Mitochondrial manganese superoxide dismutase (Mn-SOD) is the primary cellular defense against damaging superoxide radicals generated by aerobic metabolism and as a consequence of inflammatory disease. Elevated expression of Mn-SOD therefore provides a potent cytoprotective advantage during acute inflammation. Mn-SOD contains a GC-rich and TATA/CAAT-less promoter characteristic of a housekeeping gene. In contrast, however, Mn-SOD expression is dramatically regulated in a variety of cells by numerous proinflammatory mediators, including lipopolysaccharide, tumor necrosis factor-α, and interleukin-1. To understand the underlying regulatory mechanisms controlling Mn-SOD expression, we utilized DNase I-hypersensitive (HS) site analysis, which revealed seven hypersensitive sites throughout the gene. Following high resolution DNase I HS site analysis, the promoter was found to contain five HS subsites, including a subsite that only appears following stimulus treatment. Dimethyl sulfate in vivo footprinting identified 10 putative constitutive protein-DNA binding sites in the proximal Mn-SOD promoter as well as two stimulus-specific enhanced guanine residues possibly due to alterations in chromatin structure. In vitro footprinting data implied that five of the binding sites may be occupied by a combination of Sp1 and gut-enriched Krüppel-like factor. These studies have revealed the complex promoter architecture of a highly regulated cytoprotective gene.

Reactive oxygen species produced during both normal cellular function and, most importantly, as a consequence of the inflammatory response, have been implicated in the initiation and propagation of a variety of pathological states (1, 2). The superoxide dismutases (SODs) are the primary cellular defense that has evolved to protect cells from the deleterious effects of oxygen free radicals (3, 4). Three forms of SOD have been identified in eukaryotic cells: the cytoplasmic copper/zinc SOD, which is expressed constitutively in most cases, Mn-SOD expression is highly regulated by a variety of proinflammatory mediators (5–9).

Recent data have decisively demonstrated the critical cellular importance of Mn-SOD in a variety of different tissues. For example, homozygous mutant Mn-SOD mice die within 10 days of birth, exhibiting severe dilated cardiomyopathy, lipid accumulation in liver and skeletal muscle, and metabolic acidosis, as well as decreased enzyme activity of aconitase, succinate dehydrogenase, and cytochrome c oxidase, which are all extremely sensitive to alterations in the cellular redox state (10). Additionally, transgenic mice expressing elevated levels of human Mn-SOD in lung tissue were highly protected from hyperoxic injury after exposure to 95% oxygen and thus survived longer than nontransgenic littermates (11). Overexpression of Mn-SOD has also been implicated in the suppression of tumorigenicity of human melanoma cells (12), breast cancer cells (13), and glioma cells (14). Alterations in Mn-SOD levels have also been associated with a number of neurodegenerative diseases, including Parkinson’s disease (15), Duchenne muscular dystrophy, Charcot-Marie-Tooth disease, and Kennedy-Atler-Sung syndrome (16).

Mn-SOD synthesis in eukaryotic cells is up-regulated dramatically by proinflammatory mediators including lipopolysaccharide (LPS), tumor necrosis factor alpha (TNF-α), interleukins-1 and -6 (IL-1 and IL-6), and interferon-γ (6, 7, 9, 17–21). This up-regulation is blocked completely by actinomycin D, suggesting that the increase in Mn-SOD mRNA in response to LPS, TNF-α, or IL-1 may result from an increase in the rate of transcription of Mn-SOD (7–9, 18), results confirmed by nuclear run-on studies (data not shown).

Although highly regulated, Mn-SOD contains a GC-rich promoter lacking a TATA- and a CAAT-box. This promoter architecture was originally associated with housekeeping genes that are constitutively expressed (22). The additional layer of transcriptional regulation of this gene differentiates it from most housekeeping genes. Unfortunately, current knowledge about the molecular mechanisms controlling transcriptional regulation from promoters that lack a TATA- and CAAT-box is limited. Most of the studies addressing regulation of TATA- or CAAT-less promoters have focused on either the initiator element (Inr), which controls transcriptional initiation (23), or the general transcription machinery, especially TFIID (24–26) and, most recently, TFII-I (27). In addition, most studies have analyzed transcription from TATA- or CAAT-less promoters by employing naked DNA templates in vitro, a model system that may not adequately reflect the physiological situation.

In order to explore the mechanism of the transcriptional regulation of this unique TATA- and CAAT-less gene promoter, we first investigated alterations in the chromatin structure of rat Mn-SOD by mapping regions hypersensitive (HS) to cleav-

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† The abbreviations used are: SOD, superoxide dismutase; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin; LMPCR, ligation-mediated polymerase chain reaction; DMS, dimethyl sulfate; GKLF, gut-enriched Krüppel-like factor; kb, kilobase(s); bp, base pairs; Inr, initiator element; hGH, human growth hormone.

