Biochemical and Biophysical Research Communications **272,** 164–168 (2000)
doi:10.1006/bbrc.2000.2754, available online at http://www.idealibrary.com on IDEAL®

A Region in the 3′ UTR of MnSOD RNA Enhances Translation of a Heterologous RNA

Linda Knirsch and Linda Biadasz Clerch
Lung Biology Laboratory, Department of Pediatrics, Georgetown University School of Medicine, Washington, DC 20007

Received April 19, 2000

The studies reported in this paper were designed to test the hypothesis that a *cis* element located in the 3′ UTR of manganese superoxide dismutase (MnSOD) RNA, designated MnSOD-response element (MnSOD-RE), is a translational enhancer *in vivo.* NIH/3T3 cells were transfected with a posttranscriptional reporter construct in which MnSOD-RE was placed 3′ of the coding region of chloramphenicol acetyltransferase (CAT); this construct is designated CAT-RMS. Transient transfection of CAT-RMS did not change the concentration of CAT mRNA but increased CAT activity by ~400% compared to a control construct, CAT-V, which contains approximately the same size of non-MnSOD 3′ UTR sequence. Transfection of CAT-RMS had no effect on endogenous MnSOD protein, mRNA, or MnSOD RNA-binding protein activity. Because of its ability to increase translation of a heterologous RNA, MnSOD-RE may be useful in designing expression vectors for *in vitro* expression systems and *in vivo* gene therapy. © 2000 Academic Press

Key Words: **superoxide dismutase; RNA binding; antioxidant enzyme; chloramphenicol acetyltransferase; translation; reporter; posttranscriptional regulation.**

It has become widely appreciated that the control of gene expression is regulated not only at the level of transcription, but also at the posttranscriptional level by changes in mRNA stability and/or mRNA translation. Posttranscriptional regulation is an efficient means of altering protein expression quickly in response to a change in environmental signals (1). Specific regions of the mRNA sequence and their interaction with mRNA-binding proteins have been shown to play a major role in the regulation posttranscriptional regulation (2, 3). It was for this reason that our prior studies were directed at looking for the presence of an RNA-binding protein that interacted specifically with the 3′ untranslated region (UTR) of manganese superoxide dismutase (MnSOD) mRNA. MnSOD mRNA was chosen for these experiments because data in animal models indicated that posttranscriptional regulation of lung MnSOD was a critical determinant of the ability of the animal to withstand the damaging effects of pulmonary oxidant stress (4). MnSOD is transcribed in the nucleus, translated in the cytoplasm, and transported into mitochondria where it catalyzes the conversion of superoxide radical to hydrogen peroxide (5, 6). In animal models in which the lung is substantially damaged by oxidative stress there was a decrease in translational efficiency; MnSOD protein synthesis was significantly diminished in spite of an increase in the level MnSOD mRNA (7–11). Conversely, in animal models where the lung is relatively protected against the damaging effects of reactive oxygen intermediates, MnSOD protein synthesis was increased (7, 10–12). Therefore, in order to design potential therapeutic means of preventing oxygen toxicity, it became important to understand the mechanisms responsible for regulating MnSOD mRNA translational efficiency. To that end we previously reported the presence of a redox-sensitive protein in rat lung and cells of all other species tested that specifically binds to a *cis* element in the 3′ UTR of MnSOD mRNA (13, 14). Moreover, in an *in vitro* cell-free system we have shown that both the MnSOD RNA-binding protein (MnSOD-BP) and the *cis* element, which we now designate MnSOD RNA-response element (MnSOD-RE), are required for efficient translation of the mRNA (14). The experiments described in this paper were designed to test the hypothesis that the 3′ UTR *cis* element of MnSOD RNA functions as a translational enhancer element *in vivo* by transfecting NIH 3T3 cells with a posttranscriptional reporter construct.

METHODS AND MATERIALS

Animals. 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 xylazine (~10 mg/kg) plus ketamine (~75 mg/kg). Lung tissue was excised, frozen in liquid nitrogen, and stored at −70°C until use.

0006-291X/00 $35.00

Cell culture. NIH/3T3 cells (ATCC, CRL-1658) were grown in medium consisting of DMEM supplemented with 10% newborn calf serum, 2 mM glutamine, 10 μM thymidine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 ng/ml selenium.

