Myeloid zinc finger (MZF)-like, Kruppel-like and Ets families of transcription factors determine the cell-specific expression of mouse extracellular superoxide dismutase

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Extracellular superoxide dismutase (EC-SOD or SOD3) is an important protective enzyme against the toxicity of superoxide radicals that are produced under both physiological and pathophysiological conditions. We have isolated and characterized over 11 kb of the mouse EC-SOD gene and its 5′- and 3′-flanking regions. The gene consists of two exons, with the entire coding region located within exon 2. In order to study the mechanism of cell-specific gene regulation for mouse EC-SOD, we characterized 2500 bp of its 5′-flanking region using cultured cells derived from mouse lung fibroblasts (MLg), kidney medulla (mIMCD3) and hepatocytes (Hepa 1-6). Real-time PCR showed that basal expression of EC-SOD was considerably higher in MLg cells compared with the other cell types. Reporter-gene assays revealed that the proximal promoter region was sufficient to support this high expression in MLg cells. Although no

obvious TATA box was identified, our results show that a highly purine-rich region from −208 to +104 contains active binding sites for both the Kruppel-like and Ets families of transcription factors. Using electrophoretic mobility shift, DNase footprinting and reporter gene assays, we identified myeloid zinc finger 1 and gut-enriched Kruppel-like-factor-like nuclear transcription factors as repressors of EC-SOD expression, whereas nuclear transcription factors from the Ets family, such as Elf-1 and GA-binding protein α and β , were potent activators of EC-SOD transcription. We propose a model that highlights competition between Ets activators and Kruppel-like repressors within the proximal promoter region that determines the level of EC-SOD expression in a particular cell type.

Key words: antioxidant, gene expression, promoter.

INTRODUCTION

The catalytic reduction of superoxide anions to hydrogen peroxide and oxygen is carried out by three known isoforms of superoxide dismutase (SOD), CuZn-SOD (SOD1), Mn-SOD (SOD2) and extracellular (EC)-SOD (SOD3) [1–3]. The first two isoforms are widely distributed in mammalian tissues and are expressed at a relatively high level. EC-SOD is the more recently discovered enzyme, and is excreted from the cell just after synthesis and binds to the extracellular matrix [4,5]. In humans and mice, EC-SOD exists as a homotetramer with a molecular mass of 135 kDa, whereas in rats, EC-SOD exists as a dimer with a native molecular mass of 97 kDa [6,7]. The enzyme has differing affinities to proteoglycans, such as heparan sulphate, dictated by the composition of positively charged amino acids at its C-terminal end [8]. EC-SOD is secreted from cells in two forms, with or without the heparin-binding domain [9]. The removal of the C-terminal heparin-binding domain occurs after passage through the Golgi network and is mediated by the intracellular protease furin [10]. Unlike CuZn-SOD and Mn-SOD, EC-SOD expression is highly restricted to particular tissues and cell types. For example, high levels of EC-SOD expression in the lung are primarily attributed to Type II epithelial cells [11] and to the smooth-muscle region surrounding blood vessels and airways [4]. Low levels of EC-SOD are also found in plasma, lymph and

cerebrospinal fluid [8,12]. Several studies characterized EC-SOD expression in human culture cells and demonstrated that fibroblasts and glial cells are an important source of EC-SOD [13].

The role of EC-SOD in maintaining homeostasis of mammalian organisms under normal physiological conditions appears not to be crucial, but its function becomes increasingly important when organisms are exposed to increasing levels of oxidative stress [14,15]. EC-SOD appears to have an important role in homeostasis of vascular cells and can be found at very high concentrations in the interstitium of the arterial wall [16]. In the vascular wall, EC-SOD seems to prevent pathophysiological effects of superoxide. EC-SOD levels are regulated by a wide array of cytokines and growth hormones, as well as nitric oxide, angiotensin II and exercise [17–19].

To begin to understand the molecular mechanisms that regulate EC-SOD transcription, we characterized the proximal promoter of the mouse EC-SOD gene in order to identify factors that control its cell-specific expression. We show that EC-SOD is expressed at a considerably higher level in mouse lung fibroblasts compared with mouse hepatocytes and cells derived from the kidney medulla. The proximal promoter region has a very high GA content with multiple binding sites for the Kruppel-like, myeloid zinc finger (MZF)-like and Ets-like families of transcription factors. DNase I footprinting, electrophoretic mobility shift and transient transfection assays confirmed the importance

Abbreviations used: BKLf, basic KLF; EC-SOD, extracellular SOD; EKLf, erythroid KLF; EMSA, electrophoretic mobility shift assay; FAM, 6-carboxyfluorescein; GABP, GA-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GKLF, gut-enriched KLF; IL-2R α , α subunit of interleukin-2 receptor; KLF, Kruppel-like factor; LKLF, lung-specific KLF; MZF-1, myeloid zinc finger 1; PEA3, polyoma virus enhancer A factor 3; RAZ-1, SCAN-related protein associated with MZF-1B; SOD, superoxide dismutase; TAMRA, 6-carboxytetramethylrhodamine; UKLF, ubiquitous KLF.

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The sequence of the mouse EC-SOD genome has been submitted to the EMBL, GenBank®, DDBJ and GSDB Nucleotide Sequence Databases under the accession number AF039602.

of these transcription factors in cell-specific regulation of EC-SOD expression.

MATERIALS AND METHODS

Materials

Oligonucleotides were obtained from Invitrogen (Carlsbad, CA, U.S.A.). [α - ^{32} P]dATP (3000 Ci/mmol) was purchased from Amersham International (Arlington Heights, IL, U.S.A.). Anti-GKLF [(gut-enriched Kuppel-like factor (KLF)], anti-GABP α (GA-binding protein) and anti-(Elf-1) IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). All other chemicals and enzymes were from Boehringer Mannheim (Indianapolis, IN, U.S.A.), Sigma Chemical Co. (St. Louis, MO, U.S.A.), or Gibco BRL (Gaithersburg, MD, U.S.A.).

Mouse EC-SOD genomic clone

A mouse 129 genomic library (2×10^7 plaque forming units/ml) constructed in Charon 35 was obtained from Dr Oliver Smithies and Dr Nobuyo Maeda (University of North Carolina, Chapel Hill, NC, U.S.A.). From this library, we screened approx. 500 000 plaque forming units using a full-length mouse EC-SOD cDNA probe. We identified four positive clones with one of these clones containing an insert of approx. 16 kb. We have sequenced over 11 000 bp of the mouse EC-SOD genome. This sequence includes at least 5000 base pairs of the 5' region of the mouse SOD3 gene.

Cell culture

A mouse liver cell line, Hepa 1-6, a mouse lung fibroblast cell line, MLg, and mouse kidney medulla cell line, mIMCD3, were obtained from American Type Culture Collection (ATCC; Manassas, VA, U.S.A.). MLg cells were cultured in minimal essential medium (MEM) supplemented with non-essential amino acids and 10% (v/v) heat-inactivated bovine calf serum. mIMCD3 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 10% (v/v) fetal bovine serum. Hepa 1-6 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. Cells were maintained at 37 °C and 5% CO₂ in a humidified incubator.

Plasmids

The mouse EC-SOD 5'-flanking sequences were generated by a standard PCR technique using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA, U.S.A.) and corresponding primer pairs containing *Mlu*I and *Bgl*II restriction sites for the 5' and 3' primers respectively. The amplified DNA was cloned using pCR-Blunt II-TOPO kit (Invitrogen). The cloned EC-SOD promoter regions were re-cloned into pGL3-Basic vector (Promega, Madison, WI, U.S.A.) at *Mlu*I and *Bgl*II restriction sites. DNA sequencing confirmed the identity of all constructs.