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age by DNase I in both control and stimulated cells. To further delineate the binding of specific transcription factor(s) in the proximal promoter of Mn-SOD, we used in vivo footprinting coupled with ligation-mediated polymerase chain reaction (LMPCR) to screen the region surrounding the prominent promoter site and the transcriptional initiation site in both control and induced cells. Our findings have provided evidence for the molecular architecture of the Mn-SOD promoter.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The L2 rat pulmonary epithelial-like cell line (ATCC CCL 149) was grown as a monolayer in 150-mm cell culture dishes containing Ham’s modified F12K medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 10 μg/ml penicillin G, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37 °C in humidified air with 5% CO2. At approximately 90% confluence, cells were treated with 0.5 μg/ml Echerichia coli LPS (E. coli serotype 055:B5, Sigma), 10 ng/ml TNF-α (kindly provided by the Genentech Corp.), or 2 ng/ml IL-β (kindly provided by NCI, National Institutes of Health) for 0.5–8 h to induce Mn-SOD expression. Untreated cells were used as controls.

**DNase I Hypersensitive Site Studies**—We employed isolated nuclei for low resolution DNase I HS site studies and permeabilized cells to analyze HS site 1 at high resolution. To analyze DNase I HS sites at low resolution, nuclei were isolated essentially as described (28). For high resolution DNase I HS site studies, L2 cells were permeabilized in 4 ml of cold nuclear isolation buffer (60 mM KC1, 15 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 5 mM MgCl2, 0.25 mM sucrose, 1 mM dithiothreitol, and 60 mM Tris-HCl, pH 8.0) containing 0.05% lyso phosphatidylcholine (Sigma) for 1 min on ice (29). Aliquots of approximately 3 × 106 permeabilized cells/ml of nuclear isolation buffer were digested with increasing concentrations of DNase I (Worthington) at 37 °C for 4 min. Digestions were terminated by dilution to a final concentration of 1% SDS, 100 mM KCl, 1 mM EDTA, 0.1 mM EGTA, 5 mM MgCl2, 0.25 M sucrose, 1 mM dithiothreitol, and 60 mM Tris-HCl, pH 8.0, and 50 μg/ml proteinase K. Genomic DNA was purified by incubation in 1% organic extraction and one additional extraction with organic solvent, followed by precipitation with ethanol. Samples were then treated with 100 μg/ml RNase A, organically extracted, precipitated, and suspended in TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). DNase I-digested DNA was restricted with an appropriate enzyme and size-fractionated on either of two gel systems to achieve the desired resolution: a low resolution 1% agarose gel (FMC Bioproducts) in TAE buffer, pH 7.8 (40 mM Tris, 3 mM NaCl, 1 mM EDTA) to resolve DNA fragments ranging from 0.5 to 15 kilobase pairs or a high resolution procedure that utilizes a 2.5% Metaphor agarose gel (FMC Bioproducts) in TAE buffer to resolve DNA fragments ranging from 0.1 to 1 kilobase pair. The DNA was alkaline-denatured, electrotransferred to a nylon membrane, and cross-linked to the membrane with UV light (30). The membrane was prehydrated in a buffer containing 0.5 M sodium phosphate, pH 7.2, 7% SDS, 1% bovine serum albumin, and 1 mM EDTA at 65 °C for 15 min. The DNA fragments used for probe templates were isolated by restriction digestion of various subclones of the rat Mn-SOD genomic clone (31) and radiolabeled using a random primer DNA labeling system (Life Technologies, Inc.). Hybridization and autoradiography were performed as described (8).

**In Vivo Dimethyl Sulfate (DMS) Treatment**—L2 cells at 90% confluence were washed with room temperature phosphate-buffered saline buffer and then replaced with phosphate-buffered saline containing 0.5–0.25% DMS for 1–2 min at room temperature. The cell monolayer was washed with 4 °C phosphate-buffered saline to quench the DMS reaction. The cells were lysed in 67 mM EDTA, pH 8.0, 1% SDS, and 0.6 mg/ml proteinase K and incubated overnight at room temperature. Genomic DNA was purified as described for DNase I HS site studies. The DNA samples were digested with BamHI, and strand cleavage at modified guanine residues was achieved by treatment with 1 μM pipericidine at 90 °C for 30 min. Naked genomic DNA was harvested and purified from cells without any DMS treatment and digested with BamHI. The in vitro guanine-specific reaction was performed as described (30).

