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A NF-κB p65 Subunit Is Indispensable for Activating Manganese Superoxide: Dismutase Gene Transcription Mediated by Tumor Necrosis Factor-α

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Abstract Expression of the manganese superoxide dismutase (Mn-SOD) is induced by tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and lipopolysaccharide (LPS). Recently, a TNF-responsive element (TNFRE) was identified within the second intron of the murine Mn-SOD gene. The 5′ CCAAT/enhancer binding protein (C/EBP)-related region within the TNFRE was responsive to TNF, whereas the 3′ NF-κB-related region alone was not. This report describes the minimal promoter region of the Mn-SOD gene and investigates the *cis*-acting elements and *trans*-acting factors responsible for TNF-α-induced Mn-SOD gene expression. Reporter plasmid transfection studies demonstrated that inducible transcription factors enhanced the transcriptional activity of the Mn-SOD gene through the intronic enhancer region. Electrophoretic mobility shift assays demonstrated that after TNF-α stimulation, p50 and p65 NF-κB subunits bound specifically to the newly identified NF-κB transcription factor-binding site, distinct from the previously described NF-κB site, within the intronic enhancer region. In addition, site-directed mutagenesis and cotransfection studies demonstrated that the NF-κB p65 subunit enhanced the transcriptional activity of the Mn-SOD gene through the newly identified NF-κB site. These results show that a NF-κB p65 subunit is mainly involved in the molecular mechanisms controlling TNF-α-mediated Mn-SOD gene transcription. J. Cell. Biochem. 77:474–486, 2000.

Key words: manganese superoxide dismutase; transcription; nuclear factor-κB; CCAAT/enhancer binding protein; tumor necrosis factor-α

The superoxide dismutases (SODs) are important metalloenzymes that scavenge superoxide radicals through disproportionation [Fridovich, 1989]. Three types of SOD with distinctive distributions are known to exist. The copper-zinc SOD (CuZn-SOD) is found mainly in the cytosol of eukaryotes; the iron SOD (Fe-SOD) is found in prokaryotes, eukaryotic algae, and higher plants; and the manganese SOD (Mn-SOD) is found in both prokaryotes and the mitochondria of eukaryotes. Expression of CuZn-SOD and Fe-SOD is constitutive, whereas Mn-SOD is inducible by various stimuli, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), lipopolysaccharide (LPS), interferon-γ (IFN-γ), or X-irradiation [Visner et al., 1990; Hirose et al., 1993; Akashi et al., 1995]. Induction of Mn-SOD is important for protection against inflammation and oxidative stress [Fridovich, 1989].

TNF-responsive element (TNFRE) has been identified within the second intron of the Mn-SOD gene [Jones et al., 1997]. DNA-binding assays, using TNFRE, showed that C/EBP β binds to the 5′ region of the TNFRE and both C/EBP β and NF-κB bind to the 3′ region. Transfection assays demonstrated that the 5′ C/EBP-related region was responsive to TNF, whereas the 3′ NF-κB-related site was not. Kuo et al. [1999] performed extensive studies of the transcriptional regulation of Mn-SOD promoter that lacks a TATA- and a CAAT-box. These investigators used DNase I-hypersensitive (HS) site analysis, which revealed five HS sites within the proximal Mn-SOD promoter. However, no *cis*-acting element that binds NF-κB

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and responds to TNF-α has been identified in the Mn-SOD promoter.

Proinflammatory cytokines such as TNF-α and IL-1, or bacterial endotoxins such as LPS, cause rapid activation and nuclear translocation of NF-κB, a transcription factor [Baldwin, 1996; Sweet and Hume, 1996]. The classic form of NF-κB consists of a p50/p65 heterodimer and resides in the cytoplasm in the inactive form, bound by its inhibitor proteins I-κB. Upon TNF-α stimulation, signal transduction events cause phosphorylation, ubiquitination, and degradation of I-κB, resulting in the release of NF-κB, which translocates into the nucleus and regulates gene expression [Brockman et al., 1995; Brown et al., 1995; Chen et al., 1995; Ghosh et al., 1998].

In a recent report, we showed that inducible transcription factors enhanced the transcriptional activity of the Mn-SOD gene through the TNFRE mediated by LPS and IFN-γ in NIH3T3 cells and the cooperation between proteins binding to the newly identified NF-κB and C/EBP sites led to synergistic gene transcription [Maehara et al., 1999].

To identify both *cis*-acting elements and *trans*-acting factors responsive to TNF-α, we performed transient transfection studies, using various mutants of 5′ flanking DNA and the intronic enhancer of the murine Mn-SOD gene, and confirmed the transcription factors by competition and supershift electrophoretic mobility shift assays (EMSA). Our findings provide the evidence that p65 NF-κB subunit as a component of the inducible complex that binds to the newly identified NF-κB binding site within the enhancer region and help explain the mechanism by which NF-κB proteins activate transcription of the Mn-SOD gene.

MATERIALS AND METHODS

Materials

Cell culture reagents and calf serum (CS) were obtained from Gibco-BRL (Life Technologies, NY). Recombinant murine TNF-α was purchased from R&D Systems (Minneapolis, MN). Anti-C/EBP α, β, and δ; anti-CRP-1; anti-p65, p50, and p52 NF-κB; anti-SP-1 and SP-3 antibodies; a NF-κB consensus oligonucleotide; a NF-κB mutant oligonucleotide; a SP-1 consensus oligonucleotide; and a C/EBP consensus oligonucleotide were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A λ genomic library prepared from *Bam*HI, *Mbo*I, *Bgl*II, or *Sau*3A-digested DNA isolated from the spleen cells of B6/CBA F1J mice was purchased from Stratagene (La Jolla, CA).

Cell Culture

NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% CS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Plasmid Construction

The Mn-SOD promoter constructions were generated by polymerase chain reaction (PCR) amplification with the genomic Mn-SOD clone as a template [Maehara et al., 1999]. The 5′ primer (the positions of the 5′ base are indicated relative to the Mn-SOD transcription initiation site in parentheses) (−1696) 5′-ACATCAGCCATGGAGACG-3′ was used with the 3′ primer (+82) 5′-CCGACACAACATTATTGAGG-3′ in a standard amplification reaction with cycling temperatures of 94°, 58°, and 74°C. The PCR product was subcloned into the pCR™2.1 vector (Invitrogen) and digested with *Bam*HI and *Eco*RV, gel purified, and ligated into a *Bgl*II-*Sma*I-digested pGL3 basic vector (Promega, Madison, WI). The 5′ deletion constructs were generated using the Deletion Kit (Nippon Gene, Tokyo) according to the manufacturer's suggestions. These plasmids were digested with *Kpn*I and *Bss*HII, gel purified, and ligated into a *Kpn*I-*Mlu*I-digested pGL3 basic vector again (Fig. 1A). The constructions that contains the Mn-SOD intronic enhancer region were generated by PCR amplification with the genomic Mn-SOD clone as described previously [Maehara et al., 1999]. In brief, PCR amplification was carried out with the 5′ primer (+2119) 5′-CGCGGATCCGGGGGCATCTAGTGGAGAAGTA-3′ and the 3′ primer (+2420) 5′-TTTTGACGTCGACGCGAGCTCTGGCTCCACAGAAGG-3′ in the standard amplification reaction as described above. Plasmids containing mutation within the C/EBP site (mC/EBP) or the NF-κB site (mκB) in the intronic enhancer region were constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) (Fig. 1C). The PCR products were digested with *Bam*HI and *Sal*I, gel-purified, and ligated into a *Bam*HI-*Sal*I-digested pGL3 promoter vector (Promega) or pGL3 basic vector containing the murine Mn-SOD promoter

