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Characterization of the 5′ Flanking Region of the Human MnSOD Gene

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Human manganese-containing superoxide dismutase(MnSOD) is a nuclear encoded mitochondrial protein that scavenges potentially toxic superoxide radicals by dismuting O_2^- to O_2 plus H_2O_2. To understand the molecular mechanism governing the transcriptional regulation of the human MnSOD gene, I have isolated and sequenced a genomic clone containing the 5′ flanking region of the human MnSOD gene. One major transcription start site was mapped by primer extension to a guanine residue 67 base pairs upstream from the translation start site. Eight putative Sp1 binding elements and one AP1 consensus sequence, but no TATA or CAAT box, were found in the promoter region. Furthermore, a series of chimerical/CAT reporter gene constructs were used to transfect human hepatocellular carcinoma(HepG2), human neuroblastoma and human skin fibroblast cell lines to characterize the promoter and regulatory region of the human MnSOD gene. The results show that human MnSOD gene expression is governed by one promoter and that the basic promoter is located between nucleotides −34 and +38. The results also indicate that both positive and negative elements are involved in the regulation of the cell-type specific expression of the human MnSOD gene. The functional studies indicate that the Sp1 binding sites or G+C rich regions play an important role in regulation of expression of the human MnSOD gene *in vivo.*

The first enzymes involved in the detoxification of oxygen free radicals are the SODs, which convert superoxide anion into H_2O_2 and O_2. Three forms of SOD, with distinctive distributions characterized by their metal requirements are known to exist in aerobic organisms (1,2). Manganese (MnSOD) and iron (FeSOD) forms of SOD are found in prokaryotes, while eukaryotes contain both MnSOD and two copper-zinc-containing SODs (Cu/Zn SODs).

MnSOD in eukaryotic cells is strictly a mitochondrial enzyme located in the inner membrane and encoded by a nuclear gene. A large precursor form is made in the cytosol and transported into the mitochondria by an energy-dependent process (3, 4). The human MnSOD enzyme is found in higher concentrations in those tissues with the largest numbers of mitochondria (liver, heart, kidney) (5). Induction of MnSOD in eukaryotes has been observed following treatment with paraquat (6, 7), x-irradiation (8), hyperoxia (9), interleukin-1 (10), tumor necrosis factor (10, 11), phorbol ester (12), and dinitrophenol (13), suggesting that MnSOD induction is important for protection against oxidative stress. Furthermore, transcriptional run-on assays have discerned that the stimulatory effects of TNF and phorbol ester on the expression of the human MnSOD gene occur at least partially at the level of transcription (12).

The human MnSOD gene was mapped to chromosome 6q25 (14), a region of the chromosome frequently lost in some cancers as glioblastomas, lymphomas, malignant melanoma and ovarian carcinoma (15, 16). Furthermore, a diminished level of MnSOD activity occurs in a wide variety of tumor cells. The lowered activity is generally a consequence of lower amounts of enzyme protein and its mRNA (8). Moreover, the results of MnSOD mRNA turnover experiments in the tumor cells and normal cell counterparts can rule out the possibility that a decrease in stability causes the reduced level of the steady state MnSOD mRNA in tumor cells (17). It is reported that stable transfection of MnSOD cDNA into a melanoma cell line can suppress the malignant melanoma phenotype (18). To determine the MnSOD primary gene structure and mechanisms of regulation is an important first step toward further elucidation of the cause for the loss of MnSOD activity in human cancer cells. To ascertain the molecular structures and mechanisms governing the regulated

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expression of the human MnSOD gene, I have isolated and characterized the 5′-flanking region of the human MnSOD gene, and have also analyzed the functional promoter of the human MnSOD gene and defined the transcription initiation site in normal and tumor cell lines.

MATERIALS AND METHODS

Cloning and DNA sequencing the human MnSOD gene 5′ end and flanking regions. To isolate the human MnSOD genomic clones, a BamH I/EcoR I fragment of human MnSOD cDNA was used as a probe to screen two human leukocyte genomic libraries(ATCC and Promega). Approximately 4×10^6 plaques were screened in duplicate. One genomic clone (containing the 5′ flanking region and most of the human MnSOD gene) was isolated. This positive clone was purified and characterized further after restriction enzyme digestion and southern blot analysis, as previously described (19). This clone contained 15 kb genomic DNA insert and was cut by Sac I to release 3 genomic fragments, 3.2 kb, 4 kb, and 7.8 kb. These fragments were isolated and subcloned into pGEM3Z at the Sac I site. The 4 kb insert which covers 2.1 kb of 5′ flanking region, exon 1, intron 1, exon 2 and a part of intron 2 was sequenced by the dideoxynucleotide method of Sanger et al (20).

