Positive and negative regulatory elements in the upstream region of the rat Cu/Zn-superoxide dismutase gene

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Cu/Zn-superoxide dismutase (SOD1) catalyses the dismutation of superoxide radicals and neutralizes the oxidative effects of various chemicals. Deletion analysis of the upstream region of the rat *SOD1* gene revealed that the promoter contains a positive regulatory element (PRE) and a negative regulatory element (NRE), which encompass the regions from −576 to −412 and from −412 to −305 respectively from the site of initiation of transcription. These DNA elements showed enhancer and silencer activities respectively in the natural context and in a heterologous promoter system. Using an electrophoretic-mobility-shift assay and a supershift assay with a specific antibody, the *cis*-elements of the PRE and NRE were identified as binding sites for transcription factors Elk1 and YY1 (Ying-Yang 1) respectively. Consistent with the presumed roles of the PRE and NRE, Elk1 increased *SOD1* gene transcription about 4–5-fold, whereas YY1 exerted a negative effect of about 6-fold. Mutations of the Elk1- and YY1-binding sites led to diminution and elevation respectively of transcriptional activities, both in the natural context and in heterologous promoter systems. These results suggest that the transcription factors Elk1 and YY1, binding in the PRE and NRE respectively, co-ordinate the expression of the *SOD1* gene.

Key words: *cis*-element, Elk1, *SOD1*, YY1.

INTRODUCTION

Superoxide dismutase (SOD) constitutes the first co-ordinated line of defence against reactive oxygen species, which have been implicated both in the aging process and in degenerative diseases, including arthritis and cancer [1,2]. Cu/Zn-superoxide dismutase (SOD1), which catalyses the dismutation of superoxide radicals ($O_2^{-\bullet}$) to oxygen and hydrogen peroxide, is a key enzyme in the metabolism of oxygen free radicals [3]. In addition, SOD1 is distributed in the cytoplasm of various cells, whereas Mn-SOD is present in the mitochondria [3,4]. SOD1 comprises approx. 90% of the total SOD activity in adult lung. Its activity first appears shortly before birth and gradually increases during adulthood [5,6]. Hass and Massaro [7] reported that the increase in SOD1 activity from birth to adulthood is due to the rate of SOD1 synthesis slightly exceeding its rate of degradation.

The physiological significance of the induction and regulation of SOD activity has been investigated. Increased activity of Mn-SOD and enhanced expression of SOD1 have been reported in the substantia nigra of Parkinsonian patients [8,9], and mutations in the *SOD1* gene have been found in patients with familial amyotrophic lateral sclerosis [10]. In addition, Kong and Fanburg [11] showed that SOD1 in pulmonary artery endothelial cells is not influenced by hormones or changes in Ca^{2+} or cAMP levels, but is elevated by agents that generate oxygen-based free radicals, H_2O_2, exposure to hyperoxia or hypoxia, lipopolysaccharide, and certain cytokines, including tumour necrosis factors α and β1 and interleukin-1. Other studies have demonstrated that extracellular SOD is up-regulated along with inducible nitric oxide synthase after nuclear factor-κB (NF-κB) activation [12]. Hardmeier et al. [13] reported that SOD activities were also increased at a very early post-irradiation period. The ACE1 transcriptional activator protein, which is responsible for the induction of yeast metallothionein (CUP1), also controls SOD1 expression in response to copper [14]. In addition, because of a long-standing interest in the relationship of oxygen radical metabolism and SOD expression, the effects of many chemicals and biological factors that influence oxygen radical generation have been examined.

We have previously reported studies concerning the genomic structure and regulation of the *SOD1* gene [15,16]. The genomic organization of the rat *SOD1* gene is quite similar to that of human *SOD1* [16,17]. We observed that transcription factor Sp1 and CCAAT-enhancer-binding protein (C/EBP)-related factors interact with the proximal upstream region of human *SOD1* [18]; C/EBP is also a major activator of transcription of the rat *SOD1* gene in liver cells [19]. In the present investigation, we report new fundamental insights into the transcription of the *SOD1* gene. Using serial deletion analysis and transient transfection assays, we have identified a positive regulatory element (PRE) and a negative regulatory element (NRE) in the *SOD1* promoter. We have further shown, using a mobility-shift assay and site-directed mutagenesis, that the transcription factors Elk1 and YY1 (Ying-Yang 1) are involved in binding to these regulatory regions. Our results suggest that binding of the transcription factors Elk1 and YY1 in the PRE and NRE respectively are responsible for the co-ordinated expression of the *SOD1* gene.

MATERIALS AND METHODS

Cell culture and transfections

HepG2 human hepatoma cells were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum,

Abbreviations used: SOD, superoxide dismutase; SOD1, Cu/Zn-SOD; PRE, positive regulatory element; NRE, negative regulatory element; CAT, chloramphenicol acetyltransferase; tk, thymidine kinase; EMSA, electrophoretic-mobility-shift assay; NF, nuclear factor; C/EBP, CCAAT-enhancer-binding protein; YY1, Ying-Yang 1; AP, activator protein; CP, CCAAT-box-binding protein; SRF, serum response factor; SRE, serum response element.

