A Coding Region Determinant of Instability Regulates Levels of Manganese Superoxide Dismutase mRNA*

Christopher A. Davis‡, Joan M. Monnier§, and Harry S. Nick¶¶

From the ‡Department of Biochemistry and Molecular Biology and §Department of Neuroscience, University of Florida, Gainesville, Florida 32610

The mitochondria-localized manganese superoxide dismutase (MnSOD), serves a key cytoprotective role against reactive oxygen species arising from a variety of cellular processes and immunological stresses. Previous data from our laboratory suggest that the regulation of the rat MnSOD gene may occur not only at the transcriptional but quite possibly at the post-transcriptional level. To verify this hypothesis, we have attempted to identify regions within the rat MnSOD cDNA that may be functionally involved in regulating the stability of the mRNA. Using a c-fos-based promoter activation system, we have identified an ~280-nucleotide fragment within the MnSOD mRNA coding region that, when fused to a rabbit β-globin gene, destabilizes the normally stable β-globin mRNA. This cis-directed destabilization phenomenon confers its effects independent of position and stimulus. Most importantly, the MnSOD coding region determinant functions when placed in the 3′-untranslated region of the β-globin transcript, demonstrating its activity in the absence of ribosome transit. We feel that these data provide a mechanistic basis for both the basal and stimulus-dependent post-transcriptional regulation of MnSOD.

Regulated levels of antioxidant enzymes provide an initial level of cellular defense against damaging reactive oxygen species. The nucleus-encoded mitochondria-localized manganese superoxide dismutase (MnSOD) is one such enzyme providing potent cytoprotection against a variety of challenges. In addition to reversing the malignant phenotypes of specific carcinomas (1), overexpression of MnSOD has been shown to protect against exposure to radiation (2, 3), cytokines (4, 5), chemotherapeutic agents (6), ischemia/reperfusion (7), and glutamate-dependent neurotoxicity (8). Gene ablation studies generating MnSOD (−/−) mice have demonstrated the physiological importance of this enzyme. Depending on the background strain used, homozygote MnSOD (−/−) mice develop severe cardiac myopathy (9) or neurological abnormalities (10), with death resulting within 10 days or 3 weeks of birth, respectively.

Several laboratories, including our own, have provided insights into the molecular regulation of the MnSOD gene through promoter elements (11) and the identification of a bacterial endotoxin and cytokine-specific intronic enhancer element (12, 13). Nuclear run-on analysis following induction with proinflammatory mediators (11) does not reflect a complete recapitulation of induced levels of RNA observed in steady-state Northern analysis, inferring a relevant role for RNA stability in the molecular regulation of MnSOD. Interestingly, induced MnSOD transcripts do not return to basal levels following treatment with actinomycin D or cycloheximide, suggesting the involvement of de novo transcription and translation in the turnover of the message (14). These data implicate the existence and potential importance of an underlying mechanism controlling MnSOD mRNA half-life in both the basal and stimulated expression of this gene.

Modulations in mRNA stability provide a rapid mechanism for regulating cellular protein levels independent of de novo transcription. Not surprisingly, the regulation of this process is highly coordinated as evidenced by the disastrous consequences observed with v-fos. Fibroblasts expressing v-fos appear oncogenic due to the loss of a native destabilizing element within the 3′-untranslated region (UTR). The result is higher levels of the fos gene product and the observed oncogenic phenotype (15, 16). Analogously, MnSOD overexpression has not only uncovered its cytoprotective potential but demonstrated that uncontrolled expression of this antioxidant activity causes potent antiproliferative effects in a variety of cell culture (17, 18) and in vivo systems (1). Therefore, the control of MnSOD mRNA levels may be a critical physiological mechanism for overall regulation of the redox state of the cell.

Perhaps the most ubiquitous and well understood destabilizing element found within rapidly degraded mRNAs is the adenylate-uridylate-rich element (ARE). To date, at least three classes of ARE have been identified based on their presence, number of repeats of the pentamer AUUUA, and the subsequent effects of RNA deadenylation and decay (20). In addition to the AREs found with chemokine, cytokine, and proto-oncogene transcripts containing AREs, coding region determinants of stability have been identified within a variety of mRNAs including vascular endothelial growth factor (21), interleukin-2 (22), neurofilament light subunit (23), urokinase receptor (24), c-myc (25) and c-fos (26). The best characterized of these elements is the c-fos major coding region determinant of stability (mCRD). Recently, work by Grosset et al. (27) clearly defined the boundaries of the c-fos mCRD, established the importance of its position relative to the poly(A) tail, and identified and cloned several cellular factors that interact with the mCRD.