Transient transfection

Transfection assays were carried out using LIPOFECTAMINE® reagent (Invitrogen). Briefly, Hepa 1-6, mIMCD3 and MLg cells were seeded at 90% confluence on 24-well plates, incubated for 24 h before transfection and then treated with LIPOFECTAMINE® for 5 h according to the manufacturer's protocol. At 24 h after transfection, cell extracts were assayed for luciferase activity

using a Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's instructions. All experiments were performed at least in triplicate, and the data were normalized to *Renilla* Luciferase ('control' reporter) to control for differences in transfection efficiency. Co-expression experiments were performed using expression vectors containing full-length cDNAs of various transcription factors, pcDNA1-Ets-2, pcDNA1-Elf-1 (gifts of Dr J. Leiden), pcDNA3-kz-MZF-1, pcDNA3-kz-MZF-1B, pcDNA3-kz-RAZ-1 (gifts of Dr J. Morris), pcDNA1-GABP α , pcDNA1-GABP β (gifts of Dr M. Martin), pcDNA3.1-GKLF (a gift of Dr A. Rustgi) and pBK-CMV-LKLF (a gift of Dr J. Lingrel).

Gel-shift assay

These reactions were done in 10 μ l of 20 mM Hepes, pH 7.9, 1 mM dithiothreitol, 5% (v/v) glycerol, 0.2 mM EDTA, 0.05% Nonidet P40, ^{32} P-end-labelled oligonucleotide probe (40 000 c.p.m.) and 2 ng (for Ets family transcription factors) or 100 ng (for Kruppel-like transcription factors) of poly(dI-dC) · (dI-dC). The reaction was started by adding 2 μ g of either BSA or nuclear extract. In competition experiments, an excess of unlabelled oligonucleotide was added to the mixtures before the start of the binding reaction. In supershift experiments, the nuclear extracts were pre-incubated with corresponding IgG for 20 min on ice. The free and protein-bound probes were separated on 5% polyacrylamide gel in 0.25 $\times$ Tris/borate/EDTA, then dried and exposed to X-ray film.

DNase I footprinting assay

A fragment of the EC-SOD gene promoter region (−208 to +104) was excised from pGL3-mSOD3 (−208/+104) plasmid using *Mlu*I and *Sma*I restriction enzymes. A DNase I footprinting assay was performed by incubating ^{32} P-labelled probe (10 000 c.p.m.) with 9 μ g of BSA or nuclear extract from MLg or mIMCD3 cells at 25 °C for 30 min, in 20 mM Hepes, pH 7.9, 1 mM dithiothreitol, 5% (v/v) glycerol, 0.2 mM EDTA, 0.05% Nonidet P40 and 500 ng of poly(dI-dC) · (dI-dC) in a total volume of 50 μ l. After incubation, the samples were treated with 2, then 3, units of DNase I for 1 min at 25 °C. Control reactions were performed in the presence of BSA (9 μ g). All samples were phenol-extracted, ethanol-precipitated and loaded on a 6% acrylamide/8 M urea sequencing gel. A sequencing ladder was obtained by loading a sample of the probe that had been treated with formic acid and piperidine to cleave at G and A residues.

Preparation of nuclear-protein extract

Nuclear extracts from MLg and mIMCD3 cells were prepared using a mini-extraction procedure [20]. Protein concentrations were determined by a modified Bradford method (Bio-Rad, Hercules, CA, U.S.A.) using BSA as standard.

Reverse transcriptase-PCR and real-time PCR

Total RNA was prepared from the cultured cells using Trizol reagent (Invitrogen). The synthesis of single-stranded DNA from RNA was performed using SuperScript™ First-Strand Synthesis System for reverse transcriptase-PCR (Invitrogen), according to the protocol provided by manufacturer. The PCR product of EC-SOD mRNA-derived cDNA was quantified using a dye-labelled fluorogenic oligonucleotide probe with 6-carboxy-fluorescein (FAM) as a label and 6-carboxytetramethylrhodamine (TAMRA) as a quencher at the 5' and 3' end

respectively. The nucleotide sequence of the probe 5'-(FAM)-CATCAGCCACGCTGCCACCG-(TAMRA)-3' corresponds to nucleotides 242–261 of the mouse EC-SOD cDNA. This double dye-labelled probe was obtained from PerkinElmer–Applied Biosystems (Foster City, CA, U.S.A.). The EC-SOD specific primers used for PCR were the forward 5'-AGGTGGA-TGCTGCCGAGAT-3' and reverse 5'-TCCAGACTGAAATAGGCCTCAAG-3' primers. The forward and reverse primers correspond to nucleotides 200–218 and 319–341 of the mouse EC-SOD cDNA respectively. For PCR, we used *TaqMan*® Universal PCR Master Mix containing dUTP instead of dTTP. This master mixture also contained uracil-N-glycosylase (UNG), which destroys any carryover PCR products. All procedures, including data analysis, were performed on the ABI PRISM 7700 Sequence Detection System (PerkinElmer–Applied Biosystems) using the software provided with the instrument. The EC-SOD mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The GAPDH sequence was amplified and detected using *TaqMan*® Rodent GAPDH Control Reagent (VIC™ Probe) (Applied Biosystems, Foster City, CA).

The reaction mixture for PCR contains 5 μ l of single-stranded DNA diluted 1 in 5, 400 nM forward primer for EC-SOD, 400 nM reverse primer for EC-SOD, 200 nM probe for mSOD3, 40 nM forward primer for GAPDH, 40 nM reverse primer for GAPDH, 200 nM probe for GAPDH and 25 μ l of *TaqMan*® Universal PCR Master Mix in a total volume of 50 μ l.

RESULTS

Genomic organization of the EC-SOD gene

Four independent positively hybridized clones were identified from a mouse genomic library probed with radiolabelled EC-

Figure 1 Structure of the murine EC-SOD gene

The mouse EC-SOD gene was cloned and 11660 bp was sequenced and submitted to GenBank® (Accession number AF039602). (A) Structural organization of the mouse EC-SOD gene and its corresponding mRNA. The EC-SOD gene contains two exons interrupted by a large intron. The sizes of the exons and intron are shown in bp. UTR, untranslated region. (B) Sequence of the intron/exon splice junctions. Upper-case letters indicate the exon sequence and lower-case letters indicate the intron sequence. The splice junctions shown conform to previously published consensus sequences for splice junctions [21]. Nucleotides identical with the consensus sequence of the splice donor (DS) and acceptor (AS) sites are in bold (100% identity).

Figure 2 Basal expression of EC-SOD mRNA in mIMCD3, Hepa 1-6 and MLg cells

The cells were cultured to 80–90% confluence and then washed with ice-cold PBS. The RNA was purified using Trizol reagent, reverse-transcribed and quantified by real-time PCR using *TaqMan*® fluorogenic probe as outlined in the text. The data were normalized to GAPDH expression. Means $\pm$ S.D. were calculated from at least three independent experiments.

SOD cDNA. The longest of these clones contained an insert of approx. 16 kb and was used for sequencing. The DNA insert was sequenced on both strands using a combination of vector-specific, universal sequencing primers and primer walking. We compared previously published sequence data for the mouse EC-SOD cDNA [11] with our genomic sequence in order to determine genomic intron/exon structures. Based on this comparison, we determined that the mouse EC-SOD gene contains two exons and one intron (Figure 1A). Exon 1 is 245 bp in length and exon 2 is 1556 bp. They are separated by 3888 bp intron. The complete coding sequence for the precursor form of EC-SOD begins 19 bp after the start of exon 2. The intron/exon boundaries are shown in Figure 1(B) and are in good agreement with eukaryotic consensus splice sequences [21].