**LMPCR**—The LMPCR was performed as described previously (32). The following primers were used for LMPCR. For the top strand primer sets, the following were used: A, primer 1, 5'-TGTAGCGCCGGTCTCCTCCCTCTCGTTGAC-3' and primer 2, 5'-CTGTTGCGACCTACGAGCTT-3'; B, primer 1, 5'-ATGCTTCCGAGGCCCAGC-3' and primer 2, 5'-GCGCCATGCGAGCTGAC-3'; C, primer 1, 5'-GGTTAATTGCGAGGCTGAC-3' and primer 2, 5'-GGTGGGTTAGCGGAGCTGAC-3'; D, primer 1, 5'-GTGGTTACCTTCGAGGCCCAGC-3' and primer 2, 5'-CCCTAACCTCAGGGCAGCAG-3'; E, primer 1, 5'-GTGGTTTATCACTATTATGTGTTG-3' and primer 2, 5'-GGTTTATCGAGGCCCAGCAGCAG-3'. For the bottom strand primer sets, the following were used: A, primer 1, 5'-CATAGTCGTAAGGACCGTCA-3' and primer 2, 5'-GTCAGGGGAGGTGTGGTCGCG-3'; B, primer 1, 5'-CTGTTCTCTGCTAACACAG-3' and primer 2, 5'-GTCAGGGGAGGTGTGGTCGCG-3'; C, primer 1, 5'-CCCTAACCTCAGGGCAGCAG-3' and primer 2, 5'-GTCAGGGGAGGTGTGGTCGCG-3'; D, primer 1, 5'-GTGGTTTATCACTATTATGTGTTG-3' and primer 2, 5'-GGTTTATCGAGGCCCAGCAGCAG-3'; E, primer 1, 5'-GTGGTTTATCACTATTATGTGTTG-3' and primer 2, 5'-GGTTTATCGAGGCCCAGCAGCAG-3'. For primer extension, the following primers were used: A, primer 1, 5'-CATAGTCGTAAGGACCGTCA-3' and primer 2, 5'-GTCAGGGGAGGTGTGGTCGCG-3'; B, primer 1, 5'-CCCTAACCTCAGGGCAGCAG-3' and primer 2, 5'-GTCAGGGGAGGTGTGGTCGCG-3'; C, primer 1, 5'-CTGTTCTCTGCTAACACAG-3' and primer 2, 5'-GTCAGGGGAGGTGTGGTCGCG-3'; D, primer 1, 5'-GTGGTTTATCACTATTATGTGTTG-3' and primer 2, 5'-GGTTTATCGAGGCCCAGCAGCAG-3'; E, primer 1, 5'-GTGGTTTATCACTATTATGTGTTG-3' and primer 2, 5'-GGTTTATCGAGGCCCAGCAGCAG-3'. The polymerase chain reaction products were size-fractionated on a 6% denaturing polyacrylamide gel, electrotransferred to a nylon membrane, and covalently cross-linked to the membrane by UV irradiation. The membrane was hybridized with an M13 single-stranded probe using our isolated genomic clone as template and promoter-specific primers for extension, washed as described for DNase I hypersensitive site studies, and exposed to x-ray film.

**In Vitro DMS Footprinting**—The rat Mn-SOD promoter from position −467 to +32 was ligated into pCR2.1 (Invitrogen). Human Sp1 protein was purchased from Promega, and the purified gut-enriched Krüppel-like factor (GKLF) fusion protein containing the three-zinc finger DNA binding domain was kindly provided by Dr. Vincent W. Yang (Johns Hopkins University) (33).

Sp1 (26 pmol) and/or GKLF (170 pmol) was incubated with linearized pCR2.1 containing the rat Mn-SOD promoter in 10 μM Tris, pH 7.5, 75 mM KCl, 1 mM dithiothreitol, and 5 μg/ml proteinase K, followed by DMS treatment in vitro. DMS-modified protein-DNA complexes and naked template DNA were cleaved, gel-fractionated, and transferred to a nylon membrane as described above. Two oligonucleotides, 5'-ACACGGCCGTTCGCTAGCAGCC-3' and 5'-CTTGACCGAGGCCCCGAGTGGCTT-3', were used as hybridization probes as described (34) for visualization of top and bottom strand sequences, respectively.

**Transient Transfection Analysis**—Transfection efficiency was controlled by using a batch transfection method. Cell monolayers were grown to 70–90% confluence on 150-mm plates. Cells were transfected with 10 μg of each expression vector using the DEAE-dextran method (34). After 24 h, cells were trypsinized, pooled, and plated onto four separate 100-mm tissue culture plates. Inflammatory mediators were added to the medium of each plate 24 h later as described above.

Twenty-four hours later, total RNA was isolated from the cell monolayers for Northern analysis as described (8). Human growth hormone was assayed from the media of individual 100-mm plates incubated for 48 h after the addition of LPS, TNF-α, or IL-1β. Each vector was tested in more than four independent experiments, bringing the number of independently transfected plates to approximately 16 for each vector. The concentration of secreted hGH was measured using an 125I-labeled monoclonal antibody assay kit purchased from Nichols Institute Diagnostics with a lower limit sensitivity of 0.06 ng/ml. Each experimental sample was assayed in duplicate.

