Transcriptional regulation of the human manganese superoxide dismutase gene: the role of specificity protein 1 (Sp1) and activating protein-2 (AP-2)

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Manganese superoxide dismutase (MnSOD) plays an important role in regulating cellular redox conditions. Expression of MnSOD has been shown to protect against damage by oxidative stress and to suppress the malignant phenotype of human cancer cells. We have previously cloned the human MnSOD (*SOD2*) gene and analysed its 5′ proximal promoter, which has been characterized by a lack of a TATA or CAAT box and the presence of multiple GC boxes. To define further the molecular mechanisms for the regulation of MnSOD expression, multiple transcription factor-binding motifs containing overlapping specificity protein 1 (Sp1)- and activator protein (AP)-2-binding sites were identified by DNase I footprinting analysis. Functional studies in three cell lines with different levels of Sp1 and AP-2 proteins suggested that the cellular levels of these proteins may differentially regulate transcription via GC-binding motifs in the human *SOD2* promoter. Co-transfection of an Sp1 expression vector resulted in an increase in the transcription of the promoter-driven reporter gene. In contrast, co-transfection of the AP-2 expression vector caused a decrease in transcription. Direct mutagenesis analysis of Sp1- and AP-2-binding sites showed that Sp1 is essential for transcription of the human *SOD2* gene, whereas AP-2 plays a negative role in the transcription. Immunoprecipitation of Sp1 and AP-2 proteins demonstrated that Sp1 interacts with AP-2 *in vivo*. Two-hybrid analysis revealed that interaction between Sp1 and AP-2 plays both a positive and negative role in the transcription of the reporter gene *in vivo*. Taken together, our data indicate that AP-2 down-regulates transcription of the human *SOD2* gene via its interaction with Sp1 within the promoter region. These findings, coupled with our previous observation that several cancer cell lines have mutations in the promoter region of the human MnSOD gene, which lead to an increase in an AP-2-binding site and a decrease in the promoter activity, signal the importance of understanding the promoter structure and the regulation of the human *SOD2* gene by Sp1 and AP-2.

Key words: enzyme induction, oxidative stress, tumour suppressor gene.

INTRODUCTION

Much evidence implicates reactive oxygen species (ROS) as a critical etiological factor in a variety of pathological conditions, including aging [1], atherosclerosis [2] and neurodegenerative diseases, such as amyotrophic lateral sclerosis [3] and Parkinson's disease [4]. Additionally, it has been suggested that ROS may play a role in tumour development by inducing DNA damage, enhancing tumour promotion and stimulating cell proliferation [5]. Antioxidant enzymes play an important role in protecting cells from oxidative stresses. The primary antioxidant enzymes are superoxide dismutases (SODs), catalases and glutathione peroxidases. The known function of SOD is to catalyse the dismutation of the superoxide (O_2^-) radical into H_2O_2, which is further converted into water by catalase or glutathione peroxidases. In humans, there are three forms of SOD: a homodimeric copper–zinc SOD (Cu,ZnSOD or SOD1) found primarily in the cytosol; a homotetrameric manganese SOD (MnSOD or SOD2) located in the mitochondria matrix; and a homotetrameric glycosylated Cu,ZnSOD in the extracellular space (ECSOD or SOD3) [6].

Within the SOD family, MnSOD has been shown to be essential for survival of aerobic life [7]. These findings are not unexpected as mitochondria are a major subcellular source of ROS [8] and play pivotal roles in apoptosis [9]. MnSOD knockout mice develop neonatal lethality resulting from cardiomyopathy [10] and neurodegeneration [11]. Several transgenic studies have shown that MnSOD protects against oxidative stress-induced cellular apoptosis and injury [12–17]. In cell culture models, overexpressing MnSOD significantly reduces cell death mediated by the toxic effects of tumour necrosis factor ('TNF') [12], iron [13], nitric oxide [14], alkalosis [15] and chemical hypoxia [9]. In transgenic mice, expressing human MnSOD in the mitochondria protects animals from oxygen-induced lung injury [16], adriamycin-induced cardiac toxicity [17] and ischaemia-induced brain injury [13]. Additionally, numerous studies suggest that MnSOD may act as a new type of tumour suppressor (for review see [6]), by modulating the activity of redox-sensitive transcription factors [18] and specific signal mediators [19].

Human MnSOD is a 88.6 kDa nuclear-encoded mitochondrial antioxidant enzyme containing four identical subunits and one Mn^{2+} per subunit. The human *SOD2* gene was initially assigned to chromosome 6 and subsequently localized to 6q25 [20]. To study the regulation of MnSOD expression under normal physiological and pathological conditions, we have cloned the human *SOD2* gene, including a 3.4 kb 5′-flanking region and a 1.36 kb 3′-flanking region. The human *SOD2* gene is a single-copy gene containing five exons interrupted by four introns. The transcription-initiation site is preceded by a promoter, which is characterized by its lack of a TATA or CAAT box and the

Abbreviations used: ARE, antioxidant response element; AP, activating protein; DTT, dithiothreitol; SOD, superoxide dismutase; NF-κB; nuclear factor κB; ROS, reactive oxygen species; Sp1, specificity protein 1.

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presence of a GC-rich region, containing multiple specificity protein 1 (Sp1)-binding sites [21]. The human, bovine and mouse MnSOD cDNAs share more than 90% similarity [22]. The 5′-proximal region also appears to be highly conserved in the GC-rich region [23,24]. We have demonstrated that multiple Sp1 and activator protein (AP)-2-binding sites constitute the basal promoter, and that Sp1 is an important regulator for the basal expression of the human *SOD2* gene [25]. We have found in several cancer cell lines a set of unique mutations in the basal promoter region of the human *SOD2* gene [26]. These mutations lead to an increase in the AP-2-binding site and a decrease in the transcription activity of the promoter. These results suggest that Sp1 and AP-2 may have opposite roles in regulating transcription of the human *SOD2* gene. Because it has been well documented that increased expression of MnSOD suppresses tumorigenesis [5], differential regulation of the basal promoter activity may play an important role in the development of cancer. In the present study, we performed in-depth analysis of the human *SOD2* promoter and the roles of Sp1 and AP-2 on the transcriptional regulation of the human *SOD2* gene. Systematic deletion analysis using the 5′ deletion-driven luciferase reporter gene was performed to determine the relative importance of each GC-binding motif. DNase I footprinting analyses with purified Sp1 and AP-2 proteins were performed to map the corresponding binding sites in the promoter. The regulatory roles for Sp1 and AP-2 in the transcription of the human *SOD2* gene were explored further using the promoter-reporter constructs co-transfected with Sp1 or AP-2 expression vectors in VA-13, HeLa and HepG2 cells, which have different levels of endogenous Sp1 and AP-2 proteins. Site-specific mutagenesis analysis was also performed to determine the contribution of Sp1- and AP-2-binding sites in regulating the transcription of the human *SOD2* gene. Finally, immunoprecipitation coupled with Western-blot analysis of the immunoprecipitated proteins and cDNAs encoding Sp1 and AP-2 proteins linked to GAL4 or VP16 proteins in a mammalian two-hybrid system were used to verify *in vivo* interaction of Sp1 and AP-2.

MATERIALS AND METHODS

Cell culture and transfections

Human cervical carcinoma (HeLa), human hepatocellular carcinoma (HepG2) and simian-virus-40-transformed human lung fibroblast (VA-13) cell lines were obtained originally from the American Type Culture Collection (Manassas, VA, U.S.A). HeLa, HepG2 and VA-13 cells were maintained in Dulbecco's modified Eagle's medium, Dulbecco's modified Eagle's medium/Ham's F12 medium and Eagle's basal medium respectively. The media were supplemented with 10% (v/v) fetal-bovine serum, 200 μM L-glutamine and 1% antibiotics (Life Technologies, Gaithersburg, MD, U.S.A.). The cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were plated at a density of 5×10^5 cells in a 6-well plate. After an overnight culture, the cells were co-transfected with 1 nM experimental plasmid constructs and 0.1 nM *Renilla* luciferase, or with 1 μg of β-galactosidase as an internal control, using a modified calcium phosphate method. In the Sp1 and AP-2 co-transfection experiments, various concentrations of Sp1 or AP-2 expression vector were co-transfected with the MnSOD promoter-driven reporter constructs into VA-13 or HepG2 cells. After 48 h, the cells were washed twice with PBS and lysed in a passive lysis buffer (Promega, Madison, WI, U.S.A.). The samples were analysed for luciferase activity using the Dual-Luciferase® Reporter Assay System (Promega). β-Galactosidase activity was measured using *o*-nitrophenyl β-D-galactopyranoside ('ONPG'; Aldrich Chemical Co., Milwaukee, WI, U.S.A.) as a colorimetric substrate.