Analysis of EC-SOD mRNA expression in different cells

In order to evaluate the baseline level of EC-SOD expression in different cells, we used three distinct cell lines derived from mouse liver hepatocytes (Hepa 1-6), kidney medulla (mIMCD3) and lung fibroblasts (MLg). The relative EC-SOD mRNA level in these cells was 1.0 ± 0.069 for Hepa 1-6, 9.3 ± 0.36 for mIMCD3 and 268.8 ± 7.9 for MLg. The level of EC-SOD mRNA was considerably higher in MLg cells compared with Hepa 1-6 (269-fold) or mIMCD3 (29-fold) cells (Figure 2). The expression of EC-SOD in cultured cells is in good agreement with published results that described its expression in mammalian tissues and organs [11]. The highest level was observed in the lung, whereas liver and kidney medulla express considerably lower levels of EC-SOD.

Functional characterization of the 5'-flanking region of the mouse EC-SOD gene

Computer-assisted sequence analysis of the 5' promoter region identified several putative Ets binding sites (core sequence AGGAAG) within the proximal promoter region (Figure 3). The search for other putative transcription-binding sites using the TRANSFAC (Transcription Factor) database [22] revealed that

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-2553 GCTTTGTGCA TGCTAGGCAC GAGTGCTCCC TCTGTTCTAC CTTCCCACCC TTGAGAAAAAT
-2493 TGCTTTTTTT TTTTTTTTTT TTTTTTTTAA TGGAGAGCTA TGTAACCTCAG ATCAGCCTAG
-2433 GGTTCGAATC CTTCTGCCTC AGTCTCCTGA ATAGCAGGGA GCACCAGTGT GTGTCGCTGG
-2373 GCCGACCTTA TGATTGAAAT CCTGACTTGG CCATTTGCTA GCAGTGCATC TTGACCCTGT
-2313 GGTTCCTCCT TTTCTGAACC TTTTGTCTCT ATCTATAGAG TAGACGAATC AAAACTATTA
-2253 CTATCATCAA CTGTGATACT CATGATACTT TCTTGTAATA TGAGAAGATT CACATAATGA
-2193 AAGCAGAGTT TCTGGTGTGG CTGAGTGCAG GGTCTCTCCAC CCCATCCCTG TTCCACTGTA
-2133 CTTTCCCTGT ATTTCCTTTT GCATGGGTCA ACAAAGTCTT GGGGGGAGAG GGGTGGTCCC
-2073 AACTAATTAG CTTGGAGTCT CATTGTCCCA GAATGCATCA TGCTTAGACC ATGACAAATG
-2013 TAACCCAAGC CACCAGCTTT TCATCTAGGA ATAAAGTTGG CTTCTAAGAA AGAAAAGAGA
-1953 TCACAATACG TAAGTAAGGG GACTGAATAT TACAGAGATG GAGCGGGGAG GAGGAGGGCA
-1893 ATTTTCTCCT AGAGAGCATA AAAAATGCAA GTGAATTGAG GCTTGCCACT AATCCTAGAA
-1833 TCCCAGAACG AAGGAATGCT TTCCTGAGTC TTGAAGGAAA TAAATCAGG CTCATGGAAG
-1773 AGATACACAC TTAACAACGT AGCTAGGAGC TTATGCAAGA GTTACTTCGG GGCTGGAGAG
-1713 ATGGCTCAGT GGTTAGGAGC TTTTCCAGAG GTCCTGAGTT CAATTCCAG CAACCATATG
-1653 GTGGCTCAGC ACCATCTGTA ATGGGATCAG ATGGCCTCTT TTGGTGGGTC TGGAGAGAGC
-1593 AACAGTATAC GCACATAAAA TAAGTAAATA AATCTTAAAA AAAAAAGAAA GAATCACTTC
-1533 AAAGCCAGAA TGAATAAAG GATTTAACAA GATGATTGGA GAAGCCAGGA TTTGGTAGGA
-1473 TGTATGTGGG TGGTTGTGGG TGGCTGTAGG TGGTAGGTGG CTGAGGGTGG CTGGCAGCCA
-1413 TGGGCACTGG GTAGTACCTG AACTCTATA AGCTCATTG TTGAGGACAG CTACTGCCAA
-1353 CAGCAGATAA AAAGTTCAGG GCTGAATCG CTTCTAGGGG ACGCCACCTG AAGGAGGTTT
-1293 ACTGAGCTCC AGGAGGCAGC CAGAGAACAG GTTACCGAAC AAACCTCCCA CCTGCCAGGC
-1233 CTGGCGACTG GAGGTGTGAG GGGCCATTCT GTCTCTAGCT GTACCACTGA TGCTCGGTTA
-1173 TCTTCTAAG TGTGTGAACC ACATGGGGCA GAAACTGTG TAGGTTGGAG ACTCTGGTGT
-1113 GTGGTGTGGT AGGGGGCAGG TACCCAATAA CCATTTGTCA AAGGAATTCA GGGGACAGAC
-1053 AAGCGGCAGC AAGCTGGGGA TGCTTCTCTG GAAAGAGAG CCTTTTATTT CTAGAGAGTT
-993 TCTGTGATGG ACAGGAGCTC CCAACAACAA AAGAAATAAG GAGCCTGGGA TTGGAGGTTT
-933 CTGGCTCCTC CAATCTGTGT AGTCTATGTA ACAATCTTCC TGGGCAGGCC TCAGTTGGCA
-873 GAGTGCTTTC CTAACACGCA TGAAGGCCTG TGTTTGACCC CAGCGACACA CCAACGTGAT
-813 AGTGACGCCC TGCAATCCCA GCATTGGGA GCTGATGACA GGGGGATTAA AAGTTCAAGG
-753 TAATCCCCAG TTACAGATGG AATTGAAGCT AGCTTGGGTT AAATGCAACT TTGTTTCAAA
-693 AACAAACAAC CCTCCTTTGT CATTTTAATA CAACAGTGGC AACAATCATA ATGTTATTGT
-633 CACGGTCATT GTTGACTCAC TAATGTCCTT ATGAAAACCA GACACTGGCT TTACTCCTTT
-573 GATTCTCATG GGGGATGAGA GCTATTATAT TACAGATGCT GAGAGGGAGG TAAAGGGAGG
-513 GCTTCCAGGA GCACACGGGT AGGAAAAGGC AAGACTGAAG GCACACCACC ATGCCCTGCC
-453 CACACCTTGT GTGGTAGCAC AGCTCTGGAG AGAGTGTGCG ACTCAGCTGC CAAGTGCCCA
-393 TGTCTGTCTT AAACACATAC CAACATCTAG CAAAAGAAGT CAGTAGCTGA GGGAGTCAGG
-333 GGAAGAGAAA AGGCCCTTCG AGAAGAGAGC CATTGTGCCT CTTCAAACAA GACTATTTTC
-273 TTTCTCATTT CTGCTCCCTT GCTGCGTCTT GAAGGTCACT GACTGCGTCT GCACGAGCCA

-213 GGAAGTCAC GAAAGACATA CTTGGCAAGC AGAACCAGCA GTGGGAAAGG GACTGGGAGG

-153 TGGGGACAAG GAGAAGATGA AGTGGGGGAG GAGGGGGAAA AGGAAGGAGA GGAGAAGGGG
      MZF-1 MZF-1 Ets
-93 AGGAAGAGGA GGAGGAAGAA AAAGAGGAGG AGAAAGGAAT AAGAAGGGGA GAAGGAGACG
      Ets Ets MZF-1
-33 GAGTAGGAAG AGAAGGAAA AGAAGGAGGG GAGGAAGAG GAGGAGGCAG CAATTTTACC
      Ets Ets MZF-1 Ets
+28 ACAAGGGACA GCCAAGCTGG CTTTGCTTCT CTTGGCCAGC CCAATGACCT TCCCTCCATT
      Ets
+88 TGCTGACCAC TCCCCCGGGC TGGCCTCCCT GCTGCTCGCT CACATAACAG CCAGCTGGAC

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Figure 3 Nucleotide sequence of the mouse EC-SOD promoter and its 5'-flanking region

The transcription initiation site is designated as +1 (arrow). Putative binding sites for MZF-1 are underlined and binding sites for the Ets family of transcription factors are indicated by shaded boxes. The consensus sequence for GKLF is designated as an open box.

the promoter region from -208 to +104 bp has several binding sites for Kruppel-like and MZF-1 transcription factors. The putative binding sites for these proteins often overlap one another as shown in the region from -102 to -127, highlighting a potentially complex organization of the EC-SOD promoter. The highly repetitive GA sequence makes it difficult to predict the exact location of binding sites for Ets-family proteins. Never-

theless, at least seven potential binding sites for Ets binding proteins with an AGGAAG consensus sequence were identified in this region. The transcription-initiation site was determined based on a previously published mouse lung EC-SOD mRNA transcript [11].

