Manganese superoxide dismutase (Mn-SOD), a tumor necrosis factor (TNF)-inducible gene product, plays an important role in removing superoxide anions produced inside mitochondria. Two regulatory regions, the proximal promoter region (PPR), which is upstream from the transcription initiation site, and the TNF-responsive element (TNFRE), which is inside intron 2, are responsible for Mn-SOD expression. To understand how each of these regions contributes to the transcription of Mn-SOD, quantitative reverse transcription-PCR, chromatin immunoprecipitations, and in vivo nucleosome sensitivity assays were performed on the murine Mn-SOD gene. These assays demonstrate that Sp1 and nuclear factor (NF)-κB p65 are required for Mn-SOD induction by TNF. Sp1 bound the PPR constitutively, whereas NF-κB p65 and C/EBP-β bound the TNFRE only after TNF treatment. Binding of C/EBP-β to the TNFRE was dependent on the presence of NF-κB p65. The chromatin structure within the TNFRE became more accessible to nuclease digestion after TNF treatment. This accessibility required Sp1 and NF-κB p65. Treatment of cells with an inhibitor of histone deacetylation, or transient transfection with coactivator-expressing plasmids, enhanced the expression of Mn-SOD. NF-κB p65 binding was required for acetylation of histones H3 and H4 at the PPR and the TNFRE. Together, these data suggest communication between the PPR and the TNFRE which involves chromatin remodeling and histone acetylation during the induction process of Mn-SOD in response to TNF.

The importance of Mn-SOD function is illustrated by the neonatal death of mice containing targeted disruptions of their Sod2 genes (4, 5). A variety of factors, which include TNF, interleukin-1, lipopolysaccharide, interferon-γ, and x-ray irradiation, induce Mn-SOD transcription (6–8). The important role of Mn-SOD in protection against inflammation and oxidative stress emphasizes the need to regulate Mn-SOD expression precisely.

Sequence analysis, promoter mutagenesis, and in vivo genomic footprinting identified two regulatory regions separated by 2.2 kbp of DNA (Fig. 1) (9, 10). The 5′-proximal promoter region (PPr) contains numerous putative regulatory motifs that can be recognized by Sp1, AP-1, C/EBP, and NF-κB family members. Results of promoter mutagenesis experiments demonstrated that the PPR contributed only a 1.7-fold increase to the total induced expression in response to TNF, indicating that TNF responsiveness was not within this region. However, in vivo genomic footprinting suggested a role for the Sp1 binding sequences in constitutive expression of Mn-SOD. A TNF-responsive element (TNFRE) was identified within the second intron of the Mn-SOD gene. Sequence analysis of this 238-bp element revealed numerous potential transcription factor binding sites. TNF induced the occupancy of the binding sites for NF-κB, C/EBP, and NF-1 within the TNFRE. In vitro DNA binding assays showed that the 5′-region of the TNFRE bound C/EBP-β, and the 3′-region bound C/EBP-β and NF-κB. One report suggested an NF-κB binding site (originally named as C/EBPX because of its homology to a C/EBP binding site) within the 5′-region of the TNFRE which is dispensable for the responsiveness to TNF (11). The 3′-NF-κB site, however, was not independently responsive to TNF and was suggested to provide position and orientation independence to the TNFRE in a heterologous setting (10).

The intronic location of the TNFRE leads to an intriguing question about how the PPR and the TNFRE communicate over 2.2 kbp of DNA. The packaging of eukaryotic DNA into nucleosome and higher order structures of chromatin allows for the possibility that these two regulatory regions are physically close enough for direct interaction or communication through bridging molecules. The role of chromatin structure in Mn-SOD expression was suggested by the observation that the promoter of the rat Mn-SOD gene became more sensitive to DNase I after TNF treatment, indicating that some form of nucleosome remodeling occurred (12). Whether the remodeling involves acetylation of specific lysine residues on the N-terminal tails of core histones or nucleosome repositioning is not known.