RNA isolation and analysis. Total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extract method as described in (21), and poly(A)-containing RNA was prepared by oligo (dT) affinity chromatography. Five μg of total RNA were fractionated by electrophoresis on 1.2% formaldehyde agarose gels and transferred to a nylon membrane (Amersham) as described (19). Northern blot hybridization was performed at 68°C for 16–18 hours with ^{32}P-labeled human MnSOD cDNA probe at a concentration of $1.2–3\times10^6$ counts/ml. After hybridization, the filters were washed with 1% SDS and 0.1 × SSC solution for 15 minutes at room temperature. The filters were then washed twice with 1% SDS and 0.1 × SSC solution for 30 minutes at 65°C. Autoradiography was for 1–3 days at −70°C using Kodak XAR-5 film and an intensifying screen. The mRNA levels were normalized by rehybridization with a human β-actin cDNA probe.

Primer extension assays. To determine the transcription start site of the human MnSOD gene, primer extension assays were done. One end-labeled oligonucleotide, corresponding to position 107/142 of the human colon cancer MnSOD cDNA clone(17) was used as a primer, and poly(A)$^+$RNA extracted from three different cell lines including HepG2, neuroblastoma and skin fibroblasts was used as templates in two steps. In the first step, 2–5 μg of poly(A)$^+$RNA was mixed with a $1.5–3\times10^5$ cpm labeled primer and 2 μl of 10×γTth reverse transcriptase buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.01% (W/V) gelatin) in a volume of 14.4 μl. This solution was incubated at 90°C for 5 minutes and then incubated at 69°C for 5 minutes. In the second step, 2 μl of 10 mM $MnCl_2$, 1 μl of 2.5 mM dNTP and 2 μl of IU γTth DNA polymerase were added in the above reaction solution in a final volume of 20 μl. The extension reaction samples were kept at 70°C for 28 minutes, then extracted with phenol/chloroform, and analyzed on 6% sequencing gels.

Plasmid construction. The promoter/CAT chimerical constructs were generated as follows. First, a fragment containing the human MnSOD gene promoter sequence from −223 to +38 was obtained by a Pst I/SfaN I digestion of 4 kb genomic Sac I fragment. The 261 bp Pst I/SfaN I fragment was blunt-end ligated into the Pst I site of the polylinker of the promoterless plasmid pBasic-CAT (Promega). This construct was named pGCAT223. The construct pGCAT2013 (−2013 to +38) was generated by ligation of a Hind III/AfI II fragment derived from 4Kb fragment into the construct pGCAT223 at the Hind III/AfI II site. The construct pGCAT223 was digested with BanII/Hind III and AfI II/Hind III respectively. The big fragments were isolated and blunt-end ligated to themselves to generate two constructs, namely construct pGCAT114(−114 to +38) and construct pGCAT34 (−34 to +38). The construct pGCAT2013 was cut by Hind III/Spe I and a large fragment was isolated and ligated to itself to yield a new construct called construct pGCAT842 (−842/+38). The construct pGCAT5538 was produced by ligation of 3.2 Kb original genomic Sac I fragment into the construct pGCAT2013 at the Sac I site. The construct pGCAT14 was designed by ligation of a DNA synthetic fragment, corresponding to position −14/+38 of the human MnSOD gene 5′ flanking region, into the PstI site of the polylinker of the promoterless plasmid pBasic-CAT.

The pCAT-promoter plasmid, which contains the SV40 mini promoter upstream from the CAT gene was obtained from Promega. To search for enhancer sequences or transacting elements governing the regulation of the human MnSOD gene expression, pCAT promoter-derived plasmids were constructed by cloning three overlapping fragments of the genomic DNA upstream from the promoter region of the human MnSOD gene obtained from the construct pGCAT2013 digested by Pst I, Sac II, and Sac I/Sac II respectively, into the Pst I site of the pCAT-promoter. The resultant plasmids were designated as pCAT/SOD01153 (−427 to −26), pCAT/SOD01152 (−717 to −223), and pCAT/SOD01182 (−2013 to −427). The number and orientation of the inserts were determined by restriction enzyme digestion.