The 5'-promoter region of the mouse EC-SOD gene was obtained using a PCR-based method and inserted in front of a

Figure 4 Identification of functional promoter elements within the mouse EC-SOD gene

5' regions of the EC-SOD promoter, as indicated, were inserted into the luciferase reporter vector pGL3-Basic. These various promoter-reporter constructs were transiently transfected into MLg, Hepa 1-6 and mIMCD3 cells as shown. At 24 h later, luciferase activity was measured and normalized to *Renilla* Luciferase activity produced by the co-transfected control plasmid pRL-CMV. Results shown are means $\pm$ S.D. from at least two independent transfection experiments, each performed in quadruplicate.

luciferase reporter gene (pGL3-Basic). As shown in Figure 4, the construct spanning the region from -208 to $+104$ of the EC-SOD promoter exhibited a maximal 4-fold increase in the promoter activity in MLg cells, as compared with the pGL3-Basic construct. This increase in activity was only observed in MLg cells, whereas in Hepa 1-6 and mIMCD3 cells its activity was considerably lower. Progressively increasing the length of the 5'-flanking sequence up to 2500 bp shows a significant reduction in luciferase activity, but still higher than the pGL3-Basic construct alone. These deletion analyses revealed that the *cis*-regulatory elements required for basal promoter activity of the EC-SOD gene are located within an approx. 0.3 kb region around the transcription-initiation site. Specifically, the region from -208 to $+104$ was essential for the expression and elicited weak promoter activity in Hepa 1-6 and mIMCD3 cells. On the other hand, the 5'-flanking region from -2500 to -208 carried the silencer sequences of EC-SOD expression, at least in MLg cells.

In vitro analysis of the EC-SOD promoter region

DNase I footprint experiments were performed to identify regulatory elements that are important for the transcriptional activity of the EC-SOD proximal promoter. A ^{32} P-labelled 312 bp EC-SOD promoter region corresponding to nucleotides -208 to $+104$ was used as a probe in footprinting experiments using mIMCD3 and MLg nuclear extracts. As shown in Figure 5, nuclear proteins from mIMCD3 cells protected the promoter in five separate regions, referred to as A (-1 to -15), B (-55 to -64), C (-73 to -81), D (-101 to -131) and E (-141 to -165). The protected element D appears to be the strongest and spans approx. 30 bp. The proteins from MLg nuclear extract were unable to protect any of these regions under the binding conditions shown. The protected sequence E showed a weaker footprint signal and spans at least 24 bp. It is the most distally protected region. The sites A, B and C are shorter and show less prominent protection from DNase digestion. We focused our

subsequent studies on the protected region D, because it showed the strongest protection in this assay.

Electrophoretic mobility shift assays (EMSAs) and supershift assays demonstrate the presence of GKLf-like and MZF-like transcription factors in the DNA-protein complexes

In order to characterize transcription factors that interact with the EC-SOD promoter, EMSAs and supershift assays were performed. Using the 312 bp promoter region as a probe, EMSA analysis showed that broad shifted bands have a higher intensity with nuclear proteins from mIMCD3 cells compared with MLg cells (Figure 6A, lanes 2 and 3). The same probe does not show any shifted band when incubated with BSA (Figure 6A, lane 1).

Synthetic oligonucleotides used in EMSA (Table 1) were designed to cover all the protected and part of the non-protected regions within the EC-SOD promoter or to fit the known consensus sequences for the Ets or Kruppel-like families of transcription factors. The oligonucleotide SOD ($-131/-98$) spans protected region D, while SOD ($-37/-11$) corresponds to the non-protected region. The shifted DNA-protein complexes were completely eliminated by 200-fold molar excess of the unlabelled SOD3 ($-131/-98$) oligonucleotide (Figure 6A, lane 6), while SOD ($-37/-11$) oligonucleotide, as well as Ets-family-consensus oligonucleotide, failed to show any competition for binding (Figure 6A, lanes 4 and 5). Based on these data, we focused our attention on the protected region D that is represented by SOD ($-131/-98$) oligonucleotide. When SOD ($-131/-98$) probe is labelled with ^{32} P]dATP and incubated with nuclear proteins from mIMCD3 cells, it shows two distinct shifted bands (Figure 6B, lane 1, arrows). Competition experiments show that both bands were completely abolished by a 50 and 200 molar excess of self-nucleotide (Figure 6B, lanes 10 and 11) as well as several other oligonucleotides that represent different regions of the promoter, such as SOD ($-14/+14$), SOD ($-97/-72$), SOD ($-167/-140$) (lanes 2 and 3, 8 and 9,

Figure 5 DNase I footprinting assay of mouse EC-SOD3 proximal promoter region

An end-labelled 302 bp *MluI/SmaI* fragment (positions -208 to $+104$) of the mouse EC-SOD promoter was incubated with nuclear extracts obtained from mIMCD3 (lanes 3 and 4) and MLg (lanes 5 and 6) cells and subsequently digested with increasing amounts of DNase I. Several protected regions are identified as A, B, C, D and E. Lane 1, G + A, Maxam-Gilbert sequencing ladder of the probe DNA; In lane 2, the probe was incubated with BSA.

and 12 and 13 respectively). The oligonucleotide SOD ($-73/-48$) shows complete competition for the larger complex and partial competition for the faster migrating band (Figure 6B, lanes 6 and 7), while SOD ($-37/-11$) and Ets-family-consensus oligonucleotides failed to compete with either shifted complex (lanes 4 and 5, and 14 and 15). These results suggest that transcription factors that interact with protected region D also show an affinity to other sequences within the EC-SOD proximal promoter.

In order to identify the specific transcription factors that interact with protected region D, we synthesized oligonucleotides that encode consensus binding sequences for GKLF and MZF-1 transcription factors. When these oligonucleotides were used for EMSAs with nuclear extracts obtained from cultured mIMCD3 and MLg cells, they formed specific slow-migrating complexes, whereas incubation with BSA did not produce any shifted band (Figure 7A, lanes 1–6). It is important to mention that the intensity of the shifted bands was higher with mIMCD3 nuclear extract compared with MLg nuclear extracts. A similar pattern was observed for SOD ($-131/-98$) oligonucleotide, with two bands migrating at exactly the same position as for the GKLF and MZF-1 probes (Figure 7A, lanes 8 and 9). Moreover, when GKLF and MZF-1 oligonucleotides were used for competition experiments, they eliminated only the upper or lower bands, respectively (Figure 7B, lanes 2–5). Specific antibodies for Kruppel-like proteins incubated with nuclear extract considerably decreased the intensity of the slow migrating band while have no effect on the fast migrating complex (Figure 7B, lane 7). The competition and super-shift experiments indicate that MZF-1 and Kruppel-like factors interact primarily with region D, but also appear to interact with additional regions within the mouse EC-SOD promoter.