The purpose of this study was to better understand the molecular mechanism(s) that regulate the stability of the MnSOD mRNA. To this end, we have modified the c-fos short term promoter activation system previously described by Shyu et al. (28) to identify functional elements that control MnSOD mRNA half-life. We have demonstrated that destabilization of the...
MnSOD mRNA is mediated by an 280 base sequence element within the coding region of the rat MnSOD mRNA that is functional during both basal and stimulus-dependent gene expression. Most importantly, we have observed that this cis-acting, destabilizing element functions independently of ribosome transit.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—MnSOD fragments were amplified by polymerase chain reaction from a previously characterized MnSOD cDNA (29) using standard conditions and primers specific to appropriate 5′ and 3′ termini. Products are denoted by their position relative to the translational initiation site (+1) of the MnSOD cDNA (Fig. 3). For the in-frame/coding region pBSOD constructs, care was taken to ensure proper transition between each of the RNAs in the chimera so as not to introduce frameshift mutations or premature stop codons. The MnSOD fragments were ligated into either the BamHI (coding region) or BglII (3′-UTR) sites of the previously described plasmid pBBB4 (28), a kind gift of Dr. Ann-Bin Shyu (Fig. 2A). Restriction endonuclease digestion and DNA sequencing verified the integrity and orientation of each pBSOD vector.

**Cell Culture, Transfection, and Induction of the Human c-fos Promoter**—L2 cells, a rat pulmonary epithelial-like cell line (ATCC CCL 149) was grown in Kaighn's modified F-12 Ham's medium (Sigma) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc.), antibiotic/antimycotic (Life Technologies), and glutamine 0.06% (Life Technologies) at 37 °C in room air, 5% CO2. At 90% confluence, cells were split into 3 × 150-mm plates 1 day prior to transfection. Cells were exposed to a total of 30 µg of plasmid DNA, 15 µg of pBBB4 control plasmid, and 15 µg of each pBSOD vector in DEAE-dextran (13).

**Fig. 1.** Measurement of the endogenous MnSOD mRNA half-life. A, Northern blot analysis of a typical time course following continuous LPS exposure (0.5 µg/ml). B, time course and Northern blot analysis of cells exposed to LPS (0.5 µg/ml) for 5 h followed by three washes with media to remove the stimulus. C, values from densitometric analysis of the representative wash-out shown in B were analyzed using Enzfitter software. The decay fit a single exponential and provided the designated t½ of 4.2 h.

MnSOD mRNA is mediated by an −280 base sequence element within the coding region of the rat MnSOD mRNA that is functional during both basal and stimulus-dependent gene expression. Most importantly, we have observed that this cis-acting, destabilizing element functions independently of ribosome transit.

**Fig. 2.** Measurement of β-globin mRNA half-life in L2 cells. A, the rabbit β-globin genomic locus including introns, exons (boxes), and 3′-UTR (thick line) is represented on the pUC12 plasmid backbone of pBBB4. The arrow represents the direction of transcription by the minimal c-fos promoter (705 bp). The 0.42-kb MluI/BamHI fragment used for Northern blot analysis, the translational stop codon (TGA), and the BamHI (coding region) and BglII (3′-UTR) cloning sites are also depicted. B, representative Northern blot analysis of L2 cells transfected with pBBB4 illustrating the β-globin mRNA transcribed in vivo from this vector. The membrane was stripped and reprobed with cathepsin and murine c-fos cDNAs to ensure proper loading and validate the starvation and refeeding regimen. C, decay curves resulting from densitometric analysis of the data in B. The decay of the β-globin message and all chimeric transcripts henceforth fit single exponential decays from the maximal mRNA (observed 1 h after serum refeeding) using Enzfitter. The half-life was determined from the single exponential fit of the β-globin mRNA decay.
Twenty-four hours after transfection, the cells were pooled and split into 8 × 100-mm plates. After a 24-h recovery period, the cells were serum-starved for an additional 24 h by incubation with F12K plus 0.5% FBS. The serum-responsive, human c-fos promoter was then activated by incubation with medium containing 15% FBS.

RNA Isolation and Northern Analysis—Total RNA was isolated from cells at the indicated times as per the Chomczynski and Sacchi method with modifications (14). Twenty µg of RNA was fractionated on 1% agarose, 6% formaldehyde gels, electrotransferred to Zetabind membranes, and UV-cross-linked as described (30). Membranes were washed in prehybridization buffer (30) for 15 min at 60 °C. Membranes were hybridized overnight in prehybridization buffer with a radiolabeled 0.42-kb MluI-BamHI fragment of the β-globin gene contained in the vector pBBB4 (Fig. 2A), generated by random primer extension (31). All blots were probed with a radiolabeled 1.8-kb EcoRI fragment of a cathepsin cDNA to control for variations in loading. To verify that the serum-starvation/refeeding protocol afforded transient stimulation of the cell cycle-regulated c-fos promoter, we hybridized a representative pBBB4 time course with a radiolabeled 2.1-kb EcoRI fragment of mouse c-fos cDNA. After hybridization, membranes were washed and exposed to film using Fisher Biotech L-Plus intensifying screens at −75 °C.

mRNA Half-life Determination—All Northern analyses are representative of experiments repeated 2–3 times. The half-lives of the corrected pBSOD mRNA levels were determined by densitometric analysis using NIH Image. The curve-fitting software Enzfitter 2.0 (Biosoft Corp.) was utilized to calculate mRNA t 1/2 values from maximal induction following serum refeeding. The decay patterns fit a single exponential decay, and t 1/2 values were calculated from these fitted curves.