Preparation of cytoplasmic extracts. Rat lung or cultured cells were homogenized in 25 mM Tris buffer, pH 7.4, containing 0.5 mM EDTA, 1% Triton X-100, 40 mM KCl, 0.2 U/ml aprotinin, 10 μg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride. The homogenate was subjected to centrifugation at 100,000 $\times$ g for 1 h at 4°C to obtain cytoplasmic extract (S100) for use in RNA-binding assays. Protein concentration was measured using the Coomassie Plus assay (Pierce Chemical Co.) with bovine serum albumin as a standard.

Measurement of RNA-binding protein activity. A cRNA ^{32}P-labeled probe containing the MnSOD-RE was generated by *in vitro* transcription of plasmid RMS-12-Cl-16; this plasmid was made linear with Asp I to generate a 132-base probe designated RMS-NA as previously described (14). The binding of protein to RMS-NA was assessed by means of a gel retardation assay (13, 14). An ultraviolet light (uv) cross-linking assay was performed to determine the apparent molecular weight of MnSOD-BP (15).

Reporter constructs. A posttranscriptional reporter construct was generated from a plasmid vector, CAT-V, provided by Dr. B. Beutler, University of Texas, Southwestern Medical Center, Dallas, TX (16). CAT-V consists of the chloramphenicol acetyltransferase (CAT) coding region under the transcriptional control of the SV40 late promoter followed by ~250 bases of a distal sequence of the TNF 3′ UTR [a region devoid of AU-rich elements]. CAT-V was used as a control construct. The parental vector, pSVL, was purchased from Pharmacia. A CAT reporter construct containing the MnSOD 3′ UTR region was generated by removing the SacI/BamHI restriction endonuclease fragment from the 3′ UTR of CAT-V and inserting a Sac I/BamHI insert from plasmid RMS 12-Cl-16; this insert contains the Nde I to Pst I 216 base-long region of rat MnSOD 3′ UTR that was previously identified as the *cis* element that binds to MnSOD-BP and is involved in regulating MnSOD mRNA translation *in vitro* (14). The CAT-MnSOD 3′ UTR construct is designated CAT-RMS. pSV-β-galactosidase vector (Promega) was used as a positive control for monitoring transfection efficiency.

Cell transfection experiments. Transient transfection was performed using Lipofectin (Gibco/BRL) following the manufacturer's instructions. Briefly, 3T3/NIH cells were seeded in 60 mm culture dishes and allowed to achieve ~50% confluence in cell culture medium described above. Lipofectin solution containing pSV-β-galactosidase and CAT-RMS, CAT-V, or pSVL plasmid was added to cells in medium without serum or antibiotics; after 16–24 h of incubation, the adherent monolayers were washed and complete NIH/3T3 cell culture medium was added. Transfected cells were assayed 48 h post transfection. CAT activity was measured with an assay system (Promega) based on liquid scintillation counting (LSC) of CAT reaction products. β-Galactosidase enzyme activity was determined with a spectrophotometric assay system (Promega) based on the hydrolysis of o-nitrophenyl-β-D-galactopyranoside to o-nitrophenol. In each case a standard curve was done to insure that the sample data was measured in a linear range. Data were normalized to cell protein concentration.

Measurement of MnSOD protein and RNA. We measured MnSOD protein by Western analysis as previously described (8). MnSOD mRNA was quantified by a ribonuclease protection assay (RPA). To generate a ^{32}P-labled probe for MnSOD in NIH/3T3 cells, we obtained a mouse MnSOD cDNA from Dr. Peter Jones (Emory Univ. School of Medicine, Atlanta, GA). This cDNA was made linear with XbaI and antisense cRNA was transcribed with the SP6 riboprobe system (Promega) for synthesis of high specific activity radiolabeled RNA probes. For use as an internal standard 18S rRNA probe was generated from a plasmid purchased from Ambion. Total cellular RNA was isolated using Tri-LS reagent (Molecular Research, Inc.) and quantified using absorbance at 260 nm and an extinction coefficient of 0.025 $(\mu g/ml)^{-1}cm^{-1}$. The RPA was performed using the RPA II kit (Ambion). Briefly, 20 μg total cellular RNA was allowed to hybridize with the ^{32}P-labeled MnSOD and 18s rRNA probes. Unprotected probes were then digested with RNaseA/T1 and resolved on a 6% non-denaturing polyacrylamide gel. Gels were dried and exposed to hyperfilm (Amersham). For quantification, the autoradiographs were scanned by laser densitometry (Molecular Dynamics) using Image Quant Software. MnSOD RNA data are expressed as relative densitometry units (DU) of MnSOD RNA per 18s rRNA.