To determine the role of chromatin in controlling the accessibility of transcription factors to the Mn-SOD gene, we examined changes in nucleosome-hypersensitive sites, the requirement for coactivators that have the potential to modify chromatin, and examined changes in histone acetylation at both the PPR and the TNFRE of the Mn-SOD gene in response to TNF. Using
transcription factor-deficient cell lines, Sp1 and NF-κB p65 were found to be required for the induction of Mn-SOD by TNF. The TNFRE but not the PPR became hypersensitive to nucle¬ase after TNF treatment, and both Sp1 and NF-κB p65 were necessary for these changes. Chromatin immunoprecipitation assays showed that Sp1 bound PPR constitutively, whereas C/EBP-β and NF-κB p65 bound the TNFRE only after TNF induction, and the binding of C/EBP-β to DNA required NF-κB p65. In a NF-κB p65-dependent manner, TNF induced rapid changes in histone acetylation at both the PPR and the TNFRE. Overexpression of the transcriptional coactivators CBP (CAMP-responsive element-binding protein-binding pro¬tein) and p300 could enhance TNF-mediated expression, and E1a, an adenovirus inhibitor of CBP/p300 activity, blocked the induction. Taken together, these results suggest that induction of Mn-SOD by TNF requires communication between the PPR and the TNFRE, a process involving chromatin remodeling and histone acetylation that ultimately leads to the assembly of the transcription factor complex at the PPR and the TNFRE, and in turn gene activation.

MATERIALS AND METHODS

Cells and Reagents—NIH3T3 murine fibroblasts were obtained from the American Type Culture Collection. The embryonic fibroblast cell line containing targeted disruptions of the NF-κB subunit p65 was provided by Dr. D. Baltimore (California Institute of Technology, Pas¬adena, CA). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum (HyClone, Inc., Logan, UT), 1 mm l-glutamine, and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin). Human recombinant TNF-α (Genzyme, Inc., Cambridge, MA) and trichostatin A (TSA) were used at 500 units/ml and 150 ng/ml, respectively. To generate an embryonic fibroblast line that might have characteristics more similarly related to the p65−/− line, murine embryonic fibroblasts (MEF) were isolated from C57BL/6 mice and cultured. The Sp1 knockout cell line was derived from embryonic stem cells containing a homzygous deletion in Sp1 gene as described previously (13). The activation of NF-κB p65 by TNF is normal in this cell line as determined by analysis of the IP-10 gene (13). This analysis included mRNA production and in vivo genomic footprinting. No wild type line prepared in a mouse identical to the Sp1−/− is available. Sp1−/− cells were grown in α-minimum Eagle’s medium supplemented with 5% fetal bovine serum (HyClone, Logan, UT), 1 mm l-glutamine, antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin), 1 ng/ml basic fibroblast growth factor, and 4 μg/ml insulin.

Plasmids and Transient Transfection—Plasmids expressing E1a, CBP, and p300 were provided by Drs. L. Gooding (Emory University), R. Goodman (The Vollum Institute, Oregon Health Science Center, Portland), and Y. Nakatani (Dana Farber Cancer Research Center, Boston), respectively. Transient transfection of NIH3T3 cells was performed using FuGENE 6 (Roche Molecular Biochemicals) following protocols provided by the manufacturer. Cells were treated with TNF at 24 h post-transfection and assayed for Mn-SOD transcripts by real time RT-PCR as indicated.

Real Time RT-PCR—Total RNA was isolated from cells using the RNAeasy kit (Qiagen, Valencia, CA). For each sample, 2 μg of RNA was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer’s directions. Parallel reactions without reverse transcriptase were included as negative controls. 1/50th of each reverse transcription reaction was analyzed in a PCR containing 5% dimethyl sulfoxide, 1× SYBR (BioWhittaker Molecular Applications, Rockland, ME), 0.04% gelatin, 0.3% Tween 20 (pH 8.3), 3 mm MgCl2, 0.2 mM dNTP, Taq polymerase, and 50 μM of each primer. Real time PCR was conducted using a Bio-Rad i-cycler with denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 68 °C for 30 s for a total of 40 cycles. Two sets of PCR assays were performed for each sample using the following primers specific for cDNA of Mn-SOD and β-actin: 5′-TGTGTCTGGGAGTCACAGGTCAG and 3′-GODRTE5, 5′-GTCAATC-
NF-κB p65 and Sp1 are necessary for TNF-induced chromatin accessibility at the TNFRE. NIH3T3, MEF, Sp1⁻/⁻, and NF-κB p65⁻/⁻ cells were treated with TNF for the indicated times. After permeabilization, cells were digested with two concentrations of MNase. Genomic DNA was collected and subjected to ligation-mediated PCR using primer sets specific for the PPR and the TNFRE of the Mn-SOD gene shown in Table I. A and B, coding strand of the PPR; C and D, noncoding strand of the PPR; E and F, coding strand of the TNFRE; and G, noncoding strand of the TNFRE. In A–D, open boxes indicate hyposensitive regions, and solid boxes indicate hypersensitive regions. In E–G, gray boxes indicate hypersensitive regions that become hyposensitive after TNF treatment, and hatched boxes indicate hyposensitive regions that become hypersensitive after TNF treatment.