Plasmids and site-directed mutagenesis

A 39 b λ phage clone containing the entire human MnSOD gene [21] was used to generate deletion fragments from the 5′-flanking regions by PCR. All PCR products were obtained by a high-fidelity *pfu* DNA polymerase (Stratagene, La Jolla, CA, U.S.A.). The following 15 primers were used to create 5′ deletions and then subcloned upstream of the luciferase reporter gene. The underlined 9 bp indicate the *Kpn*I site in the upper-strand primers or the *Bgl*II site in the lower-strand primers. Upper-strand primers: (−3400), CGGGGTACCCTTACAATGGAGATAGTGGGCCA; (−2987), CGGGGTACCGACCATGAGGCAGCTTTGAAGACA; (−1605), CGGGGTACCAGATCACTTGAGGTCAGGCGTTCG; (−1240), CGGGGTACCCCTGTTGTGAAGCCAAGTTCAGGT; (−890), CGGGGTACCTACTGTAAACACACAAAACATGAC; (−555), CGGGGTACCGCTGGCTCTACCCTCAGCTCATAG; (−400), CGGGGTACCAAACTCAGGGGCAGGCGCCGCAG; (−210), CGGGGTACCCCTCCTTTCTCCCGTGCCCTGGGC; (−154), CGGGGTACCCAGGCACGCAGGGCACCCCCGGG; and (−76), CGGGGTACCGCCCTTGCGGCGCAGCTGGGGTCG. Lower-strand primers: (−380), GGAAGATTCTGCGGCGCCTGCCCCTGAGTTTTCC; (−190), GGAAGATCTCAGGGCACGGGAGAAAGGAGGC; (−130), GGAAGATCTACCCCGGGGGTGCCCTGCGTGCCT; (−52), GGAAGATCTGCGACCCCAGCTGCGCCGCAAGGG; (+24), GGAAGATCTGCCGAAGCCACCACAGCCACGAGT. PCR was carried out in a 50 μl master mixture containing 10 mM each of dATP, dTTP, dCTP and dGTP or 7-deaza-2′-dGTP for GC-rich regions as described previously [27]. The ends of all PCR products were filled in with a Klenow fragment of DNA polymerase I. The 5′ deletion fragments were subcloned into a pGL3 luciferase vector (Promega). DNA sequencing analysis was used to confirm the nucleotide sequences and orientations of all constructs generated.

Site-specific mutations of Sp1- and AP-2-binding sites in the promoter were designed on the basis of published data [28,29], in which the resulting mutated sites were confirmed by competitive gel-shift analysis using a Chameleon™ double-stranded site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The selection primer corresponding to a change of unique *Xba*I (underlined bases) site was GATCGCCGTGTAATTCTGGAGTCGGGGCGGCCGGCCGCTTC. The mutated primers to target each Sp1- and AP-2-binding site (underlined sequences with the mutated nucleotides in lower case) were designed as follows: (Sp1-M1), CAGCTCATAGGCCGGCTGaatGGCGCTGACCAGCAGCTAG; (Sp1-M2), CCCCGCCGGCACCCTCAGGaatGGACCGGAGGCAGGGCCTTCG; (Sp1-M3), GTGTACGGCAAGCGCGGGtaaaCGGGACAGGCACGCAGGG; (Sp1-M5), CTTTCTTAAGGCCCGCGaatGGCGCAGGAGCGGCACTC; (AP-2-M1), CCCTCAGCTCATAGGCCaaaTGGGCGGCGCTGACCAGCAGCTAG and AGGCCTCTGGACCaaaCGGCCCCaaaGCAGCGCAACCAAAACTC; (AP-2-M2), GCCTAGTGCAGCCAGATCCaaaCCGGCACCCTCAGGGGCGGACCGGAGGC and GCCGCCTCCCTTCGGaaaCGCGCCACTCAAGTACGGC; (AP-2-M3), CTCCCGTGCCCTGGGCaaaaGGTGT ACGGCAAGCGCGGGCGGGCGGGACAG; and (AP-2-M5), GCGCTTTCTTAAGGCttCGGGCGGCGCAGGAGCGGC. Sp1-M4 and AP-2-M4 were made by inserting mutations in double complementary

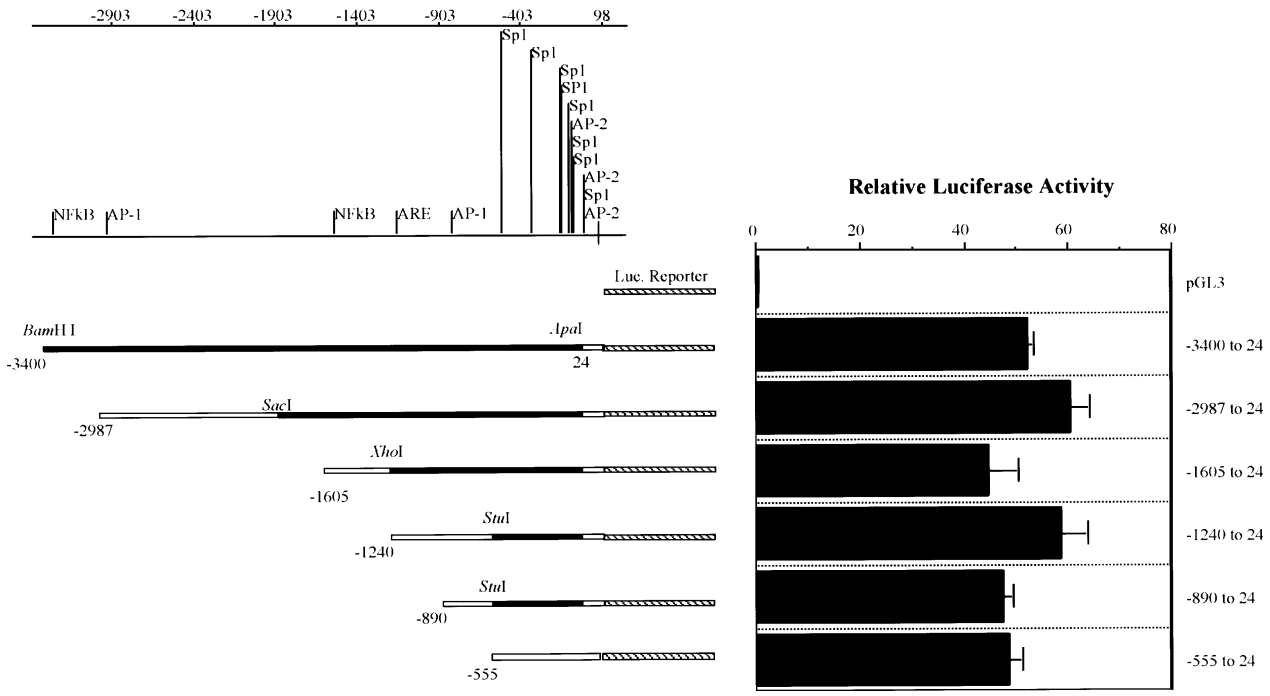

Figure 1 Effects of the 5′-flanking region of the human *SOD2* gene and its deletions on luciferase reporter activity

Left: schematic representation of the reporter gene under the control of the 5′-flanking region and its deletions. Deleted fragments were generated by restriction digestion (solid box) or by PCR (open box) to systematically remove each potential transcription factor-binding site. The luciferase reporter (Luc. Reporter) is also indicated (shaded box). The corresponding transcription factor-binding sites for NF-κB, AP1, ARE, Sp1 and AP-2 are marked. Right: deletion constructs were transiently transfected into HeLa cells and the relative luciferase activity was determined. Luciferase activities were normalized to *Renilla* luciferase as an internal control. Results are means ± S.E.M. performed in triplicate from three separate experiments.

oligonucleotides. After annealing, the double strands were cloned between *Sma*I and *Pvu*II restriction sites. Two sets of oligonucleotides were used as follows: (Sp1-M4), GGGGTTGGGCGCGGCGGGCGCGaaatGGGGCCCGCGGGGGGGGaaatGGttatGCGGTGCCCTTGCGGCGCAG and CTGCGCCGCAAGGGCACCGCataaCCatttCCCCCCCCGCGGGCCCCatttCGCGCCCGCCGCGCCCAACCCC; and (AP-2-M4), GGGGTTGGGCaattCGGGCaaGGGGCGGGGCCCaaaGGGGGGGGGGCGGGGCGGCaaTGCCCTTaaGGCGCAG and CTGCGCCttAAGGGCAttGCCGCCCGCCCCCCCCCCtttGGGCCCCGCCCCttGCCCGaattGCCCAACCCC. All mutations in the binding sites were ascertained by DNA sequencing. The internal restriction sites *Stu*I, *Nar*I, *Mst*II, *Pst*I, *Sma*I and *Pvu*II were used to link mutations for the individual sites for Sp1 and AP-2.

