THE REGULATION OF HUMAN MANGANESE SUPEROXIDE DISMUTASE GENE

SUREERUT PORNATADAVITY

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ABSTRACT

Manganese superoxide dismutase (MnSOD) is a nuclear-encoded metalloenzyme that protects cells by removing superoxide radicals in the mitochondria. Human MnSOD gene is highly regulated and responds to various stimuli. The level of MnSOD has been shown to be associated with myriad pathological diseases and is also useful in cancer therapy. Thus, knowing how to modulate endogenous MnSOD levels to control and treat disease is important. The aim of this study was to investigate the mechanism of MnSOD regulation in the hepatocellular carcinoma (HepG2) cell line by using two known reagents, TPA and HIV-1 Tat protein. In this study, we investigated the induction of MnSOD gene by TPA. The results show that the MnSOD basal promoter is responsive to TPA-activated MnSOD transcription. Sp1 protein plays a positive role in basal and TPA-activated MnSOD transcription, whereas Egr-1 protein has a negative role in basal promoter activity and no effect on TPA-mediated activation of MnSOD transcription. We further studied the suppression of MnSOD by HIV-1 Tat protein. The results show that Tat suppresses MnSOD expression from the MnSOD basal promoter by altering the distribution of Sp1/Sp3 proteins. Tat significantly increases the level of full-length Sp3, but not Sp1 and truncated Sp3. Mutational analysis of Sp3 and transcription assay in insect SL2 cells reveals that Sp3-suppressed MnSOD transcription is not just simply a competing mechanism. We found that the Sp3-repressed MnSOD promoter activity requires sufficient length and/or the structure being near the full-length Sp3. The immobilized MnSOD basal promoter reveals a distinctive protein pattern between nuclear extracts from Sp1 and Sp3-overexpressed. Moreover, we partially identified that general transcription, TAFII 130, is involved in the protein-protein interaction of Sp1 and Sp3. Taken together, the transcription of MnSOD is regulated by the transcription factors contained in the basal promoter and their interaction with other transcription factors.

KEY WORDS: MnSOD/TPA/HIV-1 Tat PROTEIN/ Sp1/Sp3/TRANSCRIPTION FACTORS
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บทคัดย่อ

Manganese superoxide dismutase (MnSOD) เป็น metalloenzyme ที่ทำหน้าที่กำจัด superoxide radicals ใน mitochondria ยีนที่ควบคุมการแสดงออกของ MnSOD อยู่ใน nucleus การแสดงออกของยีน MnSOD จะถูกควบคุมอย่างรัดกุม และสามารถตอบสนองต่อสิ่งต่างๆที่มากระตุ้น การแสดงออกของยีน MnSOD มีความเกี่ยวเนื่องกับขบวนการเกิดโรคต่าง ๆ และยังสามารถช่วยในการรักษาโรคมะเร็งได้อีกด้วย ดังนั้นการรู้กลไกที่ควบคุมการแสดงออกของยีน MnSOD จะสามารถนำมาประยุกต์ใช้ในการรักษาและควบคุมโรคได้ การศึกษานี้มีวัตถุประสงค์ที่จะศึกษากลไกที่ควบคุมการแสดงออกของ MnSOD ในเซลล์มะเร็งตับ $HepG_2$ โดยใช้สาร 2 ตัวที่มีผลต่อการแสดงออกของ MnSOD คือ TPA และ โปรตีน HIV-1 Tat ผลของการศึกษาพบว่า TPA เพิ่มระดับของ MnSOD mRNA ด้วยการเพิ่ม activity ของ MnSOD basal promoter และพบว่าโปรตีน Sp1 เป็น activator ที่ควบคุมการแสดงออกของยีน MnSOD ทั้งในสภาวะปกติ และในสภาวะที่มี TPA เป็นตัวกระตุ้นจาก ขณะที่โปรตีน Egr–1 เป็น repressor และไม่มีความเกี่ยวข้องกับการควบคุมการแสดงออกของยีน MnSOD ในสภาวะที่มีการกระตุ้นจาก TPA ผลการศึกษากลไกการแสดงออกของ MnSOD โดยใช้โปรตีน HIV-1 Tat พบว่า Tat ลดการแสดงออกของยีน MnSOD โดยลด activity ของ MnSOD basal promoter ด้วยการเพิ่มปริมาณของ Sp3 อย่างมีนัยสำคัญ ผลของการศึกษาในเซลล์แมลง SL2 และ เซลล์ HepG2 พบว่าความสามรถของ Sp3 ในการลดระดับ activity ของ MnSOD basal promoter ขึ้นอยู่กับความยาว หรือโครงสร้างที่ใกล้กับด้าน N-termini ของ Sp3 และโปรตีนตัวอื่น ๆที่ไม่พบหรือไม่ทำงานในเซลล์ SL2 นอกจากนี้ผลของการศึกษายังแสดงถึงความแตกต่างของชนิดของโปรตีนที่เกี่ยวเนื่องในการควบคุมการแสดงออกของยีน MnSOD โดย Sp1 และ Sp3 ผลการศึกษาสามารถบ่งชี้ได้ว่า TAFII130 เกี่ยวข้องกับการควบคุมการแสดงออกของยีน MnSOD สรุปโดยรวม การควบคุมการแสดงออกของยีน MnSOD จะขึ้นอยู่กับ interaction ของโปรตีนที่อยู่บน basal promoter กับโปรตีนตัวอื่นๆ

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CONTENTS

Page

CHAPTER 2: LITERATURE REVIEW

CHAPTER 3: TPA-ACTIVATED TRANSCRIPTION OF THE HUMAN MnSOD GENE: ROLE OF TRANSCRIPTION FACTORS Sp1 and Egr-1

CONTENTS (continued)

Page

MATERIALS AND METHODS

RESULTS

CHAPTER 4: DIFFERENT ROLES OF Sp FAMILY MEMBERS IN HIV-1 Tat-MEDIATED HUMAN MANGANESE SUPEROXIDE DISMUTASE SUPPRESSION IN HEPATOCELLULAR CARCINOMA CELLS

MATERIALS AND METHODS

CONTENTS (continued)

CONTENTS (continued)

LIST OF FIGURES

Page

LIST OF FIGURES (continued)

LIST OF ABBREVIATIONS

AP-2	Activator protein 2
CAT	Catalase
CuZnSOD	Copper/Zinc containing superoxide dismutase
ECSOD	Extracellular superoxide dismutase
Egr-1	Early growth response 1
EMSA	Electrophoretic mobility shift assay
GSH	Glutathione
GPx	Glutathione peroxidase
HIV-1	Human immunodeficiency virus 1
I2E	Inronic 2 enhancer region
IL-1β	Interleukin-1 beta
IP	Immunoprecipitation
MnSOD	Manganese superoxide dismutase
NF-κB	Nuclear factor kappa B
P7	MnSOD basal promoter
PKC	Protein kinase C
SOD	Superoxide Dismutase
Sp1	Specificity protein 1
Sp3	Specificity protein 3
TAR	Transactivating responsive element
Tat	Transactivating transcriptional factor
TAFII 130	Transcription factor II 130
TNF-α	Tumor necrosis factor alpha
TPA	12-O-tetradecanoylphorbol-13-acetate

CHAPTER 1

INTRODUCTION

No aerobic organism can avoid oxidants; free radicals and ROS, because they are byproducts of cell metabolism, are needed for cell survival. However, the excess of oxidants leads cells to undergo oxidative stress and the damage of cellular macromolecules such as DNA, lipid, and protein. Thus, cells need to have a tight antioxidant defense mechanism to cope with the deleterious effects of the excess oxidants. The primary antioxidant defenses in aerobic organisms include CAT, GPx, GSH, three isoforms of SOD: MnSOD, CuZnSOD, and ECSOD.

Mitochondria are an important organelle of cells because they are known as the power house of the cell and the consequences of mitochondria function make them the major source of intracellular oxidants. However, mitochondria contain MnSOD in their matrix which convert superoxide radicals into less toxic molecules. Thus, the function and the location of MnSOD make MnSOD play many critical roles in such areas as cell survival, tumor suppressor, and anti-apoptosis. Furthermore, the level of MnSOD has been shown to be associated with many pathological diseases such as cardiovascular disease, cancer, immune system decline, neurodegenerative diseases, AIDS, etc. The MnSOD level is also useful in cancer therapy. This leads to an attempt to modulate the expression of endogenous MnSOD. Although MnSOD is mitochondrial protein, it is encoded by nuclear DNA, transcripted in the nucleus, translated in the cytoplasm, and then transported to the mitochondria. MnSOD gene is characterized as a housekeeping gene; however, MnSOD is responsive to various stimuli such as tumor promoting agent, TPA, and HIV-1 Tat protein.

TPA is a phorbol ester that affects many cellular events which depend on the TPA targets. The target of TPA includes PKC and non-PKC. TPA acts as DAG by binding to the C1 domain of PKC. Non-PKC, chimaerins, PKD, RasGRPs, Munc13s, and DAG kinase gamma have regions that are homologous to the C1 domain of PKC which is recognized by TPA. One of the effects of TPA is TPA induces MnSOD expression. The TPA-responsive element has been found in the distal of MnSOD promoter. However, the DNaseI hypersensitive reveals the protection region in the basal promoter of both normal and stimulation conditions. Moreover, the mutation found in the MnSOD proximal promoter of colon cancer cell lines caused the reduction of MnSOD promoter activity. This indicates that there may be an alternative pathway for TPA-induced MnSOD expression. Thus, it is necessary to study the mechanism of TPA-induced MnSOD expression to fully understand how MnSOD regulation occurs.

Tat protein is produced by HIV-1 during its replication to increase the rate of transcription. Tat possesses many unusual properties. Tat transactivates HIV-1 by binding to the RNA, TAR element instead of to DNA as other transcription factors do. Tat is secreted from HIV-1 infected cells and uptaken by bystander cells. The uptake is dependent on the short basic region of Tat. Tat also modulates many host cellular genes including MnSOD. It has been demonstrated that HIV-infected patients undergo oxidative stress, which may result from Tat-suppressed MnSOD expression. The suppression of MnSOD in HIV-infection has been extensively investigated. However, the mechanism(s) of Tat-suppressed MnSOD expression has been a puzzle for more than a decade.

The goal of this study is to investigate the mechanisms of how MnSOD is regulated by using two known models: up- and down-regulation by TPA and HIV-1 Tat protein, respectively. This study will accumulate more understanding of MnSOD regulation which can be applied to modulate the endogenous MnSOD level to control and treat disease.

CHAPTER 2
LITERATURE REVIEW

Oxidants and oxidative stress

There are many oxidant molecules in cells, such as superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$), etc. Most of these oxidant molecules are derived from the biochemical redox reactions involving oxygen, which occurs as part of normal cell metabolism. For example, superoxide radicals are derived from electron leakage in the electron transport chain during ATP synthesis in the mitochondria (Hass and Massaro, 1987). $^{\cdot}OH$ is derived from redox cycling of metal, iron, and copper which catalyzes reaction of $O_2^{\cdot -}$ and H_2O_2 (Fenton, 1894, 1899; Harber and Weiss, 1933). There are many exogenous sources that initiate oxidant molecules, such as UV light, gamma radiation, environmental pollutants, and cigarette smoke. Whenever cells contain an excess of oxidant molecules, cells will undergo oxidative stress which leads to damage of the macromolecules such as DNA, lipid, and protein. This oxidative stress condition is more often than not a cause of a secondary complication in many disorders such as neurodegenerative diseases, AIDS, cancer, etc. Fortunately, cells are equipped with an array of antioxidant defense mechanisms that are tightly regulated to combat oxidant damage.

ANTIOXIDANT SYSTEM

The cellular antioxidant system can be divided into two categories according to the nature of their activities: antioxidant substrates and antioxidant enzymes. Antioxidant substrates, such as glutathione (GSH) and thioredoxin (TRX), which directly react with oxidant molecules and neutralize their reactivity. GSH and TRX are important to keeping the balance redox status between intra- and extracellular, whereas antioxidant enzymes, such as catalase, glutathione peroxidases, and superoxide dismutases (SODs), which convert oxidant molecules into less reactive product. SOD converts supeperoxide anion into hydrogen peroxide which further catalyzes by catalase and glutathione peroxidase into non-toxic molecule, water.

SUPEROXIDE DISMUTASE (SOD)

SOD when first isolated was thought to be a copper storage protein (Mann and Keilis, 1938). Subsequently, SOD was identified by a number of names, indophenoloxidase, tetrazolium oxidase, etc. The catalytic function of SOD was discovered by McCord and Fridovich as shown in equation 1 (McCord and Fridovich, 1969).

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 \qquad \text{equation 1}$$

$$O_2^{\cdot -} + H_2O_2 \xrightarrow{\text{iron or copper ions}} O_2 + {}^{\cdot}OH + OH^- \qquad \text{equation 2}$$

SOD is present in all aerobic organisms and most subcellular compartments that generate activated oxygen because SOD must eliminate $O_2^{\cdot -}$ before it is converted into a very toxic oxidant, such as ${}^{\cdot}OH$. For example; in Haber and Weiss reaction generates a very deleterious oxidant, ${}^{\cdot}OH$ (equation 2) which causes a chain reaction damage of lipid and DNA. SOD regulates Haber and Weiss reaction by (1) determining the relative proportions of two substrates in Haber and Weiss reaction (equation 1 and 2). (2) $O_2^{\cdot -}$ oxidizes the [4Fe-4S] clusters of dehydratases, such as aconitase, causing inactivation and release of Fe(II), which catalyzes the Haber and Weiss reaction (equation 2). Moreover, $O_2^{\cdot -}$ reacts with NO and generates a very toxic oxidant, peroxynitrite ($ONOO^-$). Thus, SOD is thought to play a central role in the defense against oxidative stress (Beyer *et al.*, 1991).

There are three isoforms of human SOD classified on the basis of their localization: copper/zinc (CuZnSOD) in the cytoplasm, manganese (MnSOD) in the mitochondria, and extracellular CuZnSOD (ECSOD) in the extracellular. These isozymes can be separated on the basis of their sensitivity to KCN and H_2O_2. MnSOD is resistant to both reagents whereas CuZnSOD and ECSOD are sensitive to both reagents.

Cytosolic CuZn superoxide dismutase (CuZnSO or SOD1)

CuZnSOD (SOD1) is a homodimeric metalloenzyme with a molecular weight ranging from 31 to 33 kDa, and it contains two atoms of copper and two atoms of zinc per molecule at active site (Steinman *et al*., 1982). Copper atoms are involved in the catalytic of $O_2^{\cdot-}$ whereas Zn atoms are thought to play a role in maintaining the stability of CuZnSOD (Fridovich, 1998). Among SODs, CuZnSOD is the most abundant enzyme in most tissues (McCord and Fridovich, 1969; Keller *et al*., 1991; Crapo *et al*., 1992). CuZnSOD is found predominantly in cytosol and in other compartments, such as nucleus and mitochondrial intermemebrane spaces (Slot *et al*., 1986; Fridovich, 1998). SOD1 gene is located on chromosome 21 (Leveanon *et al*., 1985). Mutation of SOD1 gene has been shown to be associated with the familial form of amyotrophic lateral sclerosis (FALS) (Gurney, 1994; Brown, 1995; Valentine and Hart, 2003).

Extracellular CuZn superoxide dismutase (ECSOD or SOD3)

ECSOD (SOD3) is a homolog of CuZnSOD which localizes within extracellular fluids, such as plasma and lymph (Marklund, 1982). Subsequently, three isoforms of ECSOD have been identified: ECSOD A, ECSOD B, and ECSOD C (Marklund, 1990). ECSOD is a homotetrameric glycoprotein, with a subunit molecular weight of 34 kDa and containing copper and zinc atoms (Carlsson, *et al*., 1996; Oury *et al*., 1996). ECSOD gene is encoded by chromosome 4 (Hendrickson *et al*., 1990). ECSOD has been found majority at the vessel wall (Stralin *et al*, 1995; Ocury, 1996). ECSOD shares many of the same properties attributed to CuZnSOD, such as sensitivity to KCN, the same ratio of Cu and Zn /subunit, etc. (Marklund, 1982, 1985; Tibell *et al*., 1987). However, ECSOD is expressed only in a few cell types (Marklund, 1990; Sandstrom, et al., 1993 Folz, *et al*., 1997).

Manganese superoxide dismutase (MnSOD or SOD2)

MnSOD (SOD2) is a homotetramer with a subunit molecular mass of 22 kDa with one Mn(II) / subunit. MnSOD is found mainly in the mitochondrial matrix (Weisiger and Fridovich, 1973). The contribution of MnSOD activity is dependent on the number of mitochondria. Hence, heart and liver have high activity of MnSOD, whereas red blood cells have no MnSOD activity. MnSOD is liable to denature by heat or chemicals.

MnSOD is of particular interest among SODs because of its location and properties. MnSOD is present at the site of $O_2^{\cdot-}$ generation. Mitochondria are prone to oxidative damage because they metabolize over 95% of oxygen; their DNA lack histones; and DNA repair is poor (Weisiger and Fidovich, 1973; Halliwell and Gutteridge, 1995). MnSOD is unlikely to generate potent oxidants because free Mn (II), released from inactivated MnSOD, does not participate in the Haber and Weiss reaction. (Archibald and Fridovich, 1981, 1982). In contrast, CuZnSOD generates a potent oxidant, $O_2^{\cdot-}$ in two ways: (1) CuZnSOD acts as peroxidase toward many substrates such as H_2O_2 which generates the $O_2^{\cdot-}$. (2) H_2O_2 inactivates and destroys CuZnSOD complex by eliminating histidine residue in each subunit of CuZnSOD, leading to increased free Cu(II) level in the cells. Free Cu(II) enters redox cycling in Haber and Weiss reaction and generates $O_2^{\cdot-}$ (Bray *et al*., 1974; Hodgson and Fridovich, 1975a, 1975b; Yim *et al*., 1990; Wiedau-Pazos, *et al*., 1996). This phenomenon may explain, in part, why CuZnSOD enhances the oxidative damage of macromolecules in certain disease states (Sinet, 1982; Elroy-Stein and Groner, 1988; Wiedau-Pazos *et al*., 1996). Furthermore, mitochondria dysfunction is associated with many diseases, such as aging, cancer, neurodegenerative diseases, etc.

CRITICAL ROLE OF MnSOD

Extensive investigation of MnSOD has provided much data that indicate the importance of MnSOD in an aerobic organism. In the early decades of the 20th century, MnSOD was mostly studied in association with diseases. Recently, MnSOD has been studied for its advantages in controlling and treating disease. The critical role of MnSOD is summarized.

MnSOD is essential for life

There is much evidence demonstrating that MnSOD is essential for the aerobic life of low organisms, such as bacteria, to high organisms, such as human. For example, inactivation of MnSOD gene in *Escherichia coli* increases mutation frequency and cell death when grown under aerobic conditions (Farr *et al*., 1986). The absence of MnSOD expression increases the sensitivity of *Saccharomyces cervisiae* to oxygen (van Loon *et al*., 1986). MnSOD knockout mice die shortly after birth from cardiomyopathy and neurodegeneration (Li Y *et al*., 1995; Lebowitz *et al*., 1996)

MnSOD as tumor suppressor

That MnSOD is a new type of tumor suppressor is supported by two lines of evidence. First, MnSOD activity is reduced in many types of culture tumor cells when compared to their normal counterparts (Oberley and Buettner, 1979). MnSOD level is significantly lower in the poorly differentiated hepatoma clone than in the highly differentiated clone (Borello *et al.*, 1993). The second line of evidence demonstrates the suppression of tumor properties by overexpression of MnSOD. Overexpression of MnSOD suppresses tumorigenicity (St. Clair *et al.*, 1992; Church *et al.*, 1993; Li JJ. *et al.*, 1995; Zhong *et al.*, 1997) and promotes cell differentiation (St. Clair *et al.*, 1994). Moreover, the *in vivo* expression of MnSOD also decreases tumorigenecity (St. Clair *et al.*, 1997) and suppresses metastasis of the tumor (Safford *et al.*, 1994).

MnSOD as anti-apoptosis modulator

Accumulated data have demonstrated that cells undergo oxidative stress need MnSOD for their survival. Overexpression of the MnSOD gene has been shown to prevent cell death in different cell types from many stimuli, such as TNF in cervical carcinoma cells and in MCF-7 human breast cancer cells (Wong *et al.*, 1989; Manna *et al.*, 1998), paraquat in mouse fibroblast cell line (St. Clair *et al.*, 1991), alkaline in fibrosarcoma cells (Majima *et al.*, 1998), rotenone or antimycin in fibrosarcoma cells (Kinningham *et al.*, 1999), and 3-nitropropionic acid in PC6 neuronal cells (Bruce-Keller *et al.*, 1999). There are evidences demonstrated that anti-apoptosis of MnSOD involved p53 tumor suppressor in p53-deficient mouse model (Pani *et al.*, 2000; Drane *et al.*, 2001).

MnSOD for cancer therapy

Knowing the relationship between MnSOD level and pathological diseases is useful to control and treat disease. Recently, usage of MnSOD to modulate treatment of many diseases has been under intensive investigation, and the results have been optimistic. For example, increased MnSOD protected ADR-induced cardiac injury in a mouse model. Overexpression of the MnSOD gene has been used successfully to prevent acute effects and late effects on normal tissues during lung radiation treatment (Epperly *et al.*, 1998, 1999, 2001). SOD inhibition of human leukemic cells results in increased cell death by treatment with estrogen derivative (Huang et al., 2000).

REGULATION OF MnSOD GENE

The MnSOD Gene

The MnSOD gene is nuclear-encoded and localized on chromosome 6q25 (Cregan et al., 1973; Church et al., 1992). The MnSOD gene has been completely sequenced revealing that MnSDO is a single-copy gene consisting of five exons interrupted by four introns with a typical splice junction (Wan et al., 1994). A transcription initiation site is at 74 bp upstream from the translation start site. The preceding sequence of transcription start site of the MnSOD gene contains a high GC content. MnSOD is transcribed and processed into multiple MnSOD mRNA species: 1, 4 and 6 kb. These mRNA transcripts are generated by an alternative polyadenylation process (Ho and Crapo, 1988; Church, 1990; Hurt et al., 1992). Melendez and Baglioni (Melendez and Bglioni, 1993) have studied the stability between species of MnSOD mRNA. The 4 kb transcript has a shorter half-life than the 1 kb transcript suggesting that the stability of mRNA may play a role in MnSOD regulation. MnSOD transcripts are translated to the proper protein in the cytosol and then transported to the mitochondria by the mitochondrial targeting sequence (MTS) (Hellewell et al., 1986; Ho and Crapo, 1988; Wispe et a*l*., 1989). The MTS is removed by matrix-specific protease in the mitochondria (Schatz and Butlow, 1983; Eiters and Schatz, 1988).