**Construction of Expression Vectors**—A 4.5-kb EcoRI/BglI fragment of the noncoding sequence was isolated from the 17-kb Mn-SOD genomic clone (31), and the 5' overhang ends were filled in using the large fragment of E. coli DNA polymerase I (Klenow fragment). The resulting blunt end Eco/Rag fragment was cloned into the Hincll polylinker site in a promoterless, pUC 12-based human growth hormone expression vector, pCGH, (Nichols Institute Diagnostics). Unique restriction enzyme sites were utilized to delete increasing portions of the Mn-SOD EcoRI/Es site sequence, creating the vectors illustrated in Fig. 8A.

**RESULTS**

**Chromatin Structure of Mn-SOD**—The induction of Mn-SOD mRNA by LPS, TNF-α, or IL-1β may be a consequence of de novo transcription rate, the prolongation of Mn-SOD mRNA half-life, or both. Co-treatment of induced cells with actinomycin D indicates that de novo transcription is necessary for stimulus-dependent gene expression. To address the contribution of these proinflammatory mediators to the induced steady-state message levels, nascent transcription was evaluated in both isolated nuclei and lysophosphatidylcholine-permeabi-
lized cells. These nuclear run-on studies showed that LPS, TNF-α, or IL1-β treatment increased the rate of transcription of Mn-SOD over control by 3–9-fold (data not shown).

Since de novo transcription is necessary for the induction of Mn-SOD by LPS, TNF-α, or IL-1β, we next evaluated alterations in the chromatin structure of Mn-SOD in response to these mediators. Nuclei were isolated from control and stimulated L2 cells, a rat pulmonary epithelial-like cell line, and then exposed to increasing concentrations of DNase I. Purified genomic DNA was then digested with restriction enzymes near the region of interest and size fractionated on 1% agarose gels. Fig. 1A shows a low resolution Southern analysis of DNase I-digested DNA restricted with KpnI and indirectly end-labeled with the probe designated PK, which abuts the 5’ KpnI site. HS site 1 maps to the promoter region of Mn-SOD, whereas HS sites 2–7 map within the gene itself. These seven HS sites that were found in both control and stimulated cells are shown on the restriction map of Mn-SOD in Fig. 1A. We also evaluated regions approximately 10 kilobase pairs 5’ to HS site 1 and 3’ to HS site 7 and detected no additional hypersensitive sites near Mn-SOD. In addition, DNase I digestion of deproteinated genomic DNA demonstrated that all of these HS sites are specific to in vivo chromatin structure (data not shown).

Since no differences in chromatin structure were detected between control and induced cells, we hypothesized that stimulus-specific alterations in chromatin structure may occur within each HS site. HS site 1 was chosen based on its proximity to the transcriptional initiation site and because this site is highly sensitive to cleavage by DNase I. In order to display the structure of the HS site in the promoter in more detail, we digested genomic DNA with BamHI, which cleaves near HS site 1 followed by Southern analysis with probe BH. This strategy expanded the separation of the DNase I-cleaved fragments and led to the discovery that HS site 1 was composed of three subsites (marked with stars in Fig. 1) in control and stimulated cells. Most interestingly, a fourth subsite became strongly apparent only in the samples derived from LPS-, TNF-α-, or IL-1β-treated cells, as shown in Fig. 1B.

To better resolve potential stimulus-dependent alterations in chromatin structure, we developed an approach to evaluate changes within HS site 1 at higher resolution. The DNase I cleavage pattern was analyzed by fractionation on a 2.5% Metaphor agarose gel. This gel accurately resolves fragments differing in size by as little as 2% in the range of 200–800 bp. In addition, we utilized control and LPS-, TNF-α-, or IL-1β-stimulated cells permeabilized with lysophosphatidylcholine rather than isolated nuclei in order to more efficiently retain intact protein-DNA complexes, as has previously reported (35). L2 cells were treated with LPS, TNF-α-, or IL-1β for 4 h, permeabilized as detailed under “Experimental Procedures,” and incubated with increasing concentrations of DNase I. The DNase I-cleaved genomic DNA was then purified, digested with the restriction endonucleases HincII and RsalI, and size-fractionated on 2.5% Metaphor agarose gels. Hybridization with probe BH revealed five discrete DNase I HS regions, indicated as HS subsites 1-1 to 1-5 (Fig. 2). The HS subsites 1-1, 1-2, 1-3, 1-4, and 1-4 were found in both control and treated cells, implying