DNase I footprinting analysis

In vitro DNase I footprinting analysis was performed to locate Sp1- and AP-2-binding sites in the promoter region. Two fragments (−555 to −190 and −210 to +24) were obtained by *Cla*I and *Hin*dIII digestion, and were labelled with [γ-^{32}P] to the 5′ hydroxy-terminus of nucleoside by T4 polynucleotide kinase (New England Biolabs, Beverly, MA, U.S.A.). To generate single end-labelled fragments, the labelled fragments were digested by *Kpn*I to remove the ends of the upper strands or by *Bgl*II to remove the ends of the lower strands. Purified Sp1 or AP-2 protein (Promega) was incubated with the single end-labelled fragments, and the DNA was partially digested by RNase-free DNase I. The samples were separated on a 6% (w/v) polyacrylamide sequencing gel.

Preparation of nuclear extracts and electrophoretic mobility-shift assay

Nuclear extracts were isolated from $(1–2) \times 10^7$ cells per ml of exponentially growing HeLa, HepG2 and VA-13 cells as described by Dignam et al. [30]. The cells were rinsed twice in PBS and resuspended in three pellet volumes of buffer A [10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT) and 0.2 mM PMSF]. After incubation on ice for 13 min, nuclei were obtained by centrifugation at 14000 ***g*** for 2 min at 4 °C and suspended in two pellet volumes of buffer B [20 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM DTT, 0.2 mM PMSF and 1 μg/ml protease inhibitors (pepstatin, aprotinin and leupeptin)]. After incubation on ice for 20 min, extracts were centrifuged at 12000 ***g*** for 2 min at 4 °C to remove nuclear debris. Supernatants were aliquoted and stored at −80 °C. Protein concentration was determined by a colorimetric assay (Bio-Rad, Richmond, CA, U.S.A.).

Sp1 consensus double-stranded oligonucleotides were purchased from Promega. The 5′ end of the oligonucleotides was labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase. A SOD2-AP2 probe was used to detect AP-2 binding. These probes contained a binding site for AP-2 in the rat MnSOD promoter (upper strand, 5′-AGCTCAAGCCCGCGGGCTC-3′; lower strand, 5′-TCAGAGCCCGCGGGCTTG-3′) [31]. Complementary single-stranded oligonucleotides were annealed and radioactively end labelled by [α-^{32}P]dCTP in a fill-in reaction of the 5′ overhangs with Klenow DNA polymerase (New England Biolabs). The probes were purified on a 20% (w/v) native polyacrylamide gel. The gel piece containing the band

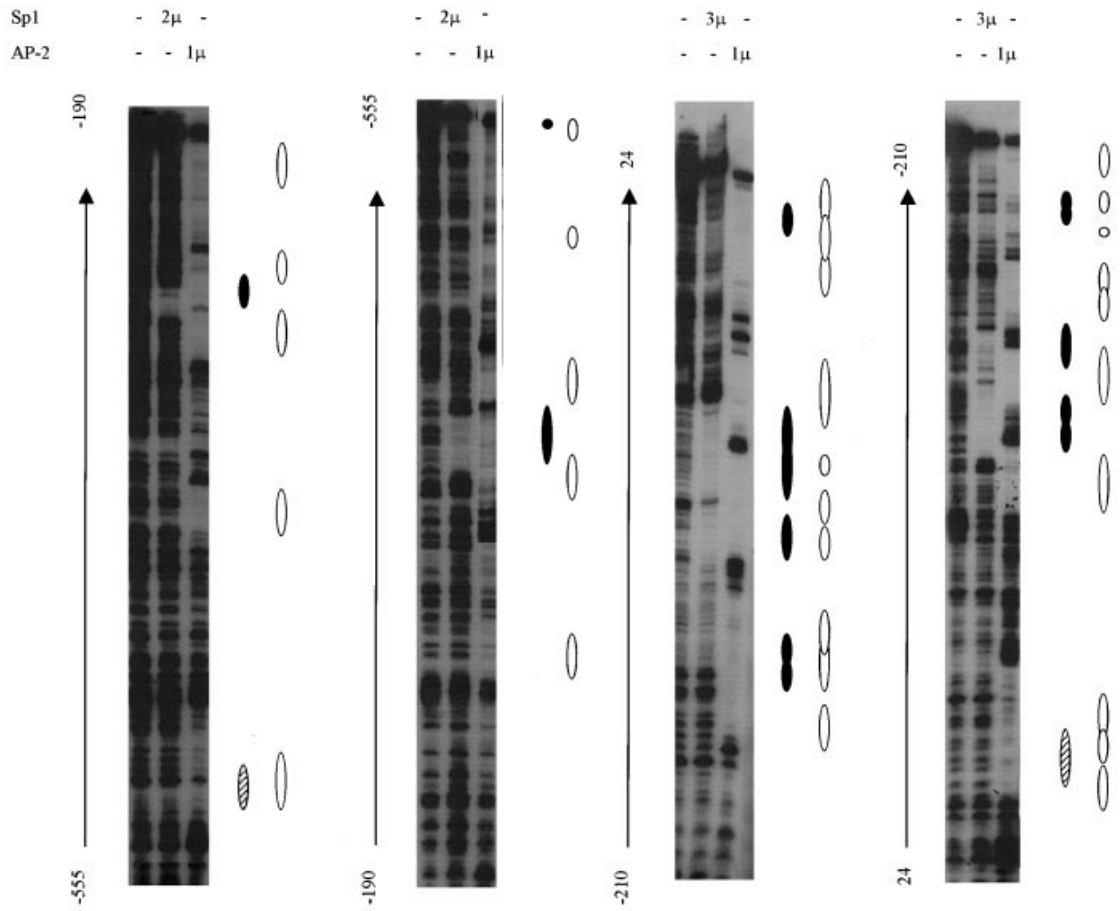

Figure 2 Localization of Sp1- and AP-2-binding sites in the promoter region of the human *SOD2* gene

DNase I footprinting analysis was performed using two single-end labelled promoter fragments with the purified Sp1 and AP-2 proteins. The length and orientation of the promoter fragments are shown on the left. The corresponding Sp1- and AP-2-binding sites are indicated on the right. Solid ovals indicate the protection by Sp1, and open ovals indicate the protection by AP-2. The shaded ovals represent Sp1-binding sites that cannot be detected, but can be detected on the complementary strand. μ, binding units.

corresponding to the double-strand DNA was excised and eluted overnight at 37 °C in 400 μl of TE buffer [10 mM Tris/HCl (pH 7.4) and 1 mM EDTA]. A portion (5 μg) of nuclear proteins from HeLa, HepG2 and VA-13 cells was incubated with ^{32}P-labelled Sp1 or AP-2 probes for 20 min at 22 °C in a binding buffer [10 mM Tris/HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 5 % (v/v) glycerol, 1 mM $MgCl_2$ and 1 mM DTT]. For supershift analysis, nuclear proteins were preincubated with ^{32}P-labelled probes for 20 min followed by incubation with 0.4 μg of anti-(Sp1) or -(AP-2) sera (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for an additional 1 h at 22 °C. Samples were separated on a polyacrylamide gel in 0.5 × Tris-borate-EDTA buffer [450 mM Tris/borate (pH 8.0) and 1 mM EDTA]. After electrophoresis, the gels were dried and exposed to Kodak film for 12–48 h at −70 °C.

Immunoprecipitation analysis

To determine whether Sp1 interacted with AP-2 *in vivo*, VA-13 cells were transfected with the expression plasmids RSV-AP2 (kindly provided by Dr Trevor Williams, Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT, U.S.A.) and pAC-Sp1 (originally provided by Dr Robert Tjian, Molecular and Cell Biology Department, Howard Hughes Medical Institute, University of California at Berkeley, Berkeley, CA, U.S.A.). Cells were co-transfected with the Sp1 and AP-2 expression plasmids or vectors controls. Cells were harvested after 36 h and nuclear extracts were prepared for immunoprecipitation. Nuclear extracts were obtained from VA-13 or HepG2 cells in RIPA buffer [9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 150 mM NaCl (pH 7.4), 1 % (v/v) Nonidet P40, 0.5 % sodium deoxycholate, 0.1 % SDS, 10 mg/ml PMSF and 1 μg/ml aprotinin]. The antibodies used included a rabbit anti-(AP-2) antibody (Santa Cruz Biotechnology) and an agarose-conjugated anti-(Sp1) antibody (Santa Cruz Biotechnology). Nuclear extracts were incubated overnight with the corresponding antibodies at 4 °C. After incubation for 4 h, Protein A/G (20 μl; Santa Cruz Biotechnology) was added to the reaction with the anti-(AP-2) antibody. Immunoprecipitates were collected by centrifuging the samples at 605 ***g*** for 5 min, and washing them four times with RIPA buffer. Samples were resuspended in the sample loading buffer, separated by SDS/PAGE [12.5 % (w/v) gel] and then transferred on to nitrocellulose. After transfer, samples were analysed by Western blotting.