Measurement of CAT RNA. CAT RNA was quantified by RPA using the RPA II kit (Ambion) as described above and the data are expressed as DU of CAT RNA per 18s rRNA. The CAT probe was generated by subcloning the XbaI/HindIII cDNA fragment of CAT from the pCAT3 basic vector (Promega) into pGem 7Z (Promega), the constructed plasmid vector is designated CAT-RPA. The CAT probe was synthesized from CAT-RPA by transcription with the T7 riboprobe system (Promega) after the plasmid was linearized with Hind III.

Statistical analysis. The densitometry values for individual animals were averaged per experimental group, and the standard error (SE) of the group mean was calculated. The significance of the difference between two groups was obtained using a two-tailed unpaired *t*-test analysis assuming unequal variances.

RESULTS AND DISCUSSION

NIH/3T3 cells contain 90 kDa MnSOD-BP. To warrant the use of NIH/3T3 cells for transfection of posttranscriptional reporter plasmids, it was necessary to show to that these cells contain MnSOD-BP and that the binding-protein has the same characteristics as MnSOD-BP in rat lung extract. Figure 1A shows that cytosolic extract from NIH/3T3 cells contains a protein that binds to the RMS-RE with the same electrophoretic mobility as protein in rat lung extract. MnSOD-BP activity in both lung and NIH/3T3 cells was redox sensitive and complex formation required the presence of free sulfhydryl groups. Binding-protein activity was eliminated by treatment with either diamide, which catalyzes the oxidation of free SH groups, or N-ethylmaleimide, which alkylates and thus blocks free SH groups (Fig. 1A). The redox-sensitivity of the MnSOD-BP is in agreement with our prior data (13). In uv cross-linking analysis of the RNA-protein interaction, one major band at ~90 kDa was identified on the autoradiograph when extracts from either rat lung or NIH/3T3 cells were used in the binding reaction indicating both samples contain MnSOD-BP of the same apparent molecular mass. These data show that NIH/3T3 cells are a suitable model in which to test the hypothesis that the 3′ UTR of MnSOD mRNA contains a translational enhancer element that interacts with MnSOD-BP to increase translational efficiency.

The MnSOD 3′ UTR cis element increases CAT activity. Translational efficiency of a specific RNA is determined by many factors including the length of the 3′ UTR and the context of the initial methionine codon. In order to eliminate as many variables as possible NIH/3T3 cells were transiently transfected with two reporter constructs between which the only difference