An expression vector, pSV40HGH-1 used to monitor transfection efficiency by co-transfection with promoter test constructs, was generated by cloning 2.2 kb BamH I/EcoR I fragment of pOGH plamide (Nicholls Institute) into the Ava I/Hind III site of pCH110 (Pharmacie, UG, Uppsala, Sweden).

Cell culture and DNA transfection. Human skin fibroblast (#8399) cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum. Human hepatocellular carcinoma (HepG2) cells and Human neuroblastoma (SK-N-SH) cells were cultured in Eagle's MEM with nonessential amino acids, sodium pyruvate, Earle's BSS and 10% fetal bovine serum. The cells were plated at a density of approximately 1×10^6 on 60 mm dishes on the day prior to the transfections. The next day, the cells were refed with 3 ml of DME/F12 with HEPES media containing

10% fetal bovine serum and incubated for 2–3 hours. Transfections were then performed by the calcium phosphate co-precipitation method (19). Each precipitate contained 15 μg of test plasmid and 2 μg of pSV40HGH-1. The cells were incubated with the precipitate for 4 hours followed by a 3-minute glycerol shock. The cells were then washed and refed with growth media and incubated at 37°C for 2.5 days. In individual experiments, duplicate dishes were analyzed for each sample. All transfection assays were done in triplicate in order to compensate for experimental variation.

Human growth hormone assay and CAT assay. Human growth hormone determinations using a radioimmunoassay kit (Nicholls Institute) were carried out on the media 60 hours after glycerol shock according to the protocol from Nicholls Institute. Meanwhile, the cells were harvested and cell extracts were prepared by four cycles of freeze-thawing in 150 μl of 0.25 mM Tris-HCl, pH 7.5. CAT activity was assayed by acetylation of chloramphenicol and solvent extraction of the radio-labeled product (22). The volume of extract used for CAT assay was determined by the results of human growth hormone assay. The CAT assays were performed in 125 μl containing 0.125 uCi of [^{14}C] chloramphenicol and 6nmol of n-butyryl-CoA at 37°C for 1 hour. The butyrylated chloramphenicol was separated from the free chloramphenicol by xylene extraction and was quantified by liquid scintillation counting. In all transfection experiments, pCAT-Basic, pCAT-promoter and pCAT-control were used as negative and positive controls, respectively. Finally, the mean activities normalized to pCAT-control were determined and used for quantitative comparison.

RESULTS

One positive clone with a 15 kb insert containing the 5′ flanking region and most of the human MnSOD gene was isolated from the human genomic DNA library as described above. This genomic insert was released from the positive clone by digestion with Sac I and yielded three fragments. These three fragments were 3.2 kb, 4 kb, and 7.8 kb in size as determined by restriction mapping and Southern blot analyses. The 4 kb fragment, which contained 2.1 kb of the 5′ flanking region and exon 1, exon 2, intron 1, and a part of intron 2 of the human MnSOD gene, was used to determine the functional promoter of the human MnSOD gene. To facilitate sequencing, restriction fragments from the 4 kb fragment were subcloned into pGEM3Z and the entire 2.1 kb BamH I fragment of the 5′ flanking region was sequenced completely. The promoter region sequences of the human MnSOD gene is shown in Fig. 1. Notably, no “TATA box” and “CAAT box” were found in the 5′ flanking region. This feature is common to the promoters of many other “housekeeping” genes as well as the bovine and the rat MnSOD gene (23, 24, 25). The sequence between nucleotide residues −200 and −1, which contains the putative promoter of the human MnSOD gene, has a G+C content of 84%. On the upstream of the human MnSOD gene coding region a number of transcription factor binding DNA elements were found, and many of which match loosely to their respectively consensus sequences (26). A prominent feature of the MnSOD gene 5′ flanking region is possession of one vestigial human Alu sequence located at positions between −1584 and −1524, which is also described in some other genes (27, 28, 29, 30, 31). More significantly, a sequence element (TAGAGACGGGGTTTCACtA) of the upstream Alu sequence (at positions −1568 to −1550) matches 18 of 19 nucleotide residues (a mismatch is in lowercase letter) to an OXPHOS enhancer (enh) consensus sequence (TAGAGANNNGGTTTCACCA) (32).