MnSOD is highly conserved in the mammalian species, in the human, mouse, rat, and bovine (Ho and Crapo, 1988; Ho et al., 1991; DiSillvestre et al., 1995; Meyrick and Magnuon, 1994; Wan et al., 1994). There are two polymorphisms found in the coding region of MnSOD gene. First, a polymorphism at amino acid 58 changed from isoleucine to threonine has less stability leading to reduce MnSOD activity (Ho Crapo, 1988; Borgstahl *et al*., 1996). The second polymorphism was identified in the MTS of amino acid –9 which changed from alanine to valine. The –9 polymorphism is defective in targeting the mitochondria (Rosenenblum *et al*., 1996). There is much evidence indicating that MTS polymorphism is associated with several diseases, such as breast cancer, Parkinson's disease, FALS, and cardiomyopathy (Shimoda-Matsubashi *et al*., 1997; Ambrosone *et al*., 1999; Tomblyn *et al*., 1998; Hiroi *et al*., 1999).

MnSOD expression

The expression of the MnSOD gene seems to be cell-type, cell-cycle, and environmental dependent. For example, human MnSOD is highly expressed in heart, liver, and spleen but not in red blood cells. MnSOD level in the guinea pig lung is high in the last period of gestation and decreases immediately after birth (Yuan *et al.*, 1996). The elegant study of environmental regulates MnSOD expression is in the *Escherichia coli* K12. This bacterium, when grown under anaerobic conditions, contains only FeSOD. However, upon exposure to a low oxygen level, it rapidly synthesizes MnSOD (Hassan and Fridovich, 1977). Several data demonstrate that human MnSOD is induced by various stimuli such as radiation, cytokines, toxins, chemicals (Oberley et al., 1987; Akashi *et al.*, 1995; Wong and Goeddel, 1987; Borg *et al.*, 1992; Fujii and Taniguchi, 1991). Most stimuli are thought to directly generate oxidants and these oxidants cause induction of MnSOD level by activation of signaling transduction pathways and transcription factors such as phosphorylation. However, some stimuli induces MnSOD expression without directly generating an oxidant. MnSOD level is lower in several pathological diseases such as cancer (Oberley and Buettner, 1979; Halliwell, 1993), AIDS (Polyakov *et al.*, 1997), neurodegenerative diseases (Fahn and Cohen., 1992; Ferrente *et al.*, 1997), aging (Ku *et al.*, 1993), and atherosclerosis (Halliwell, 1993). The mechanism of MnSOD reduction is mostly unknown, with the exception of the presence of two polymorphisms and mutation in the promoter region.

The study of MnSOD regulation is mostly at the transcriptional step because of two main factors. Cloning and sequencing analysis show no difference in the primary structure of MnSOD protein of normal and tumor cells, but the level of MnSOD transcripts was reduced (St. Clair and Holland, 1991). Nuclear run-on transcription assay and assays of steady-state MnSOD transcripts indicate that in most cases the induction of MnSOD transcripts was from the transcription de novo (Wong and Goeddel 1988; Visner *et al.*, 1990; Brog *et al.*, 1992).

Known mechanism of MnSOD transcription

MnSOD is like other genes in which the transcription is regulated by promoter and enhancer. There are 2 identified regions of the MnSOD gene that play an important role in MnSOD regulation: promoter and intronic region I2E. The MnSOD gene is characterized as a housekeeping gene because MnSOD promoter has a high percentage of GC content without TATA and CAAT box, but it contains cluster binding sites for Sp1 and AP-2 (Wan *et al.*, 1994; Yeh *et al.*, 1998). Co-transfection assay of insect cells reveals that Sp1 is a major regulator for MnSOD transcription from basal promoter whereas AP-2 plays a negative role (Yeh *et al.*, 1998). Mutation in the basal promoter has been identified in several colon cancer cell lines: HT29, U87, HT1080, etc. These mutations occur as a unique set of three substitutions: C(-102)T, insert at –93, and C(-38)G which lead to an increase in the AP-2 binding site and disrupt hair pin loop structure of the promoter. This may, in part, explain the reduction of mutated promoter activity (Xu *et al.*, 1999b).

In addition to the basal promoter, responsive elements to TPA and starvation have been identified in the distal promoter region of MnSOD. MnSOD TPA-responsive element, MSTRE, is located at –1292 to –1202 which contains a cAMP-responsive element-like sequence, TGACGTCT (Kim *et al.*, 1999). Oxidative stress caused by glucose deprivation increased MnSOD expression through Forkhead transcription factors, FOXO3a. The FOXO3a binding sites were found at position –1249 (GTAAACAA, inverse) and –997 (TTGTTTAA) in the distal promoter region of MnSOD gene (Kops *et al.*, 2002).

MnSOD expression increased in response to many cytokines. Elements of MnSOD responsive to tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1) have been found in an intronic 2 region between +1741 to +2083, I2E, fragment in both human and mouse (Jones PL *et al.*, 1997; Xu *et al.*, 1999a). This I2E fragment has the properties of traditional enhancer element that functions in an orientation- and position-independent manner. The I2E fragment contains several transcription factor binding sites: C/EBP-1, C/EBP-X, C/EBP-2, NF-κB, and NF-1 which are highly homologous between human and mouse. Most MnSOD induction by (TNF-α) and (IL-1) was mediated through the NF-κB site (Xu *et al.*, 1999a). Some chemicals that induce MnSOD expression are NF-κB independent, such as asbestos.

Some of the mechanisms of MnSOD regulation have been elucidated. However, more investigation of MnSOD regulation is needed. *In vivo* footprinting revealed several cluster protection sites in the basal promoter, and several distributed protection sites in the downstream of start sites under normal and stimulation conditions indicated that there are still unidentified mechanisms to regulate MnSOD expression (Kuo *et al.*, 1999; Rogers *et al.*, 2000). Moreover, mechanisms of most reagents known to induce or suppress MnSOD expression remain unclear such as, TPA, HIV-1 Tat protein.

12-O-TETRADECANOYLPHORBOL-13-ACETATE (TPA)

TPA is a phorbol ester compound, which is isolated from plant family *Euphhorbiaceae* such as *Poinsettia*, *Croton tiglium,* etc. The structure of TPA, $C_{35}H_{54}O_{8}$, is composed of polycyclic (4-ringed) with substitutes at positions 12 and 13 (Fig. 2-1). The hydroxyl group at the 4 position on ring C of TPA is in the beta position and indicates that TPA is the active form of phorbol ester (see Fig. 2-2). TPA has molecular weight of 603 kD. (for a review, see Silinsky and Searl, 2003).

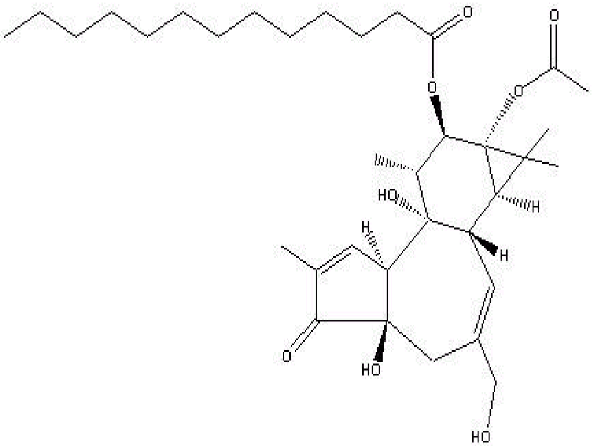


Figure. 2-1 The structure of TPA with has long chain carbon at position 12

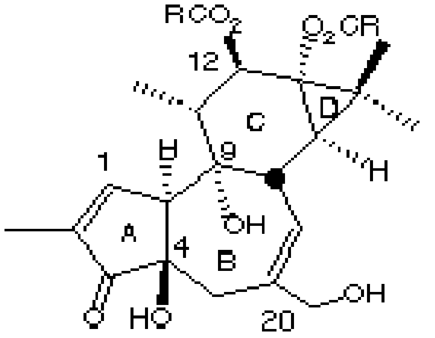


Figure 2-2. The structure of general phorbol ester with hydroxyl group in a beta position (projecting outward), active phorbol ester.

TPA has been found to produce many physiological effects on cells in a variety of tissues (Nishizuka, 1988). The most common effect of TPA that has been extensively investigated is promotion of tumor growth. This led to the discovery of the major players in mammalian signal transduction cascades, the enzyme protein kinase C (PKC) which is the target of TPA. TPA acts as the natural activator of PKC, diacylglycerol (DAG), by being bound to the same domain as DAG (Newton, 1995). The C1 domain of PKC, this C1 domain is a zinc-finger receptor enriched in cysteine residues, but this structure is not related to the zinc-finger protein of nucleic acid binding domain (Zhang *et al.*, 1995). Not all PKC isozymes respond to TPA. Atypical PKCs isozymes (aPKCζ and PKCλ/ι) could not respond to TPA because it contains only one C1 domain and lacks key residues for maintaining its active conformation (Jaken, 1996; Ron and Kazanietz, 1999).

In addition to PKC, eukaryotic cells contain five alternative types of TPA targets: chimaerins (Hall et al., 1990; Ahmed *et al.*, 1990), protein kinase D, (PKD) (Valverde *et al.*, 1994), RasGRPs (Lorenzo *et al.*, 2000), Munc13s (Brose *et al.*, 1995), and DAG kinase gamma (Shindo *et al.*, 2001). All these targets contain the region that is homologous to the C1 domain of PKC. The effect of TPA is dependent on the pathway of each target. However, TPA has been found to increase the expression of many cellular genes, such as platelet thromboxane receptor, PAI-1, WAF1, MnSOD (D' Angelo *et al.*, 1996; Arts *et al.*, 1998; Biggs and Kraft, 1999; Fujii and Taniguchi, 1991).

TRANSACTIVATING TRANSCRIPTIONAL FACTOR (Tat)

Transactivating transcriptional factor (Tat) is encoded by human immunodeficiency virus 1(HIV-1). Tat is a non-structural protein that expresses early in HIV-1 replication (Cullen, 1998). Tat is essential for viral gene expression, replication, and pathogenesis (Dayton *et al.*, 1986; Fisher *et al.*, 1986). Tat contains 86-101 amino acids depending on the HIV-1 strain. Tat is encoded by 2 separate exons disrupted by vpu and env genes (Fig. 2- 3) (Karn, 1999).

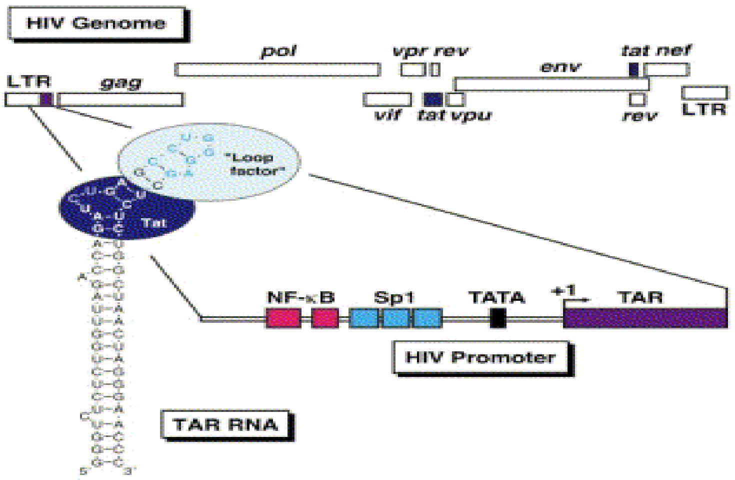


Figure 2-3. Structure of the HIV genome

The first exon of Tat is encoded for the first 72 amino acids and is organized into 3 major domains. The function of the first coding exon is the cofactor binding domain and transactivation. The basic domain contains the arginine-rich RNA binding motif. The second exon is encoded for the rest of the amino acids and contains an RGD motif. The function of the second coding exon remains unclear, but it has been proposed that it may function by being bound to the cell adhesion molecule on the cell surface. The RGD motif is usually found in many extracellular matrix proteins. There are 5 conserved regions of Tat contained in the first coding exon: N-terminal, cysteine-rich, core, basic, and C-terminal (Fig 2-4) (for a review, see Rana and Jeang, 1999).

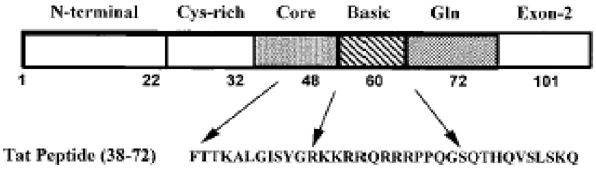


Figure 2-4. The Tat protein and the sequence of the Tat peptide that recognizes Tat with high affinity and specificity.

Tat is secreted from HIV-infected cells and uptaken by non-infected cells (Frankel and Pabo, 1988). Tat enters the cell and reaches the nucleus in a few minutes is not by cell-mediated endocytosis because the uptake is not inhibited at 4^{o}C and by endocytosis inhibitors (Derossi *et al.*, 1994; Vives *et al.*, 199; Polyakov *et al.*, 2000). There are 2 lines of evidence that indicate that Tat enters the cell by receptor or internalization. The $\alpha_v\beta_5$ integrin receptor has been shown to bind to Tat (Vogel *et al.*, 1993). Tat internalizes into the cell through the basic region of Tat (48-60), GRKKRRQRRRPPQ and the lysine residue is more critical for internalization than arginine residuals (Ruben *et al.*, 1989; Vives *et al.*, 1997). This uptake property of Tat leads to the study of intracellular delivery of non-permeable molecules such as drugs, antibodies etc (for a review, see Futaki, 2002).

Tat is an atypical transcriptional activator because it binds to the nascent leader RNA sequence, transactivating responsive element (TAR) which is located at +1 to +44 (Arya *et al.*, 1985; Rosen *et al.*, 1985; Rice *et al.*, 1988). The basic region of Tat contains the sequence that is able to specifically recognize a three-nucleotide pyrimidine 5' bulge (UCU) of TAR stem loop (Fig. 2-5) (for a review, see Taube *et al.*, 1999). It is believed that Tat increases the transcription rate by rendering the RNA polymerase II competent for elongation leading to increasing the number of mature transcripts rather than facilitating the transcriptional initiation (Feinberg *et al.*, 1991). This phenomenon results from the interaction among Tat, cyclin subunit of the positive elongation factor b (P-TEFb), cyclin T1 and cyclin-dependent kinase 9 (CDK9). The binding of Tat/P-TEFb complexes to the TAR allows the CDK9 to phosphorylate the C-terminal of RNA polymerase II leading to the transition from initiation to elongation of transcription (Jones KA *et al.*, 1997).

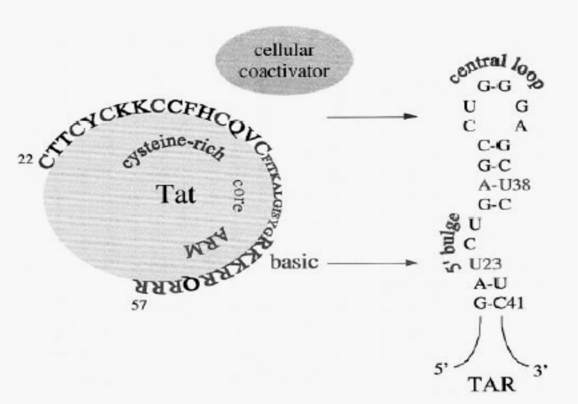


Figure 2-5. Structure of Tat and TAR

In addition to transactivating the HIV gene, Tat modulates many host cellular genes such as IL-2, Il-6, IL-8, MCP-1 and MnSOD, etc. (Ambrosino *et al.*, 1997; Ott *et al.*, 1998; Gonzalez *et al.*, 2001; Lim *et al.*, 1996; Flores *et al.*, 1993). Moreover, Tat also possesses a growth-promoting activity, stimulates the angiogenic process, and increases tumor growth and metastasis (Ensoli *et al.*, 1993; Campioni *et al.*, 1995; Corallini *et al.*, 1996).

CHAPTER 3

TPA-ACTIVATED TRANSCRIPTION OF THE HUMAN MnSOD GENE: ROLE OF TRANSCRIPTION FACTORS Sp-1 and Egr-1

ABSTRACT

Induction of manganese superoxide dismutase (MnSOD) in response to oxidative stress has been well established in animals, tissues, and cell culture. However, the role of the MnSOD promoter in stimulus-dependent activation of transcription is unknown. The MnSOD promoter lacks both a TATA and a CAAT box but possesses several GC motifs. In a previous study, we showed that the basal promoter contains multiple Sp1 and AP-2 binding sites and that Sp1 is essential for the constitutive expression of the MnSOD gene. In this study, we identified an Egr-1 binding site in the basal promoter of MnSOD. We also found that the basal promoter is responsive to 12-O-tetradecanoylphorbol-13-acetate (TPA)-activated MnSOD transcription in the human hepatocarcinoma cell line HepG2. The contributions of these binding sites and the roles of the transcription factors Egr-1, AP-2, and Sp1 in the activation of MnSOD transcription by TPA were investigated by site-directed mutation analysis, Western blot analysis, and overexpression of transcription factors. The results showed that Sp1 plays a positive role for both basal and TPA-activated MnSOD transcription, whereas overexpression of Egr-1 has a negative role in the basal promoter activity without any effect on TPA-mediated activation of MnSOD transcription.

INTRODUCTION

Manganese superoxide dismutase (MnSOD) is a highly regulated member of the SODs, which are the first line of cellular defense against the toxic effects of reactive oxygen species (Weisiger and Fridovich, 1973). Accumulated data demonstrate the critical role of MnSOD in many aspects of aerobic life. The MnSOD knockout mice die shortly after birth from either cardiomyopathy (Li Y *et al.*, 1995) or neurodegeneration (Lebovitz *et al.*, 1996), depending on the genetic background from which the animals were derived. Overexpression of MnSOD has been implicated in suppression of tumorigenicity of human melanoma (Church *et al.*, 1993), breast cancer (Li JJ *et al.*, 1995), and glioma (Zhong *et al.*, 1997); suppression of neoplastic transformation (St. Clair *et al.*, 1992); promotion of cell differentiation (St. Clair *et al.*, 1994); and prevention of hyperoxia-induced pulmonary injury (Wispe *et al.*, 1992), doxorubicin-induced cardiac toxicity (Yen *et al.*, 1997), and ischemia–reperfusion-induced brain injury (Keller *et al.*, 1998). Moreover, overexpression of MnSOD has been shown to prevent death of many cell types such as cervical carcinoma (Wong *et al.*, 1989) and breast cancer (Manna *et al.*, 1998) after tumor necrosis factor-*a* mediated injury as well as in fibrosarcoma cells exposed to rotenone or antimycin (Kiningham *et al.*, 1999).

The expression of MnSOD has been tested as a means to modulate therapeutic outcome in many diseases. For example, up-regulation or down-regulation of the MnSOD gene by antisense or sense cDNA renders the cells more sensitive or resistant to radiation (Suresh *et al.*, 1994). Overexpression of MnSOD protects the normal lung against radiation-induced fibrosis in a mouse model (Epperly *et al.*, 2000). Down-regulation of MnSOD has been used for selective killing of cancer cells (Huang *et al.*, 2000). These findings demonstrate the importance of understanding the mechanism by which MnSOD is regulated.

The MnSOD is a single-copy gene located on chromosome 6q25.3 (Church *et al.*, 1992). The gene consists of five exons that are interrupted by four introns (Wan *et al.*, 1994). The basal promoter of MnSOD lacks a TATA and a CAAT box but contains multiple overlapping Sp1 and AP-2 binding sites (Yeh *et al.*, 1998). Transcription factor Sp1 contains three zinc finger motifs in DNA-binding domains that recognize GC-rich sequences of GGGCGG (Dynan and Tjian, 1983). The factor also has an inhibitory domain in the N terminus (Murata *et al.*, 1994) and four transcriptional activation domains (A, B, C, and D). Transcriptional domains A and B are required for general and full activation, while domain C possesses very low activation (Courey and Tjian, 1988), and domain D is required for synergistic activation (Pascal and Tjian, 1991). The Sp1 protein is capable of inducing gene expression by forming homotypic, Sp1–Sp1, interactions (Mastrangelo *et al.*, 1991; Pascal and Tjian, 1991; Su *et al.*, 1991) or by forming heterotypic interactions with different classes of nuclear proteins such as TATA box-binding protein (TBP) (Emili *et al.*, 1994), C/EBP (Lee *et al.*, 1997), and YY1 (Lee *et al.*, 1993; Seto *et al.* 1993). An additional feature Sp1 may contribute to the ability to induce gene expression is that its binding may result in DNA loop formation through zinc finger motif 1 (Su *et al.*, 1991; Sjottem *et al.*, 1997).

One of the transcription factors in the zinc finger family with a binding site usually near or overlapping with Sp1 in a common GC-rich region promoter is the early growth response factor (Egr-1; also known as Krox-24, NGFI-A, and Zif268). The Egr-1 factor is the immediate–early protein that rapidly and transiently responds to a variety of stimuli, including mitogens, developmental or differentiation processes, radiation injury, or neuronal excitation. It is an 80-kDa protein containing three zinc finger motifs in DNA-binding domains that recognizes GCGG/TGGGCG sequences (for reviews, see Liu *et al.*, 1998, and references therein). The Egr-1 protein can act as an activator or repressor because it contains both repression and activation domains. Unlike most transcription factors, Egr-1 has a bipartite nuclear localization sequence (Gashler *et al.*, 1993).