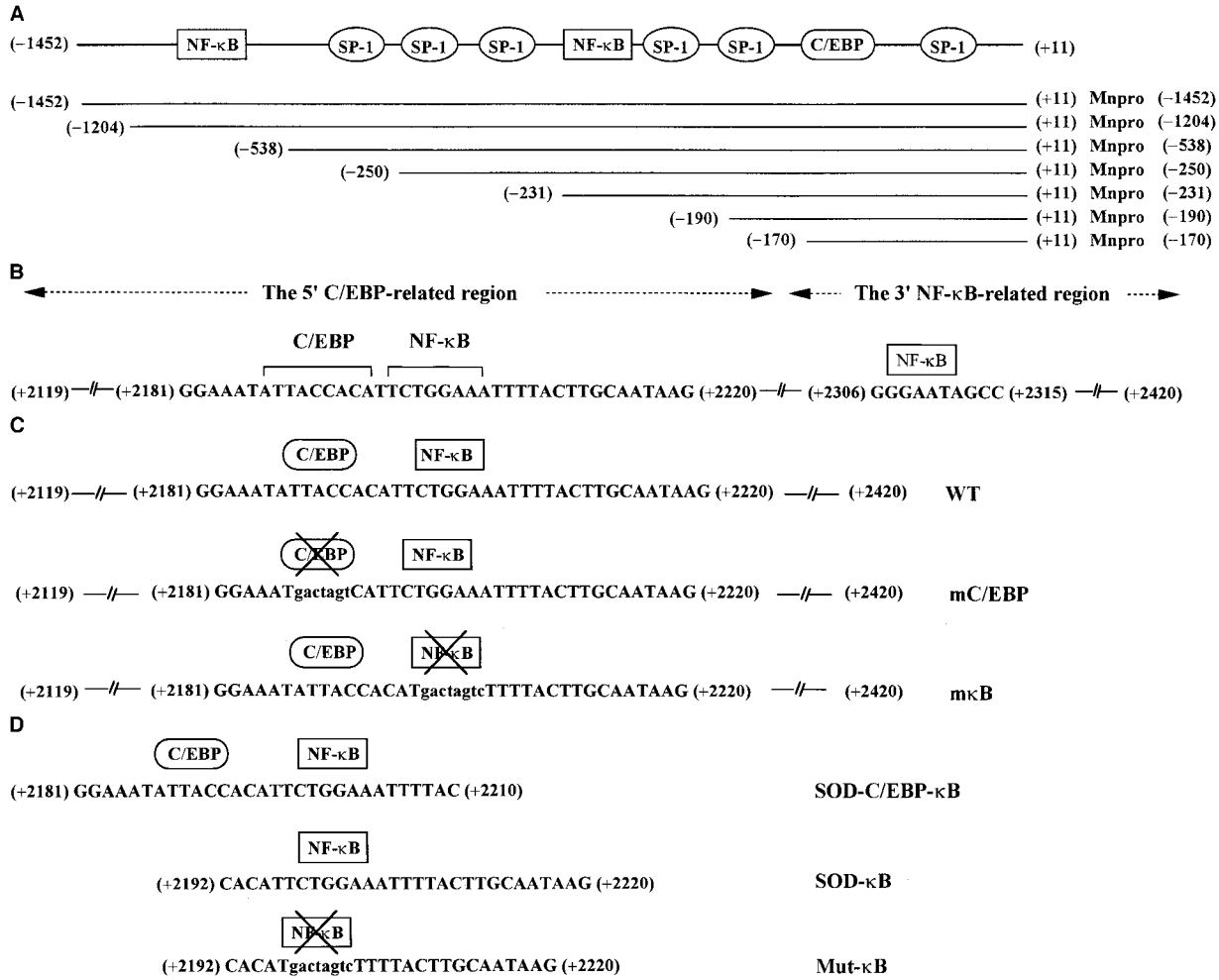

Fig. 1. Schematic representations of the promoter and the intronic enhancer of the murine manganese superoxide dismutase (Mn-SOD) gene. **A:** Luciferase reporter constructs containing the promoter and 5′ flanking DNA of the Mn-SOD gene are illustrated below a schematic of the computer-identified potential regulatory sites. The positions of the bases are indicated relative to the Mn-SOD transcription initiation site (+1). The 5′ base pair of each construct is indicated in parentheses. **B:** Schematic representation of the Mn-SOD enhancer. The locations of the sites for C/EBP and NF-κB are indicated by brackets. **C:** Sequences of wild-type, and mutants of the reporter vectors containing the intronic enhancer. Wild-type enhancer sequences are indicated by capital letters. A crossed-out site indicates that the site has been mutated to a nonbinding sequence, with the mutated sequence indicated by lowercase letters. **D:** Sequences of the oligonucleotides for EMSA.

region nucleotide −231 to +11. All plasmids were sequenced to confirm their fidelity. Expression vectors for human NF-κB p65 (encoding the full-length cDNA of p65) and human NF-κB p50 (encoding amino acids 1–399 of p105) were gifts of Drs. Nancy R. Rice (Frederick Cancer Research and Development Center, Frederick, MD), Alain Israël (Institut Pasteur, Paris), and Craig A. Rosen (Human Genome Sciences, Rockville, MD) [Ruben et al., 1991; Rice et al., 1992], and the expression vector for mouse C/EBPβ (encoding the full-length cDNA of AGP/EBP) and pcDNA3 were a gift of Dr. Sheng-Chung Lee (National Taiwan University, Taipei) [Chang et al., 1990; Lee et al., 1993].

Transfection Assays

Transient transfection of NIH3T3 cells was carried out using SuperFect reagent (Qiagen, Hilden, Germany). In general, the day before transfection, 4×10^5 cells were plated in 12-well tissue culture plates, supplemented with fresh medium before transfection. A total of 2.5 μg of DNA consisting of 2.25 μg of the indicated luciferase plasmid and 0.25 μg of the pRL-thymidine kinase control vector (pRL-TK) (Promega) per plate was mixed with 10 μl of Su-

perFect reagent and allowed to form complexes for 15 min in serum-free or antibiotic-free medium before being added to the cells. Cells were incubated with the complexes for 14 h. After transfection, the cells were placed in serum-free medium for 10 h; 1 or 10 ng/ml TNF-α, or control buffer, was then added for an additional 14 h. After being harvested, the cells were assayed by the Dual-Luciferase Reporter Assay System (Promega), using a luminometer (EG&G Berthold, Germany). For cotransfection studies, cells grown in 12-well plates were transfected with 2.25 μg of luciferase reporter plasmid and pRL-TK mixture (ratio 9:1) and 0.25 μg of the indicated expression plasmid. pcDNA3 plasmid DNA was used to adjust the total amount of expression plasmid DNA for each transfection to be equal. Promoter activities were expressed as relative light units (RLU), normalized against the activity of the pRL-TK control vector. All transfection experiments were repeated at least three times.