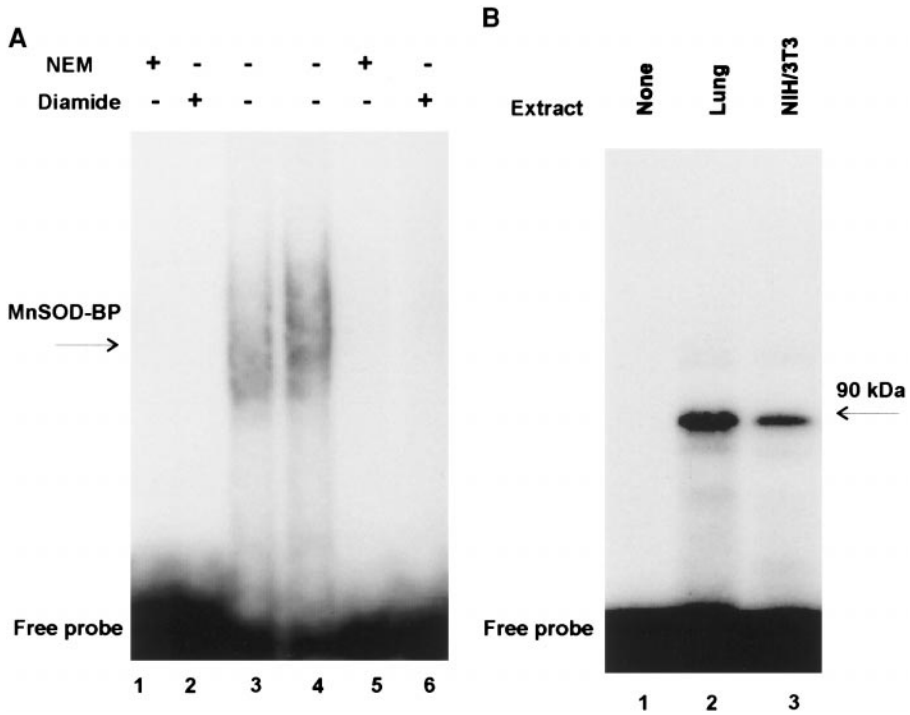

FIG. 1. MnSOD RNA-binding protein in rat lung and NIH/3T3 cells. (A) Gel retention analysis using RMS-NA probe and cytoplasmic extracts from rat lung (lanes 1–3) and NIH/3T3 cells (lanes 4–6). The arrow points to the major RNA–protein complex seen in lanes 3 and 4. Complex formation is eliminated when the extracts are treated with *N*-ethylmaleimide (NEM) lanes 1 and 5, or with diamide (lanes 2 and 6). The migration of free probe is indicated at the bottom of the autoradiograph. (B) Ultraviolet light cross-linking analysis. The molecular mass of the binding protein was estimated by calibration against ^{14}C-labeled protein standards (Amersham). The arrow indicates one major MnSOD-BP band at ~90 kDa detected in both lung and NIH/3T3 cells. The migration of free probe is indicated at the bottom of the autoradiograph.

was in the sequence present in the 3′ UTR. Using this strategy, the CAT-RMS construct could be compared with the CAT-V control and the difference in data would reflect the effect of the MnSOD 3′ UTR *cis* element that was present only in CAT-RMS. CAT-V was chosen as a control because it contains a 3′ insert of ~250 bases, that is close in size to the MnSOD 3′ UTR that is the subject of this investigation. It is known that the length of the 3′ region contributes to mRNA stability and/or translation and therefore this control

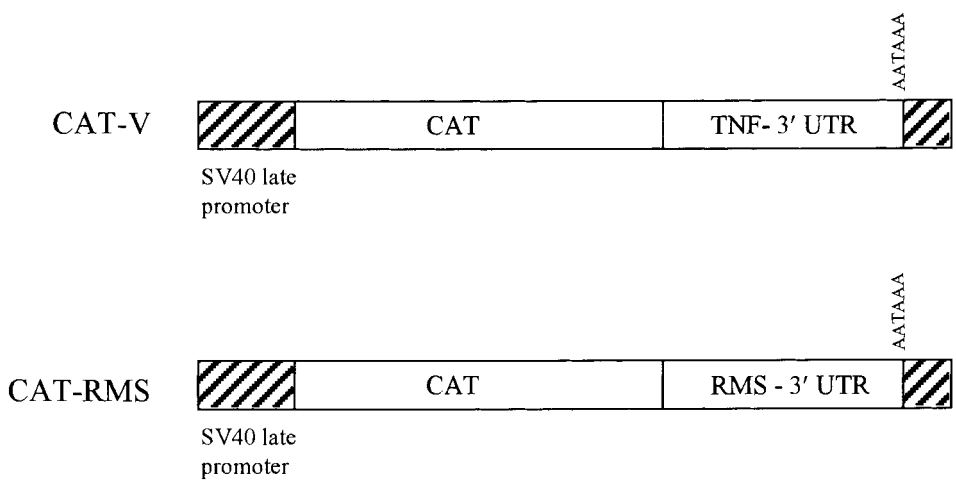

FIG. 2. Diagram of the posttranscriptional reporter vectors. The designated name is given on the left. Construction of these plasmids is described under Materials and Methods.

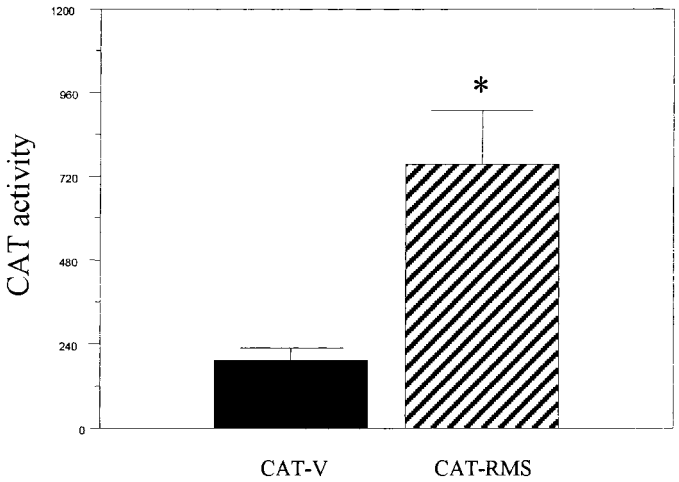

FIG. 3. CAT activity in transfected NIH/3T3 cells. The data are given as means ± SE. The solid bar shows CAT activity in cells transfected for 48 h with CAT-V; the striped bar gives data in cells transfected for 48 h with CAT-RMS. $n = 7$ trials; CAT activity was determined in duplicate for each trial. The asterisk (*) indicates $P = 0.0013$ compared with CAT activity in cells transfected with CAT-V.