In vivo two-hybrid analysis

To determine the effects of the interaction of Sp1 and AP-2 on regulation of the transcription of the reporter gene, protein–protein interaction studies were performed using a mammalian two-hybrid system (Promega). Two sets of primers were used to amplify Sp1 and AP-2 cDNA from original clones [32,33]. The resulting Sp1 and AP-2 PCR products were cloned into pBIND, which contains the GAL4-binding domain, and into pACT, which contains the VP16-activation domain, respectively. Primers with *Bam*HI and *Xba*I sites at the 3′ and 5′ ends were designed for PCR-cloning of Sp1 cDNA as follows: ACAGGTGAGCTTGACCTCACAGCC and TCAGAAGCCATTGCCACTGATATT. Primers with *Bam*HI and *Kpn*I sites in their 3′ and 5′ ends were designed for PCR-cloning of AP-2 cDNA as follows: ATGCTTTGGAAATTGACGGATAAT and GAATTCCGGGGGCACAGGGGTGTG. The fusion-protein expressing constructs were co-transfected with a luciferase reporter gene driven by a promoter composed of a TATA box and multiple GAL4-binding sites (pG5*luc*). Interaction of the two proteins, as GAL4 and VP16 fusion proteins, resulted in an increase in firefly luciferase expression over the negative controls. Data were evaluated using the Systat 6.0 for Windows statistical analysis program.

RESULTS

Multiple GC-binding motifs constitute the basal promoter of the *SOD2* gene

In order to study the regulation of the human *SOD2* gene, a *Bam*HI fragment containing a 3.4 kb 5′-flanking region has been cloned. DNA sequence analysis revealed multiple transcriptional binding sites in the 5′-flanking region, including nuclear factor κB (NF-κB), antioxidant response element (ARE), Sp1, AP-1 and AP-2, which potentially function in regulating the transcription [21]. To examine the contribution of each binding motif, a series of 5′ deletion-driven luciferase reporter constructs were transiently transfected into HeLa cells. The normalized reporter responses showed that a fragment (-555 to 24), containing clusters of Sp1 and AP-2 sites, was sufficient for a high-level transcription of the reporter gene (Figure 1). There was no significant increase in transcriptional activity with any fragment extending beyond -555 from the transcription start site, suggesting that Sp1 and AP-2 may play important roles in the constitutive expression of the human *SOD2* gene.

Localizations of Sp1- and AP-2-binding sites in the promoter region

Computational searches for transcription factor-binding sites indicated that GC boxes located upstream of the transcription-initiation site of the human *SOD2* gene provide overlapping binding sites for Sp1 and AP-2 proteins. To determine the exact binding sites for Sp1 and AP-2 on the human *SOD2* promoter, purified Sp1 and AP-2 proteins were used to protect two fragments of the promoter in both upper and bottom strands using partial DNase I digestion. Eight binding sites for Sp1 and 15 binding sites for AP-2 were identified on both upper and lower strands (Figure 2). The first and last Sp1 sites cannot be detected using upper or lower strands respectively, however, they are detectable on the complementary strands. The majority of AP-2 sites were located adjacent to Sp1 sites in both directions. Several of these Sp1- and AP-2-binding sites shared overlapping CG-binding motifs. The corresponding Sp1- and AP-2-binding sites are summarized in Figure 3. The consensus sequences for Sp1- and AP-2-binding in the promoter of human *SOD2* gene can be characterized as 5′-GGGCGG-3′ and 5′-CCGSVSSC-3′ or 5′-CCGSVSVSC-3′ respectively.

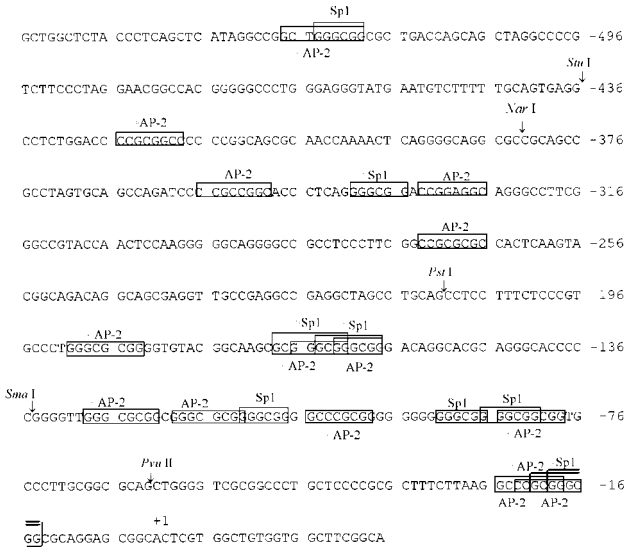

Figure 3 DNA sequences of the human *SOD2* promoter and the location of Sp1- and AP-2-binding sites in the promoter region

The sequence numbers were relative to the transcription-initiation site (+1). Several restriction enzyme recognition sites are marked. The Sp1- and AP-2-binding sites are boxed. The arrowheads indicate the strands by which consensus sequences are detected.

Two GC-binding motifs are necessary for the minimal constitutive expression

In order to investigate the role of each GC-binding motif in the transcription of the human *SOD2* gene, systematic deletion of each GC cluster was generated on the basis of the map generated by footprinting. The results from transfection of these constructs into HeLa cells indicated that all of the binding motifs were required for a high level of transcriptional activity (Figure 4). At least two binding motifs were needed to achieve minimal transcriptional activity in a position-independent manner. It should be noted that motif number 4, which contains a stretch of 70 bp, has several GC-binding domains. Deletion of this motif markedly reduced transcriptional activity. Similar results were obtained when the deletion constructs were transfected into VA-13 and HepG2 cells (results not shown).

Differential roles of Sp1 and AP-2 on the transcription of the human *SOD2 g*ene

To define how Sp1 and AP-2 regulate transcription of the human *SOD2* gene, three cell lines (HeLa, HepG2 and VA-13) were transfected with two promoter fragment-driven reporter genes. The relative levels of Sp1 DNA-binding activities in these cell lines were HeLa > HepG2 > VA-13 (Figure 5A); for AP-2 binding activity, activities were HeLa > VA-13 > HepG2 (Figure 5B). The transcriptional activities of the promoter-reporter constructs showed that transcription was regulated by cellular Sp1 and AP-2 (Figure 5C). Transcriptional activity was lowest in VA-13 cells and highest in HepG2 cells, suggesting that AP-2 may play a negative role in the constitutive repression of the human *SOD2* gene. To determine further the roles of Sp1 and AP-2 in regulating *SOD2* gene expression, the Sp1 expression