Several other potential regulatory elements for controlling the transcription of many other genes were also detected in this region. Eight hexanucleotide core sequences (GGGCGG) that correspond to consensus binding sites for the transcription factor Sp1 (33) were located (at nucleotides −342, −168, −164, −121, −113, −91, −86, and −19). One copy of the consensus octanucleotide (GTGACTAA) for the binding of AP-1 (or c-jun and c-fos protein complex), which allows activation of genes induced by phorbol esters (34, 35), was mapped to position −829. One copy of acute phase reactant regulatory elements (CTGGGA), which mediate the induction of the gene expression under acute physical stress *in vivo* (36, 37), was present in the region within −474 to −469. Moreover, two sequences (CGCGGGGCG) which closely match the Egr1 consensus sequence (GCGGGGGCG) are found in this region of the human MnSOD gene promoter(nucleotides −121 to −114 and −94 to −87) (38).

Although three mRNA specise were detected in the human MnSOD gene expression, only one transcriptional start site of the human MnSOD gene was identified by primer extension analysis. In this study, an oligonucleotide that is complementary to the sequence between +107 and +142 in

FIG. 1. Partial nucleotide sequence of the 5′-flanking region of the human MnSOD gene, the first exon with deduced amino acid sequence and the proximal first intron of the human MnSOD gene. Sp1-binding sites are indicated by arrows, which also specify orientation. Ap1 element is underlined. Acute phase reaction regulatory element is double underlined. OXPHOS enhance (enh) consensus sequence is boxed.

the human colon cancer MnSOD cDNA was used for primer extension on HepG2, neuroblastoma, and skin fibroblast cell mRNA templates and one identical major primer extension product (150 nucleotides) was identified in all above three cell lines (Fig. 2).

These three cell lines were also tested for endogenous expression of MnSOD mRNA. Cells of the three lines were grown to 100% confluence and total RNA was isolated and analyzed by Northern blot analysis using the MnSOD cDNA coding region as a probe. In addition, a human β-actin probe was also used for hybridization on the same blot to monitor amount of the mRNA used in each lane. The result of this experiment is shown in Fig. 3A. Although the MnSOD mRNA was detected in all three cell lines, the hepG2 cells contained very high levels of the 4 kb MnSOD mRNA while neuroblastoma and skin fibroblasts contained very low steady state amounts of MnSOD mRNAs. This data demonstrates that the MnSOD gene is expressed with cell specific patterns.

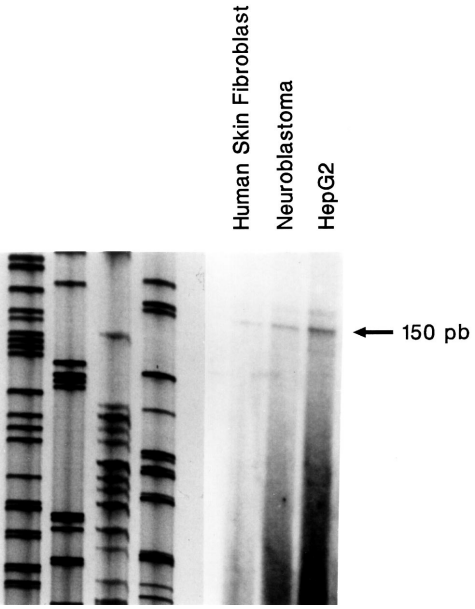

FIG. 2. Primer extension of human MnSOD mRNA. The oligonucleotide (GCTGTGCTTCTGCCTGGAGCCCA-GATACCCCAAAG) was annealed to 2 μg, 5 μg of poly(A+) RNA from HepG2, neuroblastoma and skin cells respectively, used as templates for the primer extension with rTTh DNA polymerase as described under Materials and Methods. The primer-extended products and dideoxynucleotide sequence reactions as size markers were separated on a DNA sequencing gel.