Characterization of potential Ets-binding sites

Computer-assisted searching for putative transcription-factor-binding sites within the EC-SOD proximal promoter region revealed multiple binding sites for the Ets family of transcription factors (Figure 3). At least seven binding regions for the polyoma virus enhancer A factor 3 (PEA3) transcription factor, which has been shown to interact with the core sequence AGGAA^{G/A}, were identified. The core sequence, GGAA, for the entire family of Ets proteins is observed even more frequently in the EC-SOD proximal promoter region. To demonstrate Ets interaction with these sequences, we synthesized consensus oligonucleotide that bind to Ets transcription factors. When this probe was incubated with BSA, it did not produce any shifted complexes (Figure 8A, lane 1), whereas incubation with nuclear extracts from mIMCD3 and MLg cells resulted in the appearance of a prominent, slowly migrating band (Figure 8A, lanes 2 and 3, arrow). The intensity of this complex was consistently higher when nuclear extracts from MLg cells were used. These results suggest that the amounts of Ets transcription factors in MLg cells are higher compared with the kidney mIMCD3 cells. When two oligonucleotides that span theoretically predicted Ets-binding sites were used in EMSAs, they showed a similar pattern of a single shifted band, again with a higher intensity associated with MLg nuclear extracts (Figure 8A, lanes 4–9). Ets transcription factors appear to have a lower affinity to their binding sites compared with Kruppel-like or MZF-like proteins, because increasing the concentration of the non-specific competitor poly(dI-dC)·(dI-dC) disrupted Ets complexes while having no effect on GKLF- and MZF-like shifted bands. To confirm further the specificity of binding of these oligonucleotides, a supershift EMSA was conducted by incubating MLg nuclear extracts with radiolabelled

Figure 6 EMSA of the EC-SOD proximal promoter region

(A) An end-labelled 302 bp promoter (–208/+104) was incubated with BSA or nuclear extracts from miMCD3 or MLg cells. For competition experiments, oligonucleotides, which represented two regions of the EC-SOD promoter as well as the Ets-family-consensus sequence, were added to the reactions in 200-fold excess. Neither the Ets nor the SOD (–37/–11) oligonucleotides competed for binding (lanes 4 and 5), whereas promoter region D, represented by oligonucleotide SOD (–131/–98), mostly abolished DNA–protein interactions (lane 6). (B) The similarity between binding sites found in protected region D [oligonucleotide SOD (–131/–98)] and other regions of EC-SOD promoter was examined by competitive EMSA. Two distinct bands (arrows) were consistently observed when miMCD3 nuclear extracts were incubated with SOD (–131/–98) end-labelled oligonucleotide. Unlabelled oligonucleotides, corresponding to various EC-SOD promoter regions and the Ets-family-consensus oligonucleotide, were added at 50- and 200-fold molar excess. Oligonucleotides SOD (–14/+14), (–97/–72) and (–167/–140) show complete inhibition of binding (lanes 2 and 3, 8 and 9, and 12 and 13 respectively). The oligonucleotide representing the –73/–48 region shows partial competition for binding (lanes 6 and 7), whereas oligonucleotides SOD (–37/–11) and Ets family show minimal to no ability to compete for DNA–protein complexes (lanes 4 and 5, and 14 and 15). The binding reactions were separated on 5% non-denaturing polyacrylamide gel, dried and exposed to film.

Table 1 Oligonucleotides used in EMSAs

Oligonucleotides	Sequence 5' to 3': sense strand
Ets-family consensus	GGG CTG CTT GAG GAA GTA TAA GAA T
GKLF consensus	AGG AGA AAG AAG GGC GTA GT
MZF-1 consensus	CGG CTG GTG AGG GGG AAT CG
SOD (−14/+14)	AAG AAG GAG GGG GAG GAA GAG GAG G
SOD (−37/+11)	GAC GGA GTA GGA AGA GAA GGA AAA AGA
SOD (−73/+48)	AAA GAG GAG GAG AAA GGA ATA AGA AG
SOD (−97/+72)	AGG GGA GGA AGA GGA GGA GGA AGA AAA
SOD (−131/+98)	TGG GGG AGG AGG GGG AAA AGG AGG AAG AGA A
SOD (−167/+140)	AAG GGA CTG GGA GGT GGG GAC AAG GAG A

probes in the presence of non-immune IgG or antibodies specific for the Ets family of transcription factors, such as GABP α or Elf-1. As shown in Figure 8(B), the addition of anti-GABP antibodies resulted in the complete elimination of the specific shifted band (black arrow). Interestingly, this addition of anti-GABP IgG resulted in the formation of an extra complex (marked by open arrow). One explanation for the appearance of this complex could be that the inactivation of GABP by its specific antibody allows other members of the Ets family to bind to the probes. The addition of Elf-1 specific antibodies (Figure 8B, lanes 3, 6 and 9) does not change the EMSA pattern, which suggests that the Elf-1 transcription factor either is not expressed in MLg cells, or it does not interact with these particular probes. Non-immune IgG showed no interactions with the DNA–protein complexes (Figure 8B, lanes 1, 4 and 7).

Effect of various transcription factors on EC-SOD promoter activity

We transiently co-transfected mIMCD3 and MLg cells with pGL3-mSOD3 (−208/+104) either alone or concomitantly with an expression vector that encodes transcription factors from the Ets, MZF-1 and Kruppel-like families (Figure 9). These experiments show that the GABP α –GABP β heterodimer and Elf-1 activate the EC-SOD promoter construct > 3-fold in mIMCD3 cells, whereas in MLg cells their effect was less prominent. GABP α or GABP β alone have no effect. Ets-2 transcription factor exerted very weak effects in kidney cells and no apparent effect in MLg cells (Figure 9). The Kruppel-like transcription factors, GKLF and lung-specific KLF (LKLF), showed different effects on the promoter activity. LKLF has no effect in either cell line, while GKLF demonstrated strong repression activity. Slightly weaker repression was also observed with vectors that encode the transcription factors MZF-1 and RAZ-1 (SCAN-related protein associated with MZF-1B) in MLg cells. Interestingly, MZF-1 and RAZ-1 showed no effect when transfected into mIMCD3 cells, indicating that, in these cells, the EC-SOD promoter was already repressed by endogenous transcription factors. It is also important to note that the luciferase activity of pGL3-mSOD3 (−208/+104) reporter construct was 4.5-fold higher in MLg cells compared with mIMCD3 cells (Figure 4). Overall, these results are consistent with a model that Ets transcription factors activate the EC-SOD proximal promoter, while transcription factors from the MZF-like and Kruppel-like families function as repressors (Figure 10).