MnSOD is not only expressed constitutively but also highly induced by various stimuli, including radiation (Oberley *et al.*, 1987; Akashi *et al.*, 1995), cytokines (Wong and Goeddel, 1988; Borg *et al.*, 1992), and chemical carcinogens such as 12-O tetradecanoylphorbol-3-acetate (TPA). The TPA, a protein kinase C activator, increases MnSOD transcription in many cell types (Fujii and Taniguchi, 1991). However, the mechanism by which expression of MnSOD is induced is not clear because of the lack of knowledge about transcriptional activation from the TATA-less promoter. It has been shown that TPA-induced MnSOD gene expression in a human adenocarcinoma cell line is mediated by the cAMP-responsive elementlike sequence

TGACGTCT, which is located in a region between -1292 and -1202 of the MnSOD promoter (Kim *et al.*, 1999). Furthermore, our laboratory has found that the second intronic fragment (+1741 to +2083) of the MnSOD gene is responsive to tumor necrosis factor or interleukin-1 (Xu *et al.*, 1999a) and TPA (Kiningham *et al.*, 2001) in a human lung fibroblast cell line. However, the data from DNase I-hypersensitivity analysis have revealed a protection region in the basal promoter of the MnSOD gene under both normal and stimulation conditions (Kuo *et al.*, 1999). This finding, couple with our previous demonstration that a mutation in the basal promoter causes an increase in an AP-2 binding site and reduced expression of MnSOD in cancer cells (Xu *et al.*, 1999b) suggests that the presence of GC binding sites and GC sensitive transcription factors may play an important role in both basal and activated MnSOD transcription.

We have previously identified the binding sites for and the contribution of the transcription factors Sp1 and AP-2 to the basal transcription of MnSOD (Yeh *et al.*, 1998). In the present study, we identified an Egr-1-binding site in the MnSOD basal promoter. We further investigated how Egr-1 and Sp1 participate in the transcription of the MnSOD gene from the basal promoter by TPA.

MATERIALS AND METHODS

Plasmid constructs

The Sp1 expression vector pPacSp1 was kindly provided by Dr. R. Tjian (University of California–Berkeley). Construction of the Egr-1 expression construct pCMV-Egr-1 was previously described (Muthukkumar *et al.*, 1995). The pGL3/P7 vector was constructed by cloning a P7 fragment, basal promoter (-210 to +24) of the MnSOD gene, into a promoterless pGL3 vector (Promega Corp.) containing a luciferase reporter gene. The internal control, *Renilla* (PRL-TK), was purchased from Promega. Site-specific mutation in the P7 promoter was induced by excision of the nuclotide sequence from -210 to -133 with the restriction endonucleases *Kpn*I and *Sma*I. The linearized and excised fragments were separated by electrophoresis on a 0.7% agarose gel. The linearized vector containing DNA from +24 to -133 of the MnSOD promoter was extracted and purified by a gel extraction kit (Qiagen). Subsequently, the synthetic oligonucleotide corresponding to -210 to -133 of the

MnSOD gene containing mutated bases at position -169 (from GC → AA) was inserted into the linearized vector by T4 ligase using a rapid ligation kit (Boehringer Mannheim). The nature of the mutated promoter was verified by DNA sequencing (Amersham Pharmacia Biotech).

Cell culture and transfections

The human hepatocarcinoma HepG2 cell line obtained from the American Type Culture Collection was cultured in DMEM and F12 media (1:1) supplemented with 10% FBS, insulin 1 *m*g/ml, L-glutamine, and 1% antibiotics (penicillin, streptomycin, gentamicin) (Life Technologies). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Transient transfections were performed by a modified calcium phosphate method (Graham and van der Eb, 1973). Cells were cotransfected with 1 µmol of pGL3/P7 vector and various concentrations of each expression vector. After 8 h, the medium was replaced. After 48 h, the cells were lysed, and the luciferase activity was measured by the Dual-Luc reporter assay system (Promega) using a TD-20/20 Luminometer (Turner Designs). For TPA treatments, 24 h after transfection, cells were trypsinized and replated into 12-well plates at a concentration of 1×10^5 cells/well. After an additional 24 h, cells were treated either vehicle alone or with 80 nM TPA for 24 h before the assay.

Northern blot analysis

Total RNA was isolated from HepG2 cells by a modification of guanidium thiocyanate/cesium chloride method (Glisin *et al.*, 1974). All reagents were prepared in 0.1% diethylpyrocarbonate (DEPC) water. Cells were washed twice with PBS, pH 7.4 (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4), and collected in 4 M guanidium thiocyanate. Cells were homogenized using a Dounce homogenizer. The homogenate was overlayed on a 5.7 M CsCl and 2.4 M CsCl gradient and then centrifuged at 170,000x *g* for 20 h at 21 °C. The RNA pellet was air dried and resuspended in Tris HCl EDTA (TE) buffer, pH 8.0. RNA was cleaned by extraction with a mixture of phenol:chloroform (1:3) and then precipitated with 100% ethanol at -20 °C overnight. The RNA pellet was recovered by centrifugation at 13,000x *g* for 20 min at 4 °C and washed with 70% ethanol. The RNA pellet was resuspended in TE buffer, and the concentration was determined by reading the absorbance at 260 nm. Thirty micrograms of total RNA was separated on a 1.1%

formaldehyde–agarose gel, transferred to a nylon membrane, and hybridized with the ^{32}P-labeled MnSOD cDNA. The membrane was exposed to X-ray film (Eastman Kodak) at -70 °C. The same blot was stripped by washing twice with boiling DEPC water and rehybridized with ^{32}P labeled β-actin as the loading control.

Nuclear extract preparation

Nuclear proteins were extracted following the procedure described by Garner and Revin (1981). Cells were collected and centrifuged at 100x *g* for 2 min at 4 °C. The cell pellet was resuspended in solution A (10 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 0.2 mM phenylmethylsulfonyl fluoride [PMSF]) and incubated on ice for 15 min. A 12.5-µl of 10% Nonidet P-40 was added to each sample, and the mixture was vigorously vortexed for 15 sec. The cytoplasmic and nuclear fractions were separated by centrifugation at 17,000x *g* for 30 sec at 4 °C. The nuclear pellet was subsequently resuspended in solution B (20 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25% [v/v] glycerol, 0.5 mM DTT, 0.2 mM PMSF, protease inhibitors [pepstatin, aprotinin, and leupeptin] 1 µg/ml) and incubated on ice for 20 min, followed by centrifugation at 12,000x *g* for 2 min at 4 °C. The nuclear protein concentration was determined by a BCA colorimetric assay using bovine serum albumin as the standard (BioRad Laboratories).

Western blot analysis

Western analysis was performed according to standard protocols. All antibodies were purchased from Santa Cruz Biotech Inc. Nuclear proteins (25 µg) were resolved by 10% SDSPAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell). The membrane was washed twice with TBST (100 mM NaCl, 10 mM Tris HCl, pH 7.5; 0.1% Tween 20) at room temperature. Non specific binding was blocked by incubating the membrane with 5% nonfat dry milk in TBST for 1 h at room temperature. To detect specific protein, the membrane was incubated with rabbit polyclonal anti-Sp1 antibodies (1:1000) for 1.5 h at room temperature. The membrane was washed twice with TBST and subsequently incubated with secondary antibody (horse raddish peroxidase-conjugated goat anti-rabbit IgG) (1:5000) for 1.5 h at room temperature and then wash twice with TBST and once with TBS (TBST without 0.1% Tween 20). Specific protein was identified by a chemiluminescent detection system

(Amersham). The same membrane was stripped by incubating with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris HCl, pH 6.7) for 30 min at 50°C and reblotted with Egr-1 and AP-2*a* antibodies.

Electrophoretic mobility shift assay (EMSA)

The Sp1 and AP-2 consensus double-stranded oligonucleotides were purchased from Promega. Synthetic oligonucleotides were purchased from Life Technologies. All probes were 5’-end-labeled with [γ-^{32}P]-ATP (New England Nuclear) by T4 polynucleotide kinase (Amersham). A 5-µg amount of nuclear proteins was used in each binding reaction with 4 µl of 5x binding buffer [20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 5 mM DTT, 50 mM Tris HCl, pH 7.5; 0.25 mg/ml poly(dI:dC)] and 50,000 cpm of specific probe for 20 min at room temperature. The reaction was stopped by addition of 2 µl of 10x loading buffer (25 mM Tris HCl, pH 7.5; 0.02% bromophenol blue, 4% glycerol). The DNA–protein complexes were separated from unbound DNA probe by native 6% PAGE. The gel was dried and exposed to X-ray film at -70 °C. For the competition assay, 50x of cold oligonucleotide was mixed with the nuclear proteins for 20 min at room temperature before addition of the specific probe. For the supershift assay, the binding reaction mixture was incubated with 4 µg of antibody prior to the addition of the 10x loading buffer.

Statistical analysis

All experiments were performed in triplicate and repeated at least three times, and representative findings are shown. Statistical analysis was performed by ANOVA. A p value < 0.05 indicates a significant difference between the test and the control.

RESULTS

Effect of TPA on expression of endogenous MnSOD gene

To determine whether the endogenous MnSOD gene was induced by TPA in HepG2 cells, cells were treated either with vehicle alone or with 80 nM TPA for 6 h, and RNA was extracted for Northern blot analysis. As shown in Figure 3-1, TPA significantly increased MnSOD mRNA. Densitometric measurements indicated that the level was increased four-fold after adjusting for loading with β-actin. The increase was observed for both the 1.0-kb and the 4.0-kb MnSOD transcripts.

Basal promoter contains TPA-responsive element

To identify whether a TPA-responsive element was located in the basal promoter of the MnSOD gene, the basal promoter driven luciferase expression vector was transiently transfected into HepG2 cells. The transfected cells were treated either with vehicle alone or with 80 nM TPA for various incubation times. As shown in Figure 3-2, the basal promoter activity had increased 2.8 fold at 12 h and 4.8 fold at 24 h of TPA treatment in treated cells compared with control cells. This result indicated that the basal promoter contains elements responsive to TPA.

Basal promoter contains potential Egr-1 binding sites

We have previously reported that the MnSOD gene possesses a high GC content (about 78%) in the promoter region (Wan *et al.*, 1994). A subsequent report from our laboratory showed that this region contains clusters of five binding sites for AP-2 and three binding sites for Sp1 (Yeh *et al.*, 1998). Using computer analysis, four Egr-1 binding sites were predicted at positions -169, -120, -103, and -96 (relative to the transcriptional initiation site, +1) in this basal promoter (Fig. 3-3). To determine if Egr-1 binds to the predicted site on the basal promoter, two synthetic Egr-1 oligonucleotides were designed for EMSA analysis. The first oligonucleotide corresponded to the -176/-145 region of the MnSOD basal promoter containing a predicted Egr-1-binding site at position -169. The second oligonuclotide corresponded to the -130/-90 region and contained three additional predicted Egr-1-binding sites. The EMSA revealed that the binding site at position -169 preferentially bound the Egr-1 protein (Fig. 3-4).

TPA increased Egr-1 and Sp1 but not AP-2 levels

Because the basal promoter contains the binding sites for the Egr-1, AP-2, and Sp1 transcription factors, it is possible that their levels can be increased by a variety of stimuli and subsequently activated transcription in specific genes, including MnSOD. To further investigate the role of these transcription factors in TPA-activated MnSOD transcription, we determined the levels of each transcription factor after TPA treatment. The HepG2 cells were treated with 80 nM TPA, and the level of each transcription factor was determined at various time points after the treatment. As shown in Figure 3-5, the Sp1 protein level was increased by 2.1 fold at 3 h after TPA treatment and had decreased slightly at 12 h after treatment. The Egr-1 factor was undetectable under normal culture conditions but increased within 45 min of TPA treatment. The Egr-1 reached a maximum at 90 min and rapidly declined to its normal level by 12 h after treatment. Unlike Egr-1 and Sp1, the AP-2 protein was undetectable in the HepG2 cells at all time points after TPA stimulation. Thus, it is unlikely that AP-2 has a significant effect on the TPA activation of MnSOD transcription. .

Effect of Egr-1 on TPA-activated MnSOD transcription

It has been shown that the increased Egr-1 level under stimulated conditions is capable of inducing expression of specific genes containing either TATA or housekeeping promoters in a cell type-dependent manner. Because the Egr-1 protein level increased in response to TPA treatment, Egr-1 may play a role in TPA-activated MnSOD transcription in HepG2 cells. To determine if Egr-1 mediated TPA up-regulation of the MnSOD gene, various amounts of the Egr-1 expression vector pCMV-Egr-1 were cotransfected with pGL3/P7 into HepG2 cells. As shown in Figure 3-6A, Egr-1 significantly decreased basal promoter activity ($p < 0.01$). The basal promoter activity decreased by 25% from control when the pGL3/P7 vector was cotransfected with 0.5 μg of pCMV-Egr-1 vector. To verify that the Egr-1 protein levels increased after transfection, Egr-1 was measured by Western analysis. Figure 3-6B shows that the Egr-1 protein increased in parallel with the amount of the expression vector.

To further investigate the role of Egr-1, its binding site at position -169 was altered by site-directed mutagenesis by changing two bases from CG to AA and then verifying the change by DNA sequencing (Fig. 3-7A). The binding activity of the mutated Egr-1 site was verified by EMSA. As shown in Figure 3-7B, the mutations completely eliminated the binding activity of Egr-1. To determine whether the reduction of constitutive transcription is attributable to this Egr-1 motif, various amounts of the Egr -1 expression vector pCMV-Egr-1 were cotransfected with the mutated promoter into HepG2 cells. As shown in Figure 3-8A, Egr-1 slightly but not significantly suppressed the constitutive transcription activity from the mutated promoter. To determine the role of Egr-1 in TPA-activated MnSOD expression, the mutated promoter was transiently transfected into HepG2 cells, and the cells were treated with 80 nM TPA for 24 h. As shown in Figure 3-8B, the mutated promoter did not change the level of MnSOD transcription in response to TPA treatment.

Effect of Sp1 on induction of MnSOD transcription by TPA

Transcription factor Sp1 usually serves as an enhancer for a typical TATA promoter but also is a basal activator for constitutive expression in many genes containing a TATA-less promoter. However, it is not known whether Sp1 can further activate transcription from a basal promoter such as the one in the MnSOD gene. As shown in Figure 3-5, the Sp1 protein levels increased in response to TPA treatment; therefore, Sp1 could be a candidate for the transcriptional activation of the MnSOD basal promoter. To determine whether Sp1 mediated TPA activation of MnSOD transcription, various amounts of the Sp1 expression vector, pPacSp1, were cotransfected with pGL3/P7 into HepG2 cells. As shown in Figure 3-9A, the basal promoter activity increased in a concentration-dependent manner ($p < 0.01$). The Sp1 binding activity was confirmed by EMSA. As shown in Figure 3-9B, Sp1 binding activity increased with the amount of expression vector pPacSp1 transfected. The binding activity of Sp1 in cells that were transfected with 2 μg of the expression vector pPacSp1 was comparable to that detected in the TPA-treated cells.

DISCUSSION

Increased expression of MnSOD has been shown to prevent cell death; protect against damage from hyperoxia (Wispe *et al.*, 1992) and ischemia (Keller *et al.*, 1998); suppress transformation (St Clair *et al.*, 1992), tumorigenicity (Church *et al.*, 1993; Zhong *et al.*, 1997), and metastasis (Safford *et al.*, 1994); and promote differentiation (St Clair *et al.* 1994). Attempts to improve the outcomes of cancer treatment by up- or down-regulation of the MnSOD gene in several experimental therapeutic models using gene transfection techniques have shown promising results.

The MnSOD gene is regulated by a variety of stimuli. It has been demonstrated that TPA can induce MnSOD expression by direct activation of protein kinase C (PKC) or produce reactive oxygen species that subsequently activate redox sensitive transcription factors such as AP-1 and NF-*k*B (Angel *et al.*, 1987; Edbrooke *et al.*, 1989; Fujii and Taniguchi, 1991). Recent reports from this and other laboratories suggest that TPA-responsive elements may be present in both the promoter and the

MnSOD gene. First, a region between +1292 and +1202 upstream of the transcription start site containing a CREB/ATF-1-like element (designated MSTRE) is responsive to TPA-activated transcription (Kim *et al.*, 1999). Second, we have reported that an intronic enhancer element located between +1741 and +2083 of the MnSOD gene is responsive to synergistic induction by TPA and a cytokine (Kiningham *et al.*, 2001). However, the promoter used in our studies contains only the basal promoter, which does not include the MSTRE identified by Kim *et al*. Thus, our results suggest that TPA-responsive elements may be present in the basal promoter region to facilitate the synergistic induction of the MnSOD gene by TPA and cytokines.

In the present study, our result from Northern analysis and transient transfections showed that TPA increased MnSOD mRNA in HepG2 cells at the transcription step, and this increase was mediated largely by the basal promoter. This result also suggested that the basal promoter contains binding sites for the potential activators of MnSOD transcription. Our previous studies showed that this basal promoter contains a cluster of overlapping Sp1 and AP-2 binding site (Yeh *et al.,* 1998). In this study, we have confirmed and extended our findings to include a binding site for the transcription activator Egr-1.

It is well known that the Egr-1 level increases on response to extracellular stimulations (for review, see Gashler and Sukhatme, 1995). When the Egr-1 level is increased, it may play a positive or a negative role, depending on the target gene. For example, Egr-1 displaces prebound Sp1 and activates the PDGF-A promoter in response to TPA (Khanchigian *et al*., 1995). Also Egr-1 and Sp1 cooperate to regulate induction of tissue factor gene expression by TPA (Cui *et al.*, 1996), and Egr-1 competes with Sp1 for its binding site and represses murine adenosine deaminase transcription (Sakamoto *et al.*, 1991). In this study, we found that among the four putative Egr-1 binding sites that overlapped with the Sp1 binding sites at positions -169, -120, -103, and -96 of the MnSOD promoter, the site at position -169 had a higher binding affinity for Egr-1 protein in the HepG2 cells. The reason for preferential Egr-1 binding is unknown. One of the possibilities that might influence Egr-1 binding is the number of adjacent overlapping Sp1 binding sites in each cluster of Sp1 binding motifs. The Egr-1 site at -169 overlaps with a cluster of Sp1 binding motifs that contains two overlapping Sp1 sites, whereas the remaining by interaction with transcription factors that bind directly to the GC motif, it is unlikely that an increased level of Egr-1 alone will be sufficient for the activation of MnSOD transcription. Overexpression of Egr-1 significantly decreased the constitutive expression from the normal promoter but had no significant effect on expression from the mutated promoter. Thus, the presence of Egr-1 appears to play a negative role in the constitutive transcription from the basal promoter of the MnSOD gene, as is also seen in the murine adenosine deaminase gene (Sakamoto *et al.*, 1991).

Two candidates for Egr-1 interaction are Sp1 and AP-2. However, our results showed that AP-2 is not detectable in HepG2 cells after TPA treatment. Our results are consistent with those reported by Williams *et al.*, which showed that the expression of AP-2 was tissue specific and that the AP-2 gene was repressed in HepG2 cells (Williams *et al.*, 1988). Thus, it is unlikely that AP-2 will contribute to the TPA-activated transcription of the MnSOD gene.

The Sp1 protein is ubiquitously expressed in all mammalian cells (Saffer *et al.*, 1990). It has been reported that Sp1 is associated with basal expression from TATA/CAAT-less promoters, including those of T-cell-specific MAL (Tugores *et al.*, 1997), MnSOD (Yeh *et al.*, 1998), and PDGF-A (Khachigian *et al.*, 1995). Although it is well recognized that Sp1 can serve as a transcription enhancer in a TATA-type promoter, it is not known if Sp1 can further stimulate a TATA-less promoter. Expression of Sp1 is induced or suppressed by many chemicals such as retinoic acid and TPA in a cell type dependent manner. For example, TPA increases Sp1 mRNA and protein levels in myeloma cells (D'Angelo *et al.*, 1996) but not in HeLa cells (Briggs *et al.*, 1986; Hagen *et al.*, 1992; Cui *et al.*, 1996). In the present study, we found that the Sp1 protein level increased in the HepG2 cells after TPA treatment. In addition, overexpression of Sp1 protein resulted in an increase in transcription of the MnSOD gene. These results suggest that Sp1 is a major contributor responsible for TPA-activated MnSOD transcription in HepG2 cells.

Because it has been demonstrated that the binding of Sp1 changes the DNA conformation, resulting in DNA looping (Su *et al.*, 1991; Sjottem *et al.*, 1997), or that Sp1 may interact and form tetramers (Pascal and Tjian, 1991), it is tempting to speculate that the increase in Sp1 levels in response to TPA facilitates the function of transcription activator complex within the basal promoter of the MnSOD gene.

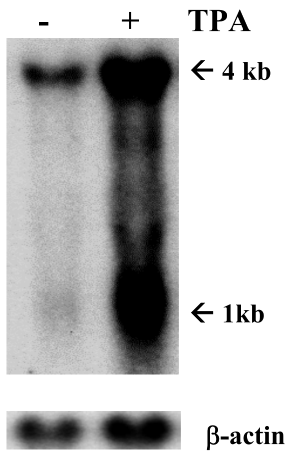


Figure 3-1. Expression of the endogenous MnSOD gene by TPA. The HepG2 cells were incubated for 6 h with either vehicle alone or 80 nM TPA, and total cellular RNA (30 μg) was analyzed by Northern analysis for MnSOD expression. The 1 kb and 4 kb MnSOD transcripts are indicated by arrows. The same blot was stripped and rehybridized with β–actin probe.

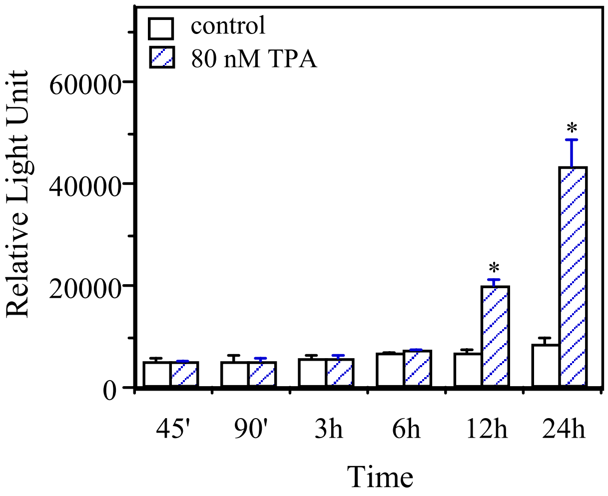


Figure 3-2. Effect of TPA on MnSOD basal promoter. The HepG2 cells were transiently transfected with the reporter gene driven by P7 (pGL3/P7). Transfected cells were trypsinized and replated for TPA treatment. Cells were treated with either vehicle alone or 80 nM TPA for 45 min, 90 min, 3h, 6h, 12h, and 24h. Basal promoter activities were determined by luciferase response in Relative Light Unit. Each data point represents the average of three independent transfections ± SEM. * Significant differences between control and treated ($p < 0.01$).