To determine whether sequences 5′ to the human MnSOD gene function as a promoter, a construct was made in the pBasic-CAT vector with a 5.5 kb (refers to −5.5 to +38) fragment of 5′ flanking region of the human MnSOD gene and was designated as pGCAT5538. Transfections of this construct into HepG2 cell line showed very high expression of CAT. In order to define whether the CAT gene was expressed in a cell-specific pattern or not, the pGCAT5538 construct was also transfected into skin fibroblast, neuroblastoma and HepG2 cell lines. As presented in Fig. 3B, the human MnSOD sequences in the pGCAT5538 construct were effective in driving the expression of CAT in HepG2, while low levels of the reporter gene product were detected in neuroblastoma and human skin fibroblast cells. The maximum level of expression was seen in the HepG2 cells and this corresponded to approximately 158% of the expression of the reporter gene seen in the same cells transfected with the pCAT control. These results correlate with the endogenous expression of the human MnSOD gene in the above three cell lines.

To ascertain in more detail the regulatory sequences required for optimum promoter activity and the minimum promoter, a series of mutant chimerical reporter gene constructs made using 5′ deletion were transfected into HepG2 cells and neuroblastoma cells. The results of the above experiments are presented in Fig. 4. The data show that maximum expression of CAT can be obtained with only 114 bp of the 5′ flanking region immediately upstream from the human MnSOD transcription start site in neuroblastoma cells, while 2013 bp of the 5′ flanking sequences needed to drive maximum expression of CAT in HepG2 cells.

The small changes in the expression level of CAT which were observed when the upstream sequences from −5500 to −2013 in HepG2 and from −5500 to −842 in neuroblastoma cells were included in the reporter constructs, were seen consistently. However, a large decrease in CAT expression was observed when constructs made by 5′ deletion from −2013 to −842 of the human MnSOD gene 5′ flanking sequences were used to transfect in HepG2. This result indicates that a significant positive element(s) is present upstream from −842, whereas a large increase in CAT