Fig. 1. Low resolution chromatin structure analysis of Mn-SOD. L2 cells were treated with 0.5 μg/ml LPS, 10 ng/ml TNF-α, or 2 ng/ml IL-1β for 4 h, and the nuclei were prepared as described under “Experimental Procedures” and incubated with increasing concentrations of DNase I (0, 0 units/ml; I, 100 units/ml; 2, 150 units/ml; 3, 200 units/ml; 4, 300 units/ml) at 37 °C for 4 min, and genomic DNA was isolated from each treatment. A, 10 μg of DNase I-digested genomic DNA from each sample was restricted with KpnI and subjected to 1% agarose electrophoresis. The probe PK (PstI–KpnI fragment) was used to indirectly end-label the genomic restriction fragments as well as the set of nested DNase I-cleaved fragments delineating each HS site. Probe PK is a 0.43-kb PstI–KpnI DNA fragment that abuts the KpnI restriction site at the 3’-end of rat Mn-SOD. The seven HS sites are numbered on the right. The restriction map of rat Mn-SOD shown above illustrates the relative positions of the probe used and the HS sites revealed. The location of the DNase I HS sites are shown with stars and numbered 1–7. HS site 1 is located within the promoter, and HS sites 2–7 are within Mn-SOD. The five exons of Mn-SOD are shown as black rectangles. The codes for the restriction endonucleases are as follows: B, BamHI; H2, HincII; H3, HindIII; K, KpnI; N, Ncol; P, PstI; R, RsalI. B, cells were treated as described for A. DNase I-digested genomic DNA was restricted with BamHI and subjected to electrophoresis in 1% agarose gel. Probe BH (BamHI–HincII fragment) was used to display an expanded view of HS site 1 with three subsites appearing in control and stimulated cells and an inducible subsite (lowest band) evident in LPS-, TNF-α-, or IL-1β-treated cells.
that these four HS subites are involved in basal expression of Mn-SOD. Most importantly, an inducible HS site, subsite 1-5, was found only in cells treated with LPS (Fig. 2). The same subsite was also observed following treatment with TNF-α and IL-1β (data not shown). This suggests that the promoter region around HS subsite 1-5 may be involved in specific alterations in chromatin structure that accompany gene activation.

**In Vivo Footprinting of the Mn-SOD Promoter**—Low resolution (Fig. 1) and high resolution DNase I HS site studies (Fig. 2) suggest that important cis-acting regulatory elements may exist in the promoter region of Mn-SOD in both control and stimulated cells. We therefore employed in vivo footprinting using DMS as a molecular probe coupled with LMPCR to resolve these cis-acting elements at single nucleotide resolution and thus display the in vivo protein-DNA contacts. The position of each LMPCR primer set is shown in Fig. 3A. In order to verify that the kinetics of transcription factor binding are stable throughout the period of induction with LPS, we tested both control and stimulated samples at 0.5, 4, and 8 h of treatment. These experiments demonstrated that the observed protein-DNA contacts are detectable as early as 0.5 h and as late as 8 h after the addition of LPS with representative examples from each time point illustrated in Figs. 3 and 4.

Fig. 3B illustrates in vivo footprinting and LMPCR results for control and 0.5-h LPS-treated samples for the top strand of the promoter from position –286 to –166 relative to the transcriptional initiation site. Fig. 3C illustrates control and 4-h LPS-treated samples for the bottom strand. As depicted in Fig. 3, numerous guanine residues exhibited altered DMS reactivity, which appeared as either diminished or enhanced hybridization signal relative to the in vitro DMS-treated DNA lanes. We have summarized this in vivo footprinting data by postulating the existence of protein binding sites at obviously clustered residues and through symmetry in the contacts and in the DNA sequence. Fig. 3, B and C, illustrates binding sites for proteins I–VI. Fig. 4, A and B, illustrates the protein-DNA contacts seen in cells stimulated for 8 and 4 h of LPS on the top and bottom strands, respectively. Binding sites II–VII are shown in Fig. 4A, while Fig. 4B illustrates binding sites VI, VIII, IX, and X. Interestingly, in addition to the guanine contact sites, we also observed consistently reproducible enhanced adenine residues marked by arrowheads in Figs. 3 and 4, which are also clustered near specific binding sites.

**Involvement of Sp1 and GKLFl with Binding Sites I–V**—A computer analysis of binding sites I–III shows a high degree of homology with the consensus DNA binding sequence and the protein-DNA contact pattern of Sp1 (36), thus suggesting that these sites may be occupied by Sp1 or Sp1-like proteins. Furthermore, binding sites IV and V contain the minimal essential DNA binding sequence, 5′-G(A/G)A/GG/C(T)-G(C/T)-3′, of a recently cloned transcription factor, GKLFl (33). This is further substantiated by Zhang et al. (37), who have presented data implicating a physical interaction of Sp1 and GKLFl. We therefore evaluated the interaction of these proteins with a plasmid (PCR2.1, Invitrogen) containing the rat Mn-SOD promoter (from –467 to +32) using in vitro DMS footprinting.

As shown in Fig. 5, either GKLFl alone (lane 1) or Sp1 alone (lane 2) is capable of binding to sites I–V. In addition, we evaluated the cooperative effects of a constant amount of Sp1 in the presence of increasing quantities of GKLFl (lanes 3–5). These data implicate the potential cooperative interaction of Sp1 and GKLFl with our 5′-most binding sites originally identified in vivo (Figs. 3 and 4). An analogous computer analysis of the region harboring binding sites VI–X has identified a number of putative candidate transcription factors. However, a comparison of the protein-DNA contact pattern as well as the physiological functions of these candidates has led us to conclude that these binding sites are most likely occupied by a set of novel basal transcription factors.