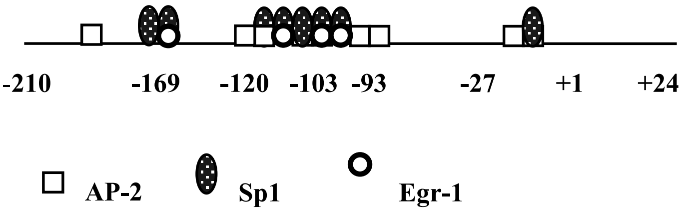


Figure 3-3. Location of Egr-1-binding sites on the MnSOD basal promoter. Schematic representation of the computer analysis revealed that Egr-1-binding sites (GCGGGGGCG) overlap with multiple Sp1 and AP-2 binding sites on the MnSOD basal promoter.

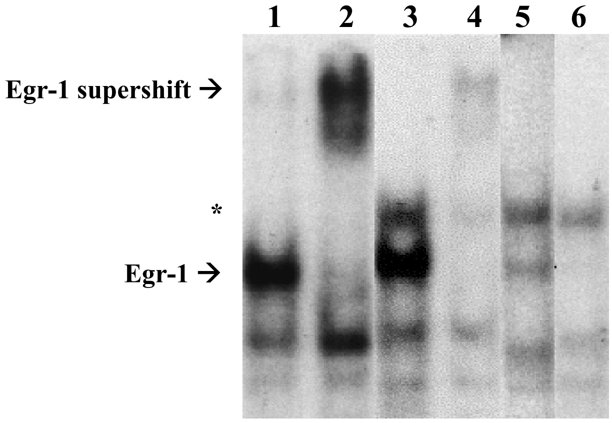


Figure 3-4. Egr-1 binding activity. Nuclear extract from cells treated with 80 nM TPA for 90 min was incubated with ^{32}P-labeled probes. Lane 1, Egr-1 consensus oligonucleotide; lane 2, Egr-1 consensus oligonucleotide and supershift with Egr-1 antibody; lane 3, oligonucleotide corresponding to the -176/-146 region of MnSOD basal promoter; lane 4, oligonucleotide corresponding to the -176/-146 region of MnSOD basal promoter and supershift with Egr-1 antibody; lane 5, oligonucleotide corresponding to -130/-90 of the basal promoter; lane 6, oligonucleotide corresponding to -130/-90 of the basal promoter and supershift with Egr-1 antibody. Asterisk (*) indicates the Sp1 band, which was previously identified by supershift with anti-Sp1 antibody (data not shown).

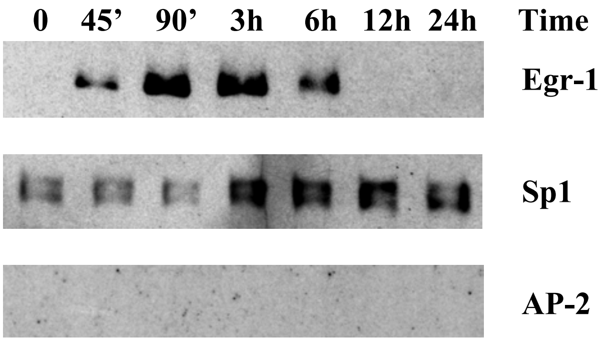
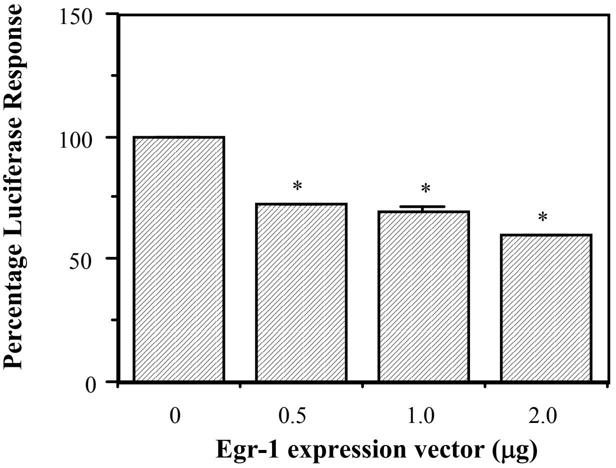


Figure 3-5. Effect of TPA on Egr-1, Sp1, and AP-2α protein levels in HepG2 cells, as shown by Western blot analysis. The HepG2 cells were treated with vehicle alone or with 80 nM TPA and collected for nuclear protein harvest at various time points as indicated. Membrane was blotted with anti-Egr-1 polyclonal antibodies. The same membrane was stripped and reblotted with Sp1 and AP-2α polyclonal antibodies.

A.



B.

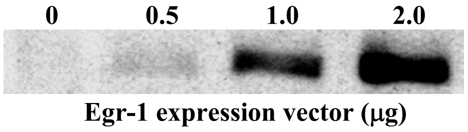


Figure 3-6A. Effect of Egr-1 on MnSOD basal promoter. The HepG2 cells were transiently transfected with pGL3/P7 along with different amounts of Egr-1 (CMV) vectors. The effect is indicated by percentage luciferase response ± SEM. *Significant differences between control and tested cells ($p < 0.01$).

3-6B. Western blot analysis of exogenous proteins. Nuclear extracts from cells transfected with an Egr-1 expression vector were blotted with anti-Egr-1 polyclonal antibody.

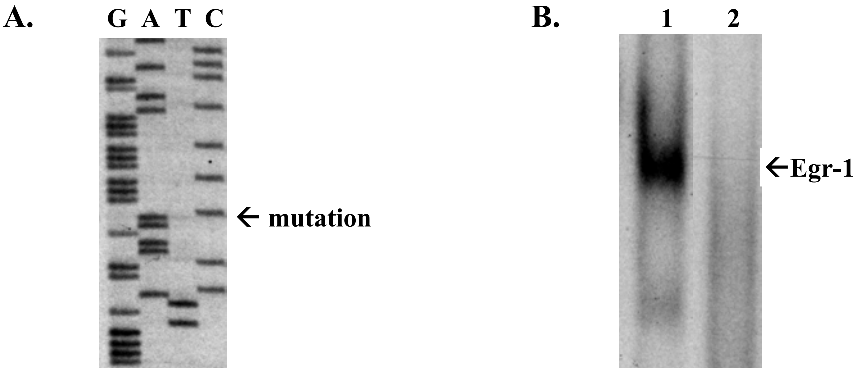
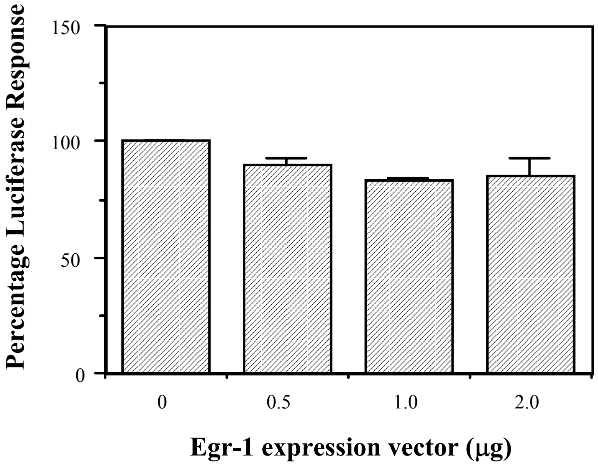


Figure 3-7A. Sequencing gel of MnSOD basal promoter. Arrow indicates an introduced mutation in the Egr-1 binding site from GC→ AA at position -169.

3-7B. Loss of Egr-1 binding activity. Nuclear extract derived from TPA-treated HepG2 cells for 45' was incubated with Egr-1 consensus oligonucleotides (Lane 1) or oligonucleotides corresponding to -210/-133 of mutated promoter (Lane 2).

A.



B.

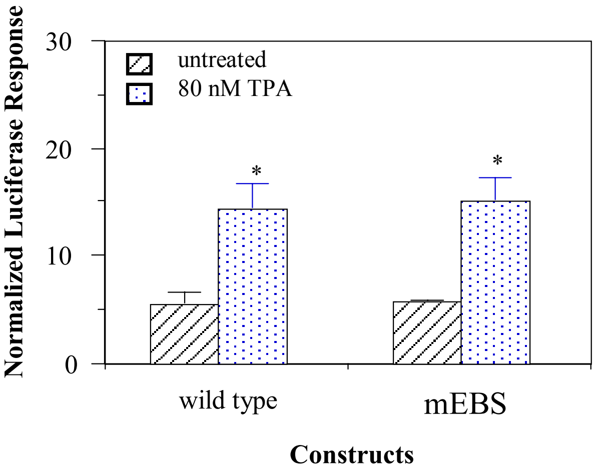


Figure 3-8A. Effect of mutated Egr-1-binding site on MnSOD basal promoter. HepG2 cells were transiently transfected with mutated promoter along with different amounts of Egr-1 (CMV) vectors. The effect is indicated by percentage luciferase response ± SEM.

3-8B. Effect of mutated Egr-1-binding site on TPA treatment. Cells were transiently transfected with either pGL3/P7 wild type or mutant and treated with 80 nM TPA for 24 h. The result is expressed as normalized luciferase response ± SEM. *Significant differences between control and treated cells ($p < 0.01$).

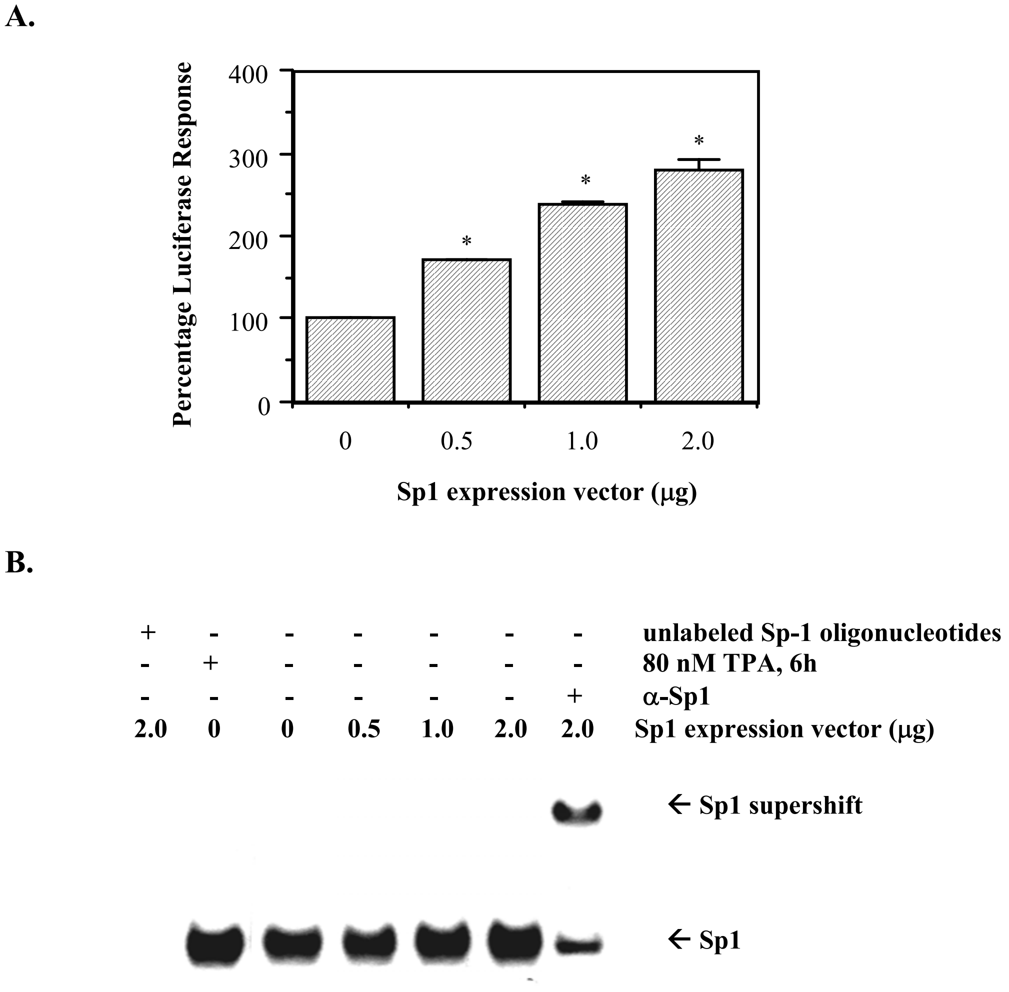


Figure 3-9A. Effect of Sp1 on MnSOD basal promoter. The HepG2 cells were cotransfected with pGL3/P7 and Sp1 (pPac) vector. The Sp1 effect is indicated by percentage luciferase respons ± SEM. *Significant differences between control and tested cells ($p < 0.01$).

3-9B. Sp1 binding activity. Nuclear extract from cells treated with 80 nM TPA for 6 h or cotransfected with increasing amounts of pPacSp1 vector was incubated with ^{32}P-labeled Sp1 probe. The Sp1 and supershift Sp1 bands are indicated.

CHAPTER 4

DIFFERENT ROLES OF Sp FAMILY MEMBERS IN HIV-1 Tat-MEDIATED MANGANESE SUPEROXIDE DISMUTASE SUPPRESSION IN HEPATOCELLULAR CARCINOMA CELLS.

ABSTRACT

The expression of manganese superoxide dismutase (MnSOD) is regulated by agents associated with cancer development. It has been shown that infection with the human immunodeficiency virus type 1 (HIV-1) is associated with the development of liver cancer and that the transactivating transcriptional factor (Tat) of human HIV-1 reduces the expression of MnSOD in several cell types. However, the role of Tat in the expression of MnSOD in hepatocellular carcinoma is unknown. Furthermore, the precise mechanisms whereby Tat suppresses MnSOD expression in hepatocellular carcinoma cells remain unclear. In this report, we build on our original observations that Tat changes the distribution of Sp family members on the MnSOD promoter which accounts for Tat-dependent changes in basal expression. In hepatic cells, Tat expression up-regulates Sp1/Sp3, which play different roles in regulating MnSOD transcription. While overexpression of Sp1 stimulates, overexpression of Sp3 represses transcriptional activity. The transcription repression effect of Sp3 is not due to Sp3 competing for the binding site with Sp1 because only the full-length Sp3 but not the truncated Sp3 suppresses MnSOD promoter activity. These findings suggest a novel mechanism by which Tat modulates the repression of the MnSOD gene and establish a link between HIV infection and liver cancer.

INTRODUCTION

Manganese superoxide dismutase (MnSOD) is a nuclear-encoded mitochondrial antioxidant enzyme that rapidly converts superoxide radicals into hydrogen peroxide and molecular oxygen (Weisiger and Fridovich, 1973). It is well established that MnSOD is essential for the survival of all aerobic life (Carlioz and Touati, 1986). *E. coli* and *S. cervisiae* lacking MnSOD function are sensitive to oxygen and show several metabolic disorders (Farr *et al*., 1986; Van Loon *et al*., 1986). Knockout mice homozygous for the MnSOD gene die shortly after birth and exhibit severe mitochondrial injury (Li Y *et al*., 1995; Lebovitz *et al*., 1996). Overexpression of MnSOD has been shown to reduce cell death and tissue injury initiated by chemical, physical, and biological agents (for a review, see St. Clair, 2001).

MnSOD activity is reduced in many types of cultured tumor cells when compared to their normal counterparts (for a review, see Oberley and Buettner, 1979). Overexpression of MnSOD has been shown to suppress radiation-induced neoplastic transformation, to induce cellular differentiation, and to suppress tumorigenicity (St. Clair *et al*., 1992, 1994; Church *et al*., 1993; Safford *et al*., 1994; Li *et al*., 1995; Zhong *et al*., 1997; Zhao *et al*., 2001). The hypothesis that MnSOD functions as a new type of tumor suppressor stems from these observations, and predicts that changes in the expression levels of MnSOD will influence tumor progression.

Infection with human immunodeficiency virus type 1 (HIV-1) is associated with the development of cancer, including Kaposi's sarcoma, non-Hodgkin's B-cell lymphoma, basal cell and squamous cell skin carcinoma, hepatocellular carcinoma, and lung cancer (Biggar *et al*., 1987; Rabkin *et al*., 1991; Engles, 2001). The oncogenic property of HIV is thought to be mediated, in part, by the Tat protein. In cell culture models, Tat is secreted by HIV-infected cells and taken up by normal cells (Frankel and Pabo, 1988; Ensoli *et al*., 1990). Tat promotes growth, stimulates the angiogenic process, increases tumor growth and metastasis, and has anti-apoptotic effects (Ensoli *et al*., 1993; Campioni *et al*., 1995; Corallini *et al*., 1996). In animal models, Tat-transgenic mice show an increase in tumorigenesis (Vogel *et al*., 1991; Corallini *et al*., 1993; Altavilla *et al*., 1999, 2000).

For efficient viral gene expression, Tat must bind to the transactivating responsive element (TAR), which is located between +1 to +44 (Arya *et al*., 1985;

Rosen *et al.*, 1985; Rice, 1988). In addition to its role in HIV-1 transcription, Tat modulates the expression of many cellular host genes including IL-6 (Ambrosino *et al.*, 1997), IL-8 (Ott *et al.*, 1998), IL-2 (Gonzalez *et al.*, 2001), MCP-1 (Lim *et al.*, 2000), and MnSOD (Flores, *et al.*, 1993; Westendorp *et al.*, 1995; Ehert *et al.*, 1996; Shatrov *et al.*, 1996). Importantly, HIV-infected patients have low levels of MnSOD (Polyakov *et al.*, 1994). Thus, Tat-mediated suppression of MnSOD may lead to increased tumorigenicity in HIV-infected patients.

The human MnSOD gene is a single-copy gene consisting of five exons separated by four introns with typically splice junction on chromosome 6q25 (Church *et al.*, 1992; Wan *et al.*, 1994). The MnSOD promoter contains a GC-rich region consisting of cluster binding sites for Sp1, AP-2, and Egr1 (Yeh *et al.*, 1998; Porntadavity *et al.*, 2001; Xu *et al.*, 2002). Our previous studies demonstrate that Sp1 is essential and sufficient for basal promoter activity (Xu *et al.*, 2002). Sp1 binds to GC boxes and related motifs, a characteristic which is also shared by members of the Sp family. Members of this family, including Sp1, Sp2, Sp3, and Sp4, have similar structural features including a zinc finger DNA-binding domain. Sp1, Sp3, and Sp4 bind to the classical GC-rich region whereas Sp2 binds to the GT-rich region (for a review, see Suske, 1999). Sp3 has three different isoforms derived from alternative internal translation start sites (Hagen *et al.*, 1992). Full-length Sp3 contains 714 amino acids. Truncated forms of Sp3 are missing 217 (M1) or 234 (M2) amino acids from their N-termini (Kennett *et al.*, 1997). Although all members of this Sp family can bind to similar GC motifs, they also exhibit different transcriptional properties. Thus, it is possible that members of this family may modulate each other’s activities on specific genes. In this report, we extend our previous studies, which demonstrated that a Tat-dependent redistribution of Sp1/Sp3 family members on the MnSOD promoter could account for repression in insect cells, to show that Sp1 and Sp3 play opposite roles in the regulation of the MnSOD promoter in HepG2 cells. We also demonstrate that full-length but not truncated Sp3 is responsible for the down-regulation of MnSOD expression by Tat in hepatocellular carcinoma cells.

MATERIALS AND METHODS

Plasmids constructs

Full-length HIV-1 Tat 86 expression vector pcDNA3-Tat was constructed as previously described (Chauhan *et al*., 2003). The pGL3/HLTR construct was obtained by cloning upstream the luciferase gene (*HindIII* restriction site of pGL3-basic plasmid (Promega, Madison, WI) with an HLTR promoter fragment isolated from pSV0CAT-HLTR (NIH AIDS Research, Rockville, MD). The biological activity of recombinant Tat protein was determined by its ability to transactivate the HLTR promoter-driven luciferase expression vector, pGL3/HLTR. Tat increased HLTR promoter activity approximately 1,000-fold. This result indicates that the recombinant Tat exhibits its biological activity in the expressed cells.

To facilitate detection, all members of Sp expression vectors contained a 10 amino acid influenza hemagglutinin epitope at their C-terminal which was recognized by monoclonal antiboby12CA5. The pCMV4-Sp1, pCMV4 full-length Sp3, and pCMV4 truncated-Sp3 were constructed as previously described (Udvadia *et al*., 1993, 1995; Kennett *et al*., 1997). The Sp3 N-terminal deletion containing 605 amino acids and zinc finger domain constructs were prepared from pCMV4 full-length Sp3 using PCR with primers starting at various amino terminal sites and the carboxyl terminal as follows: 5’oligonucleotides (605 amino acids, 5’GGGAAGCTTATGTCA GCAGATGGTCAGCAG-3’), (zinc finger domain, 5’-GGGAAGCTTATGGGGGA CCAACAACATCAA-3’), and 3’ oligonucleotides (5’-GGGAAGCTTCTAGCTAGC GTAATCTGG-3’). Each primer had the addition of a *HindIII* site, and the 3’ PCR primer incorporated DNA sequence coding for a 10-aa influenza hemagglutinin epitope. The nucleotide sequence of each construct was verified by automated DNA sequencing analysis. The internal control for transfection, pRSV-β-gal, was obtained from Dr. Mary Vore (University of Kentucky, Lexington, KY). The pGL3/P7 (MnSOD promoter) and pGL3/P7I2E (MnSOD promoter upstream with the enhancer I2E) were constructed as previously described (Xu *et al*., 1999a). The MnSOD promoter P7, is located between –210 to +24 of the MnSOD gene. The MnSOD enhancer I2E was identified as an intronic enhancer responsive to both TNF and IL-1-mediated MnSOD induction (Xu *et al*., 1999a).

Cell cultures and transfections

HepG2 cells were grown as previpusly described under materials and methods in chapter 3. Cells were transiently transfected with experimental plasmids and internal control by a modified calcium phosphate method (Graham and Van Der Eb, 1973). Forty-eight hours after transfection, cells were collected and analyzed for luciferase activities by the Reporter Assay System (Promega, Madison, WI). β-galatosidase activity was measured using o-nitrophenyl-β-galactopyranoside, ONPG (Sigma, St. Louis, MO) as a colorimetric substrate.

