondary structures formed within the 5' UTRs of mRNAs encoding proteins such as ornithine decarboxylase (1, 23, 46), transforming growth factor $\beta 1$ (37), and transforming growth factor $\beta 3$ (5) have been shown to interfere with the binding and translocation of the translational initiation complex, thus inhibiting translation. This initially suggested to us that the suppression of translation of T_{SOD-1} mRNA was due to secondary structure resulting from the additional sequence in its 5' UTR. However, the modest reduction in translational efficiency we observed with T_{SOD-1} compared with S_{SOD-1} in a cell-free translation system (Fig. 1) suggests that the secondary structure of the 5' UTR of T_{SOD-1} may only slightly contribute to the reduced level of T_{SOD-1} translation.

Since in situ hybridizations suggested that the T_{SOD-1} transcripts were not regulated by localization to specific sites in male germ cells (24b), we next tested the possibility that spe-

cific protein binding to T_{SOD-1} mRNA was a cause of the translational repression of T_{SOD-1} mRNA. To this end, we have identified a testicular protein of about 65 kDa (SOD-RBP) that binds to the 5' UTR of the T_{SOD-1} RNA by the criteria of gel retardation assays (Fig. 2), UV cross-linking (Fig. 3), and Northwestern blot assays (Fig. 8) and represses T_{SOD-1} translation in vitro (Fig. 9 and 10). The RNA-protein complex formed is highly stable in vitro and does not dissociate even at relatively high salt concentrations in the presence of detergent. We did not detect a similar protein in mouse extracts from somatic tissues including heart, spleen, lung, brain, liver, and kidney (Fig. 5), in agreement with our inability to detect any T_{SOD-1} mRNA in somatic tissues (24b). A more slowly migrating RNA-protein complex is detected in the brain, an organ containing many RNA-binding proteins of variable RNA-binding specificities.

RNA-binding proteins recognize either specific sequences or secondary structures of mRNAs. A protein of about 90 kDa regulates the translation of ferritin mRNA by binding to an iron-responsive element of about 28 nucleotides within the 5' UTR of ferritin mRNA (21, 22, 27, 38). The same protein binds to similar sequences in the 3' UTR of transferrin receptor mRNAs. A phosphoprotein which binds to highly conserved sequences (the Y and H element) of about 35 nucleotides located within the 3' UTR of mouse protamine 2 has been shown to repress translation in vitro (40, 41). Recently, Fajardo and coworkers (18) have identified two testicular proteins that bind to a 22-nucleotide sequence within the 3' UTR of protamine 1 and a 20-nucleotide region within the 3' UTR of protamine 2. In contrast, SOD-RBP appears to require most of the 114 nucleotides of the 5' UTR of T_{SOD-1} for optimal RNA-protein complex formation in vitro, since deletion transcripts of either the 5' end or the 3' end of the 5' UTR dramatically decrease or abolish the binding activity. This suggests that SOD-RBP may recognize a secondary structure formed by most of the 114 nucleotides within the 5' UTR of T_{SOD-1} . Using the computer program Fold-Squiggles to determine the predicted secondary structure and its minimum free energy, we have found a unique hairpin structure formed by the 114 nucleotides of T_{SOD-1} 5' UTR with a free energy of -39.5 kcal (-165 kJ)/mol, which is disrupted in each of the deletion constructs (data not shown). The importance of this structure for SOD-RBP binding and translational repression remains to be determined.

To explore the function(s) of SOD-RBP binding to the 5' UTR of T_{SOD-1} during spermatogenesis, we have purified SOD-RBP by RNA affinity chromatography. Estimates of the amount of SOD-RBP recovered as determined by gel retardation assays revealed that at least 70% of the RNA binding activity present in crude testicular extract can be recovered after purification (data not shown), suggesting that other testicular RNA-binding proteins, with known specificities, could also be isolated by this approach. Since aliquots of RNA affinity-purified SOD-RBP inhibit the translation of T_{SOD-1} mRNAs but not S_{SOD-1} mRNAs (Fig. 9 and 10), we propose that SOD-RBP alone or in association with additional proteins serves as a putative translational repressor in mouse testes. Since T_{SOD-1} represents only one of the many stored mRNAs in male germ cells (28, 29), it will be important to determine whether SOD-RBP can also bind to other testicular mRNAs or is specific for T_{SOD-1} . Recently, an RNA-binding protein that binds to the 3' UTR of catalase mRNA, another antioxidant enzyme of eukaryotic cells, has been reported to be present in rat lung as well as in human and mouse fibroblast cell lines (12). In mouse testes the manganese superoxide dismutase,

SOD-2, is also expressed by multiple mRNAs, one of which is under translational control (24).