was carefully chosen to eliminate problems of data interpretation that might be caused by variable size. Construction of these posttranscriptional reporter constructs is described in the material and methods section of the paper; Fig. 2 is a diagrammatic representation of CAT-V and CAT-RMS. The *cis* element of MnSOD used in CAT-RMS is the region previously shown to have protein-binding activity and to enhance translation in a cell free rabbit reticulocyte system (14). CAT activity was ~400% greater in cells transiently transfected with CAT-RMS compared with cells transfected with CAT-V (Fig. 3). No CAT activity was detected in cells transfected with the empty parental vector pSVL (data not shown). These data indicate the 3′ UTR *cis* element of MnSOD RNA increases activity of the CAT reporter in a cell culture model.

The MnSOD 3′ UTR cis element increases the CAT RNA translational efficiency. To determine whether the increase in CAT activity was a reflection of an increase in CAT RNA translation or an increase in the concentration of CAT RNA, CAT activity and RNA were measured in the same set of experiments. The relative of induction of CAT RNA translation is given in Table 1. The data for cells transfected with CAT-RMS were normalized to the data in cells transfected with CAT-V. There was no difference in the concentration of CAT RNA between CAT-V and CAT-RMS. Thus, the presence of MnSOD-RE in the 3′ UTR did not appear to alter the concentration of CAT RNA. In these experiments CAT activity was 3.4-fold higher in cells transfected with CAT-RMS than in cells transfected with CAT-V. Defining translational efficiency as CAT activity per RNA, the data indicate the presence of the 3′ UTR of MnSOD RNA results in a 3.8-fold increase in translational efficiency. These data support the hypothesis that the MnSOD *cis* element is a translational enhancer *in vivo.*

TABLE 1

Relative Translational Efficiency of CAT-RMS

Transfection vector	CAT RNA	CAT activity	Translational efficiency
CAT-V	1	1	1
CAT-RMS	0.90 ± 0.21	3.36 ± 0.90	3.77

Note. Means ± SE are given. At 48 h posttransfection, CAT-RNA was measured by RPA and the data were expressed per 18S RNA in densitometry units. CAT activity was measured by LSC and the data were expressed per mg protein. CAT RNA level and activity in cells transfected with the control vector, CAT-V, were assigned a value of 1 and the data for CAT-RMS transfection were normalized to cells transfected with CAT-V. Translational efficiency was calculated as CAT activity per CAT RNA.

The transfection of CAT-RMS had no effect on endogenous MnSOD expression. It was possible that transfection of the MnSOD 3′ UTR *cis* element in the CAT-RMS plasmid would pilfer MnSOD-BP away from endogenous MnSOD RNA. To investigate whether the transfection of CAT-RMS altered NIH/3T3 endogenous MnSOD, MnSOD-BP activity was measured by a gel retardation assay; MnSOD protein was measured by Western blot; and MnSOD RNA was measured by ribonuclease protection assays. These studies were done in cells transfected with CAT-V or CAT-RMS and the data were expressed relative to control cells transfected with pSVL, the empty parental vector. There was no difference in MnSOD-BP activity, MnSOD protein, or MnSOD RNA between cells transfected with CAT-V and cells transfected with CAT-RMS (Table 2). Thus, the relatively small amount of MnSOD-RE introduced during transient transfection did not alter normal MnSOD expression.

TABLE 2

Expression of Endogenous MnSOD in NIH/3T3-Transfected Cells

Transfection vector	MnSOD protein	MnSOD mRNA	MnSOD-BP activity
CAT-V	105.3 ± 5.6	85.7 ± 7.4	121.7 ± 12.0
CAT-RMS	96.7 ± 6.4	77.7 ± 9.5	126.0 ± 16.1
	$P > 0.05$	$P > 0.05$	$P > 0.05$

Note. Means ± SE are given. At 48 h posttransfection, MnSOD protein was measured by western analysis and the data were expressed as relative densitometry units. MnSOD RNA was measured by RPA and the data were expressed per 18S RNA in densitometry units. MnSOD-BP activity was measured by a gel retardation assay and the data are expressed in relative densitometry units. The data for all three measurements were normalized to values in cells transfected with the parental vector, pSVL.