RESULTS

Endogenous MnSOD mRNA Half-life—We have previously reported that the five transcripts of rat MnSOD mRNA observed on steady state Northern analysis arise from alternative polyadenylation of the primary transcript (29). Work from our laboratory has also shown that each of the MnSOD species are induced and degraded similarly in response to proinflammatory mediators (14). In addition, we have observed that actinomycin D treatment leads to prolonged stabilization of both basal and induced MnSOD transcripts, implicating the involvement of de novo transcription in mRNA turnover (14) and thereby precluding its use to directly assess MnSOD mRNA half-life. Therefore, in an effort to explore the potential role of mRNA stability in regulating levels of MnSOD, we utilized a stimulus-dependent wash-out approach (32) to quantitatively determine the half-life of the endogenous MnSOD mRNA.

L2 cells, a rat pulmonary epithelial-like cell line, were stimulated with the known inducer lipopolysaccharide (LPS; 0.5 µg/ml) for 5 h. MnSOD mRNA levels were evaluated temporally by Northern analysis in the continued presence of LPS (Fig. 1A) or following the removal and subsequent wash-out of the stimulus (Fig. 1B). These experiments demonstrate a role for RNA stability in regulating levels of rat MnSOD. As shown in Fig. 1C, densitometric analysis of the representative Northern data depicted in Fig. 1B provides a t 1/2 of 4.2 h for the endogenous MnSOD message.

Importance of the Coding Region or 3′-UTR in the Destabilization of MnSOD Transcripts—To localize the sequence elements involved in regulating MnSOD mRNA stability, we have employed the plasmid-based short-term promoter activation system, pBBB4 shown in Fig. 2A (26). The utility of this vector system is illustrated in Fig. 2B. L2 cells, transiently transfected with pBBB4, are subjected to serum starvation (0.5% FBS) for 24 h, effectively silencing transcription from the plasmid-based c-fos promoter. Serum refeeding (15% FBS) affords transient stimulation of the c-fos promoter and transcription of the β-globin gene. As described previously (26), transcription from the c-fos promoter is terminated within 1 h after the serum addition, allowing subsequent tracking of the stability of newly transcribed β-globin transcripts through time. Furthermore, we have not observed serum-mediated effects on MnSOD transcript levels in any of our previous experiments in a wide variety of cell systems, indicating that this short-term promoter activation system can be employed for kinetic measurements of MnSOD mRNA half-life. As shown in Fig. 2C, the β-globin reporter message is stable in L2 cells with a half-life of ~11.3 h, a figure consistent with that observed in other cell lines (33, 34). Further evidence establishing the effectiveness of the serum starvation/refeeding regimen in L2 cells is provided by the Northern analysis shown in the bottom panel of Fig. 2B, where we have stripped and reprobed the pBBB4 time course for the endogenous c-fos transcript. As seen, the promoter is inactive in the absence of serum, whereas refeeding provides an impressive burst followed by rapid turnover of the c-fos mRNA. Of note, as first described by Shyu et al. (28), the β-globin transcript undergoes an initial polyadenylation event as evidenced by the slight increase in mRNA size within 0.5 h of serum refeeding. Adenylation of the β-globin transcript is followed by.

**FIG. 3.** Summary of the MnSOD cDNA fragments inserted into pBBB4. Thin lines represent 5′- and 3′-UTRs, while thick lines denote the MnSOD coding region. The MnSOD fragments were numbered relative to the translation start site. The constructs containing MnSOD 3′-UTR, pBSOD—8/672, pBSOD181/672, and pBSOD409/672, which have been inserted into the 3′-UTR of the β-globin gene are depicted and denoted as (672).
synchronous deadenylation for ~6 h, at which time no further deadenylation is observed, and the message remains stable for at least another 5 h (Fig. 2B).

In an effort to delineate the boundaries of the MnSOD stability element, we have inserted MnSOD cDNA sequences (Fig. 3) into the c-fos/β-globin expression vector, pBBB4. To address either the potential of a position effect within the β-globin transcript or disruption of inherent β-globin stability sequences, most fragments were inserted either in the BamHI site (coding region) or the Bg/II site (3'-UTR downstream of the β-globin translation stop codon) within the β-globin gene (Fig. 2A). In addition, the insertion of the cDNA fragments both within the coding region and the 3'-UTR, has allowed us to assess the involvement of translation in the turnover of MnSOD/β-globin chimeric transcripts. DNA sequence analysis revealed the presence of an intact β-globin translation stop codon in each of the pBSOD vectors containing MnSOD sequences inserted in the Bg/II cloning site. We have therefore precluded the possibility of frameshifting and subsequent ribosome read-through into the β-globin 3'-UTR, events that lead to the rapid turnover of the human α-globin mRNA (35).