Northern blot analysis

RNA isolation and Northern blot analysis were performed as previously described under materials and methods in chapter 3.

Nuclear extract preparation

Nuclear extract was perpared as previously described under materials and methods in chapter 3.

W*estern blot analysis*

The MnSOD polyclonal antibody was obtained from Upstate Biotechnology (Charlottesville, VA). The CuZnSOD polyclonal antibody was obtained from Calbiochem (La Jolla, CA). The polyclonal antibodies against Sp1, Sp3, AP-2, Egr-1, Sp4, and PCNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibody 12CA5 against hemagglutinin was obtained from Roche Diagnostics (Indianapolis, IN). Western blot analysis was performed previously described under materials and methods in chapter 3.

Electrophoretic mobility shift assay (EMSA)

The representative GC-rich motif fragment was obtained from pGL3/P7 by cutting with restriction endonuclease *SmaI* and *PvuII* (-133/-66) and dephosphorylating with calf intestine phosphatase (New England Biolabs, Beverly, MA). The fragment was labeled with [γ-^{32}P]ATP (New England Nuclear, Boston, MA) by T4 polynucleotide kinase (Amersham Biosciences, Piscataway, NJ). The nuclear extract of HepG2 cells was incubated with 50,000 cpm probe in binding buffer [4% glycerol, 1.25 mM $MgCl_2$, 0.5 mM EDTA, 1.25 mM DTT, 10 mM Tris HCL, pH7.5, and 0.05 mg/ml poly(dI:dC)] at room temperature for 20 min. For supershift experiments, the DNA/protein complexes were further incubated with Sp1 or Sp3 antibodies for 1 h at room temperature before being loaded onto the native 6% PAGE. The protein/DNA complexes were separated on a native 6% PAGE. The gel was dried and exposed to X-ray film at -80^{o}C.

Statistical analysis

All experiments were performed in triplicate and repeated at least three times. Data were analyzed by one-way ANOVA with Bonferoni's post-test. p value < 0.05 indicates a significant difference between the test and the control.

RESULTS

Tat decreases MnSOD protein and mRNA levels in HepG2 cells

Constitutive expression of Tat reduces MnSOD protein and mRNA levels in HeLa and Jurkat cells (Flores *et al.*, 1993; Westendorp *et al.*, 1995). To investigate the effect of transient expression of Tat on MnSOD expression in HepG2 cells, cells were transfected with Tat expression vector, and the level of MnSOD mRNA was determined by Northern blot analysis (Fig. 4-1A). Densitometric analysis of normalized MnSOD mRNA with β-actin mRNA demonstrates that Tat significantly decreases MnSOD mRNA ($p < 0.05$) (Fig.4-1B). MnSOD protein level, determined by Western blot analysis, is also reduced (Fig. 4-2A). Densitometric analyses of the immunoblots (Fig. 4-2B) corroborates the Tat-mediated suppression of MnSOD expression ($p < 0.05$). These results suggest that the Tat-mediated effect might be operative in a variety of cell types.

Tat suppresses MnSOD expression from the basal promoter

We previously demonstrated that Tat suppresses basal MnSOD expression but has no effect on TNF-mediated MnSOD inducibility in HeLa cells (Westendorp *et al.*, 1995). We therefore wanted to test the effect of Tat on basal promoter-driven luciferase expression in the presence or absence of the enhancer containing the TNF-response element (Xu *et al.*, 2002). This enhancer is found in intron 2 of the MnSOD gene. Fig. 4-3A is a schematic diagram illustrating the reporter constructs used. HepG2 cells were cotransfected with different amounts of Tat expression vector and with either pGL3/P7 (MnSOD basal promoter) or pGL3/P7I2E (MnSOD promoter containing the intronic enhancer [I2E]). As shown in Figure 4-3B, Tat significantly reduces MnSOD promoter activity by 45% to 58% with 1 and 2 μg of Tat expression vector, respectively. The construct containing the enhancer shows decreased activity in the presence of Tat (43% and 52% with 1 and 2 μg of Tat expression vector, respectively) which was not significantly different from the effect of the promoter alone (Fig. 4-3C). These results suggest that Tat-mediated regulation of MnSOD expression is via the basal promoter and is independent of the intronic enhancer.

Effect of Tat on transcriptional protein levels

It is well established that Tat modulates heterologous genes, at least in part by altering the level or the binding activity of the transcription factor. The MnSOD promoter contains 78% GC, without TATA or CATT box, and multiple binding sites for Sp1, AP-2, and Egr-1. We and others have demonstrated that Sp1 is a strong activator for MnSOD promoter activity whereas AP-2 and Egr-1 are suppressors (Yeh *et al*., 1998; Zhu *et al*., 2001; Porntadavity *et al*., 2001; Xu *et al*., 2002). We also have shown that Tat alters the distribution of Sp family members in the region of the MnSOD promoter containing multiple GC boxes in HeLa cells and affects basal promoter-driven luciferase expression in Drosophila cells (Flores *et al*., 1993; Marecki *et al*., unpublished observations). Because insect cells lack many of the factors necessary for expression of mammalian genes, and because we were interested in elucidating the mechanism of Tat-mediated MnSOD expression in hepatocellular carcinoma cells, we examined whether Tat influences the expression of Sp1, AP-2, Egr-1, Sp3 or Sp4 in HepG2 cells. The levels of all these transcription factors were determined after the cells were transfected with either 1 or 2 μg of the Tat expression vector. Figure 4-4 shows that Tat increases the level of Sp1 by 11% and 18% at 1 and 2 μg of Tat expression vector, respectively, and significantly increases the level of full-length Sp3 by 32% and 39% at 1 and 2 μg of Tat expression vector, respectively. This suggests that there is a dose response to Tat, whereas Tat slightly increases truncated Sp3 (M1) by 8% at 1 and 2 μg of Tat expression vector. The levels of AP-2, Egr-1, and Sp4 are undetectable in both control and Tat-transfected cells. Thus, under the conditions of this assay, transiently expressed Tat has a positive influence on Sp3 and Sp1 expression in HepG2 cells. To test for the ability of the Sp family of proteins to bind to GC-rich boxes on the MnSOD promoter, electrophoretic mobility shift assays with a HepG2 nuclear extract and a representative GC-rich motif corresponding to nucleotides -133 to -61 of the MnSOD promoter were performed; the specificity of the binding was verified by supershift assays with antibodies to Sp1 or Sp3. Figure 4-5 shows that Sp1, full-length Sp3, and truncated Sp3 (M1) bind to a representative GC-rich motif of the MnSOD promoter (-133/-66).

Sp1 enhances and Sp3 suppresses MnSOD promoter activity

Although only the level of full-length Sp3 significantly increases after overexpression of Tat, Sp1 and truncated Sp3 also increase slightly. To determine the effect of these Sp family members on MnSOD promoter activity, HepG2 cells were cotransfected with pGL3/P7 and various amounts of pCMV4-Sp1, pCMV4-Sp3 full length, or pCMV4-truncated Sp3 (M1). Figure 4-6A shows that Sp1 significantly increases the MnSOD promoter activity in a concentration-dependent manner. In contrast, full-length Sp3 significantly decreases MnSOD promoter activity (Fig. 4-6B), while truncated Sp3 (M1) does not have a significant effect on MnSOD promoter activity (Fig. 4-6C). Thus, Sp1 and Sp3 appear to have opposing effects on MnSOD promoter activity.

Truncated Sp3 releases the suppressive effect of full-length Sp3

Since full-length and truncated Sp3 (M1) have identical DNA binding domains, and truncated Sp3 does not suppress basal MnSOD promoter activity, it is possible that the truncated Sp3 (M1) will compete with the full-length form and overcome its inhibitory effects on MnSOD promoter activity. To test this possibility, we cotransfected pGL3/P7 with pcDNA-Tat and either full-length Sp3 or truncated Sp3 (M1) into HepG2 cells. Figure 4-7 shows that in the presence of Tat, expression of Sp1 and truncated Sp3 (M1) enhances, whereas the presence of full-length Sp3 suppresses, promoter activity. Expression of truncated Sp3 (M1) partially overcomes the suppression of full-length Sp3 on MnSOD promoter activity (Fig. 4-8A). The positive effect of truncated Sp3 (M1) is further supported by its ability to enhance the effect of Sp1 on the activation of MnSOD promoter activity (Fig. 4-8B). Together, these results suggest that full-length Sp3, but not truncated Sp3 or Sp1, negatively regulates MnSOD expression.

Full-length Sp3 protein is needed for suppression of MnSOD promoter

Since only full-length Sp3 has a suppressive effect on MnSOD promoter activity, it is possible that areas in the N-terminal region are required. To test this possibility, Sp3 constructs containing 605 amino acids from the C-terminus and constructs containing only the zinc finger domain were generated by PCR amplification of full-length Sp3 cDNA (Fig. 4-9A). Expression of the predicted Sp3 proteins was verified by Western blot analysis (Fig. 4-9B). HepG2 cells were co-transfected with pGL3/P7 and various amounts of each Sp3 expression construct. The results show that truncated Sp3, the zinc finger domain only, and Sp3 containing 605 C-terminal amino acids do not significantly suppress MnSOD promoter activity (Fig. 4-10). These results indicate that amino acids present in the N-terminal region and absent on the construct containing the C-terminal 605 amino acids are needed for the suppressive effect on MnSOD promoter activity.

DISCUSSION

Accumulated data suggest that HIV-infected patients are under chronic oxidative stress which may contribute to several aspects of HIV-mediated pathogenesis, including viral replication, inflammatory response, decreased immune cell proliferation, loss of immune function, apoptosis, and increased sensitivity to drug toxicities. The survival rate of HIV-infected patients has dramatically increased with the highly active antiretroviral therapy (HAART) regimen introduced in 1996 (Mocroft and Roy, 1998). A longer life span for HIV-infected patients may result in an increasing chance for developing cancer. Since expression of MnSOD can suppress cellular transformation and cancer progression (for a review, see St. Clair 2001), understanding the mechanisms by which Tat regulates MnSOD expression may lead to development of novel interventions to reduce the risk of cancer in HIV-infected individuals.

In this study, the hepatocellular carcinoma, HepG2 cell line was chosen for the following reasons: (1) The liver is the major organ for detoxification of toxic substances including drugs used for HIV treatment. (2) Epidemiological data suggest an association of liver cancer in HIV-infected patients (Rabkin *et al.*, 1991). (3) Tat transgenic mice have a high incidence of hepatocellular carcinoma (Vogel *et al.*, 1991; Altavilla *et al.*, 1999, 2000). (4) MnSOD levels in the liver of Tat transgenic mice were reduced 50% and 70% in untreated mice and mice treated with AZT and zidovudine (Prakash *et al.*, 1997) respectively. We found that expression of Tat reduces MnSOD protein and mRNA levels in the hepatocellular carcinoma, HepG2, cells. However, the degree of suppression on MnSOD mRNA appears to be slightly less than the suppression on MnSOD protein levels. These results suggest that Tat may not only suppress MnSOD expression at the transcriptional step but may also suppress MnSOD production at the translational step. It has been proposed that the size of the Tat protein may play a role in these dual steps of controls. For an example, Tat 101 and Tat 72 can induce IL-8 protein levels (Ott *et al.*, 1998). However, only Tat 101 was capable of up-regulating IL-8 expression at the transcriptional level. Our results demonstrate that Tat 86 is able to suppress MnSOD promoter activity, which suggests the transcription controls events. Our results further demonstrate that Tat-regulated MnSOD transcription is mediated by transcription factors associated with the basal promoter because the same degree of suppression was observed whether the basal promoter alone or basal promoter containing an intronic enhancer was used.

The MnSOD promoter contains clusters of binding sites for Sp family members, AP-2, and Egr-1. However, we could not detect transcription factors AP-2, Sp4, Egr-1 in either the control or Tat-transfected HepG2 cells, indicating that these proteins are absent or not responsive to Tat-mediated mechanisms in HepG2 cells. It is not surprising that we could not detect AP-2 protein, since AP-2 expression was suppressed in the HepG2 cells (Williams *et al.*, 1988). Thus, although other and we have previously demonstrated that AP-2 plays a negative role in the expression of human MnSOD expression (Zhu *et al.*, 2001; Xu *et al.*, 2002), it is unlikely that AP-2 will be a cause for the reduced expression of MnSOD in HepG2 cells.

Previously, we have reported that mutation in the MnSOD promoters of some tumor cells leads to a significant reduction of promoter activity (Xu *et al.*, 1999b). In this study, we determined the DNA sequence of the MnSOD promoter in HepG2 cells and found no mutation (data not shown). Thus, it is unlikely that mutation in the MnSOD promoter will be a cause for Tat-regulated suppression of MnSOD transcription in HepG2 cells. Sp4 expression is tissue specific and normally expresses in the neuronal cells and certain epithelial cells (for a review, see Suske, 1999). Our finding that it is absent in HepG2 cells is consistent with this information. Using differential display, it has been reported that chronically HIV-infected cells have an increased Egr-1 mRNA level. However, the Egr-1 protein level could not be detected by Western analysis (Dron *et. al*., 1999). Our results, which demonstrate that Egr-1 protein is undetectable in both controls and Tat-transfected HepG2 cells, also exclude the possibility that Egr-1 will be a cause for the reduced expression of MnSOD in HepG2 cells. However, we cannot rule out the possibility that in a chronic exposure condition Tat may be able to induce Egr-1 levels and suppress MnSOD promoter activity.

Our results demonstrate that members of the Sp family present in HepG2 cells are capable of binding to the same site in the human MnSOD promoter. This result is consistent with the results from studies which indicate that Sp1 and Sp3 have the same DNA-binding capacity to the classical GC-rich binding proteins (Marin *et al*., 1997; Bouwman *et al*., 2000). In this study, we found that expression of Tat alters the level of Sp1, full-length Sp3, and truncated Sp3 (M1). Sp1 plays a positive role in promoter activity in the presence of Tat. In contrast, the full-length Sp3 is capable of reducing the promoter activity in the presence of Sp1. Thus, Sp1 and Sp3 appear to have different roles in Tat-regulated MnSOD transcription. It also has been proposed that truncated Sp3 (M1) is a very weak activator since it lacks activation domain A (for a review, see Suske, 1999). Our results, which demonstrate that Tat significantly increases the level of full-length Sp3 and suppresses MnSOD expression, are consistent with the possibility that Tat-mediated increases in Sp3 levels alter the Sp1: full-length Sp3: truncated Sp3 ratio in favor of full-length Sp3 function.

It has been shown that full-length Sp3 suppresses gene transcription by simply displacing Sp1 binding to the promoter (Kumar and Butler, 1997; Kwon *et al*., 1999). The MnSOD promoter contains a cluster of binding sites for Sp1 and Sp3, and thus the presence of both Sp1 and Sp3 may create competition for a binding site and lower Sp1-mediated activation. However, our results show that both full-length Sp3 and truncated Sp3 are able to bind to the same site but only the full-length Sp3 is capable of suppressing MnSOD promoter activity. These results indicate that binding to the promoter by Sp3 is not sufficient for the suppression of MnSOD promoter activity. This possibility is confirmed by the finding that the zinc finger domain of Sp3 is also unable to suppress the MnSOD promoter. Moreover, we also show that truncated Sp3 (M1) could act as a weak dominant negative for full-length Sp3 because it is capable of alleviating the negative effects of full-length Sp3 on MnSOD.

It has been reported that Sp3 may act as a transcriptional repressor by protein-protein interaction with components of the general transcription complex (De Luca *et al*., 1996). Our results suggest that it is unlikely that the suppression of full-length Sp3 is due to the inhibitory element (IKEE) located between the second glutamine-rich activation domain and the first zinc finger domain (Dennig *et al*., 1996) because the truncated Sp3 (M1) isoform also contains this element. It is also unlikely that the suppression of full-length Sp3 on MnSOD promoter activity is due to SUMO modification because the lysine residue that is needed for SUMO conjugation is IKEE, which has been previously identified as an inhibitor domain (Sapetsching *et al*., 2002; Ross *et al*., 2002). The only difference between full-length Sp3 and truncated Sp3 (M1) is the length or the structure of the N-terminal of Sp3. Full-length Sp3 may suppress MnSOD promoter activity by (1) blocking Sp1-Sp1 homotypics, (2) recruiting other co-repressors, (3) interacting with the components of transcription complex, or (4) blocking Sp1 to interact with the necessary transcription complex. Although these and other possibilities remain to be further elucidated, it is clear from this study that the N-terminal portion of Sp3 is needed for Sp3 to suppress MnSOD promoter activity.

In summary, the present studies demonstrate that the mechanism by which Tat suppresses MnSOD expression is mediated, in part, via the reduction of the basal promoter activity of the MnSOD gene. Sp1 and Sp3 play different roles in the promoter activity. While Sp1 increases promoter activity, a high level of Sp3 suppresses promoter activity. Tat significantly increases the level of full-length Sp3, which is capable of suppressing MnSOD promoter activity in the presence of Sp1. Expression of the alternatively spliced, truncated Sp3 isoform is able to alleviate the suppressive effect of full-length Sp3. These results establish a role for Tat in HIV-induced MnSOD suppression and suggest a novel mechanism for MnSOD down-regulation in HIV-associated cancer.

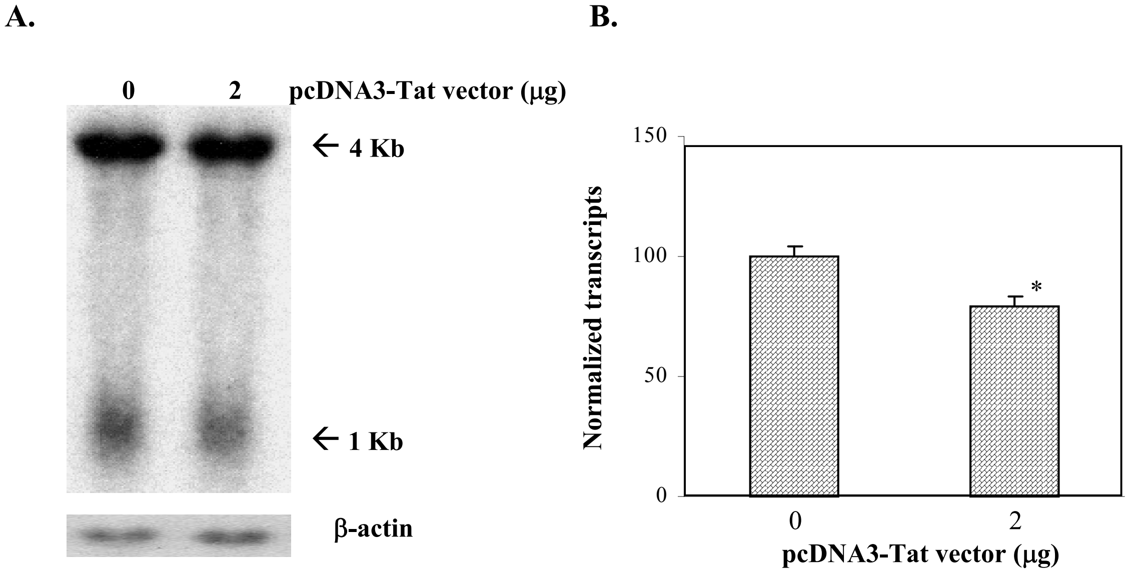


Figure 4. Tat suppresses MnSOD expression at the mRNA levels. HepG2 cells were transiently transfected with 2 μg of pcDNA3-Tat or vector alone. Cells were collected and the levels of MnSOD transcripts and protein were analyzed and quantified.

4-1A. Northern blot analysis, 30 μg of total RNA were loaded and transferred to the membrane. The membrane was hybridized with a human MnSOD cDNA probe. The same membrane was stripped and rehybridized with a β-actin probe.

4-1B. Relative intensity of total MnSOD transcript of level normalized by β-actin level. Each data point represents the average of three independent transfections ± SEM. *Significant differences between control and Tat ($p < 0.05$).

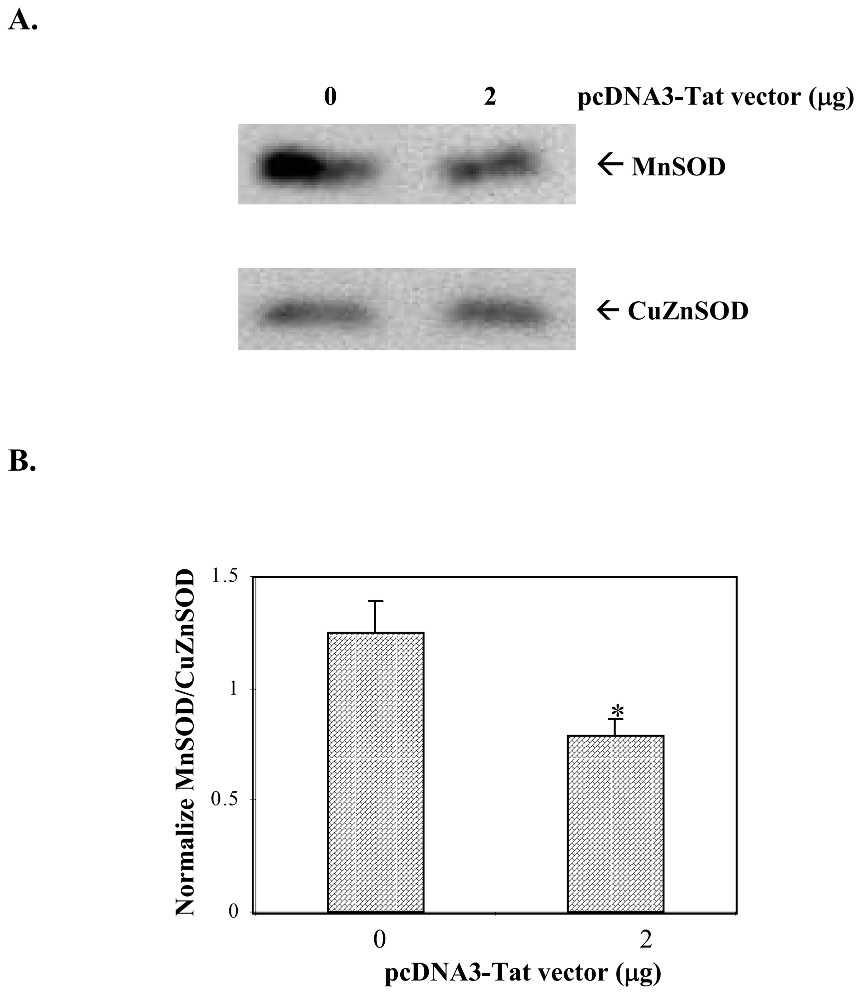


Figure 4-2A. Tat suppresses MnSOD expression at the protein levels. HepG2 cells were transiently transfected with 2 μg of pcDNA3-Tat or vector alone. Cells were collected and the levels of MnSOD protein were analyzed and quantified.