Nuclear Extract Preparation

Nuclear extracts were prepared from the cells that were left untreated or those treated with 10 ng/ml TNF-α for 20 min or for 4 h, using the rapid preparation described by Andrews and Faller [1991], with slight modification. Cells with or without TNF-α treatment were washed twice in ice-cold phosphate-buffered saline (PBS), then quickly washed in buffer A consisting of 10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 2,000 rpm, the cell pellet was resuspended in buffer A containing 0.1% Nonidet-P, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin, and incubated on ice for 10 min. The nuclei were collected by centrifugation at 5,000 rpm for 1 min at 4°C. The supernatant was removed, and the pellet was resuspended in buffer B (20 mM Hepes, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin). The nuclear pellet was incubated on ice for 30 min, followed by centrifugation at 14,000 rpm for 15 min. The protein concentrations of the nuclear fractions were determined by the Bradford assay with the Bio-Rad protein assay dye reagent, and all extracts were stored at −70°C.

Electrophoretic Mobility Shift Assays

The DNA binding reaction was performed for 20 min at 4°C in a volume of 10 μl, containing 1–2 μg of nuclear extract, 2 μl of 5× binding buffer consisting of 1×: 10 mM Tris, pH 7.5, 4% glycerol, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, and 0.05 μg/μl poly (dI-dC) with or without a 200-fold molar excess or 20 ng of an unlabeled competitor DNA. For the DNA binding reaction with SP-1-3′ and SP-1-5′ oligonucleotides, poly (dA-dT) was used in the binding buffer instead of poly(dI-dC). Synthetic complementary oligonucleotides with a G overhang were annealed and labeled with [α-^{32}P] dCTP, using the Klenow fragment. The radiolabeled probe was added for an additional incubation period of 20 min. For supershifts, nuclear extracts were preincubated with antibodies for 20 min at 4°C. DNA binding reactions were separated on 5% native polyacrylamide gels. Gels were dried and subjected to autoradiography. The following pairs of oligonucleotides were used (mutated residues are underlined) (Fig. 1D): SOD-C/EBP-κB; 5′-GGAAATATTACCAC-ATTCTGGAAATTTTAC-3′,5′-GGGTAA-AATTTCCAGAATGTGGTAATATTT-3′, SOD-κB; 5′-CACATTCTGGAAATTTT-ACTTGCAATAAG-3′,5′-GCTTATTGC-AAGTAAAATTTCCAGAATGTG-3′, Mut-κB; 5′-CACAT<u>GACTAGTC</u>TTTTACTTGCAATAAG-3′,5′-GCTTATTGCAAGTAAAA<u>GACTAGTC</u>AT-GTG-3′, SP-1–5′; 5′-GGGGTTCCCCGAG-GCGGGGCGGGGCCAAGGCCGATG-3′, 5′-GGCATCGGCCTTGGCCCCGCCCCGCCTC-GGGGAACC-3′, and SP-1-3′; 5′-GGCCGATGG-TGGGGGCGTGGCTGTAGCAAG-3′,5′-GGC-TTGCTACAGCCACGCCCCCACCATCGG-3′.

RESULTS

Mn-SOD Basal Transcriptional Activity

We have constructed 5′ end deletion mutants of the murine Mn-SOD gene. Each mutant containing its transcription initiation sites was transiently transfected into NIH3T3 cells. As shown in Figure 2, a construct containing 231 base pairs (bp) of 5′ flanking DNA exhibited high level of relative luciferase activity in the untreated cells, and the activity was increased slightly by the treatment with TNF-α. Deletion up to the position of −170 resulted in the significant decrease of the luciferase activity, indicating that the −231 to −170 region in the Mn-SOD promoter was responsible for the

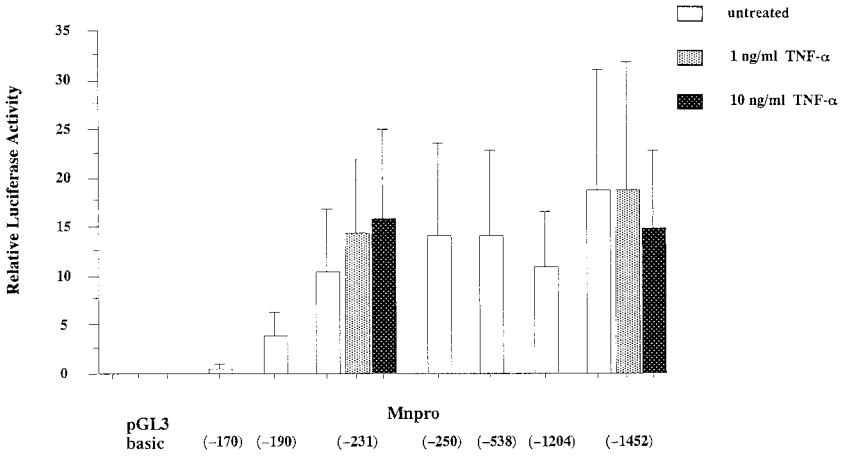

Fig. 2. Functional analysis of the manganese superoxide dismutase (Mn-SOD) promoter. NIH3T3 cells were transfected with vectors containing the 5′ flanking DNA of the Mn-SOD gene, and then treated with or without 1 or 10 ng/ml TNF-α for 14 h. The average promoter activities were generated from three separate experiments. Standard deviations of the means are indicated by the error bars. Luciferase activities were normalized for the transfection efficiency with the cotransfected pGL3-TK control vector.

basal transcription. The construct containing 1,452 bp of 5′ flanking DNA did not elevate the relative luciferase activity by TNF-α stimulation. Although sequence analysis of the region displayed putative regulatory motifs, including two NF-κB, a C/EBP, and several SP-1 binding sites (Fig. 1A) as reported previously [Jones et al., 1997], neither Mnpro (−231) nor Mnpro (−1452) exhibited an induction of the luciferase activity compatible with that of the mRNA induced by TNF-α (data not shown). These results indicated that at least 5′ flanking DNA of the Mn-SOD that we examined does not contain a *cis*-element responsive for TNF-α stimulation.

Determination of the Regulatory DNA Elements Required for Mn-SOD Transcriptional Activity Induced by TNF-α

To identified the *cis*-element responsive to TNF-α, we examined the TNFRE within the intron 2 of the Mn-SOD gene. A series of mutants within a 302-bp region of intron 2 (+2119 to +2420) were subcloned into both the pGL3 promoter vector (SVpro), which contains the basic enhancerless simian virus 40 (SV40) promoter and the Mn-SOD promoter vector (Mnpro (−231)), which contains 231 bp of the 5′ flanking DNA of the Mn-SOD gene. NIH3T3 cells were transiently transfected with one of the plasmids, and then treated with 1 or 10 ng/ml TNF-α for 14 h. Lysates from the cells transfected with SVpro produced only low luciferase activity both in untreated and treated cells (Fig. 3A). Lysates from the cells transfected with SVpro-WT (2119–2420) showed a sixfold induction after treatment with 1 or 10 ng/ml TNF-α. NIH3T3 cells were transfected with plasmids containing a mutation at either the C/EBP or the NF-κB site. These two adjacent sites were required for induction of the Mn-SOD gene transcription mediated by LPS and IFN-γ, as shown in our report [Maehara et al., 1999]. Lysates from the cells transfected with SVpro-mC/EBP containing the mutation at the C/EBP site showed a twofold induction after treatment with 1 or 10 ng/ml TNF-α; the activity of the TNF-α-treated cells transfected with SVpro-mC/EBP was one-third the activity of the TNF-α-treated cells transfected with SVpro-WT. TNF-α-induced enhancer activity was completely abolished in the cells transfected by SVpro-mκB containing the mutation at the NF-κB site.