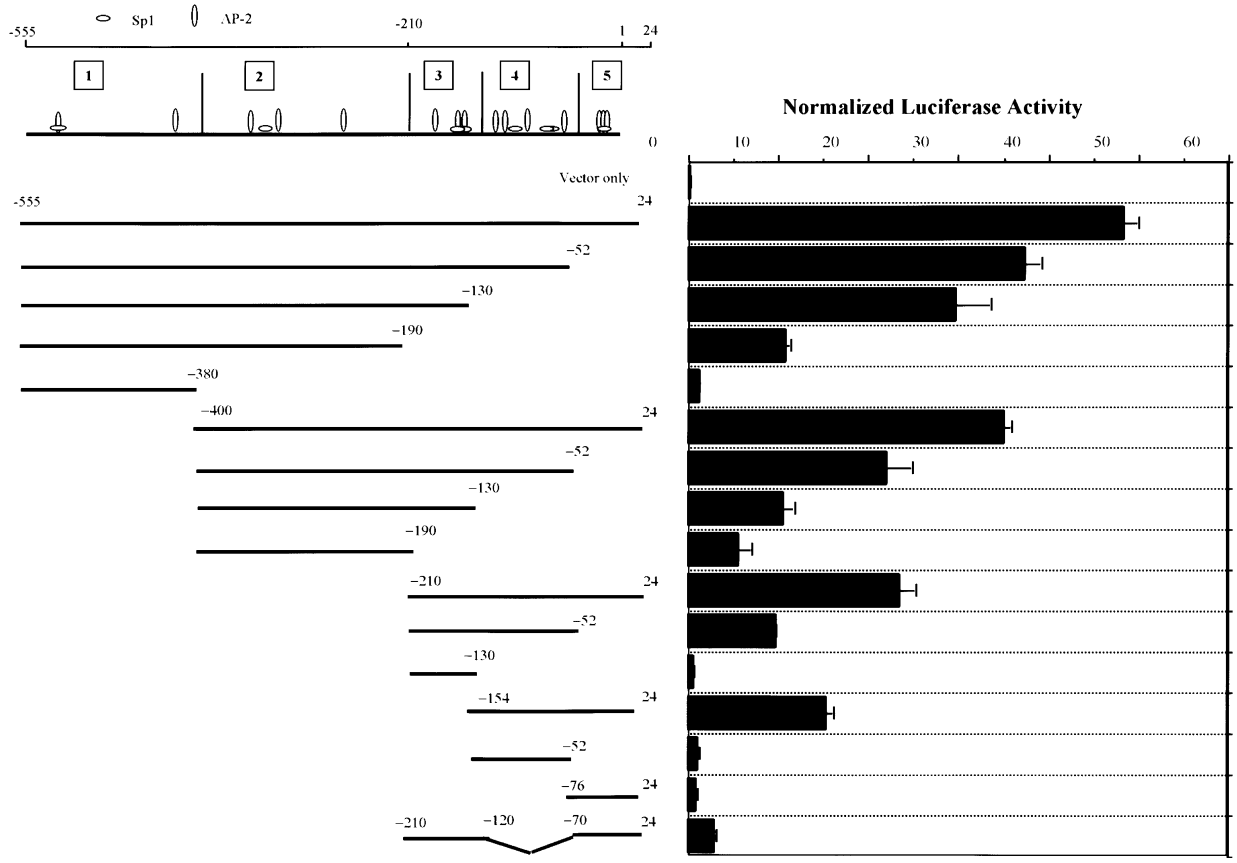

Figure 4 Effects of the selective deletions of the human *SOD2* promoter on the luciferase reporter activity

Left: schematic representation of the deletion strategies in the promoter region. Deletion of five binding domains containing eight Sp1- and 15 AP-2-binding sites in the five GC motifs are illustrated at the top. Deletion fragments were generated by PCR on the basis of the binding motifs and then subcloned into pGL3 to drive the reporter gene. Right: relative luciferase activities of all constructs were determined as described in Figure 1. Results are means ± S.E.M. performed in triplicate from three separate experiments.

construct was co-transfected with the human *SOD2* promoter-driven reporter constructs into VA-13 cells, and the AP-2 expression construct was co-transfected into HepG2 cells. Expression of Sp1 in VA-13 cells resulted in a concentration-dependent increase in transcription (Figure 6A). In contrast, expression of AP-2 in HepG2 cells resulted in a dose–dependent decrease in transcription (Figure 6B). These results indicate that Sp1 up-regulates expression of the human *SOD2* gene, whereas AP-2 down-regulates transcription of the gene.

Structural and functional analysis for Sp1 and AP-2 elements in the promoter region

Deletion analysis of the GC-binding motifs in the promoter region demonstrates that each GC-binding motif contributes to the transcription of the human *SOD2* gene (Figure 4). However, deletion of each motif also alters the length of the promoter and may affect binding of the adjacent sites. To further elucidate the role of each Sp1 and AP-2 element, site-specific mutagenesis analysis for Sp1- and AP-2-binding sites was performed. The initial fragment containing all Sp1- and AP-2-binding sites (−555 to +24) was divided into two fragments (−555 to −190 and −210 to +24), and the role of each Sp1- and AP-2-binding site was determined by its effect on the reporter gene. The results showed that mutations in the Sp1-binding sites significantly reduced transcription and that each binding site had a positive effect on the regulation of the human *SOD2* promoter activity (Figure 7). When all of the Sp1-binding sites were mutated, the transcriptional activity was completely abolished. In contrast with the results obtained from mutations of Sp1, the transcriptional activity increased when AP-2-binding sites were mutated (Figure 8). The only exception to this general feature was the mutation at the first AP-2 element in the second binding motif (Figures 4 and 8), which resulted in a decrease in transcriptional activity. Interestingly, the presence of this AP-2-binding site did not interfere with any Sp1-binding sites in the promoter. It should be noted that two AP-2-binding sites located in motif number four shared their base pairs with two Sp1-binding sites (Figures 3 and 8). Mutations of these sites showed reductions in the transcriptional activities, as the mutations also destroyed Sp1-binding sites (results not shown).

Interaction of Sp1 and AP-2 *in vitro* and *in vivo*

The results from the above experiments demonstrated that AP-2 may play a negative role in the regulation of transcription through modulation of Sp1 binding in the promoter region. Although *in vitro* DNase I footprinting indicated that Sp1 and AP-2 proteins can occupy several common GC motifs in the human *SOD2* promoter, it is not known how these proteins interact with the promoter when both are present. To determine the binding patterns of Sp1 and AP-2 on the overlapping GC

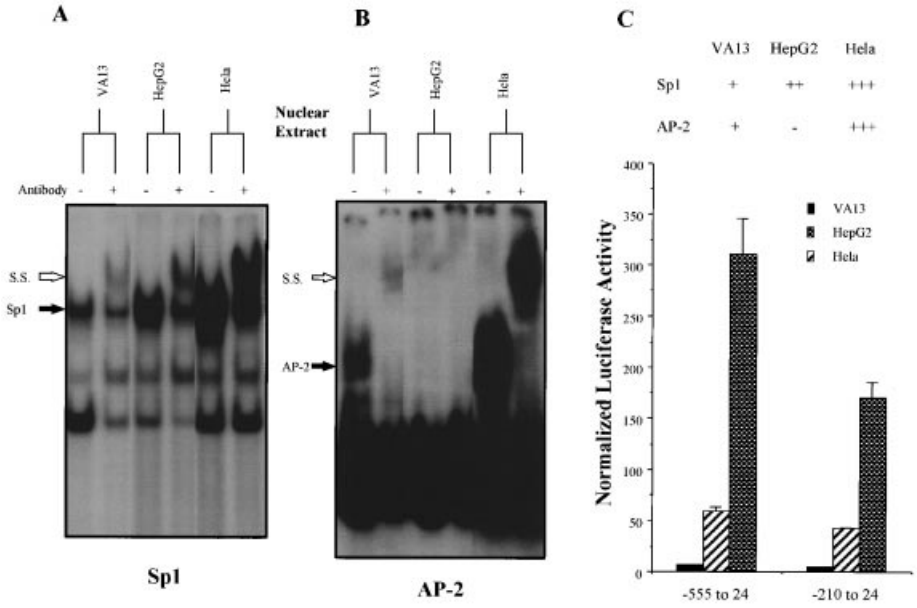

Figure 5 Effects of cellular Sp1 and AP-2 on the luciferase reporter activity under the control of the human *SOD2* promoter

The labelled Sp1 (**A**) and AP-2 (**B**) probes were incubated with nuclear extracts from VA-13, HepG2 and HeLa cells, and analysed by electrophoretic mobility-shift assay. Anti-(Sp1) or -(AP-2) antibodies were used to verify the specific Sp1 and AP-2 bands respectively. Sp1 and AP-2 and their supershifted (S.S.) bands are marked. (**C**) Relative levels of Sp1 and AP-2 in VA-13, HepG2 and HeLa cells are indicated at the top. Two promoter-reporter constructs were transiently transfected into the three cell lines. Relative luciferase activities were determined as described in Figure 1. Results are means ± S.E.M. performed in triplicate from three separate experiments.