4-2A. Western blot analysis, 50 μg of nuclear extract was loaded and transferred to the membrane. The membrane was blotted with antibody against human MnSOD protein. The same membrane was stripped and reblotted with a CuZnSOD antibody for loading control.

4-2B. Relative intensity of MnSOD protein level normalized by CuZnSOD protein level. Each data point represents the average of three independent transfections ± SEM. *Significant differences between control and Tat ($p < 0.05$).

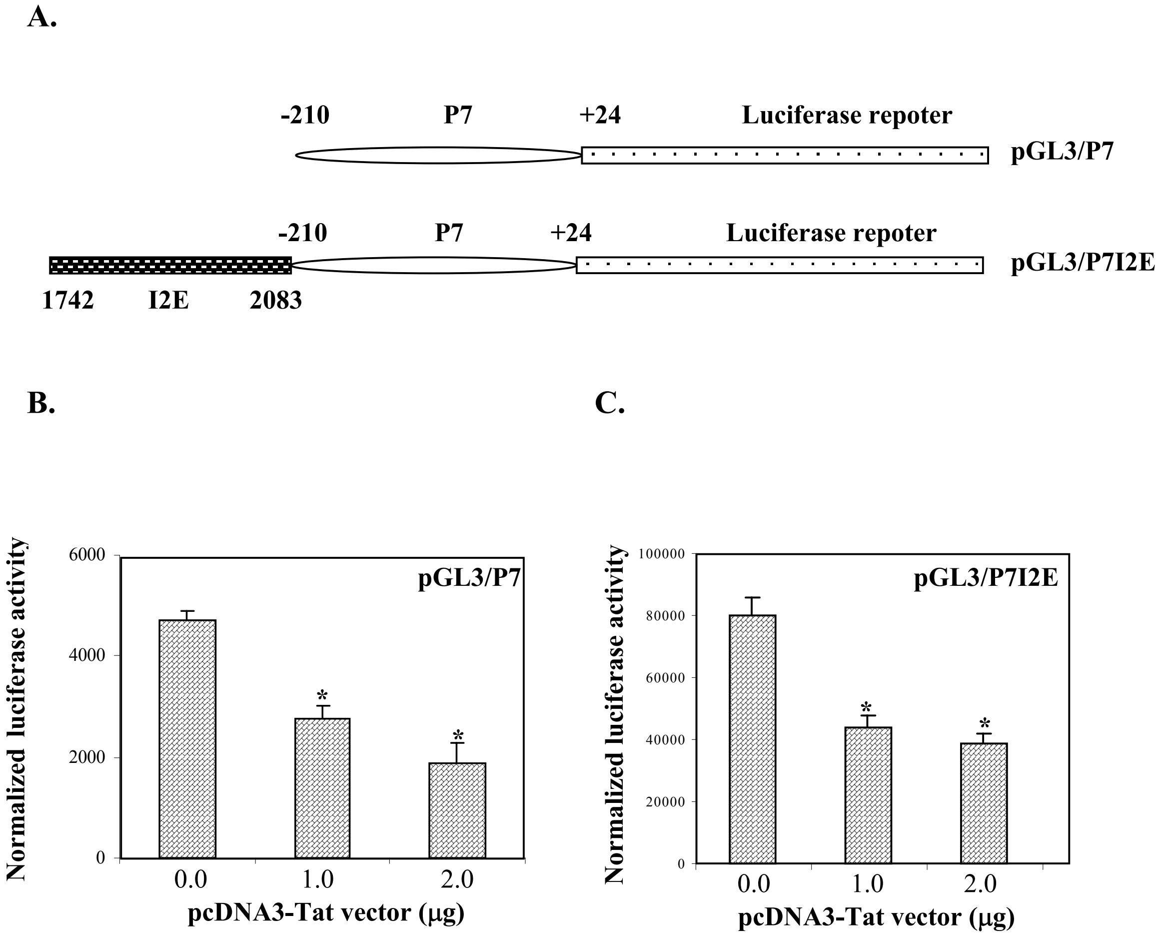


Figure 4-3. The effect of Tat on the human MnSOD gene. Fig. 4-3A. Schematic representation of the reporter gene driven by the human MnSOD promoter (P7) and P7 plus a 342 bp enhancer fragment located in the second intron of the human MnSOD gene (P7I2E) constructs. HepG2 cells were transiently transfected with indicated amounts of pcDNA3 Tat and either pGL3/P7 (Fig. 4-3B) or pGL3/P7I2E (Fig. 4-3C). Cells were collected and measured for luciferase and b-gal activities. The transfection efficiency was normalized by β-gal activity. The effect of Tat on P7 or P7I2E activity was presented as normalized luciferase activity. Each data point represents the average of three independent transfections ± SEM. *Significant differences between control and Tat ($p < 0.05$).

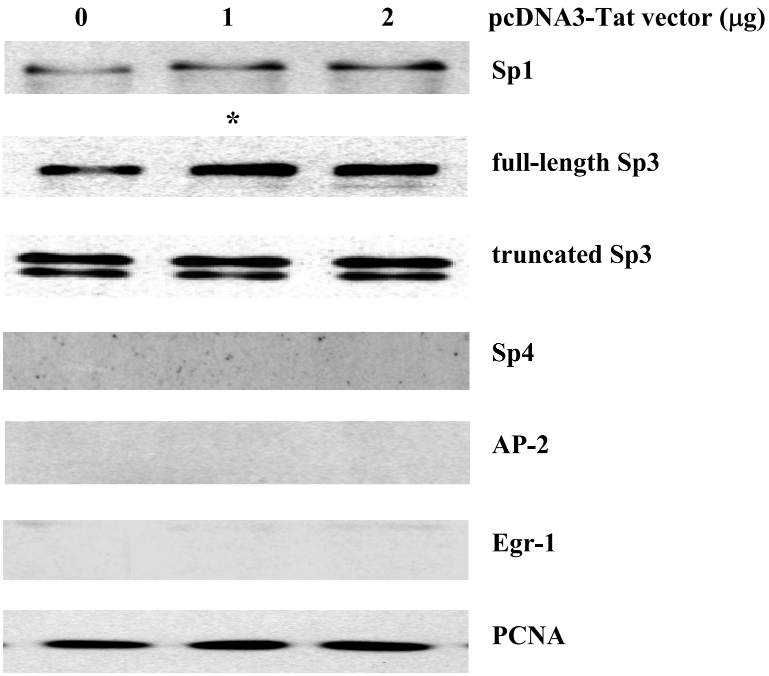


Figure 4-4. Effect of Tat on transcription factor levels. HepG2 cells were transiently transfected with indicated amounts of pcDNA3-Tat for 48 h. Cells were collected and transcription factor levels were determined by Western blot analysis. PCNA was used as the loading control. Asterisk (*) indicates significant differences between control and Tat ($p<0.05$).

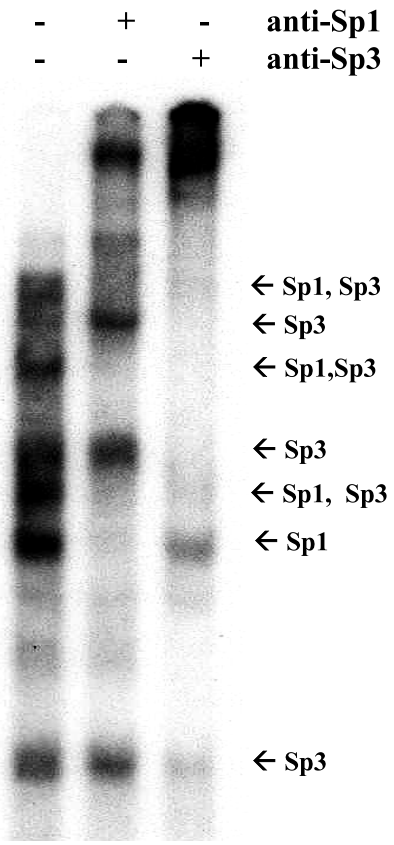


Figure 4-5. Sp1/Sp3 binding activity. HepG2 cell nuclear extract was incubated with ^{32}P-labeled representative GC-rich motif (-133/-61) of the MnSOD promoter. The locations of Sp1 and Sp3 bands were identified by supershift with antibodies against Sp1 or Sp3 proteins. The arrows indicate the identified Sp1 and Sp3 bands.

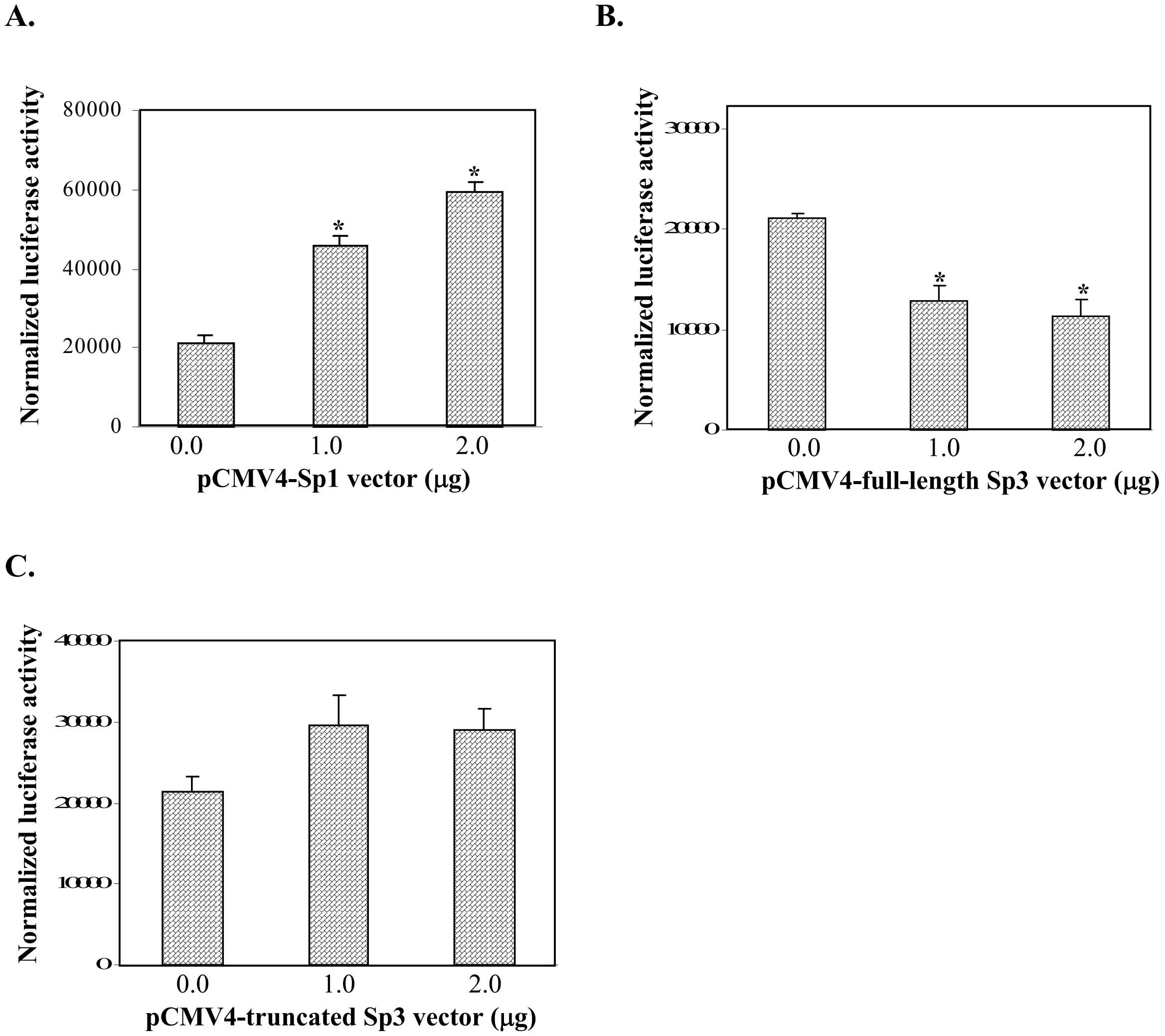
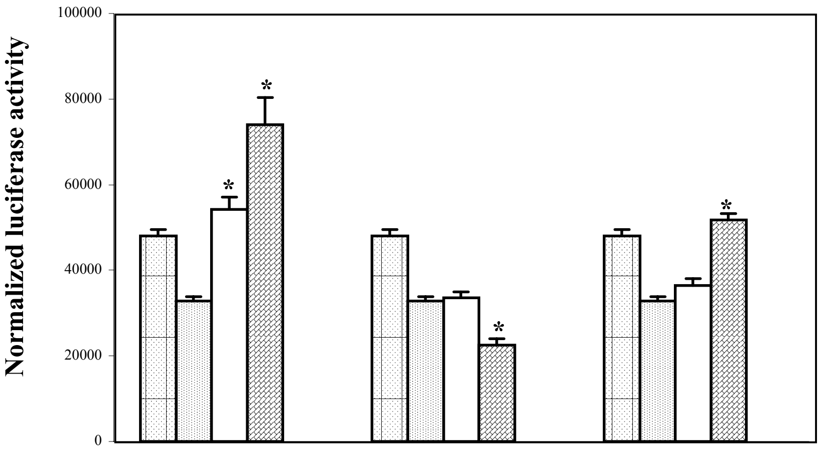


Figure 4-6. The effect of Sp1, full-length Sp3, and truncated Sp3 on MnSOD basal promoter activity. HepG2 cells were co-transfected with pGL3/P7 and the indicated amounts of pCMV4 Sp1 (Fig. 4-6A), pCMV4 full-length Sp3 (Fig. 4-6B), or pCMV4-truncated Sp3 (M1) (Fig-4-6C). Promoter activities were presented as normalized activity. Each data point represents the average of three independent transfections ± SEM. * Significant differences between control and overexpression Sp proteins ($p < 0.05$).



pcDNA3-tat	0	1	1	1	0	1	1	1	0	1	1	1	(μg)
pCMV4-Sp1	0	0	0.5	1	0	0	0	0	0	0	0	0	(μg)
pCMV4-full-length Sp3	0	0	0	0	0	0	0.5	1	0	0	0	0	(μg)
pCMV4-truncated Sp3	0	0	0	0	0	0	0	0	0	0	0.5	1	(μg)

Figure 4-7. The effect of Sp family members on the presence of Tat. HepG2 cells were co-transfected with pGL3/P7, 1 μg of pcDNA3-Tat, and the indicated amount of each member of the Sp family. The effect of each Sp family member is indicated by normalized luciferase activity using β-gal as the control for transfection. Each data point represents the average of three independent transfections transfections ± SEM. * Significant differences between 1 μg pcDNA3-Tat alone and with Sp family member ($p < 0.05$).

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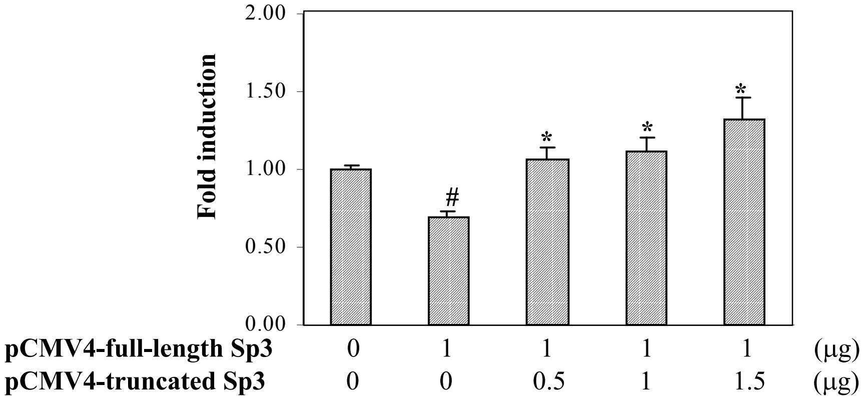
A.



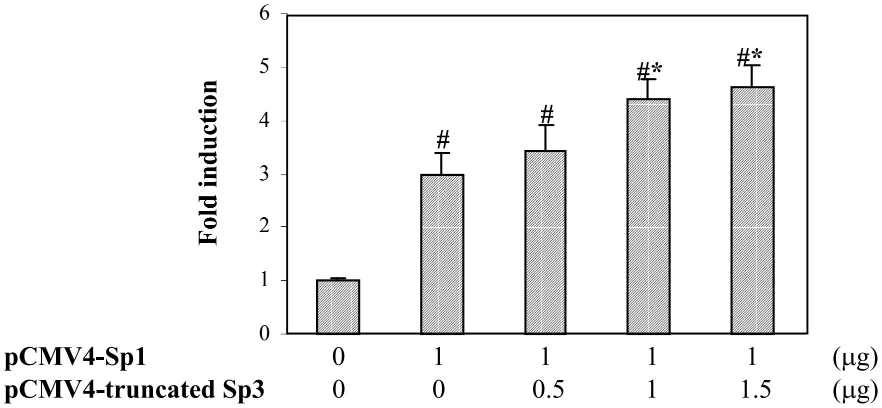


Figure 4-8A. Truncated Sp3 released the suppression effect on promoter activity. HepG2 cells were co-transfected with pGL3/P7, 1 μg of pCMV4-full-length Sp3 and the indicated amounts of pCMV4-truncated Sp3 (M1). The effect of truncated Sp3 was presented as fold increase compared to control.

4-8B. Truncated Sp3 enhanced the positive effect of Sp1 on P7 promoter activity. HepG2 cells were co-transfected with pGL3/P7, 1 μg pCMV4-Sp1, and the indicated amounts of pCMV4-truncated Sp3 (M1). The enhancement effect of truncated Sp3 is presented as fold induction compared to control. Each data point represents the average of three independent transfections ± SEM. # Significant differences between control and 1 μg pCMV4-full-length Sp3 or pCMV4-Sp1 ($p < 0.05$). * Significant differences between 1 μg pCMV4-full-length Sp3 alone or pCMV4-Sp1 alone and increasing amounts of pCMV4-truncated Sp3 ($p < 0.05$).

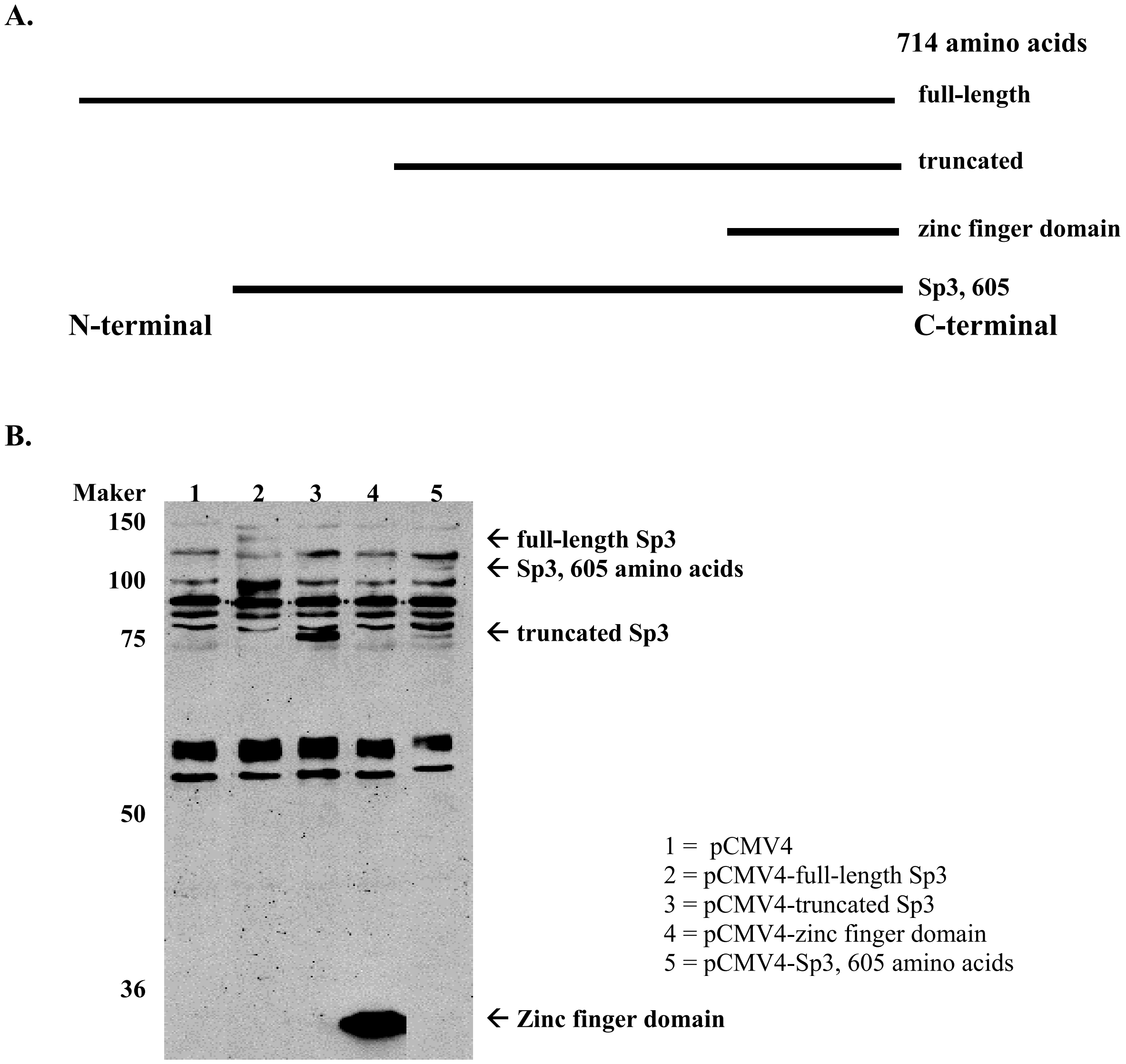


Figure 4-9A. Construction of N-deletion of Sp3 proteins. Sp3 constructs were amplified by PCR using full-length Sp3 as a template. Each fragment was cloned into the pCMV4 vector at the Hind 3 restriction site. The nucleotide sequence was confirmed by automated DNA sequencing.