All assays were performed at least three times from independent RNA preparations.

Adenovirus Infection—The adenovirus dl520 producing 12S E1A was obtained from Dr. L. Gooding. NIH3T3 cells were infected with dl520
following the procedures described previously (14). At 24 h postinfection, cells were treated with TNF for 4 h. RNA was then prepared and assayed for Mn-SOD transcript by real time RT-PCR.

Micrococcal Nuclease (MNase) Sensitivity Assay—NIH3T3 cells were split and grown to about 80% confluence. The cells were washed once with cold washing solution containing 150 mM sucrose, 80 mM KCl, 35 mM HEPES (pH 7.4), 5 mM K$_2$HPO$_4$, 5 mM MgCl$_2$, and 0.5 mM CaCl$_2$. The cells were permeabilized for 1 min at room temperature with ice-cold 0.5 mg/ml lysolecithin (Sigma) dissolved in the above washing solution. After permeabilization, the cells were washed once with MNase buffer containing 150 mM sucrose, 50 mM NaCl, 50 mM Tris (pH 7.5), and 2 mM CaCl$_2$, followed by incubation with a different amount of MNase (Roche Molecular Biochemicals) for 5 min at room temperature. The digestion reaction was stopped by the addition of stop solution containing 50 mM Tris (pH 8.0), 300 mM NaCl, 25 mM EDTA, 0.2% SDS, and 0.2 mg/ml proteinase K. After proteinase K digestion, genomic DNA was purified, and the 5'-ends of DNA were phosphorylated with T4 polynucleotide kinase. Ligation-mediated PCR was then conducted as described previously (10) using primer sets specific for the regulatory region of Mn-SOD, which are listed in Table I.

Chromatin Immunoprecipitation (ChiP)—The ChiP assay was performed as described previously (15). Briefly, formaldehyde-cross-linked nuclei lysate prepared from 4 × 10$^6$ cells was sonicated and then mixed with 5 μg of antibody prebound to protein A-Sepharose beads and immunoprecipitated nucleosome-free regions as well as in linker regions. The digestion reaction was stopped by the addition of stop solution containing 50 mM Tris (pH 8.0), 300 mM NaCl, 25 mM EDTA, 0.2% SDS, and 0.2 mg/ml proteinase K. After proteinase K digestion, genomic DNA was purified, and the 5'-ends of DNA were phosphorylated with T4 polynucleotide kinase. Ligation-mediated PCR was then conducted as described previously (10) using primer sets specific for the regulatory region of Mn-SOD, which are listed in Table I.

RESULTS

Sp1 and NF-κB p65 Are Required for the TNF Induction of Mn-SOD—Induction of Mn-SOD expression by TNF is regulated mainly at the transcriptional level in murine fibroblasts (10, 16). In vitro DNA binding assays and in vivo genomic footprinting analysis of the regulatory region of the Mn-SOD gene suggest roles for Sp1 in binding to the PPR and for NF-κB binding to the TNFRE in response to TNF (10, 11, 17). To characterize further the roles of Sp1 and NF-κB in TNF induction of Mn-SOD, the level of Mn-SOD transcript in control and TNF-treated NIH3T3, Sp1$^{-/-}$, and p65$^{-/-}$ murine embryonic fibroblasts was analyzed by real time RT-PCR (Fig. 2A). Basal Mn-SOD expression was similar among the tested cell lines. The addition of TNF rapidly induced the expression of Mn-SOD in the NIH3T3 but not in the Sp1$^{-/-}$ and p65$^{-/-}$ cells, indicating that both Sp1 and p65 are required for TNF induction of Mn-SOD in vivo. The transcription of β-actin gene, which is not regulated by TNF, was not induced (data not shown).

A freshly isolated murine embryonic cell line was also analyzed for its ability to induce Mn-SOD in response to TNF. Real time RT-PCR found that Mn-SOD mRNA was induced in this line, but with slower kinetics, peaking at 24 h (Fig. 2B). Even at 24 h of TNF treatment, no Mn-SOD mRNA was induced in either the Sp1- or p65-deficient lines (data not shown).