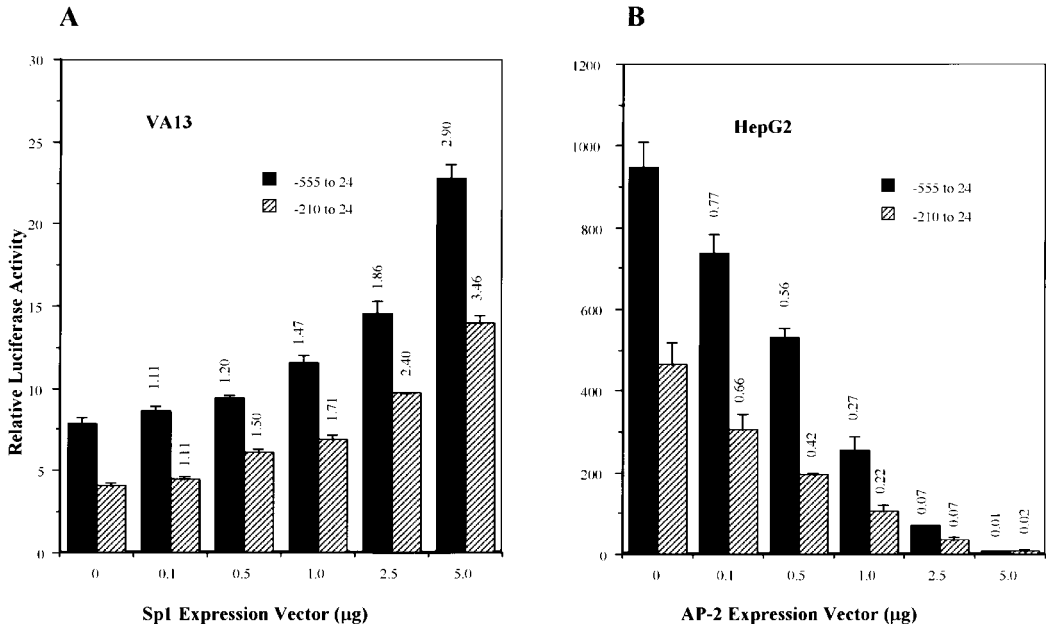

Figure 6 Positive and negative roles of Sp1 and AP-2 on luciferase reporter activity driven by the human *SOD2* promoter

The Sp1 (**A**) or AP-2 (**B**) expression vector was co-transfected with the promoter-reporter constructs into VA-13 or HepG2 cells respectively. Sp1-mediated luciferase activities were determined as described in Figure 1. AP-2-mediated luciferase activities were normalized to β-galactosidase activities as the internal control. Results are means ± S.E.M. performed in triplicate from three separate experiments. The fold increase or decrease after co-transfection with Sp1 or AP-2 relative to the vector control is indicated above the error bars.

motifs, the fourth DNA fragment in the human *SOD2* promoter was used for DNA-binding analysis using the purified Sp1 and AP-2 proteins. The results (Figure 9A) show that both Sp1 and AP-2 proteins bound to this fragment at their respective locations. The presence of AP-2 protein enhanced the intensity of the Sp1 complex (Figure 9A), whereas the presence of Sp1 protein did not have a detectable effect on AP-2 binding (results not shown). These results suggest that AP-2 can modify Sp1

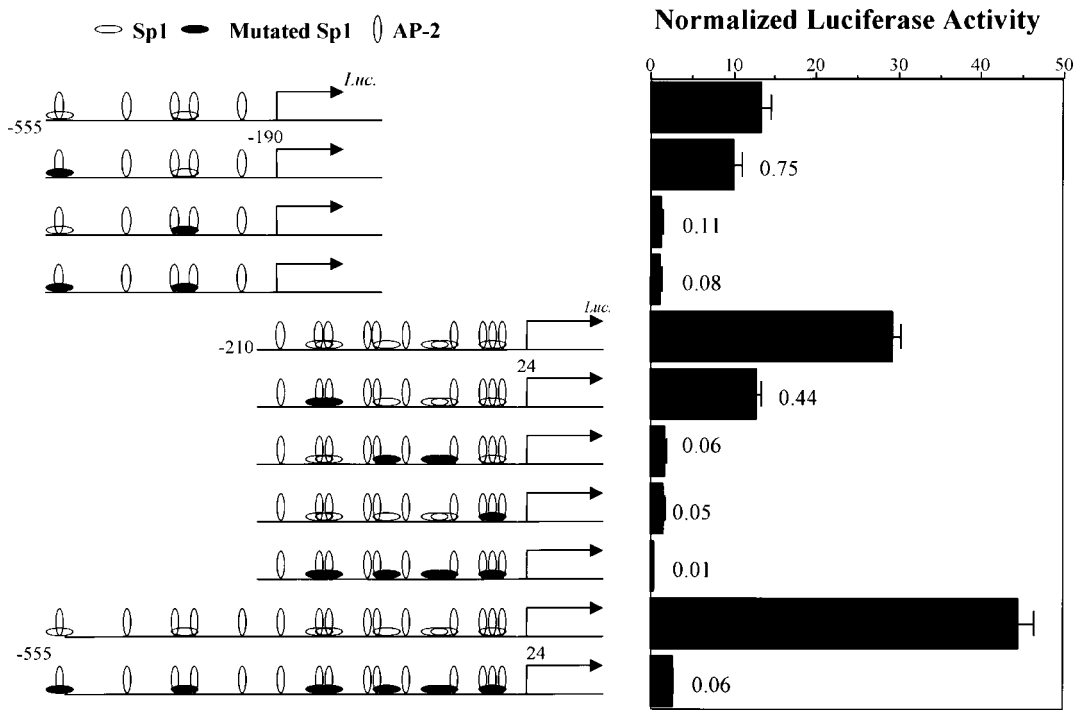

Figure 7 Effect of site-specific mutation of the Sp1-binding sites on the luciferase reporter activity

Left: schematic representation of the direct mutations of the Sp1-binding sites in the human *SOD2* promoter. Luc., luciferase. Right: relative luciferase activities were determined as described in Figure 1. Results are means ± S.E.M. performed in triplicate from three separate experiments. The fold decrease after co-transfection with Sp1 relative to the non-mutated control is indicated by the error bars.

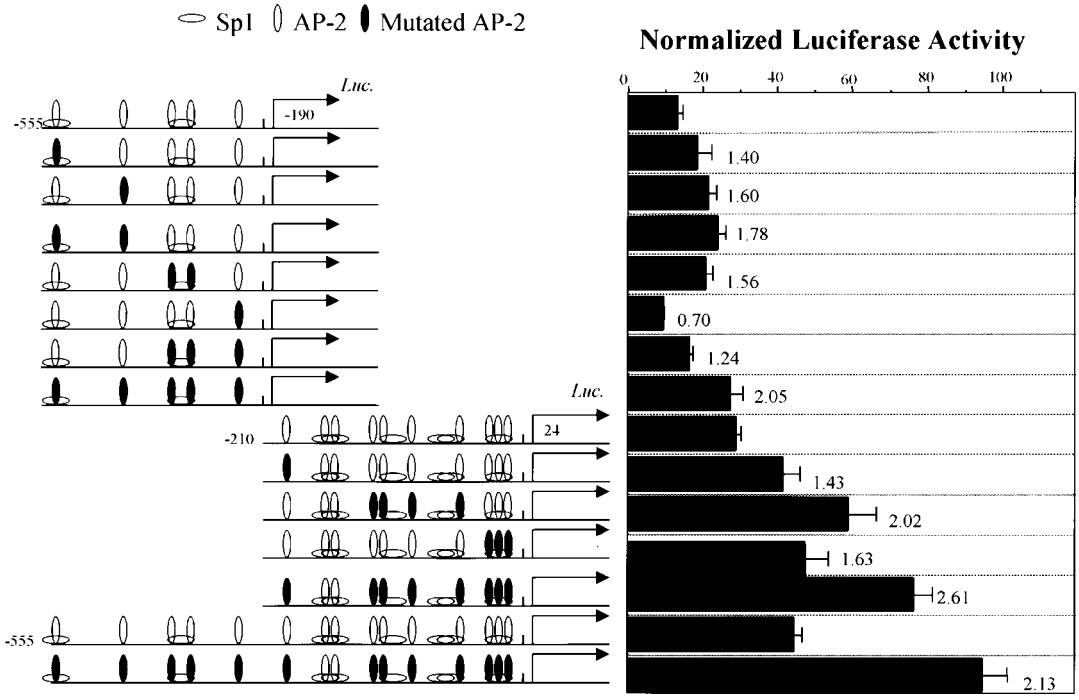

Figure 8 Effect of site-specific mutations of the AP-2-binding sites on the luciferase reporter activity

Left: schematic representation of the direct mutations of the AP-2-binding sites in the human *SOD2* promoter. Luc., luciferase. Right: relative luciferase activities were determined as described in Figure 1. Results are means ± S.E.M. performed in triplicate from three separate experiments. The fold increase or decrease after co-transfection with AP-2 relative to the non-mutated control is indicated by the error bars.