4-9B. Western blot analysis of exogenous N-deleted Sp3 protein levels, HepG2 cells were transiently transfected with 1 μg of pCMV4 expressing different lengths of the Sp3 protein. Nuclear extracts were collected and analyzed for the predicted Sp3 protein by using an antibody against hemagglutinin.

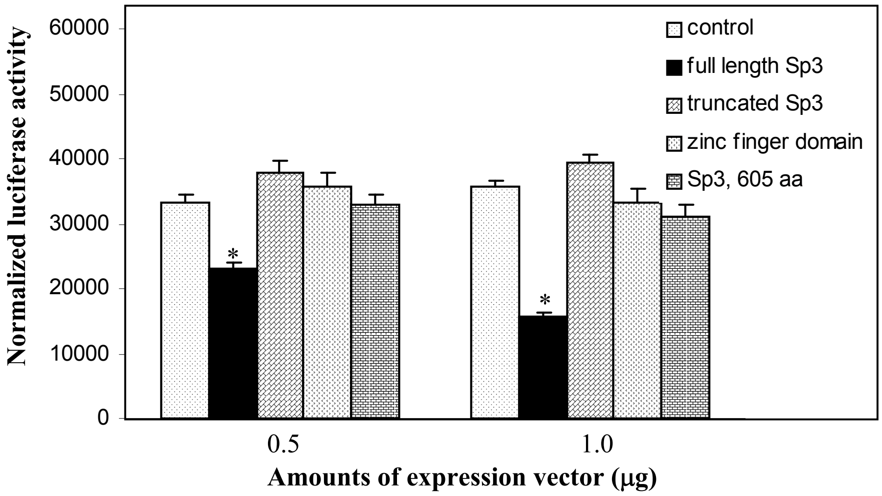


Figure 4-10. Effect of near full-length structure of the Sp3 protein on MnSOD basal promoter. HepG2 cells were co-transfected with pGL3/P7 and 0.5 or 1.0 µg of pCMV4 containing the indicated Sp3 constructs. The promoter activities are presented as normalized luciferase activity. Each data point represents the average of three independent transfections ± SEM. * Significant differences between control and test fragment ($p < 0.05$).

CHAPTER 5

INVOLVEMENT OF TAFII 130 IN Sp3-SUPPRESSED MnSOD EXPRESSION

ABSTRACT

Transcriptional regulation of nuclear-encoded manganese superoxide dismutase (MnSOD) protein in HIV-infected cells is, at least in part, dependent on specific nuclear transcription factors. Our previous investigation reveals the crucial information that HIV-1 Tat suppresses MnSOD transcription by altering the distribution of transcription factors contained in the promoter region Sp1/Sp3 proteins. Although MnSOD gene is characterized as housekeeping gene with cluster binding site of Sp1 and Sp3, the Sp3-suppressed promoter activity is not due to its ability to compete for the binding site in the MnSOD promoter. In this study, results from SL2 cells further consolidate that the competition for the binding site is unlike to be the mechanism for Sp3-suppressed MnSOD expression. We found that Sp1 physically interacts with Sp3 and this suppression requires unknown proteins that do not exist or proper function in the insect SL2 cells. Those proteins have been clearly revealed by immobilized-MnSOD promoter analysis of Sp1- or Sp3-overexpressed HepG2 nuclear extract. The result indicates that Sp1 and Sp3 interact with different set of proteins in the general transcription complex. Furthermore, we at least partially elucidate that TAFII 130 may be involved in Sp3-suppressed MnSOD transcription.

INTRODUCTION

HIV-infected patients have a high chance of developing a variety of complicated pathogeneses such as drug toxicities, secondary infection, cancer, which may result from HIV decreasing antioxidant defenses such as ascorbic acid, glutathione, and MnSOD (Pace and Leaf, 1995; Polyakov *et al*., 1994). MnSOD is a nuclear-encoded antioxidant enzyme which removes the free radicals in the mitochondria (Weisiger and Fridovich, 1973). MnSOD plays many critical roles, and its level has been found to be associated with a variety of diseases. The reduction of MnSOD level in HIV-infected cells was first observed by Wong (Wong *et al*., 1991). This reduction is caused by one of the non-structural proteins, HIV-1 Tat (Florese *et al*., 1993). Our previous findings reveal that, mediated by Sp3, HIV-1 Tat suppresses MnSOD expression from the basal promoter (see chapter 4).

Extensive study of MnSOD regulation has revealed that MnSOD promoter is a GC-rich region without TATA and CAAT box but containing multiple binding sites for Sp1, AP-2, and Egr-1 (Yeh *et al*., 1998; Porntadavity *et al*., 2001; Xu *et al*., 2002). We and other researchers have stressed that Sp1 is a strong activator for MnSOD transcription whereas AP-2, Egr-1, and Sp3 are negative regulators for MnSOD transcription (Yeh *et al*., 1998; Zhu *et al*.,2001; Porntadavity *et al*., 2001; Xu *et al*., 2002). Sp1 and Sp3 belong to the Sp family in which the zinc finger domain is the DNA binding domain (for a review, see Suske, 1999). Even though Sp1 and Sp3 bind to GC boxes with similar affinities (see, Philipsen and Suske, 1999), their effect on gene expression can be similar or different. Sp1 is a strong activator and is required for TATA-less promoter, but Sp3 can be an activator or a repressor depending on the structure and the arrangement of the promoter and cellular content (see review, Suske, 1999). Sp3 acts as a negative regulator on a variety of genes such as ODC, ADH5/FDH, and p21 (WAF/CIP1) (Kumar and Butter, 1997; Kwon *et al*., 1999; Gartel *et al*., 2001). Competition for the binding site is a well-known mechanism by which Sp3 suppresses the expression of genes containing multiple binding sites for Sp1. However, our previous result demonstrates that it is unlikely that this competition mechanism is involved in Sp3-suppressed MnSOD transcription (see chapter 4). Another possible mechanism of Sp3-suppressed gene transcription is its blocking Sp1 from its partner proteins. Sp1 is capable of interacting with itself and with other transcription proteins, including factors belonging to general transcription machinery such as TATA-box binding protein (TBP) and the TBP-associated factors hTAFII 55 and hTAFII 130. (Pascal and Tjian, 1991; Chiang and Roeder, 1995; Tanese *et al.*, 1996).

TBP and 12-14 TAFIIs are the multi-subunit components of the TFIID complex which is responsible for recognizing and binding to the core promoter elements resulting in the assembly and promotion of the preinitiation complex. TAFIIs function as the molecular bridge between activator and general transcription machinery (for a review, see Albright and Tjian, 2000). It has been proposed that Sp1 as an activator may function as a tethering moiety to recruit general transcription machinery, including RNA Pol II, to the preinitiation start site of a TATA-less promoter (Pugh and Tjian, 1990). This suggests that Sp1 may facilitate the assembly of the preinitiation complex via multiple protein-protein interactions. Any interruption from any source, of Sp1 interaction with its partner proteins or Sp1 recruitment may therefore lead to decreased in the transcription activity. In this study, we further characterize the mechanism of Sp3-suppressed MnSOD promoter activity resulting from protein-protein interaction.

MATERIALS AND METHODS

Plasmids and constructions

pCMV4-Sp1, pCMV4-full length Sp3, pPacSp1, and pPac-full length Sp3 have been described previously (Udvadia *et al.*, 1993; Udvadia *et al.*, 1995; Kennett *et al.* 1997). All members of Sp expression vectors contain a 10 amino acid influenza hemagglutinin epitope at their C-terminal which is recognized by monoclonal antibody 12CA5. The internal control for transfection, pIE1-4β-gal, was generously provided by Dr. Grace Jones (University of Kentucky, Lexington, KY). The pGL3/P7 (MnSOD promoter) was constructed as previously described (Xu *et al.*, 1999a).

Cell cultures and transient transfections

Cell of SL2 (Schneider line 2), an insect, were grown in Schneider's insect medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, Logan UT), 200 μM L-glutamine, and 1% antibiotics (Invitrogen corporation, Carlsbad, CA). The cells were grown at 25°C. The cells were plated at

1x10^6 cells/10 mls in a P100 plate the day before transfection. Cells were transiently transfected with expression vectors and internal control by the calcium phosphate method previously described by Dawid and Daolo (Dawid and Daolo, 1983). Forty hours after transfection, cells were collected and analyzed for luciferase activities by Reporter Assay System (Promega, Madison, WI). The β-galatosidase activity was measured using o-nitrophenyl-β-galactopyranoside, ONPG (Sigma, St. Louis, MO) as a colorimetric substrate. After 40 h of transfection, nuclear extracts were collected and quickly stored at -80 °C.

Human hepatocellular carcinoma HepG2 cells were grown under the condition as previously described under materials and methods in chapter 3.

Nuclear extract preparation

Nuclear extract was prepared as previously described under materials and methods in chapter 3.

Western blot analysis

The polyclonal antibodies against Sp1, Sp3, and TAFII 130 proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibody against hemagglutinin, 12CA5, was obtained from Roche (Roche Diagnostics, Indianapolis, IN). Western blot analysis was performed as previously described under materials and methods in chapter 3.

Electrophoretic Mobility Shift Assay (EMSA)

The representative GC-rich motif probe was prepared by labeling the DNA fragment corresponding to -133/-66 (*SmaI* / *PvuII*) of MnSOD promoter with [γ-^{32}P] ATP (New England Nuclear, Boston, MA) using T4 polynucleotide kinase (Amersham, Biosciences, Piscataway, NJ). The nuclear extract of SL2 cells were incubated with 50,000 cpm probe in binding buffer [(4% glycerol, 1.25 mM $MgCl_2$, 0.5 mM EDTA, 1.25 mM DTT, 10 mM Tris HCL, pH7.5, and 0.05 mg/ml poly (dI:dC)] at room temperature for 20 min. The protein/DNA complexes were separated on a native 6% PAGE. The gel was dried and exposed to X-ray film at -80°C.

Immunoprecipitation

Immunoprecipitation was performed by incubating nuclear extract with normal rabbit IgG and protein A/G PLUS-agarose (Santa Cruz, Santa Cruz, CA) in RIPA buffer (1X PBS, 1% Nonidet P-40, 0.1% SDS, 10 ug/ml PMSF, 1 mM sodium orthovanadate, and 1% protease inhibitor). After incubation for 1 h at 4°C, supernatant was collected by centrifugation at 559x g for 5 min. The supernatant was immunoprecipitated overnight at 4°C with anti-Sp1 or anti-Sp3. The protein complexes were precipitated by further incubation with protein A/G PLUS-agarose for 4 h at 4°C. The resulting precipitated complexes were washed 5 times with RIPA. The resulting precipitated complexes were separated on 10% SDS-PAGE and blotted with antibodies against Sp1, Sp3, and TAFII 130 proteins.

Immobilized template-binding assay

The P7 DNA fragment corresponding to -210/+24 of MnSOD promoter was excised by restriction endonuclease with *PstI* and *HindIII* and purified by agarose gel The *PstI*-biotinylated forward oligonucleotides containing the 3' overhang of the *PstI* restriction site, (Biotin-5'-GCC GAG GCC GAG GCT AGC C<u>TG CA</u>-3') and reverse oligonucleotides (5'-GGC TAG CCT CGG CCT CGG C-3') were annealed in NET buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris HCl, pH 7.6) by heating at 65°C for 5 min and then incubated at room temperature for 1 h. P7-biotinylated (immobilized-MnSOD promoter) was derived by ligating P7 fragment to double-stranded *PstI*-biotinylated using T4 DNA ligase (New England Biolabs, Beverly, MA). P7-biotinylated was ligated to streptavidin-beads by incubation in binding buffer (12% glycerol, 60 mM KCl, 12 mM HEPES, pH8.0, 0.12 mM EDTA, 5 mM $MgCl_2$, 5 mMDTT, 0.1% Triton X-100) at room temperature for 45 min on an end-to-end rotator. The free P7 fragment was removed from the immobilized-MnSOD promoter by centrifugation at 559x g for 5 min. Nuclear extracts were incubated with the immobilized-MnSOD promoter for 1.30 h at room temperature on an end-to-end rotator. The resulting protein complexes were washed twice with binding buffer containing 800 mM KCl and 5 times with binding buffer. The protein complexes were separated on 10% SDS-PAGE and analyzed by Silver Stain and Western blot analysis. The Western blot analysis was performed as described in the previous section. The membrane was blotted for Sp1, Sp3, and TAFII 130 proteins.

Statistical analysis

All experiments were performed in triplicate and repeated at least three times. Data were expressed as mean ± SEM. Statistical analyses of the data were carried out by one-way ANOVA with Bonferoni's post-test. P value <0.05 indicates a significant difference between the test and the control.

RESULTS

Sp1 physically interacts with Sp3

Our previous result demonstrates that the retarded bands of both anti-Sp1 and anti-Sp3 in EMSA experiments could be attributed to an interacting complex of Sp1 and Sp3 (see chapter 4). Those results imply that Sp3 may block or interfere with Sp1 functions by direct interaction with Sp1. To test this hypothesis, co-immunoprecipitation by antibodies against Sp1 or Sp3 protein was performed by using nuclear extracts from transfected HepG2 cells with either the pCMV4-Sp1 or pCMV4-Sp3 vector. As shown in Figure 5-1, when nuclear extracts derived from Sp1 protein overexpressed HepG2, cells were immunoprecipitated with Sp1 antibody, Sp3 protein was detected in the precipitated complexes (bottom left). Vice versa, Sp1 protein was also found in the complexes immunoprecipitated by Sp3 antibody (top right) from Sp3 protein overexpressed HepG2 nuclear extract. This result indicates the physical interaction between Sp1 and Sp3 proteins in HepG2 cells.

The effects of Sp1 and Sp3 on MnSOD promoter in SL2 cells

To investigate the direct role of Sp1 and Sp3 on MnSOD promoter SL2 cells were utilized. SL2 cells were co-transfected with pGL3/P7, pIE1-4β-gal, and pPac-Sp1 or pPac-Sp3. The results show that Sp1 strongly increases MnSOD promoter activity from 50- to 80-fold at 0.5 and 1.0 μg of pPac-Sp1 vector, respectively (Fig. 5-2A). Figure 5-2B shows that Sp3 significantly increases MnSOD promoter activity from 10- to 9-fold at 0.5 and 1.0 μg of pPac-Sp3 vector, respectively. The expression of exogenous Sp1 and Sp3 in SL2 cells was detected by Western blot analysis. As shown in Figure 5-2C, corresponding molecular weights of expressed Sp1 protein at approximately 80 kDa and expressed Sp3 protein at approximately 110 kDa were detected.

Required human proteins for Sp3-suppressed MnSOD promoter activity

Our previous result demonstrates that Sp3 suppresses Sp1-induced MnSOD promoter in HepG2 cells (see chapter 4). To investigate that Sp3 by itself is sufficient to reduce Sp1-activated MnSOD promoter activity, SL2 cells were co-transfected with 0.25 µg of pPac-Sp1 vector and various amounts of pPac-Sp3 vector. The result shows that Sp1 increased the MnSOD promoter approximately 33-fold at 0.25 µg of pPac-Sp1 vector. The fold induction of Sp1 on MnSOD promoter activity is not significantly reduced at any experimental concentration of pPac-Sp3 vector. This indicates that Sp3 by itself is unable to reduce Sp1-activated MnSOD promoter activity, even though the amounts of pPac-Sp3 vector were 15-fold higher than the used amounts of pPac-Sp1 (Fig. 5-3A). The binding activities of Sp1 and Sp3 were also determined by incubating these SL2 cell nuclear extracts with the oligonucleotides corresponding to the middle motif of MnSOD promoter (-133/-61). As shown in Figure 5-3B, the binding activity of Sp3 increases dose dependently. The Sp1 binding activity decreases with an increasing amount of Sp3 vector. However, the Sp3 binding activity seems to be saturated at 2.5 µg of pPac-Sp3 vector.

Sp family causes changing protein patterns of immobilized MnSOD promoter

The transcription assays of our recent findings in the insect SL2 cells and our previous results in HepG2 cells are different. This implies that the Sp3-suppressed MnSOD promoter may be involved with partner proteins. To further identify the partner protein that may be involved in the interaction with Sp1 or Sp3 for driven or suppressed MnSOD promoter, the immobilized-MnSOD promoter was incubated with nuclear extracts derived from pCMV4, pCMV4-Sp1, or pCMV4-Sp3 transfected HepG2 cells. As shown in Figure 5-4, the protein patterns changed in the control and those overexpressed with Sp1 or Sp3 proteins. Ten bands were the difference between the control and pCMV4-Sp1 in which 3 bands at approximately 36, 38 and 98 kDa presented in the pCMV4-Sp1. There were 7 bands at approximately 68, 70, 72, 80, 130, 155 and 180 kDa that had more intensity in pCMV4-Sp1 than in the control. Ten bands were the difference between the control and pCMV4-Sp3. Four additional bands at approximately 38, 54, 56 and 125 kDa were represented in the pCMV4-Sp3. The bands at approximately 70 and 78 kDa were more intense in pCMV4-Sp3 than in the control. The band at approximately 52 and 180 kDa was lighter in pCMV4-Sp3 than in the control. Two bands at approximately 55 and 68 kDa were missing in pCMV4-Sp3. There were 13 bands that differed between pCMV4-Sp1 and pCMV4-Sp3. Bands at approximately 36, 55, 68, 80, 98 and 130 kDa were missing in pCMV4-

Sp3. The intensity of bands at approximately 52, 72, 155 and 180 kDa were lighter in pCMV4-Sp3 than in pCMV4-Sp1. There were 3 additional bands at approximately 54, 56 and 125 kDa that were present in pCMV4-Sp3 and absent in pCMV4-Sp1.

The change in protein patterns implies that the different partner proteins are interactive with or recruited by Sp1 or Sp3 protein. The estimation of protein molecular weight was helpful for excluding the irrelevant proteins. However, specific molecular weight may contain many proteins. A review of existing publications led us to narrow down the Sp1 or Sp3 interactive proteins especially those proteins that function as basic transcription machinery. To identify these possible partner proteins, the same nuclear extract elution from the immobilized-MnSOD promoter was identified by Western blot analysis. As shown in Figure 5-5, there were no bands present in the nuclear extract derived from empty vector pCMV4, transfected HepG2 cells when hybridized with antibodies against Sp1, Sp3, and TAFII 130 proteins. The Sp1 and truncated Sp3 bands were more intense in the nuclear extract derived from pCMV4-Sp3 transfected HepG2 cells. In contrast, the Sp3 and TAFII 130 and full-length Sp3 were more intense in the nuclear extract derived from pCMV4-Sp1 transfected HepG2 cells.

Discussion

More than half of human genes are characterized as housekeeping genes and this type of gene controls most cell events such as proliferation, differentiation, apoptosis. However, a study in expression of housekeeping genes under normal and especially pathological conditions is urgently needed because of its unique regulation. Most housekeeping genes, including the MnSOD gene contain TATA and CAAT-less box and consist of multiple GC motifs (Dynan, 1986; Yeh *et al*., 1998).

MnSOD is one of the critical biological molecules that are reduced by HIV-1 Tat (Florese *et al*., 1993). Our previous results demonstrate that HIV-1 Tat suppresses MnSOD levels from the basal promoter by significantly increasing Sp3 levels (see chapter 4). Sp3 binds to similar binding sites with the same degree of affinity of Sp1 which is the strongly driven MnSOD promoter (see review, Suske, 1999; Xu *et al*., 2002). There is much evidence that demonstrates that Sp3 suppresses expression of most of the genes containing the cluster binding site of Sp1 by competing for the binding site with Sp1 (for a review, see Suske, 1999). However, our previous finding implies that Sp3-suppressed MnSOD promoter activity is unlikely to be this mechanism. Neither truncated Sp3 or Sp3 containing only its binding site is unable to reduce MnSOD promoter activity. This implies that Sp3 might directly interact with Sp1. In addition, our previous result from EMSA shows the retarded bands that could be shifted by both the Sp1 antibody and the Sp3 antibody (see chapter 4).

In this study, co-immunoprecipitation shows that Sp3 protein was precipitated by the Sp1 antibody and, vice versa, the Sp1 protein was precipitated by the Sp3 antibody. This indicates the physical interaction between Sp1 and Sp3 proteins in the HepG2 cells. It is known that the function of the Sp3 is gene-and cell-type specific (for a review, see Suske, 1999). The interaction of Sp1 and Sp3 may have a synergistic effect on some genes and, at the same time, this interaction may be an antagonistic expression of some other genes including MnSOD. This implies that different partner proteins may be involved.

The results from insect SL2 cells in this study contradict our previous results from HepG2 cells, in which Sp3 was unable to reduce Sp1-activated MnSOD promoter activity in insect SL2 cells whereas Sp3 reduced Sp1-activated MnSOD promoter in the HepG2 cells. This indicates that 1) only the binding of Sp3 to the MnSOD promoter is not sufficient to suppress MnSOD promoter; 2.) specific partner proteins that interact with or are recruited by Sp1 and Sp3 proteins do not exist or properly function in the insect SL2 cells. Although we have no evidence of an interaction between Sp1 and Sp3 in the insect SL2 cells, both proteins did not bind to the MnSOD promoter as dose dependent in the EMSA. MnSOD promoter activity was not reduced, even though the amount of Sp3 vector was 15-fold greater than the amount of Sp1 vector. This data consolidates our previous finding that Sp3-suppressed MnSOD promoter is not due to competition with the binding site of Sp1. This result also indicates that Sp3 is not just bound to the MnSOD promoter and simply blocks the Sp1 and then reduces MnSOD promoter. It does seem that Sp3 recruits different proteins or is unable to recruit the necessary proteins to the preinitiation site for transcription. Moreover, results also imply that this set of proteins does not exist or is not properly functioning in the SL2 cells.