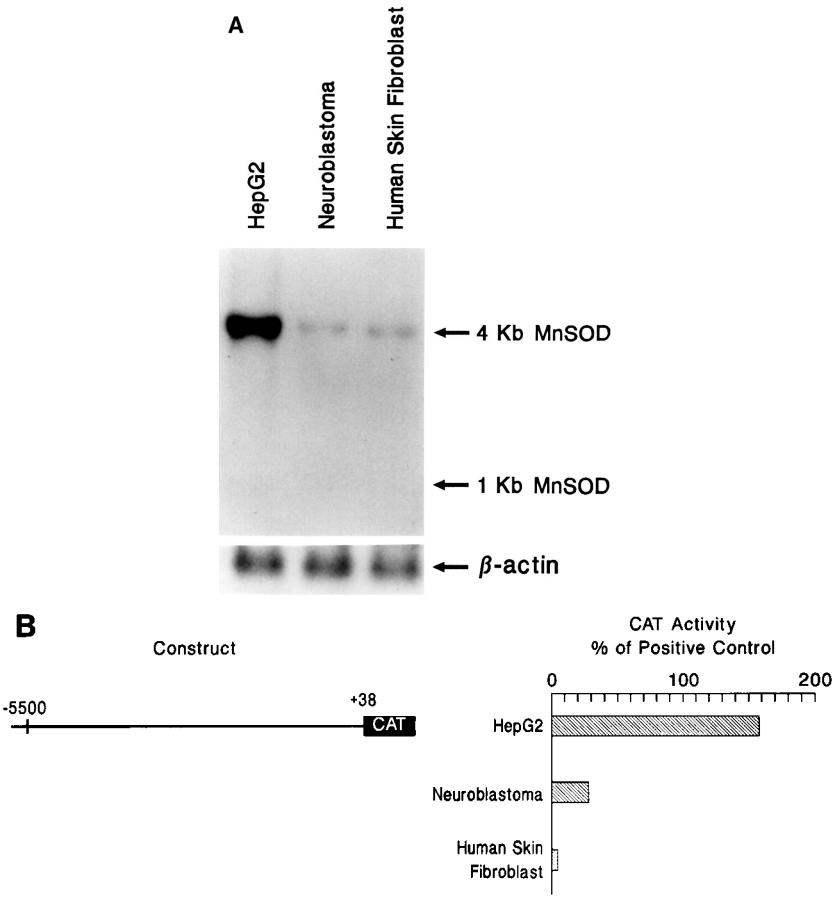

FIG. 3. Cell specific expression of the human Mnsod gene. (A) Endogenous expression of the human MnSOD gene. Northern blot hybridization analyses were performed with a human MnSOD cDNA or as a human β-actin cDNA probes and 5 μg of total RNA isolated from HepG2, neuroblastoma, and skin fibroblast cells. The mRNA species are denoted by arrows on the right. (B) Cell specific promoter activity of the human MnSOD gene 5′-flanking sequences. A portion of the 5′-end of the human MnSOD gene from +38 to −5500 was fused to the 5′-end of the CAT gene in a transfection vector. The construct was transfected into various cell types as described under Materials and Methods. CAT activity is expressed as a percent of the CAT activity in each cell type transfected with a vector containing the CAT gene driven by the SV-40 promoter and SV-40 enhancer(pCAT-Control).

expression was observed in neuroblastoma when the sequences from −842 to −233 were deleted. Thus, an important negative element is located in this region. Interestingly, pGCAT114 (−114 to +38) contains almost 45% maximal activity in HepG2 cells and maximal activity in neuroblastoma cells, and thus the most important cis-acting elements responsible for transcriptional activity of the human MnSOD gene appear to reside within this region. pGCAT34, which lacks the sequence from −114 to −34 with 3 Sp1 binding sites, contains only one Sp1 binding site and retains 6.7% of the maximum activity of CAT in HepG2 cells and 8.9% in neuroblastoma cells. Finally, the CAT activity was not detected when the construct pGCAT14, which deletes all Sp1 binding sites, was used for transient transfection in HepG2 cells and neuroblastoma cells. This result suggests that the minimum promoter governing the human MnSOD gene expression is limited within the region −34 to +38.

The data shown in Figs. 3B and 4 indicate that cell-type specific expression of a reporter gene was obtained *in vitro* with 5′ flanking sequences of the human MnSOD gene and at least one

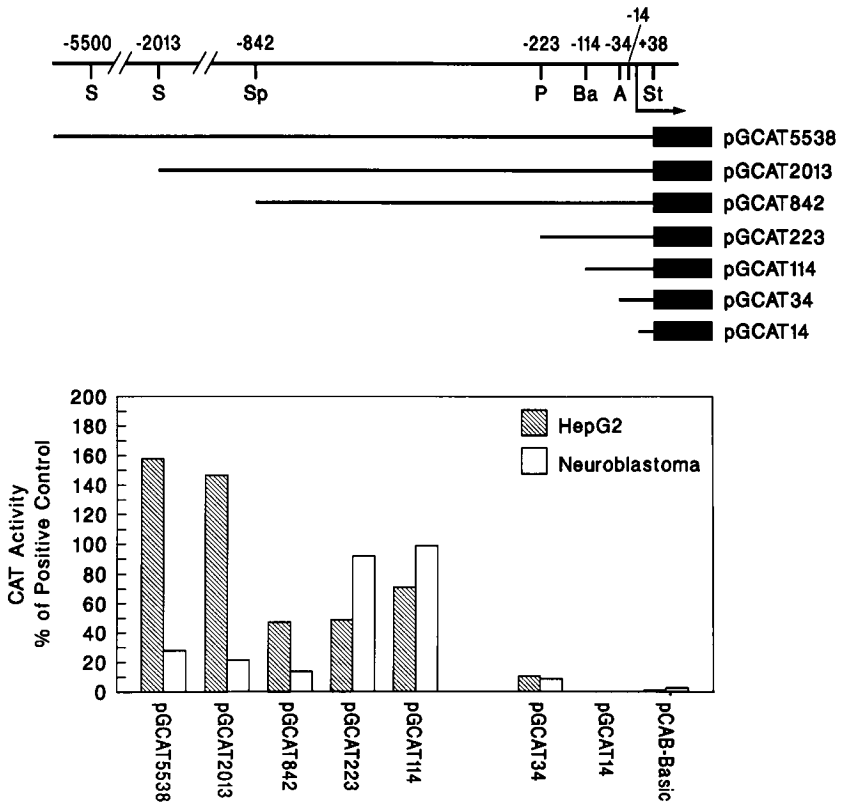

FIG. 4. Detailed analysis of the promoter activity of 5′-flanking sequences of the human MnSOD gene. The line diagram at the top is a restriction map. St, A, Ba, P, Sp, S represent restriction sites for enzymes SfaN I, AfI II, Ban II, Pst I, Spe I and Sac I. Serial deletion mutants were tested in the pBacis-CAT vector in HepG2, neuroblastoma cell lines. All constructs extend to + 38bp. The activities of the constructs are compared with the activity of pControl-CAT, which is arbitrarily taken as 100%.