To examine the interaction between the minimal promoter and the enhancer of Mn-SOD gene, transfection studies were performed with the Mn-SOD minimal promoter vector (Mnpro) containing a mutation within the C/EBP site (mC/EBP) or the NF-κB site (mκB) in the intronic enhancer region. As shown in Figure 3B, lysates from the cells transfected with Mnpro produced only low luciferase activity both in the untreated cells and in the TNF-α-treated cells. Lysates from the cells transfected with Mnpro-WT (2119–2420) showed a 10-fold induction of the relative luciferase activity after treatment with 1 or 10 ng/ml TNF-α. Lysates from the cells transfected with Mnpro-mC/EBP showed a 3- or 4.5-fold induction after treatment with 1 or 10 ng/ml TNF-α, respectively. A twofold induction in the TNF-α-treated cells transfected with Mnpro-mκB was observed relative to the activity of the construct in the

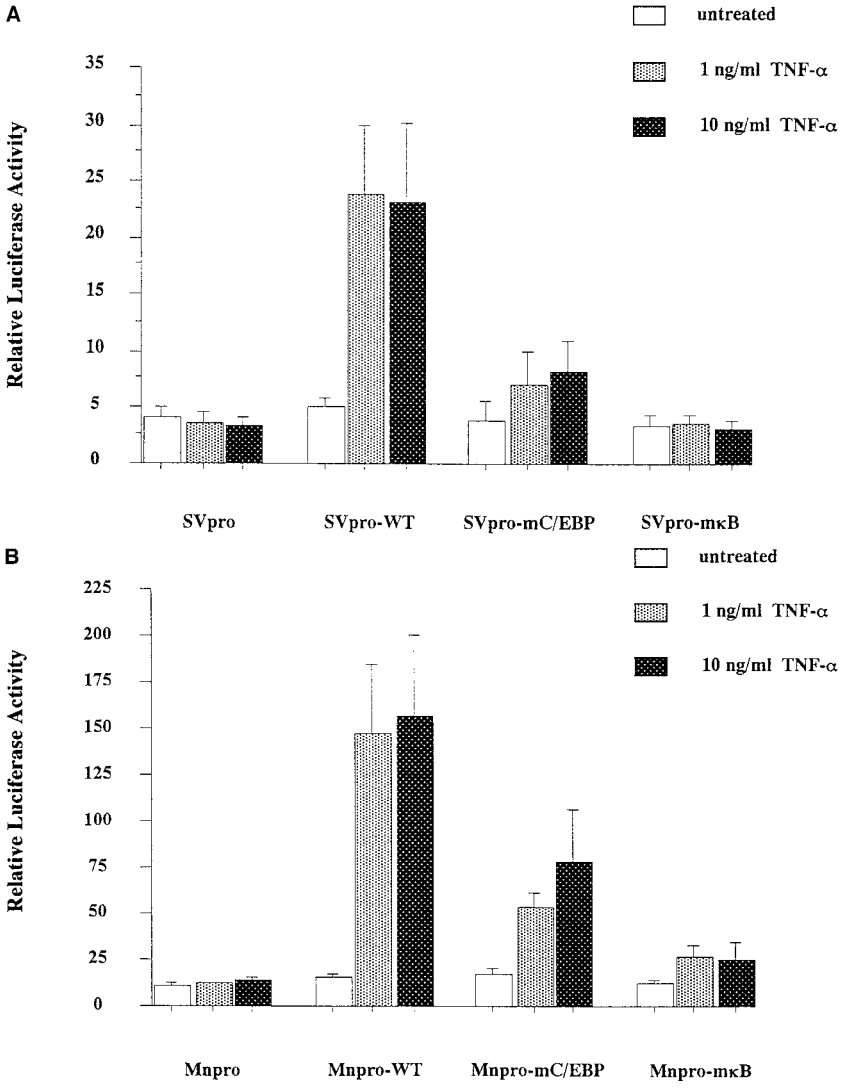

Fig. 3. Functional analysis of the manganese superoxide dismutase (Mn-SOD) enhancer. **A:** NIH3T3 cells were transfected with SVpro containing wild-type, or mutation within the C/EBP site (mC/EBP) or the NF-κB site (mκB) in the intronic enhancer region, and then treated with 1 or 10 ng/ml TNF-α for 14 h. **B:** Transfection studies were performed with the Mn-SOD promoter vector (Mnpro (−231)) containing wild-type, or mutation within the C/EBP site (mC/EBP) or the NF-κB site (mκB) in the intronic enhancer region. The average luciferase activities were generated from three separate experiments. Standard deviations of the means are indicated by the error bars.

untreated cells. These data indicate that TNF-α-induced transcriptional activation of Mn-SOD is mediated primarily by the newly identified NF-κB site, but that it depends on a potent effect by factors interacting with both the C/EBP and the NF-κB sites within the intronic enhancer region.

A NF-κB p65 Activates Mn-SOD Transcription Through the Newly Identified NF-κB Binding Site Within the Intronic Enhancer Region

To define the functional significance of these DNA-protein complexes, we performed cotransfection studies. As shown in Figure 4A, Mn-SOD promoter vector (Mnpro), Mnpro containing the 302-bp fragment in the intronic enhancer region (Mnpro-WT), or Mnpro containing mutations within the C/EBP site (Mnpro-mC/EBP) or the NF-κB site (Mnpro-mκB) was cotransfected into the NIH3T3 cells with one of the expression vectors for p65 NF-κB, p50 NF-κB, and C/EBP β, or empty expression vector pcDNA3. None of the expression vector transfected with Mnpro increased promoter activity in the NIH3T3 cells. Transfection of the cells with Mnpro-WT and pcDNA3 produced background levels of the luciferase activity. Cotransfection of p50 expression vector with Mnpro-WT, Mnpro-mC/EBP, or Mnpro-mκB did not significantly affect luciferase activity in the untreated cells. However, cotransfection of the p65 with Mnpro-WT or