Transcription usually starts with the preinitiation complex, which is recognized by TBP, and follows to form the preinitiation complex. MnSOD contains multiple binding sites for Sp1which is believed to act as an activator by recruiting and interacting with basic machinery transcription factors. Our immobilized-MnSOD promoter data reveal clearly different patterns of protein between HepG2 nuclear extracts obtained from overexpression with Sp1 or Sp3 proteins. Hampsey and Reinberg demonstrated that different TAFs are required for identical core promoters in different cell types (Hampsey and Reinberg, 1997). We would speculate that different TAFs may be required for identical core promoters in the same cell type but in different conditions. Unfortunately, there are many proteins representing the same molecular weight, so we could not identify the proteins. This requires further investigation using proteomic approaches. However, by referencing the published data, we can presume some proteins that has a high chance to interact with Sp1 protein such as TAFII 130. We were able to partially elucidate that TAFII 130 may be involved in the Sp3-suppressed MnSOD promoter since the resulting protein complexes from immobilized-MnSOD promoter with Sp1 overexpressed HepG2 nuclear extract contained a higher amount of TAFII 130 when compared with the Sp3 overexpressed HepG2 nuclear extract. The lower degree of Sp1 and TAFII 130 interaction has been demonstrated to be associated with pathological conditions such as Huntington's disease (Dunah *et al*., 2002). Unfortunately, we are unable at this time to explain why the amounts of Sp1 and Sp3 in the resulting protein complexes from immobilized-MnSOD promoter with Sp1 or Sp3 overexpressed HepG2 nuclear extract contain reversed amounts of these 2 proteins. In addition, we cannot rule out the possibility that other coactivator complexes, such as SMCC, TRAP, ARC, are involved in addition to general transcription TAFs, even though whether those other coactivator complexes can substitute for TFIID remains unclear (see review, Albright and Tjian, 2000).

The findings of this study and the previous study in chapter IV lead us to speculate that 1) The interaction of Sp3 with other proteins may interfere with Sp1 bridging to the necessary basic machinery and this Sp3 interaction may need the N-terminal part of Sp3 protein; 2) Sp3 blocks Sp1 from bridging to the necessary machinery, and Sp3 has to be big enough in size. Furthermore, our data from this study are a starting point in the protein-protein interaction area of the Sp family for MnSOD transcription. This finding and further study may apply to other housekeeping genes in general.

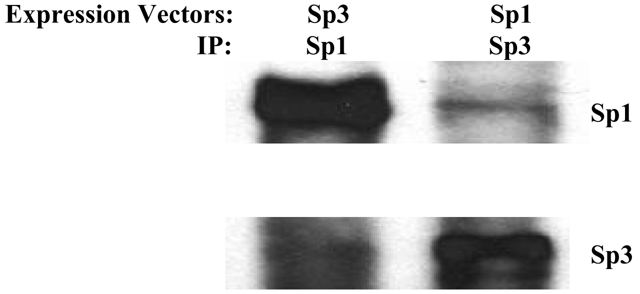


Figure 5-1. Physical interaction between Sp1 and full-length Sp3 proteins in HepG2 cells. HepG2 cells were overexpressed with pCMV4-Sp1 or pCMV4-full-length Sp3. After 48 h of transfection, nuclear extract were collected and immumoprecipitated (IP) with Sp1 or Sp3 antibody. The resulting protein complexes were separated on 10% SDS-PAGE and immunoblotted with Sp1 antibody and Sp3 antibody. (Note: coimmunprecipitate with Sp1 antibody was represented from 2 sets of experiment and coimmunoprecipitae with Sp3 antibody was represented from 6 sets of experiment.

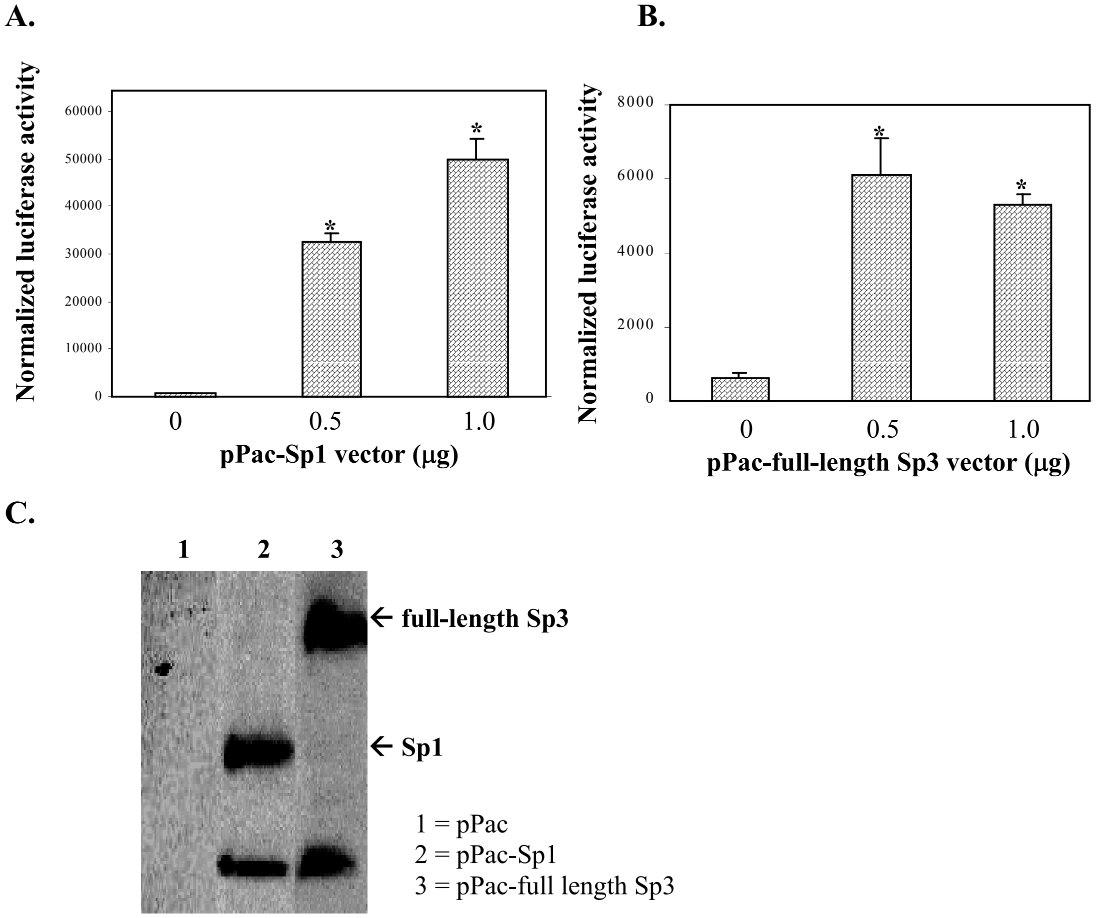


Figure 5-2. The effect of Sp1 and full-length Sp3 on the MnSOD basal promoter activity in SL2 cells. SL2 cells were co-transfected with pGL3/P7 and indicated amounts of Pac-Sp1 (Fig. 5-2A) or pPac-full length Sp3 (Fig. 5-2B). Forty-eight hours after transfection, cells were collected and measured for luciferase activity and β-gal activity. Promoter activity was represented as normalized luciferase activity and is expressed as the mean ± SEM of triplicates from three individual experiments. *Significant differences between control and over-expression ($p < 0.01$). Figure 5-2C. Exogenous levels of Sp1 and full-length proteins in SL2 cells were detected using hemagglutinin antibody.

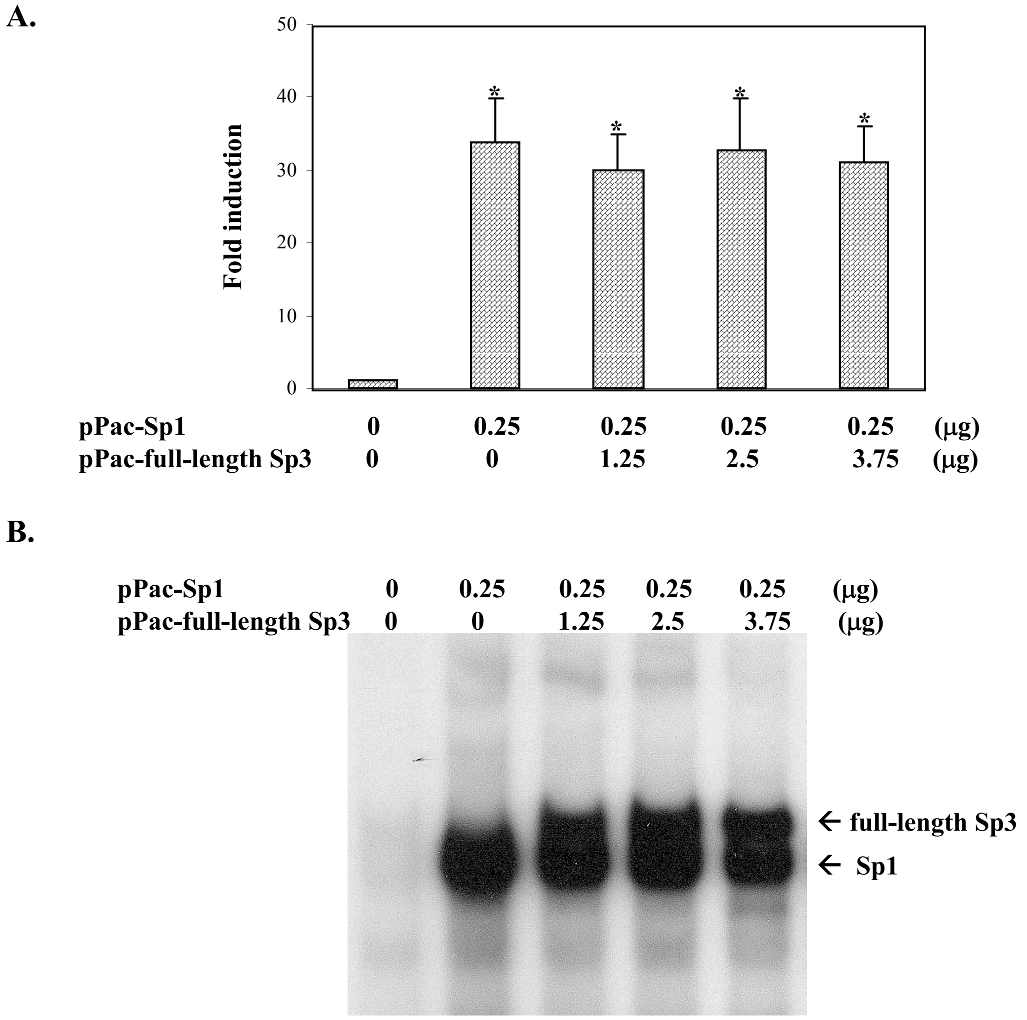


Figure 5-3. The effect of excess overexpression of full-length Sp3 on Sp1-activated MnSOD basal promoter.

5-3A. SL2 cells were co-transfected with pGL3/P7, 0.25 ug of pPac-Sp1, and increasing amounts of pPac-full length Sp3. The effect of full-length Sp3 was determined as fold induction from the pPac control vector. Each data point represents the average of three transfections ± SEM. *Significant differences between control and increasing amounts of full-length Sp3 vector ($p < 0.01$).

5-3B. Binding activity of Sp1 and Sp3 in SL2 cells nuclear extract. Indicated amounts of expression vector corresponded to the amounts in the transfection experiment were incubated with representative GC-rich motif (-133/-61) of MnSOD promoter. The identified bands are indicated.

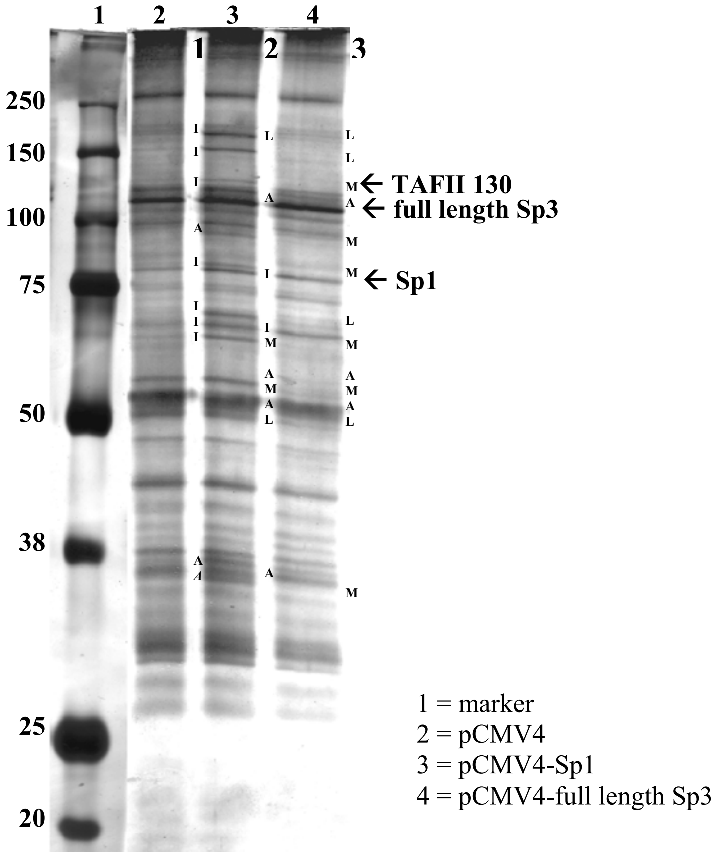


Figure 5-4. Possible protein interaction on MnSOD promoter. Nuclear extract from the transfected HepG2 cells with pCMV4-Sp1 or pCMV4-Sp3 were incubated with immobilized MnSOD promoter. The resulting protein complexes were separated on 10% SDS-PAGE. Gel was stain with silver nitrate. The molecular weight was marked on the left. The arrows indicate the possible proteins by relative to their molecular weight. The number 1 between pCMV4 lane and pCMV4-Sp1 lane indicated the difference of resulted band between those two lanes. The number 2 between pCMV4-Sp1 lane and pCMV4-full-length Sp3 lane indicated the difference of resulted band between those two lanes. The number 3 next to pCMV4-full-length Sp3 lane indicated the difference of resulted band of pCMV4-Sp3 to pCMV4 Sp1. The letter A and M mean additional and missing bands, respectively. I and L mean more intense and lighter bands, respectively.

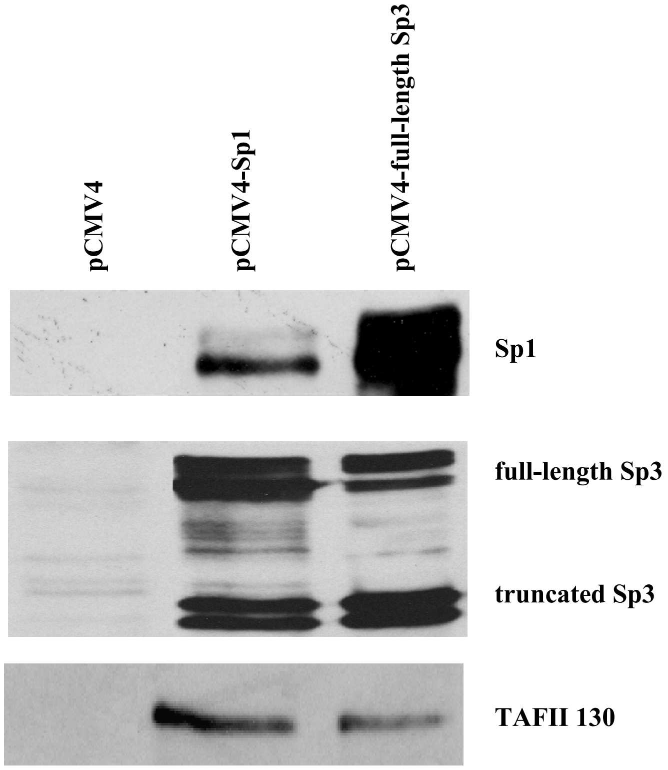


Figure 5-5. Western blot analysis of resulting protein complexes. The same resulting protein complexes (as shown in Fig. 5-4) were separated on 10% SDS-PAGE. Gel was transferred on to the membrane and blotted with Sp1, Sp3, and TAFII 130 antibodies.

CHAPTER 6

SUMMARY AND FUTURE STUDIES

Oxidative stress is an unwanted condition for cells, however in the nature, sometimes cells have defect in antioxidant system or an increased of oxidants. This oxidative stress condition is known to be a cause of a secondary complication in several pathological disorders such as aging, artherosclerosis, cancer, neurodegenerative diseases etc. There are several ways to reduce oxidative stress such as, increase antoxidant substrates by dietary supplements or increase antioxidant enzymes by increasing enzyme levels using enzyme mimetic or modulating endogenous enzyme level. MnSOD is an important antioxidant enzyme located at the major source of oxidants in the mitochondria (Weisiger and Fridovich, 1973). Unlike other metalloantioxidant enzymes, MnSOD does not generate strong oxidant by itself or its break down products; Mn(II) (Bray *et al.*, 1974; Archibald and Fridovich, 1981, 1982; Yim *et al.,* 1990). The lower level of MnSOD has been shown to associate with pathological disorders as caused by oxidative stress. Moreover, overexpression of MnSOD protein showed the possibility for cancer therapy. MnSOD gene is a housekeeping gene, but MnSOD responses to several stimuli. Most stimuli increase MnSOD mRNA level through newly synthesis (Wong and Goddel, 1988; Visner *et al.*, 1990; Brog *et al.*, 1992). MnSOD mRNA level was low in cancer cell lines even though there are no mutation in their MnSOD gene and no change in their mRNA stability (St. Clair and Holland, 1991). These indicated that the transcription step of MnSOD is one of the keys for controlling MnSOD expression. Thus, to modulate MnSOD expression, information for understanding the mechanism of how MnSOD expression at the transcriptional step is required. In this study, two models; up- and down-regulated MnSOD expression, are investigated by using two known reagents; TPA and HIV-1 Tat protein

In the up-regulated model by TPA, the result revealed that TPA began to increase MnSOD mRNA both 1 and 4 kb in hepatocellular carcinoma (HepG2) cells. We found that TPA-induced MnSOD transcription was mediated largely from the basal promoter. MnSOD basal promoter contains a cluster binding sites for Sp1 and AP-2 protein (Yeh *et al*., 1998). In this study, we identified 4 putative Egr-1 binding sites which overlap with Sp-1 binding site at; -169, -120, -103, and –96 by computational analysis. However, the binding site at -169 is preferential site for Egr-1 protein. We found that TPA increased transcription factors containing in the MnSOD basal promoter Sp1 and Egr-1 proteins but not AP-2 proteins. An overexpression of Sp1 protein increased Sp1 binding activity resulted in increasing MnSOD basal promoter activity. TPA treatment induced Sp1 binding activity as was seen in overexpression of Sp1 protein. While an overexpression of Egr-1 reduced MnSOD basal promoter activity. We also found that TPA increased activity of MnSOD basal promoter containing mutation of Egr-1 binding site at -169 with similar degree to the wild type MnSOD basal promoter. Our result suggested that Sp1 is a major activator for constitutive and TPA-induced MnSOD transcription whereas an overexpression of Egr-1 suppresses MnSOD basal promoter activity.

The down-regulated model in this study utilized HIV-1 Tat protein. We found that transiently expressed-Tat protein reduced MnSOD mRNA and protein levels in our cell model, HepG2 cell. However, the effect was less than the effect as seen by constitutively Tat-expressed in HeLa and Jurkat T cells as well as in mouse model (Flores e*t al*., 1993; Westendorp *et al*., 1995; Parkash *et al*., 1997). Degree of Tat suppressed MnSOD was stronger on the MnSOD protein than MnSOD mRNA. Our result from transcription assays of MnSOD promoter and I2E demonstrated that Tat-suppressed MnSOD transcription from the MnSOD basal promoter. Tat significantly increased full-length Sp3 level but not other known transcription factors containing on the basal promoter; Sp1, truncated Sp3, AP-2, and Egr-1 protein. We found an interesting mechanism of Sp1/Ap3 for MnSOD regulation. Full-length Sp3–suppressed MnSOD basal promoter activity and reduced Sp-1 induced MnSOD basal promoter activity. These Sp3 effects are likely independent from competing for the binding site with Sp1. Because the zinc finger binding domain and truncated Sp3 were not reduced MnSOD basal promoter activity. However, we found that actually this Sp3-suppressed depended on the length or the structure near by the N-terminal of Sp3 protein. Furthermore, the Sp3-suppressed Sp1-induced *M*nSOD basal promoter activity in HepG2 cells was abolished in the insect SL2 cells. This indicated that Sp3 suppression required third proteins or mediators that does not exist or malfunction in the insect cells. Comparing nuclear extract derived from Sp1- or Sp3-overexpressed, we found difference pattern of resulting proteins eluted from MnSOD basal promoter. This indicated that different sets of protein are recruited by Sp1 and Sp3 proteins. Results from Tat down-regulation suggested that Sp1 protein is a strong activator whereas Sp3 protein is a strong repressor. The length or structure near by the full-length of Sp3 protein and mediators or third proteins attributed to suppress MnSOD transcription by Sp3 protein.

The result form these two models in this study suggested that 1.) MnSOD expression can be modulated at the transcription level. 2.) MnSOD basal promoter is not a simple promoter, but it contains inducible and suppressible responsive elements to TPA and Tat protein. 3.) Sp1 protein is a strong activator and capable to increase MnSOD basal promoter activity. 4.) Egr-1 and full-length Sp3 proteins are the negative regulator. 5.) Truncated Sp3 protein seems to play a positive role in MnSOD transcription 6.) Sp3-suppressed MnSOD basal promoter requires the Sp3 structure or mediators 7.) Difference cellular conditions utilize different set of protein for protein-protein interaction to regulate MnSDO transcription.

Taken together, the information from this study can be applied to modulate endogenous MnSOD level by 1.) Increase Sp1 activity with natural reagents such as medicinal plants. 2.) Suppress Sp3 activity by reducing full-length Sp3 protein or eliminating the madiator or the third protein. However, the exact structure near by the full-length of Sp3 protein needs to be scrutinized in order to release Sp3-suppressed MnSOD basal promoter activity. The mediators for Sp1 or Sp3 must be identified. This study induces more challenge future study to identify the set of proteins for MnSOD transcription in different cell conditions and look for reagents that increase Sp1 protein level without any side effects.

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