DISCUSSION

EC-SOD is expressed at high levels in a variety of cell types, such as lung alveolar type II epithelial cells, alveolar macrophages and

Figure 7 Identification of Kruppel-like transcription factors as a proteins interacting with protected region D

(A) Radiolabelled DNA probes encoding consensus binding sequences for GKLF and MZF-1 transcription factors, as well as SOD (−131/−98), were incubated with MLg and mIMCD3 nuclear extracts. The GKLF oligonucleotide showed a single shifted band which was consistently of greater intensity when incubated with mIMCD3 nuclear extracts compared with MLg nuclear proteins (lanes 2 and 3). The MZF-1 probe showed similar results, except that the shifted band migrated slightly faster (lanes 5 and 6). The oligonucleotide SOD (−131/−98), representing protected region D, showed two bands (lanes 8 and 9) with a migration pattern similar to that of the GKLF and MZF-1 probes, which is readily apparent on shorter-exposed film (results not shown). BSA was used as a control for non-specific binding (lanes 1, 4 and 7). (B) Competition assays were performed with 50- and 200-fold excess of unlabelled oligonucleotides to analyse the specificity of Kruppel and MZF-1 binding to SOD (−131/−98) oligonucleotide. The consensus GKLF binding sequence completely eliminated the slow migrating band (lanes 2 and 3), whereas the consensus MZF-1 binding sequence completely eliminated the faster-migrating band (lanes 4 and 5). The polyclonal anti-GKLF antibodies partially interferes with the formation of the slow-migrating complex, while non-immune IgG does not change the binding pattern (lanes 6 and 7). The non-immune (n.i.) or GKLF-specific antibodies were pre-incubated with nuclear extract on ice for 20 min before the assay. The binding reactions were separated on a 5% non-denaturing polyacrylamide gel, before the dried gel was exposed to the film. Specific DNA–protein complexes are indicated by arrows.

chondrocytes [23]. Exceptionally higher amounts of EC-SOD can be found in the vascular system of mammals around smooth-muscle cells [8,16]. Rat neuroblastoma and glioma, as well as

Figure 8 Binding of Ets transcription factors to the EC-SOD promoter region

(A) End-labelled oligonucleotides, which corresponded to the Ets-family-consensus sequence, SOD (–37/–11) and SOD (–97/–72), were incubated with mIMCD3 and MLg nuclear extracts or with BSA. All three oligonucleotides show one major shifted band of a similar size whose intensity is greater when nuclear proteins from MLg cells were used (arrow). The incubation with BSA did not produce any specific slow-migrating bands. (B) Supershift EMSA, designed to identify GABP as a nuclear factor binding to the EC-SOD promoter. ^{32} P-labelled probes were incubated with nuclear extract prepared from MLg cells. Antibodies against GABP α , Elf-1 or non-immune (n.i.) goat immunoglobulins were added and incubated for an additional 15 min. Only antibodies specific to GABP α show complete elimination of the specific band marked by an arrow (lanes 2, 5 and 8). The open arrow showed the appearance of an unidentified but faster-migrating band. Non-immune IgG- and Elf-1-specific antibodies do not show any changes (lanes 1, 3, 4, 6, 7 and 9). The binding reactions were separated on 5% non-denaturing polyacrylamide gel, dried, and exposed to X-ray film.

Sertoli cells, also appear to secrete high amounts of EC-SOD [24,25]. The analysis of a large panel of human cell lines show that EC-SOD is expressed at a high level in all 25 investigated fibroblast cell lines and in a few glial cell lines [13]. In the kidney,

Figure 9 Regulation of EC-SOD proximal promoter by the MZF-like, Kruppel-like and Ets families of transcription factors

The cultured mIMCD3 or MLg cells were co-transfected either with the EC-SOD promoter construct pGL3-mSOD3 (–208/+104) alone or concurrently with plasmids encoding different transcription factors, as indicated. The GABP heterodimer (GABP α + β) and Elf-1 transcription factors activated the EC-SOD proximal promoter construct in mIMCD3 cells (upper panel) and, to a lower extent, in MLg cells (lower panel). The MZF-1 transcription factors repressed the luciferase activity in MLg cells, while having no effect in mIMCD3 cells. The GKLF transcription factor decreased luciferase activity in both cells. Transcriptional activities were measured and expressed as a percentage from pGL3-mSOD3 (–208/+104) activity. The activity of the pGL3-mSOD3 (–208/+104) construct was 4.5-fold higher in MLg cells compared with mIMCD3 cells (see Figure 4). Data are the means $\pm$ S.D. of at least three independent experiments. Luc, luciferase.

EC-SOD is localized mostly to the cortex region, with markedly lower levels in the medulla (I. N. Zelko, H. B. Suliman, R. B. Doctor and R. J. Folz, unpublished work). In the liver, EC-SOD is expressed at low but detectable levels [5]. In order to elucidate the molecular mechanisms governing cell-specific expression of EC-SOD, we analysed its expression pattern in several cell lines derived from mouse lung fibroblasts (MLg), liver hepatocytes (Hepa 1-6) and kidney medulla (mIMCD3). The level of EC-SOD mRNA in fibroblasts is considerably higher compared with other cells. Although high levels of EC-SOD were previously documented for human dermal fibroblasts [13], no results have been published for EC-SOD expression in mouse lung fibroblasts. Our observations of EC-SOD expression in these three cell lines are in good agreement with the EC-SOD expression patterns found in mouse tissues. Taken together, these results indicate

Figure 10 Schematic diagram illustrating the molecular mechanism for cell-specific transcription of mouse EC-SOD

Ets-like and Kruppel-like families of transcription factors compete for binding to the mouse EC-SOD proximal promoter region.

that we have found a reliable cell culture system that is suitable for the study of cell-specific expression of EC-SOD.

The present study also revealed several *cis*-elements and *trans*-acting factors involved in the constitutive and cell-type-specific expression of EC-SOD. Deletion analysis of the 5'-flanking region suggests that the 312 bp region, corresponding to nucleotides -208 to +104 relative to the major transcription start site, is responsible for cell-specific expression. Computer-assisted analysis identified multiple putative binding sites for both the Ets and Kruppel-like families of transcription factors that cluster around the transcription initiation site. The Ets family of transcription factors represents a diverse group of proteins having in common an 85-amino-acid Ets domain that typically bind to DNA sequences containing a GGAA core, but also demonstrate preferences for surrounding nucleotides [26]. There are many known members of this family, including Ets-1, Ets-2, PEA3, Elf-1, Elk-1 and -2, GABP α and PU.1/Spi-1. Many members of the Ets family are expressed in haematopoietic cells and regulate cell growth and differentiation. Some Ets proteins can function as transcriptional activators, while others are potent silencers of transcription [27]. Ets transcription factors are involved in the regulation of > 200 genes, and this number is increasing [28]. The present results show that at least two members of the Ets family are involved in the cell-specific expression of EC-SOD. One member is the GA-binding protein, which is able to activate the EC-SOD promoter when co-expressed in mammalian cells. GABP consists of an α - and a β -subunit which interact with DNA mostly as a heterodimer. GABP-binding sites are present in many TATA- and CAAT-less promoters and can initiate the assembly of the pre-initiation RNA polymerase complex [29,30]. We identified several potential GABP binding sites containing two repeats of the GGAA core sequence. EMSA studies and co-transfection experiments suggest that GABP positively up-regulates EC-SOD promoter activity and is probably involved in the formation of the pre-initiation complex. The GABP protein closely resembles nuclear respiratory factor 2, which has also been shown to bind to purine-rich sequences (GA repeats) and is expressed in erythroid cells and fibroblasts [31–33]. GABP transcriptional activity is redox-regulated through the oxidation/

reduction of cysteine residues located within the DNA binding and dimerization domains of the GABP- α subunit [34]. The second nuclear transcription factor which activates the EC-SOD proximal promoter is Elf-1. Elf-1 is a protein that is expressed at high levels in T-cells and is known to regulate expression of several T-cell genes, including granulocyte/macrophage colony-stimulating factor (GM-CSF) [35], the α subunit of interleukin-2 receptor (IL-2R α) [36], and CD4 [37]. Our studies suggest that Elf-1 activates the proximal EC-SOD promoter to the same degree as GABP. The transcription factors from the Ets family may also regulate inducible transcription of the EC-SOD gene by cytokines and growth hormones. It has been shown that Ets-1 and GABP bind to the growth-factor-regulatory sites of the rat prolactin promoter [38] and Elf-1 regulates induction of IL-2R α by interleukin-2 [36].