TNF Induces Chromatin Accessibility at the TNFRE—Chromatin structure and its remodeling in response to extracellular signals have been shown to play a crucial role in transcriptional regulation of interleukin-12, interleukin-4, interferon-γ, human immunodeficiency virus-1, the Saccharomyces cerevisiae Pho5 genes, and the murine mammary tumor virus (18–24). To determine whether changes in chromatin structure contribute to the regulation of Mn-SOD expression, MNase sensitivity assays were performed. MNase preferentially cuts DNA in nucleosome-free regions as well as in linker regions between nucleosomes. Digestion with two different concentrations of MNase was performed on TNF-treated and control NIH3T3 cells after permeabilization with lysolecithin. Permeabilization procedures prevent potential artifacts formed during nuclei preparation (22, 25). The MNase-digested genomic DNA was purified and analyzed by ligation-mediated PCR using primers that amplified the PPR and the TNFRE of the Mn-SOD gene. Strong hypersensitive banding patterns were formed on the PPR coding strand (Fig. 3, A–D) (−32 to +41) and noncoding strand (−48 to −1), indicating that the region was accessible to the nuclease and is in an “open” configuration. An MNase-resistant region (∼259 to −206 and −168 to −32 on the coding strand, and −257 to −161 and −87 to −48 on the noncoding strand) was found to overlap the proposed Sp1-binding sites of the PPR. Detection of this hypersensitive region was not a consequence of sequence preference for MNase cleavage, as naked DNA was cleaved by MNase at this region. Combining the data into a summary schematic (Fig. 4) suggests that the MNase resistant region spans −227 bp just upstream from the transcription initiation site.

MNase digestion at the TNFRE (Fig. 3, E–G) revealed three hypersensitive regions on the coding strand (+2135 to +2145, +2240 to +2342, and +2393 to +2473), and two on the noncoding strand (+2127 to +2141 and +2218 to +2362). These regions overlap the binding sites of C/EBP-2, NF-κB, and NF-1. In addition, a strong hypersensitive region overlapping C/EBP-1 and C/EBPX was detected (+2145 to +2220 and +2162 to +2229 on the coding strand, and +2185 to +2197 on the noncoding strand). Within 30 min of TNF treatment, a slight increase in MNase cleavage was observed within the potential nucleosomal region on the coding strand of the PPR, whereas the hypersensitive bands (−48 to +41) on the coding and noncoding strands maintained their intensity (Fig. 3, A and C). In contrast, pronounced changes were found inside the TNFRE after 30 min of TNF induction. On both strands of the TNFRE, TNF-induced hypersensitive bands were observed.
asterisks become hypersensitive after TNF treatment. The in MNase cleavage within the PPR coding strand (Fig. 3) is shown. For the PPR, open boxes and solid boxes indicate MNase-hyposensitive and -hypersensitive regions, respectively. For the TNFRE, gray boxes indicate the hypersensitive regions that become hyposensitive after TNF treatment, and hatched boxes indicate hyposensitive regions that become hypersensitive after TNF treatment. The asterisks (*) above the hatched boxes indicate TNF-induced changes that also occur in the Sp1−/− cells.

within the three hyposensitive regions, whereas the hypersensitive bands overlapping C/EBP-1 and C/EBPX became less pronounced (Fig. 3, E–G). The alterations in the patterns are represented in the summary diagram (Fig. 4). The hypersensitive banding patterns induced by TNF might indicate repositioning, destabilization, or disruption of nucleosomes at those regions. Assembly of a transcription factor complex at C/EBP-1 and C/EBPX could reduce the MNase accessibility to that region, leading to hyposensitive changes. It is important to note that the MEF line produced pattern similar to those of the NIH3T3 line, suggesting that both of these lines represent a wild type condition.

NF-κB p65 and Sp1 Are Necessary for TNF-induced Chromatin Remodeling—The RT-PCR experiments in Fig. 2 demonstrate that Sp1 and NF-κB p65 were required for the induction of Mn-SOD by TNF. To determine whether the TNF-induced changes in chromatin structure observed above are also dependent upon Sp1 or NF-κB p65, MNase hypersensitivity assays were performed on Sp1−/− and NF-κB p65-deficient fibroblast cell lines. In the absence of TNF treatment, the banding patterns at the PPR and the TNFRE in both mutant cells were indistinguishable from those detected in wild type cells. After TNF treatment, the p65−/− cell line failed to display any TNF-induced changes in the MNase sensitivity pattern (Fig. 3), whereas the Sp1−/− cell line showed only slight increases in MNase cleavage within the PPR coding strand (Fig. 3A) and one hyposensitive region of the TNFRE (+2240 to +2342 and +2233 to +2282 on the coding strand, and +2218 to +2362 on the noncoding strand; Fig. 3, E–G). In addition, the TNF-induced hypersensitive region (+2135 to +2145) was still detected upstream from the C/EBP-1 and C/EBPX in Sp1−/− cells (Fig. 3F). These results suggest that both Sp1 and p65 are required for the observed chromatin structure changes in NIH3T3 and MEF wild type cells. These changes are functionally related to Mn-SOD induction by TNF and potentially are accomplished through interaction between the PPR and the TNFRE.