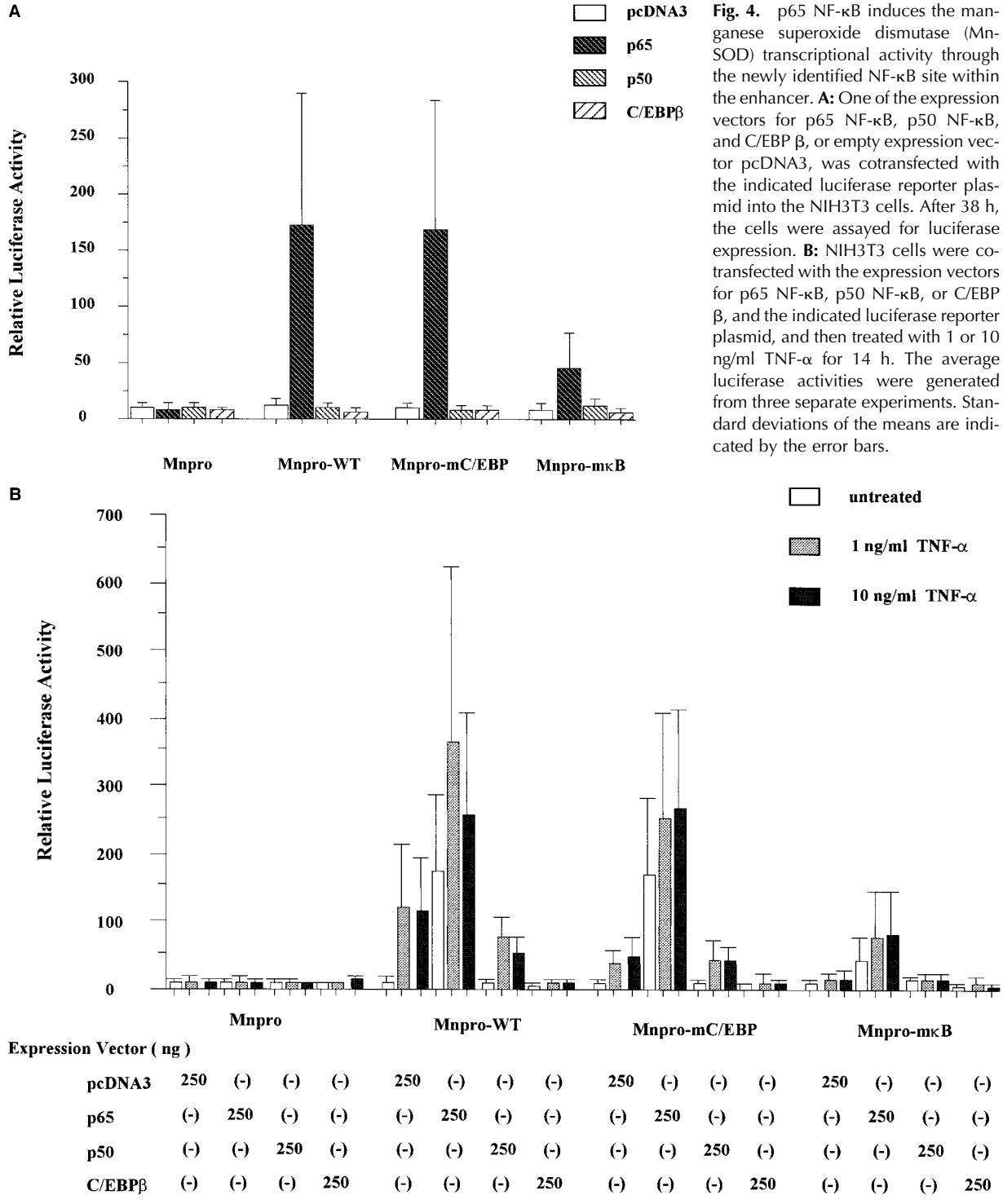

Fig. 4. p65 NF-κB induces the manganese superoxide dismutase (Mn-SOD) transcriptional activity through the newly identified NF-κB site within the enhancer. **A:** One of the expression vectors for p65 NF-κB, p50 NF-κB, and C/EBP β, or empty expression vector pcDNA3, was cotransfected with the indicated luciferase reporter plasmid into the NIH3T3 cells. After 38 h, the cells were assayed for luciferase expression. **B:** NIH3T3 cells were cotransfected with the expression vectors for p65 NF-κB, p50 NF-κB, or C/EBP β, and the indicated luciferase reporter plasmid, and then treated with 1 or 10 ng/ml TNF-α for 14 h. The average luciferase activities were generated from three separate experiments. Standard deviations of the means are indicated by the error bars.

Mnpro-mC/EBP into the cells produced a 14- or 15-fold increase in the luciferase reporter activity, suggesting that p65 NF-κB may direct transcriptional activity from the newly identified NF-κB binding site.

Cotransfection of p65 expression vector with Mnpro-mκB into the cells produced a small increase of the luciferase activity over background activity. In addition, C/EBP β expression vector did not augment luciferase reporter