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penicillin G sodium (100 units/ml), streptomycin sulphate (100 μg/ml) and amphotericin B (250 μg/ml). HepG2 cells were plated at a density of 1×10^6 cells/60 mm-diam. plate, approx. 18 h prior to transfection. An equal amount of each vector construct was transfected into the cells by the calcium phosphate/DNA co-precipitation method [20]. pRSV β-gal plasmid (5 μg) was introduced in all experiments in order to correct for transfection efficiency.

Plasmid construction

To obtain a nested set of deletions in the 5′-flanking region of the rat *SOD1* gene, deletion by ExoIII (Promega Corp., Madison, WI, U.S.A.) was performed. A 1.7 kb *Bam*HI/*Sma*I fragment (nucleotides −1633 to +85) of the rat *SOD1* gene [15] was inserted into pBLCAT2 [21]. Unidirectional 5′ deletion mutants, which are resistant to ExoIII digestion, were produced by cutting the 5′-end of the *SOD1* promoter with *Sph*I and *Bam*HI, followed by treatment with ExoIII. The end-points of deletion were confirmed by DNA sequencing using a Sequenase kit (U.S. Biochemical Corp.). To test the function of the PRE, a DNA fragment (nucleotides −576 to −412) was cloned into the upstream (*Hin*dIII/*Bam*HI) or downstream (*Sma*I/*Eco*RI) region of the herpes simplex virus thymidine kinase (tk) gene promoter in both orientations. The fragment was also inserted into the upstream region of the *SOD1* minimal promoter (−55 SOD) in both orientations. To construct plasmids NRE(−412 to −305)tkCAT and NRE(−412 to −305)SODCAT, the DNA fragment (from −412 to −305) was cloned into the upstream (*Hin*dIII/*Pst*I) or downstream (*Sma*I/*Eco*RI) region of the tk promoter in both orientations. The pmRSP-576 plasmid was constructed by PCR with primers 5′-AAACCTGCAGAGAGGGGTTG-3′ (containing a *Pst*I restriction site), 5′-GATCGGCTTGCCTACTCTTAGCAAGG-3′ (containing a mutated Elk1 site) and 5′-TCTGTAGAAATGCGGAT-3′ (containing an *Xba*I restriction site), using plasmid pRSP-576. The PCR product was digested with *Pst*I and *Xba*I and cloned into pRSP-576. Elk1-tkCAT and mElk1-tkCAT were constructed by inserting trimerized double-stranded wild-type Elk1 (5′-CATCGGCTTGCCTAGGAAGCGCAAGG-3′) and mutant Elk1 (5′-GATCGGCTTGCCTACTCTTAGCAAGG-3′) oligonucleotides respectively into the *Bam*HI site of pBLCAT2. Plasmid YY1-tkCAT was constructed by inserting a trimerized double-stranded YY-1 oligonucleotide (5′-GATCGAGCATCCATCTTGGCTCAC-3′) into the *Bam*HI site of the pBLCAT2 plasmid. The mutant plasmid (mYY1-tkCAT) has mutations in the YY1-binding site, which were generated by oligonucleotide-directed *in vitro* mutagenesis (5′-GATCGAGCATAACGCTTGGCTCAC-3′). The pmRSP-412 construct was made by PCR amplification using synthetic oligonucleotide primers 5′-AAAACCTGCAGAGACACAGAGG-3′ (which contained a *Pst*I restriction site), 5′-CACCGAGCATAACGCTTGGCTCAC-3′ and 5′-TGTCGTAGAAATGCGGAT-3′ (which contained an artificial *Xba*I site). The amplified fragment was digested with *Pst*I and *Xba*I, isolated by agarose-gel electrophoresis and ligated to the chloramphenicol acetyltransferase (CAT) vector fragment isolated from pRSP-412, which was co-digested with *Pst*I and *Xba*I. All constructed plasmids were confirmed by DNA sequencing.

β-Galactosidase and CAT assays

The CAT assay was performed as described previously [22]. At 4 h after transfection, the transfected cells were washed twice with PBS and harvested. The pelleted cells were resuspended in 100 μl of 0.25 M Tris/HCl (pH 7.9) and lysed by three cycles of freezing and thawing. After removal of cell debris by centrifugation, cell extracts were normalized for β-galactosidase activity and then assayed for CAT activity on the basis of β-galactosidase activity. Extracts were incubated with 0.025 μCi of [^{14}C]chloramphenicol, 0.25 M Tris/HCl (pH 7.6) and 0.4 mM acetyl-CoA for 1 h at 37 °C. The reaction was terminated by adding 1 ml of ethyl acetate. The organic layer was analysed by TLC with chloroform/methanol (19:1, v/v). After autoradiography, CAT enzymic activity was quantified by measuring the conversion of chloramphenicol into its acetylated forms using a BAS radioanalytical imaging system according to the manufacturer's instructions. All experiments were repeated at least three times.