Computer-assisted analysis of the 5'-flanking region of the EC-SOD gene also reveals several putative binding sites for MZF-1 protein. MZF-1 transcription factor is expressed in haematopoietic progenitor cells and contains 13 zinc fingers, which are clustered in two domains. These domains are divided by glycine- and proline-rich sequences [39,40]. The first cluster has four zinc-finger repeats, while the second cluster is formed by nine zinc fingers. Both DNA-binding domains interact with similar DNA sequences that contain a core of four or five guanine residues: the core sequence for the first domain is 5'-AGTGGGA-3' and for the second domain is 5'-CGGGNG-AGGGGA-3'. MZF-1 protein has been shown to repress the promoter activity of CD34 and *c-myc* genes in haematopoietic and non-haematopoietic cells and to act as a silencer of haematopoiesis-specific gene expression [41]. MZF-1 transcription factor is also critical for the regulation of cell proliferation and apoptosis [42,43]. MZF-1 has an alternatively spliced isoform, MZF-1B, which encodes an additional 257 residues that is involved in the formation of a complex with RAZ-1 protein. The exact role of RAZ-1 has not been determined, but it has been assumed that MZF-1B activity is mediated by specific protein-protein interactions with RAZ-1 [44]. We identified at least four putative MZF-1 binding sites in the EC-SOD promoter and demonstrated that overexpression of MZF-1 and RAZ-1 inhibits promoter activity in MLg cells. On the other hand, in mIMCD3 cells, where EC-SOD promoter appears to be already repressed, these transcription factors were unable to reduce reporter gene levels further.

The Kruppel-like transcription factors are represented by a broad class of proteins bearing three zinc fingers at their C-terminal end which interact with purine-rich DNA sequences. A majority of these proteins share additional homology with the *Drosophila* segmentation gene product Kruppel [45]. The Kruppel-like family consists of numerous transcription factors, with the best known being Sp1, and the most recently discovered being erythroid KLF (EKLF), LKLF, GKLF, ubiquitous KLF (UKLF) and basic KLF (BKLF). All of these proteins are involved collectively in transcriptional regulation of a wide array of genes during development and differentiation (reviewed in [46]). In the present study, we identified at least one sequence in position -131/-113 having high similarity with the GKLF putative binding site. Taking into consideration that GKLF has affinity for a broad variety of GA-rich sequences [47], it seems reasonable to assume that Kruppel-like proteins may interact with other sites within the EC-SOD promoter. The competition experiment with different oligonucleotides representing various regions of the EC-SOD promoter supports this model (Figure 6). The possible role of other Kruppel-like factors, such as EKLF, BKLF and UKLF, is not known at this time and will require further elucidation.

The molecular mechanism by which these transcription factors determine tissue-specific expression of EC-SOD remains speculative at present. However, cell-specific regulation of EC-SOD transcription appears to lie not only within the promoter sequence itself, but also with the balance of transcription factors that are expressed in a particular cell type. Taking into consideration that the Ets and Kruppel-like families are represented by numerous transcription factors, it is difficult to determine the exact contribution that each of these plays in EC-SOD expression. Based on our EMSA experiments, the Ets family of transcription factors have a lower binding affinity compared with Kruppel-like and MZF-like proteins. To express EC-SOD at a high level, several factors should be considered: the levels of repressors, such as Kruppel and MZF-1, should be low and the level of activators, such as Ets transcription factors, should be relatively high. The ability of Ets-binding sites to control tissue-specific EC-SOD expression may reflect the general organization of TATA-less promoters that have closely spaced multiple Ets repeats. Although this explanation for transcriptional regulation of EC-SOD is generally simplified and does not take into account other possible regulatory mechanisms, it provided the basis for future research related to the tissue-specific expression of EC-SOD.