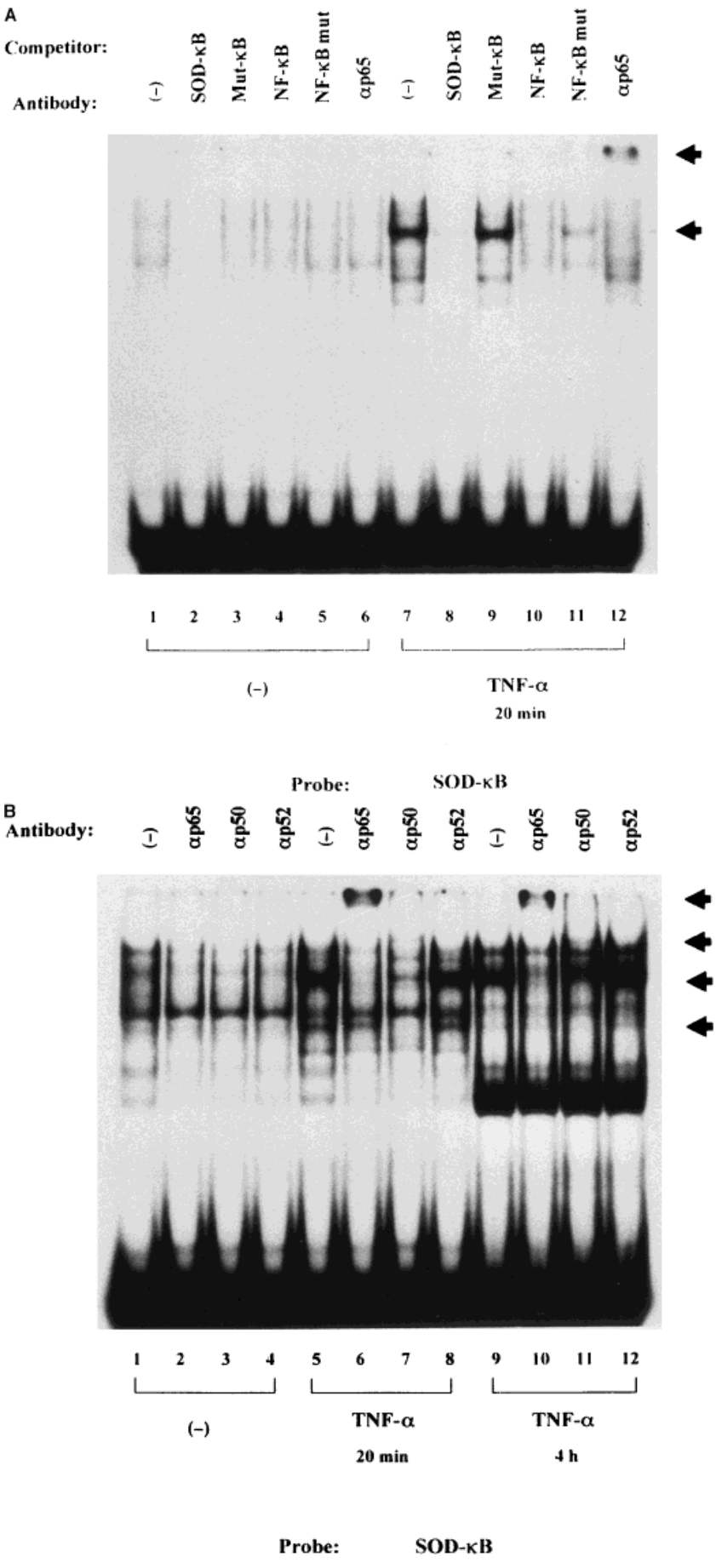

Fig. 5. Identification of NF-κB proteins binding in vitro. **A:** Electrophoretic mobility shift assay (EMSA) using a ^{32}P-labeled SOD-κB probe incubated with the nuclear extracts from NIH3T3 cells that were left untreated (**lanes 1–6**) or treated with 10 ng/ml TNF-α for 20 min (**lanes 7–12**). A 200-fold molar excess of a SOD-κB oligonucleotide (**lanes 2, 8**), a 200-fold molar excess of a Mut-κB oligonucleotide (**lanes 3, 9**), 20 ng of NF-κB consensus oligonucleotide (**lanes 4, 10**), or 20 ng of NF-κB mutant oligonucleotide (**lanes 5, 11**) was used as an unlabeled competitor. Antibody raised against p65 NF-κB subunit (**lanes 6, 12**) was added to the extracts before the addition of the probe. **B:** Supershift EMSA using a ^{32}P-labeled SOD-κB probe incubated with the nuclear extracts from NIH3T3 cells that were left untreated (**lanes 1–4**) or treated with 10 ng/ml TNF-α for 20 min (**lanes 5–8**) or for 4 h (**lanes 9–12**). Antibodies to the p65 subunit (**lanes 2, 6, 10**), the p50 subunit (**lanes 3, 7, 11**), or the p52 subunit (**lanes 4, 8, 12**) were added to the extracts before the addition of the probe. The SOD-κB-protein complexes and the supershifted complexes are indicated by arrows.

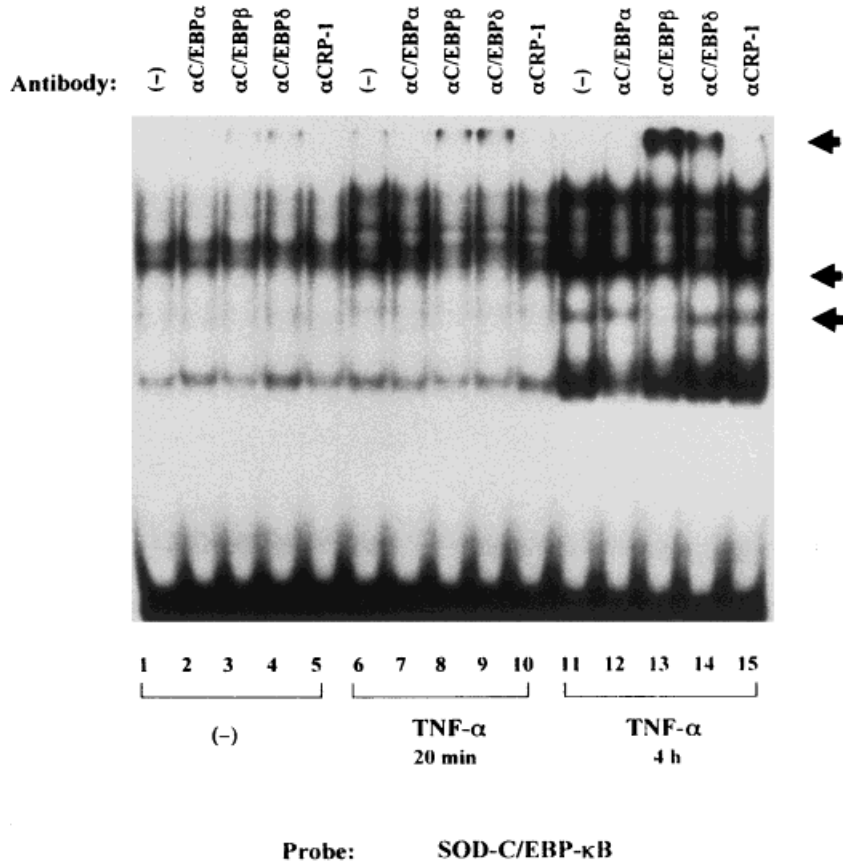

Fig. 6. Gel-shift analysis of the SOD-C/EBP-κB-protein complexes. Supershift EMSA using a ^{32}P-labeled SOD-C/EBP-κB probe incubated with the nuclear extracts from NIH3T3 cells that were left untreated (**lanes 1–5**) or treated with 10 ng/ml TNF-α for 20 min (**lanes 6–10**) or for 4 h (**lanes 11–15**). Antibodies to C/EBP α (**lanes 2, 7, 12**), C/EBP β (**lanes 3, 8, 13**), C/EBP δ (**lanes 4, 9, 14**), or CRP-1 (**lanes 5, 10, 15**) were added to the extracts before the addition of the probe. Arrows indicate the SOD-C/EBP-κB-protein complexes and the supershifted complexes.

activity in the untreated cells. Similar results were obtained in cotransfection studies using a series of SVpro mutants instead of the Mnpro mutants (data not shown).

The effects of the p65 expression vector were further increased in TNF-α-treated cells transfected with Mnpro-WT (2119–2420), Mnpro-mC/EBP, or Mnpro-mκB (Fig. 4B). However, p65 expression vector did not affect the luciferase activity in the treated cells transfected with Mnpro. These results were consistent with those of the transient transfection studies using only luciferase reporter vectors. Thus, we have confirmed the crucial site that is highly responsive to p65 NF-κB, activated by TNF-α within the intronic enhancer region.

p65 and p50 NF-κB Bind to the Newly Identified NF-κB Site In Vitro

We performed competition and supershift EMSA using the SOD-κB probe. Binding of the complexes to the SOD-κB probe was sequence specific, as it was blocked by an excess of an unlabeled SOD-κB oligonucleotide (Fig. 5A, lane 8) but not by an excess of an unlabeled oligonucleotide containing a mutation within the NF-κB site (Mut-κB) (Fig. 5A, lane 9). We also used a NF-κB consensus oligonucleotide as an unlabeled competitor. This oligonucleotide prevented formation of the SOD-κB-protein complexes (Fig. 5A, lane 10), suggesting a possible involvement of NF-κB in the assembly of the SOD-κB-protein complexes. To confirm the possibility, supershift analysis was carried out with the SOD-κB probe. Antibody to p65 or p50 supershifted the complexes from the cells treated with TNF-α (Fig. 5B, lanes 6, 7, 10, and 11), and a significant supershift was seen with the nuclear extracts stimulated by TNF-α for 20 min (Fig. 5B, lane 6). Competition and supershift EMSAs using the SOD-κB probe demonstrated that p65 and p50 NF-κB family proteins could bind specifically to the newly identified NF-κB site in vitro. These results show that despite the lack of identification of canonical NF-κB sites, the NF-κB site partici-