positive element(s) and one negative element(s) regulate this cell-type specific expression. To examine the ability of the 5′ flanking sequences to enhance or reduce transcription from a heterologous promoter. The fragments corresponding to the −427/−26 (antisense orientation), −717/−223 (sense orientation) and −2013/−427 (antisense orientation) were cloned into the enhancer position of pCAT-promoter vector containing the enhancerless SV40 promoter. The constructs were then transfected into HepG2 and neuroblastoma cells, and CAT expression of these constructs was compared to that observed from the vector alone. A ∼3.2-fold increase in expression of CAT was obtained in HepG2 while a ∼4-fold decrease in expression of CAT was present in neuroblastoma cells when a construct containing the −2013/−427 sequences was used for transfection in both cell lines (Fig.5).

DISCUSSION

I have cloned and characterized the 5′ flanking region of the human MnSOD gene as a starting point for the analysis of the mechanisms controlling its tissue- or cell-specific expression, developmentally induced expression, and decreased activity in human cancer cells. Mapping of the transcriptional start site indicated that human MnSOD transcription is initiated at the same site in HepG2, neuroblastoma, and human skin fibroblast cells. The transcription start site was identified at the guanidine residue 67 bases upstream of the translation initiation site, inconsistent with

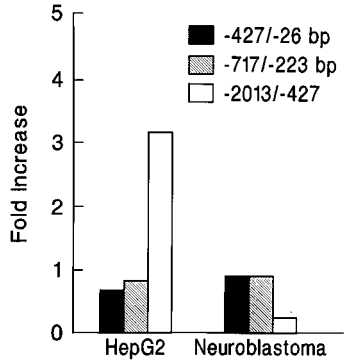

FIG. 5. Enhancer or silencer activity of the 5′-flanking sequences of the human MnSOD gene acting with the enhancerless SV40 promoter on the transcription of CAT in the pCAT-promoter vector. Sequences from −427 to −26, −717 to −223 and −2013 to −427 were tested in the opposite orientation. Fold changed refers to the increase or decrease of CAT activity in pCAT/SOD01153, pCAT/SOD1152 and pCAT/SOD01182 over the enhancerless pCAT-promoter vector alone.

consensus sequences for initiation of transcription of most eukaryotic mRNAs (39), but consistent with the transcription initiation site identified for the rat and bovine homologue of the MnSOD gene (24, 25). The data also establishes that MnSOD mRNAs from each of the 3 cell types are transcribed from the same initiation site. A comparison of sequence in the 5′ flanking region with that of the rat or bovine MnSOD genes reveals a number of features that were conserved during evolution. The nucleotide sequences immediately upstream from the site of transcription initiation of the human MnSOD gene and the rat MnSOD gene lack “TATA box” and “CAAT box”.

The sequences between nucleotide residues −200 and −1 containing the putative promoter of the MnSOD gene is highly G + C rich, with a G + C content of 76% in rat, 80% in bovine and 84% in humans. Both rat and human MnSOD genes possess one copy of the AP-1 site in the 5′ flanking regions. Eight Sp1 binding sites are present on both of the human and the bovine MnSOD promoters, while two copies of Sp1 binding core sequence are found at positions −14 and −238 of the rat MnSOD gene. Interestingly, the sequence less than 20 bp upstream from the site of transcription initiation contains one Sp1 binding site in both human and rat. Two copies of SV40 core enhancer sequence (40) and one copy of the consensus sequence of the adenovirus EIA gene enhancer (41) are only found in the 5′ flank region of the rat MnSOD gene, whereas the sequence far upstream from the human MnSOD promoter region contains one copy of an OXPHOS enhancer (enh) consensus sequence located in an Alu element region (32) which is not found in the 5′ flanking region of rat MnSOD gene.