Electrophoretic-mobility-shift assay (EMSA)

The double-stranded oligonucleotides corresponding to the Elk1-binding site and the YY1-binding site were synthesized by Gibco-BRL. The oligonucleotides were labelled with [γ-^{32}P]ATP and polynucleotide kinase. Nuclear extracts were prepared by the method of Dignam et al. [23]. DNA-binding reactions were carried out in a 15 μl volume which typically contained 10 mM Hepes (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 10% (v/v) glycerol and 500 ng of poly(dI-dC) (Sigma). Prior to the reaction with DNA, an equal amount (10 μg) of nuclear extract prepared from HepG2 cells was mixed and incubated for 20 min. In the supershift assay, the specific polyclonal antibodies against YY1 and Elk1 (Santa Cruz Biotech. Inc.) were added to the reaction mixture after the binding reaction. The DNA-binding reaction was started by adding 10000 c.p.m. of probe to the pre-incubated reaction mixture, followed by an additional incubation for 15 min at room temperature. For competition assays, the binding reaction was performed with an unlabelled probe or competitor DNA fragments. Samples were loaded on a 4% (w/v) polyacrylamide gel (acrylamide/bisacrylamide, 49:1, w/w) in 0.5 × TBE (44 mM Tris, 44 mM boric acid and 1 mM EDTA). After electrophoresis, gels were dried and exposed to X-ray film. All experiments were repeated at least three times.

RESULTS

Analysis of deletion mutants

To analyse the transcriptional regulation of the *SOD1* gene in hepatoma cell lines, plasmids carrying parts of the *SOD1* promoter region of various lengths fused to the CAT gene were constructed. Serial deletions from position −1633 to −55 using restriction endonuclease, ExoIII and S1 nuclease revealed at least three important *cis*-acting elements in the promoter region of the *SOD1* gene. DNA fragments comprising nucleotides −576 to −412 (depicted as PRE in Figure 1) and −305 to −55 were found to enhance CAT activity by 6- and 7-fold respectively compared with basal *SOD1* promoter activity. In contrast, a DNA fragment comprising nucleotides −412 to −305 reduced CAT activity about 7-fold, implying that the silencer element is involved in the transcription of the *SOD1* gene in HepG2 cells (depicted as NRE in Figure 1).

Of the two PREs, the DNA fragment from −576 to −412 was subjected to further analysis, since the DNA fragment from −305 to −55 contains the enhancer elements for Sp1, AP2 (activator protein 2), NF-IL6 and CP2 (CCAAT-box-binding protein 2), and has been studied previously [15,19,24]. To test the function of the PRE, the PRE fragment was cloned into the upstream or downstream region of the heterologous *tk* promoter and the basal promoter of the *SOD1* gene in both orientations

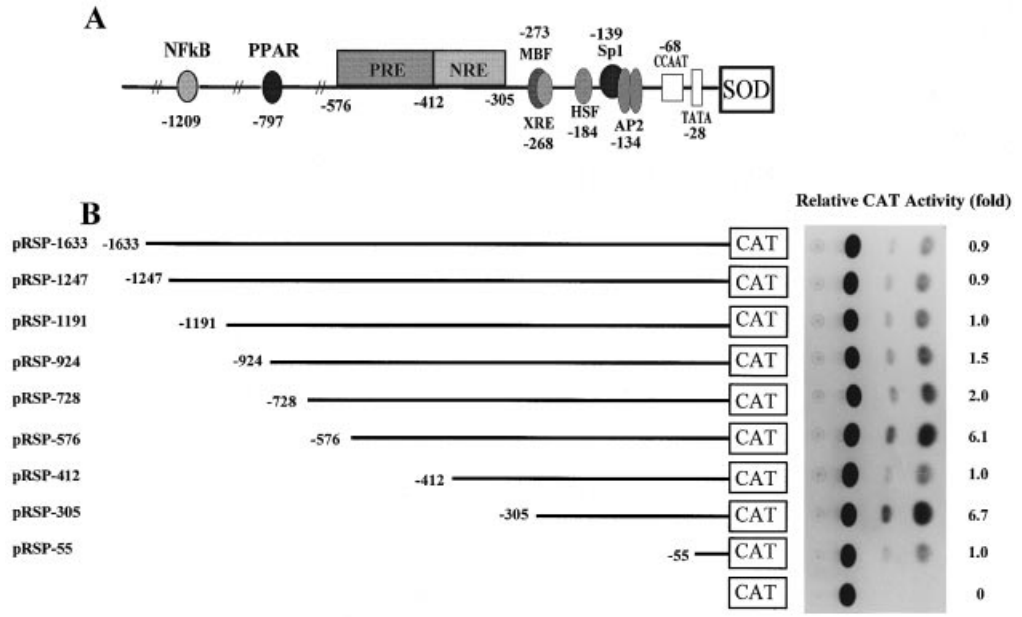

Figure 1 Deletion analysis of the upstream region of the *SOD1* promoter for the identification of positive and negative *cis*-elements