**Detection of Stimulus-specific Enhanced Guanine Residues**—As described previously, an LPS-, TNF-α-, or IL-1β-inducible HS subsite 1-5 maps upstream of the constitutive HS sites in the Mn-SOD promoter (Fig. 2). We further examined this region by DMS in vivo footprinting and LMPCR. We treated L2 cells with LPS for 30 min, 1 h, 4 h, or 8 h; with TNF-α for 1 h or 4 h; and with IL-1β for 4 h. Fig. 6A illustrates representative data for LPS treatment with the same results observed for TNF-α and IL-1β (data not shown). We were able to detect enhanced guanine residues at positions –404 and –403 on the top and bottom strand, respectively, only following treatment with LPS (Fig. 6A).

As a matter of completeness, recent studies by Das et al. (38) have reported that there is a relationship between the activation of NF-kB and the elevated steady-state levels of Mn-SOD mRNA by TNF-α or IL-1 in lung adenocarcinoma (A549) cells. In contrast, Borrello and Demple (39) have recently presented equally convincing data demonstrating that Mn-SOD gene transcription can occur independently of NF-kB activation. To address the importance of NF-kB in the regulation of Mn-SOD gene expression, we identified a putative NF-kB binding site from –353 to –344 by computer analysis, which displays a perfect identity to the proposed consensus sequence (40). However, as shown in Fig. 6B, we did not detect any alteration in guanine reactivity to DMS in vivo on either strand for this putative NF-kB binding site in LPS-treated cells. The same
results were obtained for both the TNF-α- and IL-1β-treated samples (data not shown).

Detection of an Enhanced Cytosine Residue at +51—The Inr (YYA11N(T/A)YY) (41) and downstream promoter element (DPE) ((A/G)G(A/T)CGTG) (24), typically located at approximately +30, have been shown to be important core elements for the regulation of TATA-less promoter genes. A computer analysis of these downstream sequences has revealed a reverse Inr-like sequence between −240 and −234 as well as a putative DPE sequence between +156 and +162 of the Mn-SOD promoter. As a result of the reported significance of these elements to gene transcription, in vivo footprinting was performed to further examine the region downstream to the transcription initiation site. Interestingly, we observed an enhanced adenine residue at position −238 on the bottom strand (Fig. 4B) within the Inr-like sequence and an enhanced cytosine residue at position +51 (Fig. 7), also on the bottom strand just upstream of the putative DPE site. Furthermore, treatment with LPS (Fig. 7) dramatically increases the intensity of methylation by DMS at this cytosine residue, which obviously coincides with elevated gene transcription.

Promoter Deletion Analysis of the Mn-SOD—To address the functional significance of the cis-acting elements identified by in vivo footprinting, we used unique restriction sites within the Mn-SOD 5'-flanking sequence to create a series of promoter deletions in a human growth hormone (hGH) reporter plasmid, as depicted in Fig. 8A. We evaluated promoter function by using vectors containing various Mn-SOD promoter fragments following transfection into L2 cells, with measurement of both secreted hGH levels (Fig. 8B) and hGH mRNA levels (Fig. 8C).

To ensure that hGH in the medium reflected the majority of total hGH produced in the transfected cells, we compared hGH in the cell monolayer with secreted hGH and found that 97% was secreted into the medium, whereas only 2–3% of total hGH was retained in the cells (data not shown). In order to exclude the possibility that induction of hGH is somehow due to the
vector itself, we demonstrated that LPS, TNF-α, and IL-1β had no effect on a vector containing the minimal thymidine kinase promoter (TKGH) (data not shown) as compared with the results with a 4.5-kb fragment of the Mn-SOD promoter (Eco/E, Fig. 8).

The results of multiple transfection experiments are shown in Fig. 8, B and C. As the Mn-SOD promoter was progressively shortened, the deletions had no effect on either basal or stimulated hGH expression (mRNA or protein) until the Mn-SOD promoter fragment was shortened from the SacII restriction site to the NaeI site, at which point both basal and stimulated expression were lost. Protein levels (Fig. 8B) and Northern analysis (Fig. 8C) of hGH were comparable, suggesting that the cytokine treatments did not significantly affect translation, post-translational modification, or secretion.