Since each of the five rat MnSOD transcripts has been shown to be coordinately induced and degraded (29), we began our study with the smallest MnSOD cDNA represented on steady state Northern analysis. This fragment, containing 8 bases of the 5'-UTR, the entire coding region, and 533 bases of the 3'-UTR, is denoted pBSOD–8/1199. This fragment also contains the MnSOD stop codon (position 666) and was inserted into the Bg/II site of the β-globin gene to avoid the potential for chimeric RNA destabilization by nonsense-mediated decay (36). Engineered pBSOD (β-globin/MnSOD) vectors were co-transfected with pBBB4 into L2 cells. The transfected cells were then serum-starved (0.5%) for 24 h. To induce the serum-responsive c-fos promoter that drives expression of β-globin (denoted pBBB4) and the hybrid MnSOD/β-globin (pBSOD) mRNAs, transfected cells were then incubated with media containing 15% serum. Total RNA was isolated at various time points following refeeding, and Northern analysis was performed using fractionation on a 1% agarose-formaldehyde gel. The membranes were then hybridized with a radiolabeled MluI-BamHI fragment of pBBB4 corresponding to exons 1 and 2 of β-globin (Fig. 2A). Fig. 4A shows a representative Northern analysis of cells co-transfected with pBSOD–8/1199 and pBBB4. As shown, the insertion of MnSOD sequences –8/1199 destabilizes the normally stable β-globin mRNA. The presence of two chimeric transcripts (Fig. 4A, arrows) is a result of alternative polyadenylation occurring at the poly(A) site located at position 811 within the MnSOD cDNA (I) or the β-globin poly(A) site (II). Densitometric analysis and subsequent curve fitting of each transcript (I or II) reveals a chimeric mRNA t1/2 of 1.6 h (Fig. 4C).

The rat MnSOD mRNA 3'-UTR does not contain conventional components involved in mRNA stability, such as AREs, or structural determinants like those found in histone (37, 38), IGFII (39), and transferrin (40) mRNAs. However, recent studies have identified elements within the 3'-UTR that may function as a translational enhancer (41). In an effort to explore and further map the destabilizing domain found in the –8/1199 fragment, we co-transfected L2 cells with pBSOD628/1199 and pBBB4. This fragment (628/1199) inserted into the Bg/II site of pBBB4 contains 37 bases of the coding region, the translation stop codon, and an additional 533 bases of the MnSOD 3'-UTR. As shown in Fig. 4B, the addition of bases 628/1199 had no effect on the stability of the resulting hybrid β-globin mRNA (t1/2 = 11.5 h; Fig. 4C). Further, as the insertion of this fragment does not destabilize the reporter β-globin message in cis, we conclude that the t1/2 of 1.6 h for the pBSOD–8/1199-derived chimeras is not a result of a disruption of inherent β-globin sequence elements responsible for maintaining mRNA stability.

To map the MnSOD stability element identified in pBSOD–8/1199, we generated the vector pBSOD–8/665, where the entire MnSOD coding region lacking only the translational stop codon was inserted in frame (BamHI) in pBBB4. In addi-
**FIG. 5.** The MnSOD destabilizing element is located within the coding region. A, Northern blot analysis of cells co-transfected with pBBB4 and the entire MnSOD coding region (pBSOD−8/665) inserted in the coding region of β-globin. B, Northern analysis of cells transfected with pBBB4 and the MnSOD coding region inserted in the 3′-UTR of β-globin. The bottom panel depicts cells transfected with antisense MnSOD coding region (pBSOD672−8) inserted in the 3′-UTR of β-globin. C, representative Northern analysis of cells co-transfected with pBBB4 and the pBSOD−8/672 (in the 3′-UTR of β-globin) followed by treatment with actinomycin D (4 μM) 1 h after serum refeeding. D, mRNA decay curves of the pBSOD−8/665 (solid), pBSOD−8/672 (short dashes), and pBSOD9−8/672ct D (long dashes) time courses. The pBBB4 curves from each representative co-transfection experiment are depicted using the same line format, solid (coding region (cr)) or dashed (3′-UTR (utr)) as their respective chimeric transcript.

**FIG. 6.** 3′ deletions of the MnSOD coding region determinant of instability. A, Northern analysis of cells co-transfected with pBBB4 and the MnSOD−8/463 fragment inserted into the coding region of β-globin (pBSOD−8/463cr). B, Northern analysis of cells co-transfected with pBBB4 and (−8/463) inserted into the 3′-UTR of β-globin (pBSOD−8/463utr). C, mRNA decay curves derived from the data in A and B. D, co-transfection of pBBB4 and the −8/230 fragment inserted into the coding region of β-globin (pBSOD−8/230cr). E, co-transfection of pBBB4 and the −8/230 fragment inserted into the 3′-UTR of β-globin (pBSOD−8/230utr). F, mRNA decay curves derived from the data presented in D and E. The line format in C and F is as described in the legend to Fig. 5.
To further localize the cis-acting MnSOD CRD, we created a series of deletion constructs spanning the MnSOD coding region. Fig. 6, A, B, D, and E, shows representative Northern analyses of two 3' deletions of the MnSOD coding region, pBSOD–8/463 and pBSOD–8/230, inserted in either the coding region (BamHI; Fig. 6, A and D) or 3' UTR (BglII, Fig. 6, B and E) of β-globin. As shown, insertion of the 5'-half of the MnSOD coding region (~8/463) within the coding region of β-globin results in a chimeric half-life of 1.4 h (Fig. 6A). Deletion of sequence from positions 463–230 reduces the destabilizing effects of the MnSOD element (t1/2 = 5.8 h). In addition, the data in Fig. 6B demonstrate that this cis-acting element retains its ability to function when positioned in the 3'-UTR of β-globin (t1/2 = 1.3 h). Graphical representations summarizing the half-lives for both of these constructs are depicted in Fig. 6A. The inability of the 8/230 fragment to confer cis-mediated destabilization when placed in the β-globin coding region provides evidence that the destabilization observed with pBSOD–8/665 and that ~8/463 was not due to nonsense-mediated decay of the β-globin mRNA. In addition, we were able to induce nonsense-mediated decay by inserting two tandem stop codons within the coding region of β-globin immediately upstream of the ~8/230 fragment (data not shown).