To examine the relative levels of p65, Sp1, and C/EBP in the different cell lines, Western blot analysis was performed (Fig. 5). The results showed that Sp1−/−, NIH3T3, and the MEF cell lines contained equal levels of p65. Additionally, p65−/− and the MEF cell lines contained equal levels of Sp1, whereas NIH3T3 cells contained about one-third of the level. The Sp1-deficient cell line showed the appropriate band for the mutant protein. Equal levels of C/EBP-β expression were observed in NIH3T3, Sp1−/−, and p65−/− cell lines.

Transcription Factor Assembly on Chromatin in Response to TNF—Family members of Sp1, NF-κB, and C/EBP have been shown to bind regulatory regions of Mn-SOD through in vitro analysis (10, 11, 17). In vivo genomic footprinting also confirmed the occupancy of several consensus binding sequences for Sp1 at the PPR and for NF-κB and C/EBP at the TNFRE (10). To determine whether these transcription factors bind to the Mn-SOD gene under in vivo conditions, ChIP assays coupled with quantitative real time PCR analysis of the precipitated DNA were performed. Antibodies against Sp1, NF-κB p65, and C/EBP-β were used. The results showed that p65 and C/EBP-β were used to bind the TNFRE in response to TNF with 13.8-fold and 30-fold increase in the amount of DNA precipitated in TNF-treated cells compared with control cells, respectively (Fig. 6). In addition, the binding of the two factors occurred as early as 30 min post-TNF treatment. A small increase in p65 binding to the PPR was also detected. No significant change in the amount of Sp1 antibody-precipitated PPR DNA was observed after TNF treatment. Compared with the no antibody control, only a background level of Sp1 antibody-precipitated DNA containing the TNFRE was detected, demonstrating that Sp1 does not bind directly to this region. These results are consistent with the model that TNF induces p65 and C/EBP-β to bind the TNFRE, whereas Sp1 constitutively binds the PPR.

C/EBP-β Binds the TNFRE in a p65-dependent Manner—To investigate further factor assembly at the Mn-SOD gene, ChIP assays using antibodies against Sp1, p65, and C/EBP-β were performed on Sp1- and p65-deficient cell lines. The results showed that p65 failed to bind the Mn-SOD regulatory regions in both mutant cell lines, whereas Sp1 only bound the PPR constitutively in p65−/− cells. In contrast, C/EBP-β was still induced by TNF to bind the TNFRE in Sp1−/−, but not p65−/− cells (Fig. 6). However, the magnitude of C/EBP-β binding in Sp1−/− cells was about 4-fold less than that in NIH3T3 cells. The failure of C/EBP-β binding to the TNFRE in p65−/− cells was not the result of the availability of C/EBP-β because sim-
ilar amounts of C/EBP-β were expressed in NIH3T3, Sp1−/−, and p65−/− lines (Fig. 5). These results suggest that 1) the presence of p65 is required for the binding of C/EBP-β to the TNFRE of Mn-SOD, and 2) efficient assembly of transcription factors on the TNFRE requires Sp1 binding to the PPR located 2.2 kbp upstream.

E1a Represses Mn-SOD Induction by TNF—In addition to potential direct interactions between the regions, it is possible that transcriptional coactivators could contribute to the observed dependence of the TNFRE on the binding of Sp1 to the PPR. Moreover, the activation of gene transcription can be facilitated by transcription factor-mediated recruitment of coactivators to promoters (for review, see Ref. 26). The adenovirus protein E1a is known to repress transcription of some cellular genes by inhibiting the function of CBP/p300 (27, 28). To investigate the requirement of E1a-sensitive coactivators for Mn-SOD expression, NIH3T3 cells were either infected or not with adenovirus expressing E1a. After 24 h of infection, cells were treated with TNF for 4 h. RNA was then isolated and subjected to quantitative real-time RT-PCR using primers specific to Mn-SOD and β-actin cDNA. The results showed that adenovirus infection repressed TNF induction of Mn-SOD by 70% (Fig. 7A). The level of β-actin mRNA was not altered by adenovirus infection (data not shown). To determine whether this observation is caused directly by E1a and to rule out any effects of adenovirus infection, the consequence of transiently expressing E1a in NIH3T3 cells was also tested. The results showed that E1a alone was able to repress TNF induction of Mn-SOD by 50% (Fig. 7B). It is intriguing that a dose-dependent decline was not observed. Although transfection efficiency is a factor in the experiments and provides some explanation for the lack of full repression, the data could also suggest that an E1a-insensitive coactivator in addition to CBP/p300 can function in this system.