(**A**) Schematic representation of *cis*-elements in the promoter region of *SOD1*. PPAR, peroxisome-proliferating activator receptor; MBF, metal-binding factor; XRE, xenobiotic response element; HSF, heat-shock factor; CCAAT, CAT box; TATA, TATA box. Notice that the PRE is located between nucleotides −576 and −412, and the NRE is located between nucleotides −412 and −305. The numbers represent the left-hand ends of the consensus sequences of each transcription-factor-binding site. (**B**) Serial deletions from positions −1633 to −55 by using ExoIII and S1 nuclease digestion was carried out as described in the Materials and methods section. The end points of the deletions were determined by sequencing, and the numbers (−1633, −1247, −1191, −924, −728, −576, −412, −305, −55) indicate the 5′-ends of the promoter fragments relative to the transcription start site. These derivatives were introduced into HepG2 cells, and after 48 h the lysates were prepared for CAT assays. Transfection efficiency was normalized as described in the Materials and methods section. Results are the means of at least three independent experiments that differed by $< 5\%$.

(Figure 2A). As a control, cells were transfected with pBLCAT2, which is a CAT vector containing the herpes simplex virus *tk* promoter [21]. In each case, plasmids containing the correct orientation of the PRE enhanced CAT activity approx. 5–8-fold. Reduced CAT activity, rather than total repression, occurred with the inverted orientation (Figure 2A). These results indicate that the PRE shows enhancer activity in an orientation-dependent and position-independent manner.

When the DNA fragment from −412 to −305 of the upstream region of the *SOD1* gene was deleted, the promoter activity in HepG2 cells was increased by more than 6-fold (Figure 1). To confirm this inhibitory function, the fragment (from −412 to −305) was cloned, using the same strategy as for the PRE, into the *tk* promoter and the basal promoter of the *SOD1* gene (Figure 2B). Transfection of these constructs into HepG2 cells followed by CAT assays showed that this region exerted silencer activity, resulting in a 5–8-fold decrease in promoter activity compared with the control (tkCAT) promoter. These results show that the NRE demonstrates silencer activity in an orientation-dependent and position-independent manner.

An Elk1-binding site is the important element of the PRE

In order to identify a *cis*-element, we adopted two methods: a mobility-shift assay and a transcription efficiency test in the heterologous *tk* promoter and natural context systems with the PRE. The 92 bp DNA fragment (from −540 to −448), which encompasses the Elk1-binding site −526 region, formed a complex which was competed out by the unlabelled probe (Figure 3A). In order to check any other possibilities, we synthesized several oligonucleotides (Elk1, AP2 and Sp1 sites) corresponding to the feasible binding sites in the PRE for the mobility-shift assay. In contrast with that for Elk1, the AP2 and Sp1 oligonucleotides did not compete with the DNA–protein complex, probably due to the lower sequence identity (results not shown). When labelled Elk1 was used as a probe, a clear complex was formed which was competed out by the unlabelled probe (Figure 3B, lanes 2 and 3) and by the PRE DNA fragment (Figure 3B, lane 4). No competition was observed with a mutant Elk1 oligonucleotide (Figure 3B, lane 5). This specific binding of Elk1 was confirmed by the supershift experiments using a specific anti-Elk1 antibody (Figure 3B, lanes 6 and 8).

To test whether Elk1 plays a role in the enhancing activity of the PRE, we constructed an expression plasmid containing trimerized Elk1 elements (Elk1-tkCAT) or the mutant Elk1 sequence (mElk1-tkCAT) in the heterologous *tk* promoter system. The CAT activity of the wild-type plasmid, Elk1-tkCAT, was increased about 5-fold, whereas the plasmid containing the mutant sequence, mElk1-tkCAT, showed little or no change in promoter activity (Figure 3C). We also constructed plasmids with wild-type and mutated Elk1-binding sites in the natural context. The wild-type plasmid increased transcription about 5-fold, whereas the mutation abolished the enhancing activity (Figure 3D). Thus the effect of the mutant Elk1 site was similar to that of deletion of this site. These results confirmed that the Elk1-binding site is responsible for the activity of the PRE.