5' deletions of the MnSOD cDNA were also generated in an effort to delineate the boundaries of the destabilizing element. Fig. 7, A and B, demonstrates that insertion of sequences
The coding region of MnSOD resides within positions 181--463. A, Northern analysis of cells co-transfected with pBBB4 and the 181/463 fragment of MnSOD inserted into the coding region of β-globin (pBSOD181/463cr). B, representative Northern analysis of cells co-transfected with pBBB4 and the 181/463 fragment of MnSOD inserted into the 3′-UTR of β-globin (pBSOD181/463utr). C, decay curves of the data presented in A and B. D and E, Northern analysis of cells co-transfected with the 5′ (181/377) and 3′ (267/463) halves of the MnSOD CRD (181/463) inserted into the coding region of β-globin (pBSOD181/377 and pBSOD267/463, respectively). F, mRNA decay curves of the data presented in D and E. The line format in C and F is as described in the legend of Fig. 5.

Fig. 8. The MnSOD CRD resides within positions 181--463. A, Northern analysis of cells co-transfected with pBBB4 and the 181/463 fragment of MnSOD inserted into the coding region of β-globin (pBSOD181/463cr). B, representative Northern analysis of cells co-transfected with pBBB4 and the 181/463 fragment of MnSOD inserted into the 3′-UTR of β-globin (pBSOD181/463utr). C, decay curves of the data presented in A and B. D and E, Northern analysis of cells co-transfected with the 5′ (181/377) and 3′ (267/463) halves of the MnSOD CRD (181/463) inserted into the coding region of β-globin (pBSOD181/377 and pBSOD267/463, respectively). F, mRNA decay curves of the data presented in D and E. The line format in C and F is as described in the legend of Fig. 5.

Derived from the 3′-half of the MnSOD coding region (pBSOD181/665 and pBSOD181/672) retained the ability to direct destabilization when inserted in the coding region (t1/2 = 1.4 h) or the 3′-UTR (t1/2 = 1.3 h) of β-globin. However, removal of nucleotides between positions 181 and 409 resulted in t1/2 values for the MnSOD/β-globin chimeras that were similar to that for β-globin alone (coding region (pBSOD409/665) t1/2 = 12.5 h, 3′-UTR (pBSOD409/672) t1/2 = 14.8 h; Fig. 7, D and E). The data summarized in Figs. 6 and 7 strongly implicate the importance of MnSOD sequences from 181 to 463 in mediating destabilization of β-globin mRNA. Fig. 8 represents data generated from co-transfections of pBSOD181/463 and pBBB4 where this region has been evaluated in both the coding (Fig. 8A) and 3′-UTR of β-globin (Fig. 8B). The insertion of nucleotides 181--463 of MnSOD destabilizes the β-globin message in cis with a slightly longer half-life for the MnSOD/β-globin chimera (coding region = 3.5 h, 3′-UTR = 2.9 h) as compared with that of the larger chimeras (1.5 h).

5′ and 3′ deletions of the 181/463 element were subsequently evaluated for their ability to confer destabilization of the β-globin transcript. Fragments from 181 to 377 (pBSOD181/377) or 267 to 463 (pBSOD267/463) were evaluated in the coding region of β-globin as shown in Fig. 8, D and E, respectively. Fig. 8F shows a graphical summary of these studies demonstrating that further deletion of the 181--463 region disrupts the ability of this element to destabilize the β-globin reporter mRNA. These fragments were also evaluated in the 3′-UTR (B/gIII) with identical results (data not shown), suggesting the necessity of sequence elements contained within both halves of the 181--463 fragment to direct cis-mediated destabilization. We have also studied a number of smaller fragments internal to this region, which did not affect the β-globin half-life (data not shown).