CBP and p300 Enhance TNF Induction of Mn-SOD—To investigate further the potential roles of CBP and p300 in Mn-SOD expression, the effect of their overexpression was analyzed. Vectors overexpressing CBP or p300 were transiently transfected into NIH3T3 cells followed by treatment with TNF for 2 h. RNA was then isolated and quantitatively analyzed by real-time RT-PCR (Fig. 7C). Overexpression of each of the two coactivators led to a modest 2-fold increase in Mn-SOD expression, and the increase was in a dose-dependent manner. Expression of β-actin was not altered by coactivator overexpression (data not shown). These results suggest that CBP/p300 may function as coactivator for Mn-SOD induction by TNF.

Inhibition of Histone Deacetylation Is Sufficient to Activate Mn-SOD Expression—The changes in nuclease sensitivity patterns and the potential for coactivator involvement as indicated by the above experiments suggest the possibility that acetylation of the histone N-terminal tails, which is known to be associated with the activation of gene expression, may also play a role in this system. TSA, a histone deacetylase inhibitor, was shown to induce the expression of the TNF-induced gene, MCP-1 (29). To determine whether inhibition of histone deacetylation alone can enhance Mn-SOD transcription, NIH3T3 cells were treated with TSA. RNA was isolated and applied in quantitative real-time RT-PCR analysis using primers specific for Mn-SOD and β-actin cDNA (Fig. 8). The results showed that addition of TSA alone to cell cultures resulted in a moderate 3.5-fold induction of Mn-SOD mRNA after a 16-h treatment with TSA. These results suggest that inhibition of histone deacetylation alone is sufficient to induce the expression of Mn-SOD gene.

TNF Induces Histone H3 and H4 Acetylation at Both the PPR and the TNFRE—To determine whether TNF induction leads to acetylation at the Mn-SOD gene, ChIP assays were performed on NIH3T3 cells using antibodies specific to p65, C/EBP-β, and Sp1. No antibody controls were included. The precipitated DNA was analyzed for the presence of the PPR and the TNFRE regions of Mn-SOD by quantitative real-time PCR. All data were normalized to the amount of chromatin added to each precipitation reaction and expressed as normalized precipitated DNA in ng. The average of three independent chromatin preparations ± S.E. is shown.

Fig. 5. Expression of p65, Sp1, and C/EBP-β in different cell lines. An equal amount (50 μg) of cell extract protein from NIH3T3, MEF, Sp1−/−, and p65−/− cells was loaded onto a 10% SDS-polyacrylamide gel, and Western blotting was performed using antibodies specific to p65, Sp1, or C/EBP-β.

Fig. 6. TNF induces assembly of the transcription factor complex. ChIP assays coupled with real-time PCR were performed on NIH3T3, Sp1−/−, and NF-κB p65−/− cells using antibodies specific to p65, C/EBP-β, and Sp1. No antibody controls were included. The precipitated DNA was analyzed for the presence of the PPR and the TNFRE regions of Mn-SOD by quantitative real-time PCR. All data were normalized to the amount of chromatin added to each precipitation reaction and expressed as normalized precipitated DNA in ng. The average of three independent chromatin preparations ± S.E. is shown.

Fig. 7. Effect of adenovirus infection on the expression of Mn-SOD. NIH3T3 cells were treated with TNF for 4 h. RNA was then isolated and subjected to quantitative real-time RT-PCR using primers specific to Mn-SOD and β-actin cDNA. The results showed that adenovirus infection repressed TNF induction of Mn-SOD by 70% (Fig. 7A). The level of β-actin mRNA was not altered by adenovirus infection (data not shown). To determine whether this observation is caused directly by E1a and to rule out any effects of adenovirus infection, the consequence of transiently expressing E1a in NIH3T3 cells was also tested. The results showed that E1a alone was able to repress TNF induction of Mn-SOD by 50% (Fig. 7B). It is intriguing that a dose-dependent decline was not observed. Although transfection efficiency is a factor in the experiments and provides some explanation for the lack of full repression, the data could also suggest that an E1a-insensitive coactivator in addition to CBP/p300 can function in this system.