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REFERENCES

- McCord, J. M. and Fridovich, I. (1988) Superoxide dismutase: the first twenty years (1968–1988). *Free Radical Biol. Med.* **5**, 363–369
- Fridovich, I. (1995) Superoxide radical and superoxide dismutases. *Annu. Rev. Biochem.* **64**, 97–112
- Zelko, I. N., Mariani, T. J. and Folz, R. J. (2002) Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radical Biol. Med.* **33**, 337–349
- Oury, T. D., Chang, L. Y., Marklund, S. L., Day, B. J. and Crapo, J. D. (1994) Immunocytochemical localization of extracellular superoxide dismutase in human lung. *Lab. Invest.* **70**, 889–898
- Folz, R. J. and Crapo, J. D. (1994) Extracellular superoxide dismutase (SOD3): tissue-specific expression, genomic characterization, and computer-assisted sequence analysis of the human EC SOD gene. *Genomics* **22**, 162–171
- Marklund, S. L. (1982) Human copper-containing superoxide dismutase of high molecular weight. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7634–7638
- Sandstrom, J., Carlsson, L., Marklund, S. L. and Edlund, T. (1992) The heparin-binding domain of extracellular superoxide dismutase C and formation of variants with reduced heparin affinity. *J. Biol. Chem.* **267**, 18205–18209
- Carlsson, K. and Marklund, S. L. (1988) Extracellular superoxide dismutase in the vascular system of mammals. *Biochem. J.* **255**, 223–228
- Enghild, J. J., Thøgersen, I. B., Oury, T. D., Valnickova, Z., Hojrup, P. and Crapo, J. D. (1999) The heparin-binding domain of extracellular superoxide dismutase is proteolytically processed intracellularly during biosynthesis. *J. Biol. Chem.* **274**, 14818–14822
- Bowler, R. P., Nicks, M., Olsen, D. A., Thøgersen, I. B., Valnickova, Z., Hojrup, P., Franzusoff, A., Enghild, J. J. and Crapo, J. D. (2002) Furin proteolytically processes the heparin-binding region of extracellular superoxide dismutase. *J. Biol. Chem.* **277**, 16505–16511
- Folz, R. J., Guan, J., Seldin, M. F., Oury, T. D., Enghild, J. J. and Crapo, J. D. (1997) Mouse extracellular superoxide dismutase: primary structure, tissue-specific gene expression, chromosomal localization, and lung *in situ* hybridization. *Am. J. Respir. Cell Mol. Biol.* **17**, 393–403
- Marklund, S. L., Holme, E. and Hellner, L. (1982) Superoxide dismutase in extracellular fluids. *Clin. Chim. Acta* **126**, 41–51
- Marklund, S. L. (1990) Expression of extracellular superoxide dismutase by human cell lines. *Biochem. J.* **266**, 213–219
- Folz, R. J., Abushama, A. M. and Suliman, H. B. (1999) Extracellular superoxide dismutase in the airways of transgenic mice reduces inflammation and attenuates lung toxicity following hyperoxia. *J. Clin. Invest.* **103**, 1055–1066
- Carlsson, L. M., Jonsson, J., Edlund, T. and Marklund, S. L. (1995) Mice lacking extracellular superoxide dismutase are more sensitive to hyperoxia. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6264–6268
- Stralin, P., Carlsson, K., Johansson, B. O. and Marklund, S. L. (1995) The interstitium of the human arterial wall contains very large amounts of extracellular superoxide dismutase. *Arterioscler. Thromb. Vasc. Biol.* **15**, 2032–2036
- Stralin, P. and Marklund, S. L. (2000) Multiple cytokines regulate the expression of extracellular superoxide dismutase in human vascular smooth muscle cells. *Atherosclerosis* **151**, 433–441
- Fukai, T., Siegfried, M. R., Ushio-Fukai, M., Cheng, Y., Kojda, G. and Harrison, D. G. (2000) Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. *J. Clin. Invest.* **105**, 1631–1639
- Fukai, T., Siegfried, M. R., Ushio-Fukai, M., Griendling, K. K. and Harrison, D. G. (1999) Modulation of extracellular superoxide dismutase expression by angiotensin II and hypertension. *Circ. Res.* **85**, 23–28
- Lahiri, D. K. and Ge, Y. (2000) Electrophoretic mobility shift assay for the detection of specific DNA–protein complex in nuclear extracts from the cultured cells and frozen autopsy human brain tissue. *Brain Res. Protoc.* **5**, 257–265
- Senapathy, P., Shapiro, M. B. and Harris, N. L. (1990) Splice junctions, branch point sites, and exons: sequence statistics, identification, and applications to genome project. *Methods Enzymol.* **183**, 252–278
- Wingender, E., Chen, X., Hehl, R., Karas, H., Liebich, I., Matys, V., Meinhardt, T., Pruss, M., Reuter, I. and Schacherer, F. (2000) TRANSFAC: an integrated system for gene expression regulation. *Nucleic Acids Res.* **28**, 316–319
- Su, W. Y., Folz, R., Chen, J. S., Crapo, J. D. and Chang, L. Y. (1997) Extracellular superoxide dismutase mRNA expressions in the human lung by *in situ* hybridization. *Am. J. Respir. Cell Mol. Biol.* **16**, 162–170
- Willems, J., Zwijzen, A., Slegers, H., Nicolai, S., Bettadapura, J., Raymakers, J. and Scarce, T. (1993) Purification and sequence of rat extracellular superoxide dismutase B secreted by C6 glioma. *J. Biol. Chem.* **268**, 24614–24621
- Mruk, D. D. and Cheng, C. Y. (2000) *In vitro* regulation of extracellular superoxide dismutase in sertoli cells. *Life Sci.* **67**, 133–145
- Nye, J. A., Petersen, J. M., Gunther, C. V., Jonsen, M. D. and Graves, B. J. (1992) Interaction of murine ets-1 with GGA-binding sites establishes the ETS domain as a new DNA-binding motif. *Genes Dev.* **6**, 975–990
- Lelievre, E., Lionneton, F., Soncin, F. and Vandenbunder, B. (2001) The Ets family contains transcriptional activators and repressors involved in angiogenesis. *Int. J. Biochem. Cell Biol.* **33**, 391–407
- Sementchenko, V. I. and Watson, D. K. (2000) Ets target genes: past, present and future. *Oncogene* **19**, 6533–6548
- Bottinger, E. P., Shelley, C. S., Farokhzad, O. C. and Arnaout, M. A. (1994) The human β 2 integrin CD18 promoter consists of two inverted Ets *cis* elements. *Mol. Cell. Biol.* **14**, 2604–2615
- Yu, M., Yang, X. Y., Schmidt, T., Chinenov, Y., Wang, R. and Martin, M. E. (1997) GA-binding protein-dependent transcription initiator elements. Effect of helical spacing between polyomavirus enhancer factor 3 (PEA3)/Ets-binding sites on initiator activity. *J. Biol. Chem.* **272**, 29060–29067
- Brown, T. A. and McKnight, S. L. (1992) Specificities of protein–protein and protein–DNA interaction of GABP α and two newly defined ets-related proteins. *Genes Dev.* **6**, 2502–2512
- Virbasius, J. V., Virbasius, C. A. and Scarpulla, R. C. (1993) Identity of GABP with NRF-2, a multisubunit activator of cytochrome oxidase expression, reveals a cellular role for an ETS domain activator of viral promoters. *Genes Dev.* **7**, 380–392
- Chui, D. H., Tang, W. and Orkin, S. H. (1995) cDNA cloning of murine Nrf 2 gene, coding for a p45 NF-E2 related transcription factor. *Biochem. Biophys. Res. Commun.* **209**, 40–46
- Martin, M. E., Chinenov, Y., Yu, M., Schmidt, T. K. and Yang, X. Y. (1996) Redox regulation of GA-binding protein- α DNA binding activity. *J. Biol. Chem.* **271**, 25617–25623
- Wang, C. Y., Bassuk, A. G., Boise, L. H., Thompson, C. B., Bravo, R. and Leiden, J. M. (1994) Activation of the granulocyte-macrophage colony-stimulating factor promoter in T-cells requires cooperative binding of Elf-1 and AP-1 transcription factors. *Mol. Cell. Biol.* **14**, 1153–1159

- 36 Lecine, P., Algarte, M., Rameil, P., Beadling, C., Bucher, P., Nabholz, M. and Imbert, J. (1996) Elf-1 and Stat5 bind to a critical element in a new enhancer of the human interleukin-2 receptor alpha gene. *Mol. Cell. Biol.* **16**, 6829–6840
- 37 Sarafova, S. and Siu, G. (1999) A potential role for Elf-1 in CD4 promoter function. *J. Biol. Chem.* **274**, 16126–16134
- 38 Schweppe, R. E. and Gutierrez-Hartmann, A. (2001) Pituitary Ets-1 and GABP bind to the growth factor regulatory sites of the rat prolactin promoter. *Nucleic Acids Res.* **29**, 1251–1260
- 39 Morris, J. F., Hromas, R. and Rauscher, 3rd, F. J. (1994) Characterization of the DNA-binding properties of the myeloid zinc finger protein MZF1: two independent DNA-binding domains recognize two DNA consensus sequences with a common G-rich core. *Mol. Cell. Biol.* **14**, 1786–1795
- 40 Hromas, R., Collins, S. J., Hickstein, D., Raskind, W., Deaven, L. L., O'Hara, P., Hagen, F. S. and Kaushansky, K. (1991) A retinoic acid-responsive human zinc finger gene, MZF-1, preferentially expressed in myeloid cells. *J. Biol. Chem.* **266**, 14183–14187
- 41 Perrotti, D., Melotti, P., Skorski, T., Casella, I., Peschle, C. and Calabretta, B. (1995) Overexpression of the zinc finger protein MZF1 inhibits hematopoietic development from embryonic stem cells: correlation with negative regulation of CD34 and c-myc promoter activity. *Mol. Cell. Biol.* **15**, 6075–6087
- 42 Robertson, K. A., Hill, D. P., Kelley, M. R., Tritt, R., Crum, B., Van Epps, S., Srouf, E., Rice, S. and Hromas, R. (1998) The myeloid zinc finger gene (MZF-1) delays retinoic acid-induced apoptosis and differentiation in myeloid leukemia cells. *Leukemia* **12**, 690–698
- 43 Hromas, R., Morris, J., Cornetta, K., Berebitsky, D., Davidson, A., Sha, M., Sledge, G. and Rauscher, 3rd, F. (1995) Aberrant expression of the myeloid zinc finger gene, MZF-1, is oncogenic. *Cancer Res.* **55**, 3610–3614
- 44 Sander, T. L., Haas, A. L., Peterson, M. J. and Morris, J. F. (2000) Identification of a novel SCAN box-related protein that interacts with MZF1B. The leucine-rich SCAN box mediates hetero- and homoprotein associations. *J. Biol. Chem.* **275**, 12857–12867
- 45 Schuh, R., Aicher, W., Gaul, U., Cote, S., Preiss, A., Maier, D., Seifert, E., Nauber, U., Schroder, C., Kemler, R. et al. (1986) A conserved family of nuclear proteins containing structural elements of the finger protein encoded by Kruppel, a *Drosophila* segmentation gene. *Cell* **47**, 1025–1032
- 46 Bieker, J. J. (2001) Kruppel-like factors: three fingers in many pies. *J. Biol. Chem.* **276**, 34355–34358
- 47 Shields, J. M. and Yang, V. W. (1998) Identification of the DNA sequence that interacts with the gut-enriched Kruppel-like factor. *Nucleic Acids Res.* **26**, 796–802

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