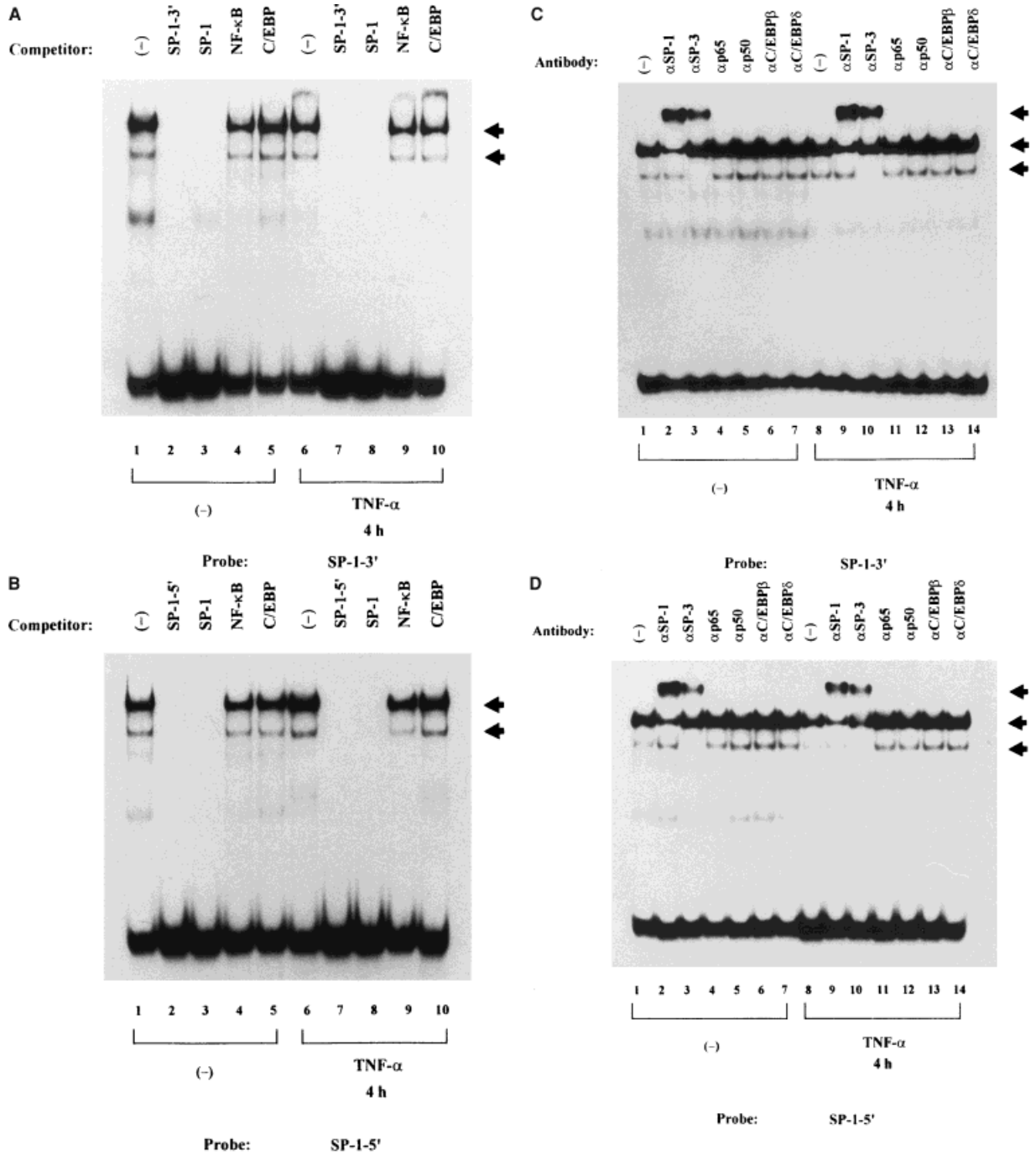

Fig. 7. SP-1 family proteins bind to the minimal promoter region of the manganese superoxide dismutase (Mn-SOD). **A:** EMSA using a ^{32}P-labeled SP-1-3′ probe incubated with the nuclear extracts from NIH3T3 cells that were left untreated (**lanes 1–5**) or treated with 10 ng/ml TNF-α for 4 h (**lanes 6–10**). A 200-fold molar excess of a SP-1-3′ oligonucleotide (**lanes 2, 7**), 20 ng of SP-1 consensus oligonucleotide (**lanes 3, 8**), 20 ng of NF-κB consensus oligonucleotide (**lanes 4, 9**), or 20 ng of C/EBP consensus oligonucleotide (**lanes 5, 10**) was used as an unlabeled competitor. **B:** Competition EMSA using a ^{32}P-labeled SP-1-5′ probe. **C:** Supershift EMSA using a ^{32}P-labeled SP-1-3′ probe. Antibodies to SP-1 (**lanes 2, 9**), SP-3 (**lanes 3, 10**), p65 NF-κB subunit (**lanes 4, 11**), p50 (**lanes 5, 12**), C/EBP β (**lanes 6, 13**), or C/EBP δ (**lanes 7, 14**) were added to the extracts from the untreated cells (**lanes 1–7**) or the TNF-α-treated cells (**lanes 8–14**) before the addition of the probe. **D:** Supershift EMSA using a ^{32}P-labeled SP-1-5′ probe. Arrows indicate the SP-1-3′-, or the SP-1-5′-protein complexes and the supershifted complexes.

pates directly in the TNF-α inducibility of Mn-SOD enhancer activity through its interaction with NF-κB family factors.

C/EBP Proteins Bind to the Adjacent Site to the NF-κB Binding Site In Vitro

Supershift EMSAs were carried out with ^{32}P-labeled SOD-C/EBP-κB oligonucleotide, using antibodies to C/EBP α, β, δ, and CRP-1. Anti-C/EBP β and δ antibodies were able to supershift the medium-size bands of complexes (Fig. 6), especially complexes from the cells treated with TNF-α for 4 h (lanes 13 and 14). However, anti-C/EBP α or anti-CRP-1 antibodies had no effect (Fig. 6, lanes 2, 5, 7, 10, 12, and 15). We performed supershift analysis using ^{32}P-labeled SOD-κB oligonucleotides containing only the NF-κB site, instead of the SOD-C/EBP-κB probe. When the nuclear extracts prepared from the untreated and TNF-α-treated cells were incubated with the SOD-κB probe, none of the antibodies to C/EBP bound to the complexes (data not shown). These results indicate that not only β, but also the δ isoform of C/EBP, binds to the C/EBP site in vitro.