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TNF Induces Histone H3 and H4 Acetylation at Both the PPR and the TNFRE—To determine whether TNF induction leads to acetylation at the Mn-SOD gene, ChIP assays were performed on NIH3T3 cells using antibodies specific for acetylated H3 or H4 histone (Fig. 9). The results showed that TNF induced a maximum of 1.3-fold increase in acetylation of H3 and 3-fold increase in acetylation of H4 at the PPR. A moderate increase of 2-fold in acetylation of both H3 and H4 was detected at the TNFRE after TNF treatment. These changes occurred in a time course consistent with the factor binding after TNF treatment. It should be noted the histone H3 acetylation in the PPR is very high in the constitutive state and is 10 times the level of the basal level at the TNFRE (data not shown). Histone H3 and H4 acetylation of the glyceraldehyde-3-phosphate dehydrogenase gene were unchanged during the TNF treatment, indicating
that the modest changes in histone acetylation were specific for the *Mn-SOD* gene. These results demonstrate that TNF induction results in acetylation of H3 and H4 at both the PPR and the TNFRE.

**NF-kB p65 Is Indispensable for Histone Acetylation at Both the PPR and the TNFRE**—To understand the role of Sp1 and NF-kB p65 in histone acetylation of the *Mn-SOD* gene, ChIP assays were performed on Sp1−/− and p65−/− cell lines (Fig. 9). The results showed no increase in histone acetylation at the PPR and the TNFRE in p65−/− cells after TNF treatment. This suggests that TNF-induced changes in histone acetylation are dependent upon NF-kB p65 binding. In contrast, a moderate increase in histone H3 and H4 acetylation was observed at the PPR and the TNFRE in p65−/− cells, which was similar to that observed in the NIH3T3 cell line. The simplest interpretation of these data is that Sp1 plays no direct role in increasing histone acetylation at the TNFRE in response to TNF. These results are consistent with the model that in response to TNF the PPR and the TNFRE communicate to facilitate factor assembly on the *Mn-SOD* gene through modification of the local chromatin structure.

**DISCUSSION**

The data presented above demonstrate that changes in chromatin structure are involved in the induction process of *Mn-SOD* transcription in response to TNF. Using Sp1- and NF-kB p65-deficient cell lines, genetic correlations among the activities of Sp1, NF-kB p65, and *Mn-SOD* gene regulation by TNF were provided. The binding of these factors to their respective regions within the *Mn-SOD* gene was fully demonstrated by ChIP assays. An E1a-sensitive coactivator also played a role in *Mn-SOD* expression. The combined results of the nuclease digestion and ChIP assays suggest that TNF induces an open chromatin structure within the TNFRE and histone acetylation at the PPR and the TNFRE. These changes were completely dependent on NF-kB p65. Because NF-kB p65 assembly in the TNFRE requires Sp1 binding to the PPR, the results also suggest communication between the two regions during TNF induction.

Based on the above observations and previous reports (9, 10), the following model for TNF-induction of the *Mn-SOD* gene is proposed. During constitutive or basal *Mn-SOD* expression, histone H3 acetylation at the PPR is high, suggesting that the region is accessible. Binding of Sp1 to the GC boxes in the PPR facilitates basal expression of *Mn-SOD*. In the basal state, the TNFRE is likely to be partially accessible as suggested by the detection of the MNase-hypersensitive banding pattern at C/EBP-1/C/EBPX and hyposensitive banding pattern at C/EBP-2/NF-kB/NF-1. After TNF treatment, NF-kB is translocated into the nucleus and can bind the TNFRE. NF-kB likely binds to two sites within the TNFRE, C/EBPX and the NF-kB site, both of which were shown to bind NF-kB *in vitro* (10, 11). Based on the observation that NF-kB p65 failed to bind the TNFRE in Sp1−/− cells, the binding of Sp1 to the PPR stabilizes initial NF-kB binding to the TNFRE. Once bound, NF-kB recruits a coactivator or coactivator complex with histone acetyltransferase activity, such as CBP/p300. Interactions between NF-kB and CBP have been documented in other systems (30, 31). The recruited coactivator acetylates histone H3 and H4 at the TNFRE and the PPR. Concurrently NF-kB promotes the binding of C/EBP-β to the C/EBP-1 region. This is supported by ChIP assay data showing that in Sp1−/− but not in p65−/− cell line, C/EBP-β bound the TNFRE after TNF induction. *In vitro*
assays also demonstrated that NF-κB p65 stimulates the DNA binding activity of C/EBP-β (32). Additional chromatin structural changes may also occur. For example, C/EBP-β has been shown to recruit the SNF/SWI remodeling complex (33). If recruited in this system, SNF/SWI could alter chromatin structure within the TNFRE, resulting in the opening of C/EBP-β/NF-κB/NF-1 region, as well as its surrounding area. A result of this reorganization is the increase in factor binding. The combined activities of factors within the complex are likely to result in the increased recruitment of the basal transcription machinery to the PPR.