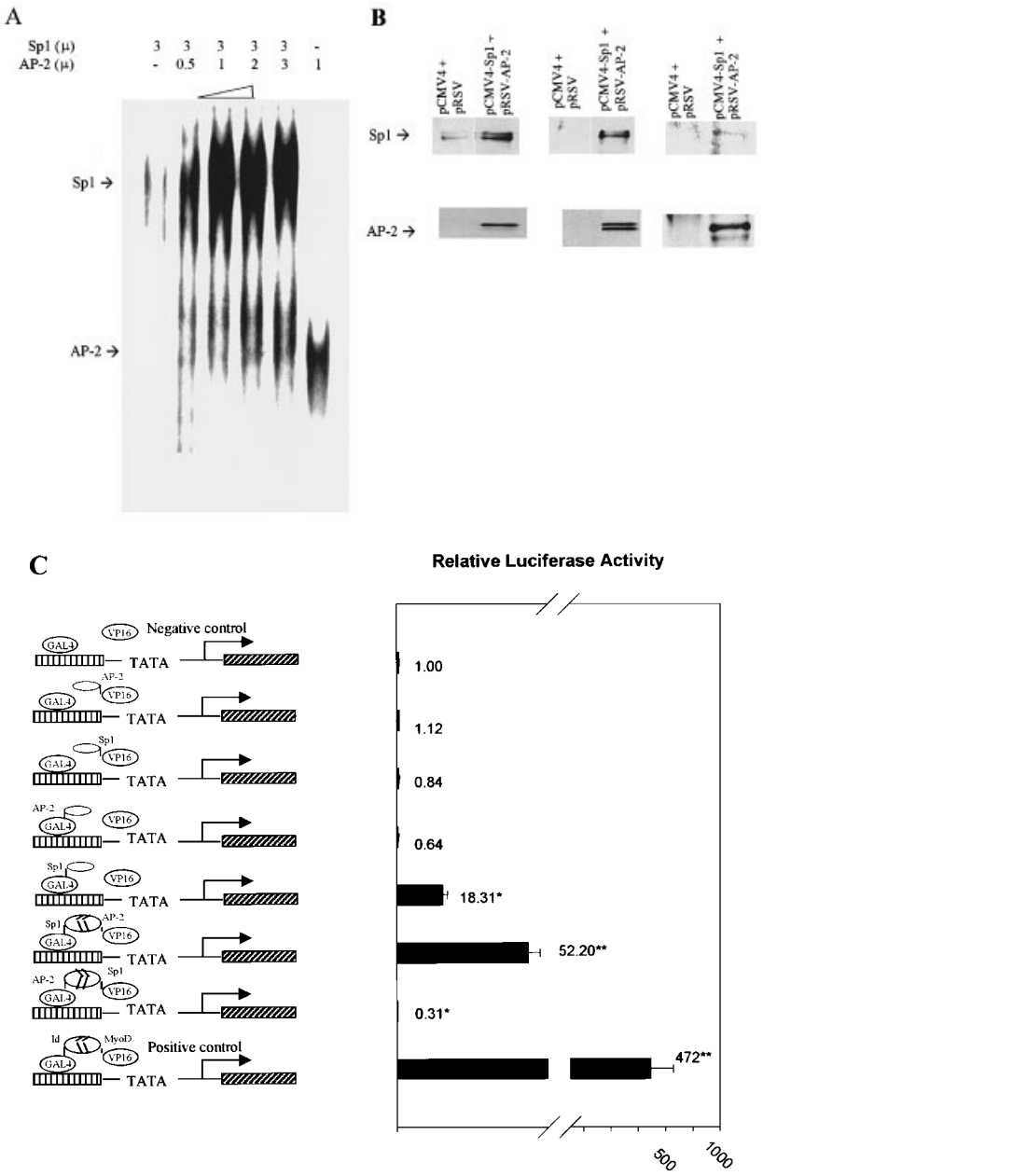

Figure 9 ***In vitro*** **and** ***in vivo*** **analyses of interaction of Sp1 and AP-2 proteins**

(**A**) The fourth GC motif of the human *SOD2* promoter was used as a probe, with or without 3 binding units (μ) of Sp1 and various concentrations of AP-2. Locations of the Sp1 and AP-2 complexes are indicated on the left. μ, binding units. (**B**) Interaction of Sp1 and Ap-2 proteins *in vivo*. Cells were transfected with 6 μg of the control vectors (pCMV and pRSV) or 6 μg of pCMV-Sp1 and pRSV-AP-2 expression vectors. The levels of Sp1 and AP-2 in nuclear extracts were determined by Western blotting (left panel). Nuclear extracts were immunoprecipitated with anti-(Sp1) (middle panel) or anti-(AP-2) (right panel) sera. Polyclonal antibodies and proteins in the resulting precipitates were detected by Western-blot analysis. Membranes were hybridized with anti-(Sp1) sera (top) and re-probed with anti-(AP-2) sera (bottom). (**C**) Interaction between Sp1 and AP-2 in a mammalian two-hybrid system. Left: schematic representation of the integrated Sp1 and AP-2 constructs. Sp1 and AP-2 cDNA were linked to GAL4 or VP16 to generate fusion proteins and were co-transfected with a luciferase reporter gene (hatched box) controlled by several upstream GAL4-binding sites (lined box). Id and MyoD were used as a positive control. Right: relative luciferase activities were determined as described in the legend to Figure 1. Results are means $\pm$ S.E.M. performed in triplicate from three separate experiments. The fold increase or decrease after co-transfection relative to the negative control is indicated above the error bars. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the negative control.

binding, which may interfere with the transcriptional activity of Sp1. These results, and those obtained from overexpression of AP-2 (Figure 7), suggest that AP-2 plays a negative role in the transcription of the human *SOD2* gene.

To determine whether Sp1 and AP-2 interact *in vivo*, we co-transfected VA-13 and HepG2 cells with the Sp1 and AP-2 expression plasmids or control vectors. Immunoprecipitation of the resultant nuclear extracts followed by Western blotting of the precipitates was performed using either the anti-(Sp1) or -(AP-2) antibodies. The results in Figure 9(B) show that both Sp1 and AP2 co-immunoprecipitated with each other, suggesting that Sp1 and AP-2 interact *in vivo*.

To explore the potential mechanism for AP-2-mediated repression of Sp1 transcription *in vivo*, Sp1- and AP-2-encoding regions were linked to the GAL4-binding domain or VP16 activation domain and co-transfected with pG5*luc*, which contains the firefly luciferase reporter gene driven by a TATA box and multiple GAL4 elements (Promega). The VA-13 cell line was used to study the interaction of Sp1 and AP-2 proteins, as these cells express low levels of both endogenous Sp1 and AP-2. As shown in Figure 9(C), when Sp1 cDNA, linked to the GAL4-binding domain, and AP-2 cDNA, linked to the VP16 activation domain, were used, the transcription activity increased in the same manner as the positive control containing Id and myogenic-determination gene D (MyoD) genes. In contrast, the relative luciferase activity was reduced when AP-2 cDNA was linked to GAL4 and Sp1 cDNA was linked to VP16. The results suggest that AP-2 is capable of interacting with Sp1 to enhance GAL4-mediated transcription of the reporter gene. This is consistent with the findings of Pena et al. [34], who demonstrated that AP-2 interacts with Sp1 to enhance transcriptional activation of the *CYP11A1* gene. However, our present results also suggest that AP-2 is capable of interacting with Sp1 to repress GAL4-mediated transcription of the reporter gene when the orientation of Sp1 and AP-2 is alternated. This finding is consistent with the orientation preference of the interaction between Id and E12 proteins as well as Fos and Jun proteins as reported previously [35].

DISCUSSION

Promoter of the human *SOD2* gene

Although much progress has been made in determining the function of SOD2 in protecting against oxidative damage mediated by various environmental and pathological conditions, little is known about how this important gene is regulated. We [21] and others [22–24] have shown previously that the promoter of the *SOD2* gene consists of several GC boxes, but lacks TATA or CAAT boxes, which are typical characteristics of the promoters of housekeeping genes. In the present study, we analysed systematically a 3.4 kb 5′-flanking region of the human *SOD2* gene. Transcriptional analysis of the binding sites located on distal upstream transcription factor-binding sites, including NF-κB, AP-1 and ARE in HeLa cells, revealed that these elements have no effect on the constitutive transcription of the human *SOD2* gene.