Many examples of translational regulation by RNA-protein interactions have been reported (1, 53, 56, 57). RNA-protein interactions have been proposed to maintain mRNAs in translationally inactive states by blocking the unwinding of the secondary structure of 5' UTRs by eIF-4A and -4B (56, 57), by preventing mRNAs from associating with ribosomal initiation complexes (63), or by blocking the movement of the ribosomal complex towards the mRNA initiation sites (39). Parkin and Sonenberg (53) and Richter and Smith (58) have shown that RNA-protein interactions maintain mRNA stability and can developmentally regulate the expression of mRNAs. Inhibition of translation resulting in the stabilization of mRNA molecules has also been reported (1). Although SOD-RBP binds to the 5' UTR of T_{SOD-1} and can repress its translation in vitro, we can only postulate that SOD-RBP has a similar function in vivo. Whether it possesses additional functions, such as mRNA localization (26), remains to be investigated.

In addition to the testicular RNA-binding proteins that interact with the 3' UTRs of mRNAs (18, 40, 41), p48 and p52, homologs of the *Xenopus* FRG Y2, p54 and p56 which function both as transcription factors and mRNA-masking proteins (48, 49), are present in the testis (42). The testicular 48- and 52-kDa proteins not only bind to all RNA sequences including the 3' UTR of mP2 mRNA (42, 64) but also bind to a conserved element in the promoter of testis-specific genes including protamine 2 (51). Although sequence analysis of the two SOD-RBP peptides of 9 and 20 amino acids has not detected complete homology to any known protein in the GENEEMBL database, 6 consecutive amino acids that match a sequence in a transcription factor (16) suggest that SOD-RBP could be another RNA-binding protein from the testis that also binds to DNA.

Why does SOD-RBP need to be expressed? A proper level of SOD-1 in eukaryotic cells is important for normal cell function. Studies with human and mouse cell lines containing integrated human SOD-1 cDNA revealed that, although the overexpression of SOD-1 makes cells more resistant to O_2^- challenges, it also enhances lipid peroxidation as a result of the accumulation of hydrogen peroxide (17). In a transgenic mouse line expressing human SOD-1, overexpression of SOD-1 causes clinical symptoms similar to Down's syndrome (6). Reduced levels of SOD-1 are also deleterious, since nearly 20% of amyotrophic lateral sclerosis patients have SOD-1 deficiencies as a result of mutations (15, 59). These studies suggest that precise regulation of SOD-1 is needed to maximize the scavenging of toxic free oxygen radicals and to avoid cell damage caused by overexpression of SOD-1.

These observations lead to the question of how SOD-RBP binding to the 5' UTR of T_{SOD-1} could modulate SOD-1 protein synthesis in the testes. The importance of SOD-1 in developing spermatids and spermatozoa has been noted in several species, including humans, rats, and rabbits (3, 7, 32). Van Loon et al. (65) have shown that late stages of germ cells (elongating spermatids) have a greatly reduced capacity to repair DNA damage compared with earlier stages of germ cells (spermatocytes and round spermatids) and thus have more risk of danger from free radicals. Lasso et al. (43) have observed that the partial loss of SOD-1 activity makes spermatozoa more susceptible to O_2 toxicity, culminating in their loss of motility. In addition, the fertilization ability of spermatozoa appears inversely correlated with levels of superoxide radicals (2). Rat male germ cells have been reported to have high SOD activities with meiotic pachytene spermatocytes and postmeiotic round spermatids and spermatozoa containing 38 to 56%

more SOD activity than testicular somatic cells (7). We find that mouse pachytene spermatocytes, round spermatids, and elongating spermatids contain relatively similar levels of both SOD-1 protein and enzymatic activity (24a). This suggests that the SOD-1 levels and the translation of the abundant postmeiotically expressed T_{SOD-1} mRNA are closely regulated in the differentiating germ cells. This regulation may be essential to prevent SOD-1 overexpression and properly titrate levels of SOD-1 protein in the developing spermatids. We propose that SOD-RBP prevents overexpression of SOD-1 in round spermatids, the cell type in which T_{SOD-1} mRNA is translationally inactive, and allows synthesis from T_{SOD-1} mRNA in elongating spermatids, the cell type in which T_{SOD-1} mRNA is translated. The loss of most of the S_{SOD-1} mRNA in elongating spermatids may necessitate this activation of T_{SOD-1} to maintain proper SOD-1 levels (24b). This regulatory mechanism could operate by a reversible binding of SOD-RBP to the 5' UTR of T_{SOD-1} mRNA. Additional proteins that bind to RNA or to SOD-RBP may facilitate these changes by serving as activators. Cellular superoxide levels may regulate the SOD-RBP-RNA binding interactions similarly to how ferritin synthesis is regulated by the level of cellular iron (67), or SOD-RBP may undergo posttranslational modifications as with testis brain RBP (26, 41).

In conclusion, we have identified and purified a testicular 65-kDa protein that specifically binds to the 5' UTR of T_{SOD-1} mRNA and represses T_{SOD-1} mRNA translation *in vitro*. We propose that this RNA-protein interaction in postmeiotic male germ cells provides a means to precisely regulate the level of SOD-1 expression in the mammalian testis.

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