**DISCUSSION**

*Mn-SOD* has characteristics similar to most housekeeping genes, such as a GC-rich promoter, which lacks both TATA and CAAT boxes. In contrast to most housekeeping genes, however, *Mn-SOD* is dramatically regulated in a variety of cell types by numerous proinflammatory stimuli. There are a few examples of other housekeeping genes that can be regulated or induced by nutrients or hormones, such as the dihydrofolate reductase, *HMGCoa* reductase (22), pyruvate dehydrogenase β (42), and insulin-like growth factor-I receptor (43). GC-rich promoters lacking both a TATA and CAAT box have also been associated with other inducible and tissue-specific genes, such as the urokinase-type plasminogen activator receptor (44), *Pim-I* (45), *CD7* (46), and *MAL* genes (47).
NF-κB was used for LMPCR. Lanes G, C, and L are in vivo DMS-treated control, and LPS-exposed (30 min) cells, respectively. Each C and L lane represents an individual plate of cells. The enhanced guanine residue is marked by a star. The nucleotide positions relative to the transcriptional initiation site are illustrated on the left. Identical results were obtained following stimulation with TNF-α or IL-1β (data not shown). A, lack of NF-κB binding on the promoter region of Mn-SOD. Primer set K was employed for LMPCR for the top strand (−359 to −350), and primer set D was used for the bottom strand (−355 to −338). Lanes are designated as in Fig. 3. The sequence of the putative NF-κB binding site perfectly matches its consensus DNA binding sequence, GGG(G/A)(C/A/T)T(T/C)(T/C)CC (40). However, we did not find any protein bound to this putative NF-κB binding site in vivo on either DNA strand of the Mn-SOD promoter. The same results were observed for TNF-α- or IL-1β-treated cells (data not shown).

Recently, significant progress has been made on the structure and function of TATA-less promoters (reviewed in Ref. 48). Most of these studies involved identification of Inrs and characterization of general transcription factor(s) by using in vitro systems. For example, TFII D, TFII-I, YY1, or the core RNA polymerase II was found to bind to the Inr, thus aiding in the nucleation of the preinitiation complex (48). This preinitiation complex is thought to interact with upstream activators, such as Sp1, and/or enhancer elements to facilitate transcriptional initiation at TATA-less promoters. Furthermore, Burke and Kadonaga (24) have identified a DPE, (A/G)(A/T)CGTG, located at +30, which was shown to be important for the regulation of TATA-less promoter genes. Another potential core element possibly associated with TATA-less promoters was localized to both sides of the transcriptional initiation site of the rat catalase gene (49).

To date, however, we have limited information on the general machinery involved in the transcription of genes lacking both a TATA and CAAT box. Specific transcription factors such as Sp1 and the Wilms’ tumor suppressor (WT1) have been associated with the developmental and neoplastic down-regulation, respectively, of the TATA/CAAT-less insulin-like growth factor-I receptor (43). Sp1 has also been associated with the regulation of numerous TATA/CAAT-less genes including urokinase-type plasminogen activator receptor (44), T-cell-specific MAL (47), and human Pim-1 (45). Unfortunately, our knowledge of the molecular architecture of an inducible TATA/CAAT-less promoter is quite limited. We believe, therefore, that our chromatin and in vivo footprinting studies on the transcriptional regulation of Mn-SOD have defined a collection of constitutive transcription factors that may delineate the general architecture of an inducible TATA/CAAT-less promoter.

Our data on chromatin structure and in vivo DMS footprinting are summarized in Fig. 9. HS subsites 1-1 to 1-4 are constitutive HS sites, which are present in both control and stimulated cells. Their relative positions have been mapped to within ±50 bp and flank the location of three large cis-acting protein binding regions (binding sites I–III, IV–VIII, and IX–X in Figs. 3 and 4). Our in vivo footprinting results have been compared with the vast transcription factor literature, including existing consensus DNA binding sequences as well as available DMS in vivoin vitro footprinting or methylation interference data. The above analyses led us to postulate that binding sites I–V may be occupied by either Sp1 or GKL F. This was supported by our in vitro footprinting studies (Fig. 5), which document the ability of both GKL F and Sp1 to interact with these binding sites. The in vitro DMS protection data is also summarized in Fig. 9 and illustrates the high conservation of guanine contacts as compared with the in vivo results. It is also obvious from the data in Fig. 5 that GKL F and Sp1 may in
some manner compete or possibly cooperate in the binding of these five sites. Attempts to identify the proteins that interact with binding sites VI–X have led to the conclusion that these sites are most likely occupied by a novel set of trans-acting factors.

The Inr (YYA\(^1\)N(T/A)YY) (41) and DPE ((A/G)G(A/T)CGTG) (24) located at approximately 130 were shown to be important for the regulation of TATA-less promoter genes. Using computer analysis, we located a reverse Inr-like sequence (240 to 234) and a putative DPE sequence (156 to 162) in the Mn-SOD promoter. Even with the reported significance of these elements in other genes, we did not observe any protected or enhanced guanine residues on either strand as far as 118 bp. However, we did observe both an enhanced adenine residue at position −38 within the Inr-like sequence (Fig. 4B) and an enhanced cytosine residue at position +51 upstream to the putative DPE sequence (Fig. 7), as summarized in Fig. 9. DMS-dependent methylation of cytosine residues has been associated with single-stranded DNA (50) and may correlate with an altered DNA structure near the start of transcription. Furthermore, the intensity of this enhanced cytosine residue was significantly stronger in the LPS-treated cells compared with control cells, where the level of transcription is dramatically elevated. We therefore believe that these results may define protein or structural alterations specific to Inr- and DPE-like elements, with further confirmation requiring alternative molecular probes or mutagenesis studies.