A key finding of the present study is that a fragment of approx. 550 bp containing only GC-binding motifs is sufficient for a high level of constitutive transcription of the human *SOD2* gene. Although any fragment consisting of at least two GC-binding motifs is able to support a low level of basal expression, the -555 to $+24$ fragment is necessary for high-level expression. Analysis of the distal deletion constructs indicated that this region confers promoter activity comparable with that observed from fragments containing additional potential transcription factor-binding sites. Within the GC-rich region of the human *SOD2* promoter, all of the GC-binding motifs contribute to the transcriptional activity. Although most of these GC-binding motifs can be replaced by their neighbouring GC-binding motifs for promoter activity, the motif most proximal to the transcription start site is more important than the distal sites. These results suggest that additional GC-binding motifs provide binding sites for co-operation between GC-binding transcription factors in order to activate transcription of the human *SOD2* gene. Since the present results from our footprinting analysis indicate that Sp1 and AP-2 bind to adjacent overlapping sites, it is highly likely that Sp1 and AP-2 might interact within the promoter region to regulate the transcription of the human *SOD2* gene.

Sp1 is the positive regulator of the human *SOD2* gene

Sp1 is a 95 kDa zinc-finger protein that was originally identified as a transcription factor from HeLa cells and recognizes the GC box (GGGCGG) in a variety of viral and cellular promoters [36]. Functional studies of the Sp1 protein have shown that three consecutive zinc-finger domains in the C-terminus interact with DNA, whereas at least one of the two glutamine-rich domains appear to be responsible for mediating transcriptional activation [37]. The Sp1-dependent promoter usually contains multiple GC boxes, and Sp1 recognition consensus sequences are often found within several hundred bp upstream of the transcription-initiation site [38]. Sp1 binds to a GC-box element and selectively activates mRNA synthesis by RNA polymerase II from housekeeping genes [39,40], which plays a key role in regulating transcription of housekeeping genes [41–43].

The data in the present study clearly establish that Sp1 is essential and sufficient for transcription of the human *SOD2* gene: (1) eight Sp1-binding sites have been identified by DNase I footprinting with the purified Sp1 protein; (2) deletion or mutation of any of the Sp1-binding sites results in a loss of transcription; and (3) expression of Sp1 protein leads to a concentration-dependent increase in transcription activity. Sp1-responsive promoters usually contain multiple Sp1-binding sites, and a single binding site appears to be sufficient for some promoters to be regulated by Sp1. However, it has been demonstrated that Sp1–Sp1 interaction with promoters containing multiple Sp1-binding sites is important for modulation of promoter activity [43]. In this regard, our data, which indicate that at least two Sp1 motifs are required for a minimum transcriptional activity and multiple Sp1-binding sites found in the human *SOD2* promoter are required for high-level activation of the gene, suggest that Sp1–Sp1 interaction may be involved in regulating expression of the human *SOD2* gene.

Role of AP-2 in the regulation of the human *SOD2* gene

Although Sp1 is a ubiquitous transcription factor present in all mammalian cells, it has been demonstrated that Sp1-binding affinity and transcriptional properties can be altered by interaction with other cofactors. Binding sites for other transcription factors, such as AP-1 [41], NF-κB [44], CCAAT-enhancer-binding protein ('C/EBP') [45] and AP-2 [46–48], are often found near Sp1 recognition motifs, suggesting that these factors may interact with each other to modulate transcription. Our present results indicate that AP-2 interacts with Sp1 to modulate the constitutive regulation of the human *SOD2* gene. Interestingly, AP-2-binding sites have been found in almost all Sp1-dependent promoters. The 52 kDa AP-2 protein is a DNA-specific binding protein that serves as a transcription factor regulating the expression of many mammalian genes. Consensus sequences for AP-2 were originally identified as CCSC(A/G)GGC within simian virus 40 enhancer sequences [49]. The AP-2 protein also binds GC-rich recognition sequences present in *cis*-regulatory regions and stimulates transcription in a binding site-dependent manner [50,51].

Regulation of mammalian gene expression by AP-2 presents an attractive system to study the regulatory roles by interaction between AP-2 and other transcription factors. One study has reported that AP-2 acts positively to increase transcription of AP-2-dependent promoters in a tissue-specific manner [52]. On the other hand, several studies have demonstrated that AP-2 might suppress transcription of Sp1-dependent housekeeping

promoters by competing with Sp1 for binding sites [46,53]. In the present study, 15 AP-2-binding sites were found in the GC-rich region of the human *SOD2* gene. These AP-2-binding sites overlap with Sp1-binding sites, suggesting that AP-2 may regulate transcription by modulating Sp1 binding. Our DNase I footprinting analyses, using purified AP-2 and Sp1 proteins, showed that AP-2 binding is stronger than Sp1, indicating that AP-2 may be more efficient at targeting the GC-rich promoter. Our current finding that expression of AP-2 strongly suppresses the promoter activity in the presence of Sp1 supports this view. It is remarkable that expression of AP-2 in HepG2 cells drastically represses promoter activity in a dose–dependent manner and that the transcriptional activity in HepG2 cells is stronger than that in HeLa cells, which have a higher level of Sp1 protein. These results suggest that AP-2 is a strong negative regulator of the human *SOD2* gene. Mutations of AP-2-binding sites, which eliminated AP-2 repression of the human *SOD2* promoter activity in the transfected HeLa cells, further support this notion. These results are consistent with and complementary to those reported previously by Zhu et al. [54], using a family of authentic and dominant-negative mutants of AP-2 to demonstrate that AP-2 plays a negative role in the expression of the *SOD2* gene.

However, it should be noted that mutation of the AP-2-binding site which does not overlap with any Sp1 site was unable to reduce promoter activity. Thus AP-2 binding may enhance transcription when the binding of AP-2 does not overlap with Sp1 binding.

Mechanisms of transcriptional regulation of the human *SOD2* gene by Sp1 and AP-2

Transcriptional interference, in which overexpression of a transcription factor results in inhibition of other transcription factors, is often found in the regulation of eukaryotic gene expression. Genes that contain GC-rich promoter entities generally have multiple Sp1-binding sites, which couple with binding sites for other transcription factors. Several recent studies indicate that AP-2 represses Sp1-dependent promoters through interference with the activation of initiation of transcription by Sp1 via specific steric interference machinery. For example, Chen et al. [53] have shown that K3 keratin gene transcription is regulated by the ratio of Sp1 to AP-2 in differentiating rabbit corneal epithelial cells. They proposed that the E element in the K3 promoter is initially occupied by AP-2 in undifferentiating cells, but is later replaced by Sp1 in differentiating cells due to the increased ratio of Sp1 to AP-2. However, this hypothesis is not supported by our present findings and those reported by Zhu et al. [54]. Firstly, the DNase I protection studies showed that Sp1 and AP-2 are able to bind the promoter simultaneously, suggesting that AP-2 does not interfere with Sp1 in binding to GC boxes of the promoter (results not shown). Secondly, the promoter activity was also decreased when a distant AP-2-binding site was mutated. Finally, an AP-2 variant, which lacks the dimerization domain that is necessary for DNA binding, was unable to repress SOD2 expression, whereas the AP-2 variant, which lacks the activation domain, was effective in repressing SOD2 expression [54].

Although the steric hindrance model for AP-2-mediated repression of Sp1-dependent promoters is reminiscent of the most frequently observed mechanisms for transcriptional interference in both prokaryotic and eukaryotic genes, our present data indicate that interaction between Sp1 and AP-2 may play a more important role in AP-2-mediated repression of human *SOD2* gene expression. Firstly, the intensity of the Sp1–DNA complex was increased by AP-2 in a dose-dependent manner when the two proteins were added simultaneously to the fourth binding motif on the human *SOD2* promoter. Secondly, Sp1 co-immunoprecipitated with AP-2, suggesting an interaction between Sp1 and AP-2 *in vivo*. Thirdly, two-hybrid analysis demonstrated that interaction of Sp1with AP-2 *in vivo* could lead to an increased or decreased expression of the reporter gene depending on the orientation of Sp1 and AP-2 linkages. Taken together, our present data suggest that AP-2 plays a negative role in the transcription of the human *SOD2* gene by interacting with Sp1.

The expression of the *SOD2* gene is essential for the survival of aerobic life and the development of cellular resistance against oxygen-radical-mediated toxicity. In the present study we describe the distinct regulatory roles of Sp1 and AP-2 in the transcriptional regulation of the human *SOD2* gene. These results, coupled with our previous finding [26] that several cancer cell lines have mutations in the promoter region which lead to an increase in an AP-2-binding site and a decrease in the promoter activity, and those reported by Zhu et al. [54,55], demonstrating that AP-2 proteins down-regulate SOD2 expression and constitutive activation of AP-2 is associated with decreased SOD2 expression, signal the importance of understanding the regulation of the human *SOD2* gene. Since increased expression of the *SOD2* gene has been shown to alter intracellular redox conditions leading to the suppression of some cancer phenotypes, understanding how the constitutive expression of the human *SOD2* gene can be controlled may lead to the design of new therapy for diseases associated with alteration of cellular redox status.

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