CONCLUSION

The results reported in this paper support the hypothesis that the MnSOD 3′ UTR *cis* element is a translational enhancer. This hypothesis was based on prior *in vitro* studies that were done in a cell-free rabbit reticulocyte system comparing MnSOD mRNA containing the 3′ UTR with MnSOD mRNA in which the 3′ UTR was deleted (14). Importantly, the present study expands the work to an *in vivo* cell culture model and shows that MnSOD-RE is capable of increasing translational efficiency of a reporter RNA. These data indicate not only that MnSOD-RE and MnSOD-BP are potential therapeutic targets for altering MnSOD during periods of oxidative stress but also that MnSOD-RE is effective in increasing the translation of a chimeric expression construct to increase the yield of protein. This latter ability may prove useful in designing transgenic vectors for gene therapy or in designing vectors for expression of recombinant proteins in cell systems.

ACKNOWLEDGMENTS

This work was supported in part by National Heart, Lung, and Blood Institute grants HL20366 and HL47413 and a Career Investigator Award of the American Lung Association to Linda Clerch, Ph.D.

REFERENCES

1. Hargrove, J. L., and Schmidt, F. H. (1989) The role of mRNa and protein stability in gene expression. *FASEB J.* **3,** 2360–2370.
2. McCarthy, J. E. G., and Kollmus, H. (1995) Cytoplasmic mRNA-protein interactions in eukaryotic gene expression. *TIBS* **20,** 191–197.
3. Siomi, H., and Dreyfuss, G. (1997) RNA-binding proteins as regulators of gene expression. *Curr. Opin. Genet. Dev.* **7,** 345–353.
4. Clerch, L. B., and Massaro, D. (1997) Lung antioxidant enzyme gene expression and tolerance to hyperoxia. *in* Oxygen, Gene Expression, and Cellular Function (Clerch, L. B., and Massaro, D., Eds.), pp. 399–424, Marcel Dekker, Inc., New York, NY.
5. Fridovich, I. (1978) The biology of oxygen radicals. *Science* **201,** 875–880.
6. Fridovich, I. (1983) Superoxide radical: An endogenous toxicant. *Annu. Rev. Pharmacol.* **23,** 239–257.
7. Clerch, L. B., Massaro, D., and Berkovich, A. (1998) Molecular mechanisms of antioxidant enzyme expression in lung during exposure to and recovery from hyperoxia. *Am. J. Physiol.* **274,** L313–L319.
8. Berkovich, A., Massaro, D., and Clerch, L. B. (1996) Pertussis toxin alters the concentration and turnover of manganese superoxide dismutase in rat lung. *Am. J. Physiol.* **271,** L875–L879.
9. Clerch, L. B., Neithardt, G., Spencer, U., Melendez, J. A., Massaro, G., and Massaro, D. (1994) Pertussis toxin treatment alters manganese superoxide dismutase activity in lung. *J. Clin. Invest.* **93,** 2482–2489.
10. Clerch, L. B., and Massaro, D. (1993) Tolerance of rats to hyperoxia: Lung antioxidant enzyme gene expression. *J. Clin. Invest.* **91,** 499–508.
11. Dirami, G., Massaro, D., and Clerch, L. B. (1999) Regulation of lung manganese superoxide dismutase: Species variation in response to lipopolysaccharide. *Am. J. Physiol.* **276,** L705–L708.
12. Clerch, L. B., and Massaro, D. (1992) Rat lung antioxidant enzymes: Differences in perinatal gene expression and regulation. *Am. J. Physiol.* **263,** L466–L470.
13. Fazzone, H., Wangner, A., and Clerch, L. B. (1993) Rat lung contains a developmentally regulated manganese superoxide dismutase mRNA-binding protein. *J. Clin. Invest.* **92,** 1278–1281.
14. Chung, D. J., Wright, A. E., and Clerch, L. B. (1998) The 3′ untranslated region of manganese superoxide dismutase RNA contains a translational enhancer element. *Biochemistry* **37,** 16298–16306.
15. Clerch, L. B. (1995) A 3′ untranslated region of catalase mRNA composed of a stem-loop and dinucleotide repeat elements binds a 69-kDa redox-sensitive protein. *Arch. Biochem. Biophys.* **317,** 267–274.
16. Han, J., Brown, T., and Beutler, B. (1990) Endotoxin-responsive sequences control cachectin/tumor necrosis factor biosynthesis at the translational level. *J. Exp. Med.* **171,** 465–475.