A YY1-binding site is the important element of the NRE

To find the transcription-factor-binding site in the NRE, an EMSA was performed with the labelled DNA fragment (from −412 to −305) and HepG2 nuclear extracts. As shown in Figure

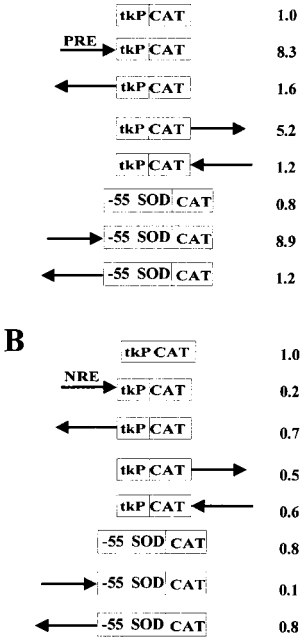

Figure 2 Identification of the PRE and the NRE as an enhancer and a silencer respectively in the heterologous promoter system and in the natural context

(**A**) The PRE (−576 to −412) was cloned into the upstream (*Hin*dIII/*Bam*HI) or downstream (*Sma*I/*Eco*RI) region of the heterologous *tk* promoter in both orientations, as indicated. The transcription activity in the natural context was determined by inserting the PRE into the upstream region of the *SOD1* minimal promoter (−55) in both orientations. Samples of 5 μg of each of the CAT reporter plasmids were transfected into HepG2 cells. Results are means of at least three independent experiments, which differed by $< 5\%$. (**B**) Inhibition by the NRE in the heterologous promoter system and in the *SOD1* minimal promoter. Using the same strategy as in (**A**), the NRE DNA fragment was cloned into the upstream (*Hin*dIII/*Pst*I) or downstream (*Sma*I/*Eco*RI) region of the *tk* and *SOD1* minimal promoters in both orientations. β-Galactosidase activity was used as a control for transfection efficiency, and CAT activity was measured and calculated as a percentage of that with the control plasmid (100%). Results are means of at least three independent experiments, which differed by $< 5\%$.

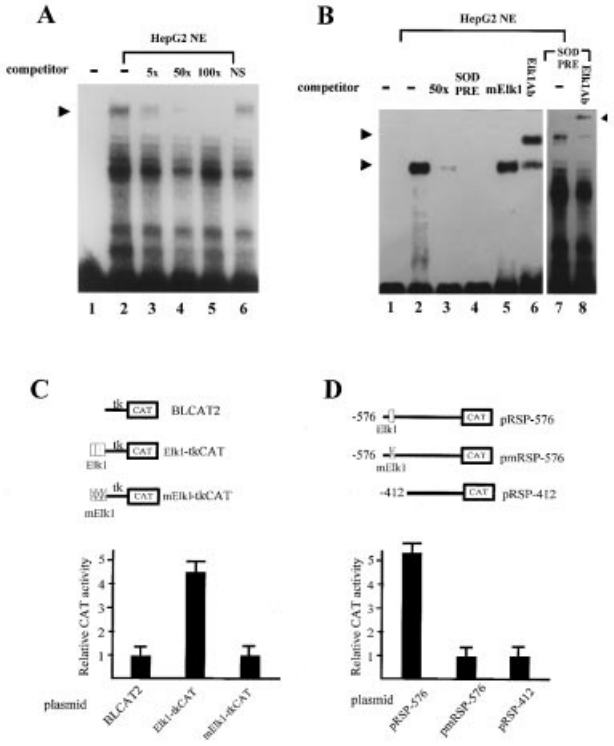

Figure 3 An Elk1-binding site is the important *cis*-element of the PRE

(**A**) EMSA for the PRE specific binding protein. The PRE fragment (−540 to −448) probe was end-labelled with ^{32}P, incubated with HepG2 nuclear extracts (NE) and analysed by EMSA in the presence or absence of unlabelled competitor. Lane 1, free probe; lane 2, bound reaction; lanes 3–5, reactions with unlabelled competitor as indicated (fold excess); lane 6, competition with non-specific (NS) DNA (−1633 to −1191). (**B**) Binding of transcription factor Elk1 to its binding site in the PRE region. ^{32}P-labelled Elk1 probe corresponding to nucleotides −537 to −516 of the *SOD1* promoter was used. Specificity of binding was determined by the competition reaction. Lane 1, free probe; lane 2, bound reaction; lane 3, competition with 50× unlabelled competitor; lane 4, competition with 100× DNA fragment (−576 to −412); lane 5, competition with mutated Elk1; lanes 6 and 8, EMSA with Elk1-specific antibody; lane 7, DNA–protein complex for PRE probe. An arrowhead at the right indicates the position of the band supershifted by the Elk1-specific antibody. (**C**) Transcription activation through the Elk1 site in the heterologous promoter system. Two independent clones, Elk1-tkCAT and mElk1-tkCAT, containing the wild-type and mutated Elk1 minimal elements respectively, were cloned at the upstream site of a heterologous promoter (BLCAT2) and transfected into HepG2 cells. Results are means ± S.D. of three independent experiments. (**D**) Transcription activation through the Elk1 site of the PRE in the natural context. Schematic diagram of the Elk1 site of the PRE (pRSP-576) and the mutated Elk1 of the PRE (pmRSP-576) in the natural context. Equal amounts (5 μg) of the plasmids pRSP-576, pmRSP-576 and pRSP-412 were transfected into HepG2 cells. The protocol for the synthesis of pmRSP-576 is described in the Materials and methods section. Results are means ± S.D. of three independent experiments.