The essential role of Sp1 in Mn-SOD activation by TNF was unexpected because the constitutive expression level of Mn-SOD in the Sp1−/− cell line was similar to that in the wild type (data not shown). Other factors, such as Sp3, may substitute for the activity of Sp1 during basal Mn-SOD transcription in Sp1−/− cells. Binding of Sp3 to the PPR has been shown by in vitro analysis (11). However, Sp1 activity is required for TNF-induced expression of Mn-SOD, indicating specific interactions between Sp1 and other Mn-SOD-specific factors. ChIP assays have shown that NF-κB p65 does not bind the TNFRE in the absence of Sp1, suggesting that Sp1 may stabilize the binding of NF-κB to DNA. Similar phenomena have been observed for the TNF induction of the MCP-1 gene (13, 34). The DNA binding regions of Sp1 and NF-κB are separated by 2.2 kbp of DNA sequence, which poses the question of how Sp1 and NF-κB may physically interact. The linear distance between Sp1 and NF-κB binding sites could be substantially reduced if 2.2 kbp of the spacer DNA between Sp1 and NF-κB is packaged into ~11 nucleosomes. This distance might allow Sp1 and NF-κB to contact each other directly or communicate through bridging molecules, such as CBP/p300. The reduced binding of C/EBP-β to the TNFRE in Sp1−/− suggests that NF-κB p65 is essential for stable C/EBP binding but also leaves open the possibility that C/EBP-β aids in interactions with the PPR.

Numerous reports have shown the importance of NF-κB in TNF-induced gene activation (for review, see Ref. 35). By studying TNF induction of Mn-SOD in a NF-κB p65-deficient cell line, we found that Mn-SOD was not induced, indicating that NF-κB p65 is required for Mn-SOD induction, and the NF-κB sites in the TNFRE are specific for p65-containing isoforms. A previous report has suggested that TNF-induction of Mn-SOD does not require NF-κB (36), a conclusion contradictory to ours. In that report, an IκB inhibitor failed to repress Mn-SOD induction by TNF, leading to the conclusion that NF-κB is not necessary for TNF-induced Mn-SOD expression. The inconsistency could be the result of different cell lines used in the two studies. An alternative interpretation is that multiple kinase pathways are activated during TNF signal transduction, and treatment of cells with one IκB inhibitor may not completely block phosphorylation of IκB and therefore the translocation of NF-κB.

Because in ChIP assays the cross-linked chromatin DNA is sonicated into fragments with an average size of about 600 bp it is difficult for such assays to differentiate the functions of the two NF-κB sites in the TNFRE because of the small distance (about 100 bp) between them. Likewise, the roles of the two C/EBP sites in the TNFRE cannot be determined individually. The ChIP data confirm the role of the TNFRE in response to TNF induction, and NF-κB as well as C/EBP-β only bind the TNFRE. Small TNF-induced increases in the PPR DNA fragments precipitated by the p65 antibody might be caused by the
assembly of a complex bridging the PPR and the TNFRE during Mn-SOD induction. Cross-linking of the complex would allow coprecipitation of the PPR DNA with the TNFRE DNA by the antibodies. Alternatively, it was reported that the 5’-promoter region of Mn-SOD had sites that could bind NF-κB in vitro (10). Although these sites are incapable of driving full TNF-induced expression of Mn-SOD, they may in fact bind NF-κB p65 and participate in expression. The current data would be consistent with weak binding of NF-κB to these sites.

Post-translational modification of histones has been correlated with several biological functions (37). In the case of acetylation, hyperacetylation correlates with gene transcription, whereas hypoacetylation is associated with gene silencing and heterochromatin structure. Three lines of evidence suggest a role for histone acetylation in Mn-SOD induction. First, a requirement for coactivators with histone acetyltransferase activity was suggested by E1A-mediated repression of Mn-SOD mRNA level. Second, inhibition of histone deacetylase and the fact that coactivator overexpression increased the Mn-SOD induction. Finally, ChIP assays showed that histone acetylation at the PPR and the TNFRE occurs rapidly upon Mn-SOD induction by TNF. ChIP assays of histone acetylation in NIH3T3 cells. This result is consistent with the role of the PPR and the TNFRE in response to TNF. Although both Sp1 and NF-κB p65 have sites that could bind NF-κB.

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