These data indicate that some common molecular mechanisms governing the regulated expression of the MnSOD gene are shared among these mammalian species, but they also possess other species-specific mechanisms. In the current study, I have been able to establish that 5.5 kb of 5′ flanking sequences contain functionally important promoter elements and that this DNA segment confers a pattern of cell-specific expression that correlates well with the pattern of expression of the endogenous gene. To show this, a 5.5 kb fragment of 5′ flanking region of the human MnSOD gene was fused into a CAT-reporter gene (pGCAT5538) and tested for transient expression in the HepG2, neuroblastoma, and skin fibroblast cell lines. I observed a very strong expression in HepG2 cells while the expression of the CAT-reporter gene was significantly lower in neuroblastoma and human skin fibroblast cells. To identify sequences in the human MnSOD gene 5′ flanking region responsible for cell-specific expression, 5′ deletion constructs were tested for transcriptional activity in HepG2 and neuroblastoma cells. A positive activity was shown by sequences between bp−2013 and −842, and this activity was present only in HepG2 cells. Negative activity was also narrowed to a region within −842 and −223 in the neuroblastoma cells. Furthermore, both negative

and positive transacting sequences function with the heterologous SV40 promoter constructs in an orientation-independent and distance-independent fashion. In future work, the interaction between the putative enhancer factor or the silence factor and its target DNA sequence will be examined by a method which can pinpoint the DNA contact residues, define the precise sequence specificity, and yield information about the physical properties.

In the experiments reported here, I studied in more detail the 152 bp fragment(from −114 to +38) that supports the maximum activity of the human MnSOD promoter. This region contains a cluster of three contiguous Sp1 binding sites and another Sp1 consensus sequence located at 18 bp upstream from the transcription start site. The activity of the human MnSOD promoter was dramatically reduced when the nucleotides −114 to −34 (which include the 3 contiguous Sp1 binding sites) were removed. However, the remaining 72 bp fragment from −34 to +38 still retained 6.7% of maximal transcription activity of the human MnSOD gene in HepG2 cells and 8.9% of maximal promoter activity in neuroblastoma cells, since this region still possesses one Sp1 motif located immediately upstream of the transcription initiation site. The promoter activity of the human MnSOD gene disappeared completely when the last Sp1 motif was deleted. The data suggest that the RNA polymerase binding site and the transcription initiation element reside within −34 to +38, and that Sp1 is the major positive transcriptional factor governing the human MnSOD gene promoter activity.

Sp1 was initially identified as a factor involved in specific expression of SV40 early RNA (42). In the past decade, the binding sequences for Sp1 have been identified in the (proximal) promoters of many cellular genes. In model systems of TATA-lacking promoters, Sp1 has been shown to be able to mediate transcription initiation by influencing the formation of a competent transcription initiation complex (43). Although no natural or synthetic promoter has been described where a single Sp1 binding sequence functions as a minimal promoter, Sp1 always functions with a second element, which can consist of additional Sp1 binding sequences or an initiator (Inr) sequence (44, 45, 46, 47). Furthermore, the functional importance of the upstream Sp1 sites in those TATA-less genes appears to depend on their relative locations in the promoters. It has been shown that Sp1 motifs located immediately upstream of transcription initiation sites of a number of genes exert positive enhancing effects (42, 43), whereas those located far upstream appear to have minimal effects (47). In keeping with these, the human MnSOD promoter appears to be dramatically stimulated by an upstream DNA region containing a GC-rich sequence with 3 contiguous Sp1 consensus binding sites (−114 to −86), and the first Sp1 binding sequence at −18/−12 in the human MnSOD promoter may play a central role in transcription initiation, because the CAT activity was completely inhibited when the construct pGCAT14, which deletes the Spi binding site, was transfected in HepG2 or neuroblastoma cells. The other 4 Sp1 motifs located upstream from −114 are shown to have no effects on the promoter activity of the human MnSOD gene.

In summary, I have characterized the 5′ flanking region and promoter of the human MnSOD gene. The three MnSOD mRNAs are transcribed from a single transcription initiation site governed by a single promoter. Our results suggest that the human MnSOD promoter region is highly regulated and that the major activity of the MnSOD promoter requires an intact G+C region located immediately upstream of transcription initiation site which contains four functional Sp1 binding sites. One far upstream enhancer and one transacting negative element are needed to mediate cell-type specific expression of the human MnSOD gene. Roles of these regulatory elements of the 5′ flanking region may be important in the regulation of expression of this gene in differentiation and in transformed cells as well.

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