SP-1 Family Proteins Bind to the Minimal Promoter Region

To identify *trans*-acting factors that interact with the minimal promoter region of Mn-SOD, EMSAs were carried out with nuclear extracts prepared from the NIH3T3 cells that were either untreated or treated with 10 ng/ml TNF-α for 20 min or for 4 h. Since the transfection studies indicated that the −231 to −170 bp region in the Mn-SOD promoter was responsible for the basal transcription, we performed competition and supershift EMSAs with ^{32}P-labeled SP-1-3′ (containing the nucleotide −192 to −163) or SP-1-5′ (containing the nucleotide −219 to −185) oligonucleotides. In addition, the nucleotide −231 to −170 encompasses the putative transcription factor binding sites, two SP-1, a C/EBP, a NF-κB (Fig. 1A) by sequence analysis [Jones et al., 1997]. Nuclear extracts from the untreated or the treated cells interacted with ^{32}P-labeled SP-1-3′ or SP-1-5′ oligonucleotides, forming two bands (Fig. 7A, B). Binding of the complexes to the SP-1-3′ or SP-1-5′ probes was blocked by an excess of an unlabeled SP-1-3′ (Fig. 7A, lanes 2 and 7), or SP-1-5′ oligonucleotides (Fig. 7B, lanes 2 and 7), respectively. We also used a SP-1, a C/EBP, or a NF-κB consensus oligonucleotide as an unlabeled competitor. Although the putative C/EBP binding site is contained in the SP-1-3′ oligonucteotide, and the putative NF-κB site in the SP-1-5′ oligonucleotide (Fig. 1A), a C/EBP or a NF-κB consensus oligonucteotide failed to prevent formation of the SP-1-3′ (Fig. 7A, lanes 5 and 10) or SP-1-5′ protein complexes (Fig. 7B, lanes 4 and 9), respectively. Only a SP-1 oligonucleotide prevented formation of the SP-1-3′ (Fig. 7A, lanes 3 and 8) and SP-1-5′ protein complexes (Fig. 7B, lanes 3 and 8), suggesting a possible involvement of SP-1 family proteins in the assembly of these complexes. To confirm the possibility, supershift analysis was carried out with the SP-1-3′ or SP-1-5′ probe. Both antibodies to SP-1 and SP-3 supershifted the SP-1-3′ and SP-1-5′ protein complexes, whereas anti-C/EBP β and δ, or anti-p65 and p50 NF-κB antibodies had no effect (Fig. 7C, D). These results indicate that the SP-1 family proteins bind to the distinct two binding sites within the minimal promoter region of the Mn-SOD gene, and are required for the basal transcription of the gene. Although the nucleotide −231 to −170 encompasses the putative C/EBP and NF-κB binding sites, both C/EBP and NF-κB proteins failed to bind to the region. These results were consistent with those of the transfection studies.

DISCUSSION

In a previous report, C/EBP β and NF-κB binding sites were identified within the TNF-responsive element (TNFRE) in the second intron of the Mn-SOD gene [Jones et al., 1997]. Although these investigators identified a NF-κB-binding site within the 3′ NF-κB-related region (Fig. 1B), they concluded that NF-κB did not act as a transcription factor for Mn-SOD gene transcription through the site. In this study, we identified the critical *cis*-acting element interacting with NF-κB, distinct from the previously described NF-κB transcription factor-binding site resided in the 3′ NF-κB-related region.

The importance of the C/EBP site and the newly identified NF-κB site that regulate Mn-SOD gene transcription was demonstrated by the ability of mutations at these sites to substantially reduce transcriptional activity induced by TNF-α (Fig. 3A, B). Mutation of the NF-κB site reduced TNF-α-mediated transcriptional activity to nearly the background level,

suggesting that the NF-κB site is indispensable for TNF-α-mediated transcriptional activation through the intronic enhancer region. Computer searches show that there are several transcription factor-binding motifs around the NF-κB site, such as C/EBP-related, c-Ets-related, and signal transducers and activators of transcription (STAT)-related binding sites. EMSA with C/EBP β and δ antibodies indicated that C/EBP β and δ do not bind to the NF-κB site, but only to the C/EBP site (Fig. 6). Despite the lack of identification of a classical NF-κB-binding site, we provided the first evidence that NF-κB proteins that were activated by TNF-α, could bind to the newly identified NF-κB site, using competition and supershift EMSAs (Fig. 5A, B).

Activation of the transcription factor NF-κB by TNF-α is essential for eliciting an effective response, as many of the TNF-α-regulated genes contain binding sites for NF-κB. We have examined the effects of NF-κB family proteins on the induction of Mn-SOD gene transcription by the cotransfection assay. Our studies indicate that p65 NF-κB plays the central role in activating the Mn-SOD transcription through the newly identified NF-κB binding site within the enhancer region (Fig. 4). Although p50 NF-κB showed an affinity to the NF-κB site by EMSA, p50 NF-κB did not significantly affect the transcriptional activity. p65 may be necessary for the activation, because p65 contains two transactivation domains in the C-terminal region of the protein [Ghosh et al., 1998; Fujita et al., 1992; Schmitz et al., 1994], whereas p50 is devoid of the domains.

C/EBPs are a family of transcription factors that regulate genes involved in acute phase response, cell growth, and differentiation [Chang et al., 1990; Lee et al., 1993; Birkenmeier et al., 1989; Akira et al., 1990]. Expression of C/EBP β and C/EBP δ is elevated in several tissues as a part of acute-phase response. Both C/EBP β and C/EBP δ showed strong affinity for the C/EBP site by EMSA (Fig. 6); however, C/EBP β did not induce the transcriptional activity (Fig. 4). We examined the role of the other C/EBP isoforms in cotransfection studies. Cotransfection of C/EBP δ expression vector with SVpro-WT into the cells activated the Mn-SOD transcription (data not shown), suggesting that C/EBP δ may affect the transcriptional activity through the C/EBP-binding site. Interestingly, several promoters of immune or acute-phase-responsive genes, such as the IL-6 gene [Isshiki et al., 1990], the interleukin-8 (IL-8) gene [Mukaida et al., 1990], and the angiotensinogen gene [Brasier et al., 1990], have adjacent or overlapping binding sites for NF-κB and C/EBP. Such interactions are implied by the close proximity of the NF-κB- and C/EBP-family binding sites in the Mn-SOD enhancer. Clarification of the roles for distinct NF-κB family proteins, C/EBP family proteins, and other potential factors requires further examination.

In this study, we used two types of luciferase reporter vector: pGL3 promoter vector (SVpro), which contains the basic enhancerless SV40 promoter, and pGL3 basic vector, which contain the murine Mn-SOD promoter region nucleotide −231 to +11 (Mnpro). Both SVpro and Mnpro produced low background luciferase activity in the untreated cells and did not increase activity by stimulation with TNF-α, or by cotransfection of p65 (Figs. 3, 4), suggesting that these promoters do not contain the site mediated by TNF-α, or p65. Both constructs containing the second intron (2119–2420), SVpro-WT, and Mnpro-WT, producing the high activities by stimulation with TNF-α, or by cotransfection of p65, indicate that the intronic enhancer region may function independent of these promoters. Mn-SOD contains a GC-rich promoter lacking a TATA- and a CAAT-box [Jones et al., 1995; Kuo et al., 1999]. EMSAs and the transfection studies showed that SP-1 and SP-3 can bind the two distinct sites within the nucleotide −231 to −170. This is required for basal transcriptional activity (Figs. 2, 7), suggesting that SP-1 family proteins may act primarily on basal Mn-SOD gene expression.

In summary, we provide a refined characterization of two distinct *cis*-acting elements in the second intronic region of Mn-SOD gene. A NF-κB p65 subunit is indispensable for activating Mn-SOD gene transcription mediated by TNF-α through the newly identified NF-κB site within the enhancer region. Our findings help explain the mechanism by which NF-κB proteins activate transcription of the Mn-SOD gene underlying immunological and inflammatory processes.

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