4(A), a DNA–protein complex appeared (Figure 4A, lane 2) which disappeared upon competition with unlabelled NRE (Figure 4A). The shifted band did not disappear when using non-specific competitor DNA comprising nucleotides −532 to −437 (Figure 4A, lane 5) or −305 to −74 (Figure 4A, lane 6). The minor bands may be non-specific complexes, since we used a relatively long DNA fragment as probe.

To characterize further the protein-binding site in the NRE, we analysed the DNA sequence from −412 to −305 and found four possible putative transcription-factor-binding sites (for YY1, NF1, MyoD and AP1) in the NRE. We synthesized oligonucleotides corresponding to each factor-binding site for the EMSA. A DNA–protein complex was formed only with the oligomer for the YY1-binding site (G^{-378}AGCATCCATCTTG-GCTCAC^{-359}), and was competed out by the unlabelled YY1 oligomer (Figure 4B), whereas other oligomers did not form any significant bands, probably due to the lower sequence identity (results not shown). We subsequently tested whether the YY1-oligonucleotide–protein complexes disappeared in the presence of competitors and was supershifted by an antibody against YY1. As shown in Figure 4(B), the specific DNA–protein complexes disappeared when either the YY1 oligomer or the NRE DNA fragment was added (Figure 4B, lanes 3/4 and 6/7). However, no competition was observed with the mutant YY1 oligomer or with a non-specific oligonucleotide (AP1) (Figure 4B, lanes 5 and 9). The binding of YY1 was further confirmed by the observation that the YY1–DNA complex was supershifted by the addition of an anti-YY1 antibody, with both a YY1 oligonucleotide probe (Figure 4B, lane 8) and an NRE probe (Figure 4B, lane 11). These results strongly imply that the important element of the NRE is the YY1-binding site.

To link the *in vitro* binding data to a functional role, we tested the transcriptional efficiency of the YY1-binding site both in the heterologous promoter and in the natural context systems. Figure 4(C) shows that the YY1-binding site in a heterologous *tk* promoter (YY1-tkCAT) repressed transcriptional activity approx. 5–6-fold, whereas the mutant YY1 site (mYY1-tkCAT) had no effect (Figure 4C). When the pmRSP-412 plasmid,