The Destabilizing Effects of the MnSOD CRD Are Not Modulated by Stimulation with Lipopolysaccharide—To address the physiological significance of the MnSOD CRD identified in this study, we have also explored the role of this element in the presence of a transcriptional stimulus. These studies were designed to address whether the stimulus LPS, known to induce MnSOD at both the steady state Northern (14) and nuclear run (11) levels, is also involved in the post-transcriptional regulation of the gene. The MnSOD cDNA construct pBSOD181/672, previously shown to confer rapid destabilization of the β-globin transcript, was co-transfected with pBBB4 as described above. However, in this experiment, the serum-starved cells were co-treated with 15% FBS and LPS (0.5 μg/ml) followed by Northern analysis. As can be seen in Fig. 9A, co-treatment with LPS did not have an effect on the stability of the MnSOD/β-globin chimera, yielding essentially an identical half-life (1.3 h) to that observed in the absence of LPS (Fig. 7). These results indicate that the MnSOD CRD regulates the half-life of both basal and induced MnSOD transcripts and thus functions constitutively to modulate the intracellular redox state.

A summary of the MnSOD cDNA fragments used in this study, their corresponding vector names, and mRNA half-lives both in the coding region and 3′-UTR of β-globin is shown in Table I. These results demonstrate that the minimal CRD is contained within nucleotides 181--463 of the MnSOD mRNA.
In an effort to more accurately address the role that mRNA turnover, we have used the short term promoter activation system (26), pBBB4. This system allows for the identification and kinetic analysis of mRNA decay events (43) and has been used successfully to describe the turnover of several mRNAs including the c-fos (28) and c-myc (25) transcripts. Using this system, we have identified an ~280 base element residing within the coding region of the MnSOD mRNA that confers cis-mediated destabilization in the absence of ribosome transit. Our results have also demonstrated that this element functions during both basal and stimulus-dependent MnSOD gene expression.

The presence of coding region stability elements was first recognized and described in the tubulin (44), c-fos (26), and c-myc (25, 45) transcripts. In the newly emerging tubulin peptide, it was shown that the first 12 or 13 nucleotides coding for Met-Arg-Glu-Ile must be recognized for tubulin to confer instability on its own mRNA (44). Alternatively, the c-fos mRNA contains a coding region element whose ability to destabilize reporter RNAs is inactivated by the insertion of upstream stem-loop structures, indicating that the cis-acting determinants must be translated or at least associated with ribosomes to confer instability (27, 34). In addition, the c-fos element may be translated out-of-frame and retain its cis-directed destabilizing effects, indicating that the RNA itself is important in directing message destabilization (46). Similar experiments suggest that the c-myc coding region element must also be translated to be efficiently degraded (25, 47).

Grosset et al. (27) demonstrated that overexpression of the protein factors shown to bind the c-fos and c-myc destabilization mechanisms results in an increase in the stability of chimeric globin messages containing the mCRD. Following ribosome transit through the coding region element, a putative protein bridge between the factors associated with the mCRD and the poly(A) tail is putatively disrupted, resulting in deadenylation, and subsequent decay of the message. Additionally, a 70-kDa protein shown to bind the c-myc coding region element is presumed to protect the poly(A) tail of the mCRD from endonucleolytic cleavage (48). Along the same line, coding region elements of instability have also been recently characterized in the interleukin-2 mRNA (22), vascular endothelial growth factor mRNA (21), the human papillomavirus type 16 L2 coding region (49) as well as the neurofilament light subunit mRNA (23); however, these studies have not differentiated the importance of translation in the destabilization mechanism analogous to the studies with c-fos (26) or c-myc (45).

The destabilizing mechanism we have documented for the MnSOD mRNA involves a CRD which differs from that of both the c-fos and c-myc determinants as it can still confer cis-directed effects when inserted downstream of the β-globin translational stop codon (BgII; Fig. 2A). Given that the majority (possibly greater than 95%) of ribosomes dissociate from mRNAs once the stop codon is positioned in the aminoacyl site of the ribosome (19), our data imply that the inherent activity of the MnSOD CRD does not require ribosome transit to confer cis-directed destabilization. In addition, it is unlikely that the relatively small percentage of read-through ribosomes would account for a peptide-mediated destabilization mechanism as observed with the tubulin transcript. Furthermore, the MnSOD CRD functions independently of additional elements, based on the lack of destabilizing determinants in other parts of the coding region or 3’-UTR of the MnSOD mRNA (Fig. 4B).

We have also attempted to address the importance of deadenylation in the process of CRD-mediated destabilization, by analyzing the comparative decrease in transcript size, as shown previously with this reporter system (28, 34). The analysis of this data indicates that the MnSOD CRD has very little
effect on the rate of chimeric deadenylation as compared with that of the β-globin transcript alone.