In addition to the 10 basal binding sites identified by in vivo footprinting, we also detected two stimulus-specific enhancements as shown in Fig. 6A. These sites also coincide with the position of the inducible hypersensitive site (HSS1-5, Fig. 2) as summarized in Fig. 9. Although computer analysis of this re-
region revealed an identity with the NF-IL-6 consensus DNA binding sequence, 5′-(A/C)TTNCNN(A/C)A (51), reported methylation interference data (51) for NF-IL-6 is not completely consistent with our guanine enhancements seen in vivo.

In order to assess the importance of these basal binding sites as well as the enhanced guanine residues, we have further investigated the chromatin structure and in vivo/in vitro DMS footprinting studies. The Mn-SOD promoter sequence is depicted from position -419 to +62 relative to the transcriptional initiation site (+1). HSS1-1 to HSS1-5 represent HS subsites 1-1 to 1-5 within HS site 1 defined by the high resolution DNase I HS site studies. The position of each HS site was defined by the fragment’s migration relative to molecular markers within an accuracy of ±50 base pairs. The results of in vivo and in vitro DMS footprinting data are marked by circles and triangles, respectively. The protected guanine residues are marked as open circles or triangles; the enhanced guanine residues are marked as filled circles or triangles. The arrows represent enhanced adenine residues, with the star designating the enhanced cytosine residue at position +51. Each bar represents an individual binding site with Roman numeral designation. The Inr-like and DPE-like sequences are boxed. The positions of the restriction sites used for promoter deletion analysis are also depicted. The rat Mn-SOD promoter sequence differing from the published sequence (52) is underlined. B, model of the in vivo architecture of the Mn-SOD promoter. The top portion illustrates the presence of 10 basal binding sites and a nucleosome(s) associated with the boundary 5′ to binding site I. The bottom portion depicts our interpretation of the induced state of the Mn-SOD promoter. The alteration of chromatin structure associated with the boundary 5′ to binding site I is due to a displacement of a nucleosome(s) upon stimulus treatment. In addition, we postulate that, based on our DNase I analysis, a more open and accessible chromatin structure is evident following stimulation. The spacing between each binding site is approximately scaled. The thin arrow represents the basal expression, and the thick arrow represents the induced expression of Mn-SOD.
evaluated their functional importance using promoter deletion analysis coupled with transient transfection in L2 cells (Fig. 8). A detailed analysis of the promoter including specific deletion of the stimulus-dependent enhanced residues as well as removal of the putative NF-κB sequence has revealed that these regions are not functionally required for TNF-α-, IL-1β-, or LPS-dependent induction of Mn-SOD. Moreover, we have also identified a cytokine- and LPS-specific intronic enhancer element that maps to HS site 2, which also functions independently of both the enhanced guanine residues and the putative NF-κB sequence in the promoter region, based on functional experiments (data not shown).

In Fig. 9B, we propose a model based on our chromatin structure and in vivo DMS footprinting studies. A strong 5′ boundary for HS site 1 is observed in control cells (Fig. 1), whereas following stimulation, the boundary is replaced with an additional HS site (subsite 1-5, Fig. 2) as well as two stimulus-specific enhancement of DMS reactivity at these guanine residues, relates to their possible involvement in the observed alterations in chromatin structure (Fig. 2). Specifically, we are proposing that these enhancements result from or are a consequence of the displacement of a nucleosome in stimulus-treated cells based on the appearance of the HS subsite 1-5 at the 5′ boundary of HS site 1, as shown in Fig. 9B. It is possible, therefore, that the enhanced guanine residues detected in vivo reflect a chromatin structure that allows for proper access of the promoter by the transcription factors involved in enhancer activity. This could result from either the binding of a transcription factor or through changes in DNA structure that result in an enhancement of DMS reactivity, a situation that is important in vivo but may not be necessary in a transient transfection with plasmids due to lack of chromatin structure.

As the primary defense against free radical-mediated damage, Mn-SOD serves an important cytoprotective role during both normal cell function and inflammation associated with a wide variety of pathological disease states. Our chromatin structure, in vivo and in vitro footprinting studies, and our data surrounding the Inr and DPE-like elements have revealed the complex architecture of a highly regulated, GC-rich, and TATA/CAAT-less promoter in which 10 basal binding sites were identified. An understanding of the regulation of Mn-SOD at the molecular level is not only of great clinical significance but can also serve as a model system for the elucidation of the regulatory mechanisms important for regulated TATA/CAAT-less promoters.

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