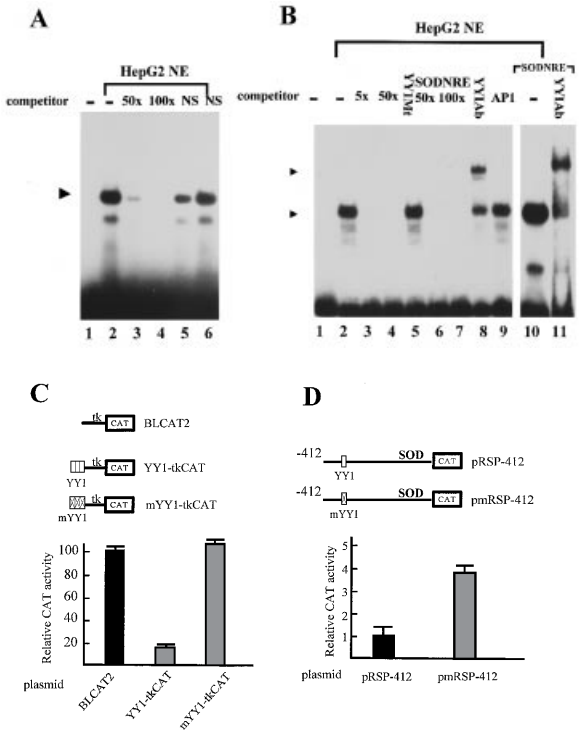

Figure 4 A YY1-binding site is the important *cis*-element of the NRE

(**A**) EMSA for the NRE specific binding protein. Samples of 10 μg of HepG2 nuclear extracts (NE) were subjected to EMSA with the ^{32}P-labelled NRE fragment (-412 to -305). Formation of complexes (lane 2) was shown to be specifically inhibited by the unlabelled oligonucleotide at the indicated molar excess of competitors (lanes 3 and 4). Competition reactions with non-specific (NS) competitors -532 to -437 (lane 5) and -305 to -74 (lane 6) were also carried out. (**B**) EMSA with a ^{32}P-labelled YY1 oligonucleotide probe encompassing the -378 to -359 region of the *SOD1* promoter. Arrowheads indicate the positions of the major DNA–protein complex (lane 2) and of the band supershifted by the YY1-specific antibody (lanes 8 and 11). Unlabelled YY1 (lanes 3 and 4), mutant YY1 (lane 5), an NRE DNA fragment (lanes 6 and 7) and AP1 (lane 9) were used as competitors. A YY1-specific antibody was used for the supershift assay (lanes 8 and 11). The DNA–protein complex for the NRE is also indicated (lane 10). (**C**) YY1-mediated repression through the YY1 site in the heterologous promoter system. Samples of 5 μg of each of the heterologous reporter plasmids BLCAT2, YY1-tkCAT and mYY1-tkCAT (the latter two plasmids have three copies of the consensus YY1 oligonucleotide and three copies of a mutated YY1 oligonucleotide respectively in front of the *tk* promoter) were transfected into HepG2 cells. Results are means $\pm$ S.D. of three independent experiments. (**D**) Transcription repression through the YY1 site of the NRE in the natural context. The plasmid pmRSP-412 was cloned by PCR amplification using a synthetic mutated YY1 oligonucleotide as described in the Materials and methods section. Results are means $\pm$ S.D. of three independent experiments.

containing the mutant YY1 oligonucleotide in the natural context, was transfected into HepG2 cells, promoter activity increased by more than 4-fold (Figure 4D). This is consistent with the results from the heterologous *tk* promoter system. These findings again strongly suggest that the YY1-binding site is responsible for the silencing function of the NRE.

DISCUSSION

The upstream region (-1729 bp) of the *SOD1* gene was analysed using the FASTA program for the presence of putative binding sites for *trans*-acting factors (Figure 1A). The common CCAAT box was located in the proximal region of the promoter of the human *SOD1* gene [15], but an inverted CCAAT sequence was found at the -73 region, which has a major function in the basal transcription of the rat *SOD1* gene [19]. There are also AP2-, NF-κB- and Sp1-binding sites. The AP2 sequence responds to PMA and cAMP [25,26]. Sp1 is a ubiquitous transcription factor that binds selectively to GC-rich regions and is involved in maintaining a basal transcription level. The Sp1-binding site is located in the *SOD1* promoter close to the AP2-binding site, and functions as a basal transcription activator with C/EBP-related factors. Four tandem repeats of the heat-shock element were also found in the proximal promoter of the rat *SOD1* gene, and sites for a metal-binding factor (MTF1) were also found. However, the molecular mechanisms by which DNA elements mediate the regulation of *SOD1* transcription have not yet been reported.

Using transient transfection with deletion derivatives, we found that strong enhancer and silencer regions are present in the 5′-flanking region of the rat *SOD1* gene (Figures 1 and 2). These DNA segments acted as potential positive and negative elements in the natural context and in heterologous promoter systems (Figure 2). A PRE was found at positions -576 to -412. This PRE sequence showed enhancer activity in an orientation-dependent manner. The reverse orientation of the PRE sequence resulted in reduced CAT activity, which was somewhat unexpected. Previous studies on the enhancers of the interferon-β and T-cell-receptor-α genes provided important biochemical details of the organization and stereospecificity of the enhancer (for a review, see [27]). Recently, Kim and Maniatis [28] reported a similar observation, i.e. that the orientation and spatial arrangements of target enhancer regions were important for the activation of transcription in the interferon-β enhancer.

Another PRE was found in the proximal regulatory region (positions -305 to -55), as indicated in Figure 1. This region contained the binding sites for metal-binding factor, Sp1, AP2 and heat-shock factor, and the inverted CCAAT box. Since Sp1 is known to be expressed in a wide variety of cells and is related to the transcription of housekeeping genes, it might not influence the transcriptional level of *SOD1* to a great extent in cells under normal conditions. From a more detailed analysis of these regions, the major positive effect was due to the inverted CCAAT box and its binding protein. Recently we showed that a functional C/EBPα-binding site is located in the CP2 site of the *SOD1* promoter, and that C/EBPα activated the transcription of the *SOD1* gene through direct binding [19]. We determined that positive regulation by CP2 is one of the major functions in the basal transcription of the rat *SOD1* gene.

The 107 bp DNA fragment from -412 to -305 in the *SOD1* promoter was shown to possess silencer activity (Figure 1). This function was confirmed by transient transfection experiments using various vector constructs (Figure 2B). Previously, Liang et al. [29] suggested that a gene in chromosome 7 of mice, named *Nmo-In*, might encode a *trans*-acting negative effector of the Cu/Zn-SOD gene. This negative factor was also responsible for the negative regulation of the other oxidative stress response genes. However, the factor was not identified. We assumed that the negative factor that binds to the promoter of the *SOD1* gene is a global repressor for oxidative stress response genes. From this interpretation, further analysis was carried out to clarify whether the core sequence of the NRE and its corresponding binding factor(s) are involved in the transcriptional repression of this gene (see below).