Interestingly, destabilization of the endogenous MnSOD mRNA does require de novo translation in that MnSOD mRNAs levels are increased in the presence of the protein synthesis inhibitor, cycloheximide, and superinduced with cycloheximide and LPS co-treatment (14). The importance of de novo translation as well as transcription is also substantiated by the sustained mRNA induction of LPS-stimulated cells following co-treatment with actinomycin D, which was previously documented by Northern analysis for the endogenous MnSOD (14). We have extended this latter observation by showing that treatment with actinomycin D increases the half-life of chimeric deadenylation as compared with mRNA does require de novo translation in that MnSOD mRNA capable of directing cis-mediated destabilization. As part of a chimeric β-globin/MnSOD mRNA, this element decreases the inherent β-globin transcript half-life in a position-independent manner to that more indicative of the endogenous MnSOD. Further, since the element retains full destabilizing capabilities when placed downstream of the β-globin translation stop codon, it presumably does not require ribosome transit to exert its cis-mediated effects. Taken together, these data demonstrate the identification of an MnSOD CRD that most likely does not involve translation events to direct mRNA decay and can confer its functional effects during both basal and stimulus-dependent MnSOD gene expression. We are currently pursuing experiments to further delineate the necessary sequence elements of the MnSOD CRD, taking into consideration the spacing of the fragments with respect to the poly(A) tail. We are also interested in exploring the potential involvement and identity of trans-acting factors that may interact with the CRD. The application of this knowledge may prove beneficial in disease pathologies where reactive oxygen species have been implicated, since we postulate that the MnSOD CRD may play a significant role in maintaining cellular redox homeostasis.

TABLE I

| Vector       | MnSOD cDNA Fragments | $t_{1/2}$ (coding region) | $t_{1/2}$ (3' UTR) |
|--------------|----------------------|---------------------------|--------------------|
| pBSOD-8/1199 | -8/230               | 199                       | 1.6                |
| pBSOD628/1199| -8/230               | 199                       | 1.6                |
| pBSOD-8/665/72| -8/665               | 665                       | 11.5               |
| pBSOD-8/672 (actino D) | 672               | 665                       | 16.7               |
| pBSOD672/8 (antisense) | 8 | 665                       | 1.7                |
| pBSOD-8/463 | -8/463               | 463                       | 1.4                |
| pBSOD-8/230 | -8/230               | 230                       | 5.8                |
| pBSOD181/665/72| 181/665            | 665                       | 1.3                |
| pBSOD181/463 | 181/463             | 463                       | 1.4                |
| pBSOD409/665/72| 409/665            | 665                       | 1.3                |
| pBSOD181/463 | 181/463             | 463                       | 1.3                |
| pBSOD181/377 | 181/377             | 377                       | 12.5               |
| pBSOD267/463 | 267/463             | 463                       | 14.8               |

REFERENCES

1. Church, S. L., Grant, J. W., Ridnour, L. A., Oberley, L. W., Swanson, P. E., Meltzer, P. S., and Trent, J. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3113–3117
2. Eastgate, J., Moreb, J., Nick, H. S., Suzuki, K., Taniguchi, N., and Zucali, J. R. (1993) Blood 81, 639–646
3. Epperly, M. W., Epstein, C. J., Travis, E. L., and Greenberger, J. S. (2000) Radiat. Res. 154, 365–374
4. Wong, G. H., and Goeddel, D. V. (1988) Science 242, 941–944
5. Hohmeier, H. E., Thigpen, A., Tran, V. V., Davis, R., and Newgard, C. B. (1998) J. Clin. Invest. 101, 1811–1820
6. Mantympaa, P., Sitionen, T., Guttmorn, T., Saily, M., Kinnula, V., Savolainen, E. R., and Koistinen, P. (2000) Br. J. Haematol. 106, 574–581
7. Chen, Z., Liu, B., He, Y. S., Vincent, R., Chua, C. C., Handly, R. C., and Chua, B. H. (1998) J. Mol. Cell Cardiol. 30, 2281–2289
8. Gonzalez-Zulueta, M., Ensz, L. M., Mukhina, G., Lebovitz, R. M., Zawacka, R. M., Engelhardt, J. F., Oberley, L. W., Dawson, V. L., and Dawson, T. M. (1998) J. Neurosci. 18, 2050–2055
9. Li, Y., Huang, T. T., Carleton, E. J., Melov, S., Ursell, P. C., Olson, J. L., Noble, L. J., Yoshimura, M. P., Berger, C., Chan, P. H., Wallace, D. C., and Epstein, C. J. (1995) Nat. Genet. 11, 376–381
MnSOD mRNA Stability Element

10. Lebovitz, R. M., Zhang, H., Vogel, H., Cartwright, J., Dionne, L., Lu, N., Huang, S., and Matzuk, M. M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 9782–9787
11. Kuo, S., Chesrown, S. E., Mellett, J. K., Rogers, R. J., Hsu, J. L., and Nick, H. S. (1999) *J. Biol. Chem.* 274, 3345–3354
12. Jones, P. L., Ping, D., and Ross, J. M. (1997) *Mol. Cell. Biol.* 17, 6970–6981
13. Rogers, R. J., Chesrown, S. E., Kuo, S., Mennier, J. M., and Nick, H. S. (2000) *Biochem. J.* 347, 233–242
14. Visner, G. A., Dougall, W. C., Wilson, J. M., Burr, I. A., and Nick, H. S. (1990) *Oncogene Res.* 5, 1–12
15. Lee, W. M., Lin, C., and Curran, T. (1988) *Mol. Cell. Biol.* 12650–12654
16. Raymond, V., Atwater, J. A., and Verma, I. M. (1989) *Oncogene Res.* 5, 1–12
17. Li, N., Oberley, T. D., Oberley, L. W., Zhong, W. J. (1998) *J. Cell. Physiol.* 175, 359–369
18. Rodriguez, A. M., Carrico, P. M., Mazurkiewicz, J. E., Melendez, J. A. (2000) *Free Radic. Biol. Med.* 28, 801–813
19. Caskey, C. T. (1975) *Adv. Protein Chem.* 27, 243–276
20. Xu, N., Chen, C. Y., and Shyu, A. B. (1997) *Mol. Cell. Biol.* 17, 4611–4621
21. Dibbens, J. A., Miller, D. L., Damert, A., Risau, W., Vadas, M. A., and Goodall, G. J. (1999) *Mol. Cell. Biol.* 19, 907–919
22. Ragheb, J. A., Deen, M., and Schwartz, R. H. (1999) *J. Immunol.* 163, 120–129
23. Canete-Soler, R., Schwartz, M. L., Hua, Y., and Schlapfer, W. W. (1998) *J. Biol. Chem.* 273, 12650–12654
24. Shetty, S., and Idell, S. (1999) *Mol. Cell. Biochem.* 199, 189–200
25. Herrick, D. J., and Ross, J. (1994) *Mol. Cell. Biol.* 14, 2119–2128
26. Shyu, A. B., Greenberg, M. E., and Belasco, J. G. (1989) *Genes Dev.* 3, 60–72
27. Grzeszczuk, R., Chen, C. Y., Xu, N., Sonenberg, N., Jacquemin-Sablon, H., and Shyu, A. B. (2000) *Cell* 103, 29–40
28. Shyu, A. B., Belasco, J. G., and Greenberg, M. E. (1991) *Genes Dev.* 5, 221–231
29. Hurt, J., Hsu, J. L., Dougall, W. C., Visner, G. A., Burr, I. M., and Nick, H. S. (1992) *Nucleic Acids Res.* 20, 2885–2890
30. Church, G. M., and Gilbert, W. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 1991–1995
31. Feinberg, A. P., and Vogelstein, B. (1984) *Anal. Biochem.* 137, 266–267
32. Melendez, J. A., and Baglioni, C. (1993) *Free Radic. Biol. Med.* 14, 601–608
33. Albert, J. A., Rundell, K., and Stiles, C. D. (1994) *J. Biol. Chem.* 269, 4532–4538
34. Shiu, A. C., Wellington, C. L., Shyu, A. B., Chen, C. Y., Greenberg, M. E., and Belasco, J. G. (1994) *J. Biol. Chem.* 269, 3441–3448
35. Chkheidze, A. N., Lyakhov, D. L., Makeyev, A. V., Morales, J., Kong, J., and Liebhaber, S. A. (1999) *Mol. Cell. Biol.* 19, 4572–4581
36. Hilleren, P., and Parker, R. (1999) *Annu. Rev. Genet.* 33, 229–260
37. March, W. F., and Pandey, N. (1988) *Trends Biochem. Sci.* 13, 49–52
38. Stauber, C., and Schumperli, D. (1988) *Nucleic Acids Res.* 16, 9399–9414
39. van Dijk, E. L., Sussenbach, J. S., and Holzhuizen, P. E. (2000) *J. Mol. Biol.* 300, 449–467
40. Klausner, R. D., Rouault, T. A., and Harford, J. B. (1993) *Cell* 72, 19–28
41. Chung, D. J., Wright, A. E., and Clerch, L. B. (1998) *Biochemistry* 37, 16298–16306
42. Mates, M. (2000) *Toxicology* 153, 83–104
43. Loa, P. T., Chen, C. Y., Xu, N., and Shyu, A. B. (1999) *Methods* 17, 11–20
44. Caron, J. M., Jones, A. L., Rall, L. B., and Kirschner, M. W. (1985) *Nature* 317, 648–651
45. Wisdom, R., and Lee, W. (1991) *Genes Dev.* 5, 232–243
46. Wellington, C. L., Greenberg, M. E., and Belasco, J. G. (1993) *Mol. Cell. Biol.* 13, 5034–5042
47. Pistoi, S., Roland, J., Babbin, C., and Morello, D. (1988) *Mol. Cell. Biol.* 16, 3117–3126
48. Bernstein, P. L., Herrick, D. J., Prokipcak, R. D., and Ross, J. (1992) *Genes Dev.* 6, 642–654
49. Sokolowski, M., Tan, W., Jellne, M., and Schwartz, S. (1998) *J. Virol.* 72, 1504–1515
A Coding Region Determinant of Instability Regulates Levels of Manganese Superoxide Dismutase mRNA
Christopher A. Davis, Joan M. Monnier and Harry S. Nick

J. Biol. Chem. 2001, 276:37317-37326.
doi: 10.1074/jbc.M104378200 originally published online August 6, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104378200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 49 references, 26 of which can be accessed free at
http://www.jbc.org/content/276/40/37317.full.html#ref-list-1