A specific DNA–protein complex was formed in an EMSA with the PRE (Figure 3A), and this disappeared on addition of unlabelled competitor DNA. Only the oligonucleotide for Elk1 competed with the PRE DNA–protein complex. To test the relationship between the binding of Elk1 and the function of the PRE in the *SOD1* promoter, we constructed plasmids bearing point mutations in the Elk1-binding site and tested them in the natural context and in heterologous promoter systems. As

expected, mutations of this positive regulatory site in each plasmid construct resulted in a failure to activate *SOD1* expression in both systems (Figures 3C and 3D). We conclude from these results that Elk1 appears to play a positive regulatory role by binding specifically to its conserved site in the *SOD1* promoter.

The transcriptional induction of several genes in response to serum growth factors has been described, in part, by the formation of ternary complexes that contain the serum response factor (SRF) and one of several ternary-complex factors such as Elk1 and SAP [30–32]. Promoters containing the serum response element (SRE) are activated by oxidative stress induced by H_2O_2 or xanthine/xanthine oxidase treatment of cells [33–36]. H_2O_2 and the diverse antioxidants cause a transient phosphorylation of Elk-1 on a site identical to its transactivation domain. However, the promoter activity of the SRF gene was tissue-specific, being 80-fold greater in primary skeletal myoblasts than in liver-derived HepG2 cells. Furthermore, a minimal SRF promoter containing a SRE was not active in HepG2 cells [37]. These observations in HepG2 cells imply that the function of Elk1 in *SOD1* transcription as an enhancer is independent of the effect of an Elk1–SRF interaction. These results therefore demonstrate that the transcription factor Elk1, which is known to be activated by a signal cascade in response to oxidative stress, contributes to *SOD1* gene transcription as a major enhancing factor.

Various constructs of the NRE in the heterologous *tk* promoter and *SOD1* minimal promoter systems showed inhibitory effects on transcription. The NRE DNA–protein complex was competed out by unlabelled NRE DNA and by YY1 oligonucleotide. Furthermore, a specific anti-YY1 antibody supershifted the DNA–protein complex efficiently (Figure 4B, lane 8). In the functional analysis of YY1 in the heterologous *tk* promoter and the natural context systems, repressive functions of this element were observed (Figures 4C and 4D). This repression was relieved by the introduction of mutations in the YY1-binding site in the heterologous promoter system and in the natural context. Repression of transcription by YY1 has been observed in the long terminal repeat of Moloney murine leukaemia virus [38], the adeno-associated virus P5 promoter [39], the human papilloma virus-18 promoter [40] and the human cytomegalovirus major immediate-early enhancer/promoter [41]. With regard to viral promoters, it was suggested that negative regulation by YY1 may play a role in maintaining the viral latency which inhibited the transcription of immediate-early and early genes [42]. On the other hand, YY1 activates the transcription of the c-*myc* promoter [43] and the ribosomal L30 and L32 promoters [44]. In addition, YY1 has been described as an initiator-binding protein [39]. The functional versatility of YY1 has been explained in several investigations. Bushmeyer et al. [45] showed that the ability of YY1 to both activate and repress transcription was due to the presence of distinct activation and repression domains. Another model to explain the functional versatility of YY1 is based on interactions with cellular or viral regulatory proteins [46].

It is known that SOD1 is expressed at high levels in liver, and its distribution is consistent with the relative protein concentration and activity of SOD [47]. These observations provided the relationship between the tissue distribution of SOD1 and the role of tissue-specific transcription factors. Under the proximal promoter, C/EBPα and Sp1 were major activators of the transcription of the *SOD1* gene [18,19]. On induction, such as by ginsenoside Rb_2, *SOD1* was strongly activated through the AP2-binding site [24]. The observations described above support the idea that the *SOD1* gene promoter has the potential to respond to normal and changing circumstances.

Transcriptional activation of *SOD1* by Elk1 is elicited by its direct binding to the Elk1-binding site in the PRE of the *SOD1* promoter, whereas the activation of several genes, including c-*fos*, in response to SRF has been explained by the formation of a ternary complex on the SRE [31,48]. At present, the interaction of YY1 with other cellular factors, such as SRF, has not been observed with the *SOD1* promoter. One possible interpretation is that YY1 is able to repress transcription by acting on the initiator-complex factors, as suggested by Margolis et al. [49], even in the absence of promoter-bound cellular factors; this repression may be relieved by the proper signals that mediate *SOD1* induction.

The CAT activity value for pRSP-576 in Figure 1 shows that transcription was activated approx. 6-fold by the PRE even in the presence of the NRE, implying that the strength of the PRE overrode the effect of the NRE. We conclude from these observations that transcription of the *SOD1* gene is basically oriented for positive regulation. In the induced state, Elk1 plays a more influential role in *SOD1* transcription. Furthermore, we have observed strong induction of the *SOD1* gene through the Elk1-binding site by an oxidative stress, such as H_2O_2 treatment (H. Y. Yoo, M. S. Chang and H. M. Rho, unpublished work).

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