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Regulation of human metallothionein-III gene expression by p300 and SP-1

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Contents

Contents I
List of Figures III
List of Abbreviations V
Abstract ····································
I. Introduction 1
II. Materials & Methods7
$\rm II$ -1. Chemicals and Materials $\cdots \cdots 7$
II-2. Cell culture
II-3. Plasmid construction7
II-4. Site-directed mutagenesis
II-5. Isolation of RNA and RT-PCR
II-6. Transfection and luciferase and β -galactosidase assays 8
II-7. Statistical analysis9
III. Results 14
III-1. Up-regulation of human MT-III expression by TSA
in SH-SY5Y cells
Ⅲ-2. Effects of ZnCl ₂ and TSA on human MT-I and
MT-III promoter activities
III-3. Effects of $ZnCl_2$ and p300 on human MT promoter
activities

III-4. Effects of HDAC 1, HDAC 11, and p300 on human
MT-III promoter activities
III-5. Effect of p300 on human MT-III deletion Promoter
luciferase gene expression
III-6. Effect of SP-1 on human MT-III-419 promoter
luciferase gene expression25
III-7. Effects of SP-1 and p300 on human MT-III promoter
or human MT-III point mutation promoter activities27
IV. Discussion ····································

v.	References	

List of Table and Figures

Table. 1. Nucleotide sequence homology of between human
MT-I and MT-III10
Table. 2. Primers and reaction conditions for amplication
of human MT-I and MT-III
Fig. 1. Stereo drawing of the Metallothionein crystal structure
and Electrostatic potential map of the surface
of Metallothionein4
Fig. 2. Structure of Trichostatin A
Fig. 3. Features of Repressed and Active chromatin
Fig. 4. Human MT-III promoter luciferase vector
construction used in transfection experiments12
Fig. 5. Construction of p300, HDAC 1, and HDAC11 expression
vector used in transfection experiments
Fig. 6. Human MT-III up-regulation by TSA in SH-SY5Y cells 15
Fig. 7. Effects of Zn and TSA on human MT-I and III promoter
luciferase gene expression
Fig. 8. Effects of Zn and p300 on human MT promoter
luciferase gene expression
Fig. 9. Effects of HDAC 1, HDAC 11 and p300 on human MT-III $$
promoter luciferase gene expression
Fig. 10. The putative binding sites for transcription factor
of human MT-I and MT-III promoter and the
cloned regions23
Fig. 11. Effects of p300 on human MT-III deletion
promoter luciferase gene expression

Fig.	12. Effects of SP-1 on human MT-III-419 promoter
	luciferase gene expression
Fig.	13. Mutation of the SP-1 site present in the human
	MT-III promoter28
Fig.	14. Effects of SP-1 and p300 on human MT-III promoter
	or human MT-III point mutation promoter

List of Abbreviations

MT	metallothionein
TSA	trichostatin A
SP-1	stimulation protein-1
HDAC	histone deacetylases
MRE	metal regulatory elements
MTF-1	MRE binding transcription factor-1
CNS	central nervous system
FBS	fetal bovine serum
RT-PCR	reverse transcriptase polymerase chain reaction

ABSTRACT

Regulation of human metallothionein-III gene expression by p300 and SP-1

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Metallothionein (MT) gene family consists of several members (MT I, II, III) that are tightly regulated during development. Human MT I and MT II are expressed in many tissues, including the brain, whereas human MT III is expressed mainly in the central nervous system. Human MTs are thought to play roles both in the intracellular regulation of the essential trace elements (zinc or copper) and in the protection from of a variety of stress conditions. Human MT I and MT II expression are regulated by metals like as zinc or cadmium. However the transcriptional regulation mechanism of human MT III is still obscure, although human MT III is expressed in the brain and the prostatic cancer regions. In this study, the objectives of the present study was to investigated the transcription factor of strength and specificity of human MT-III promoter region, and designed sever point mutations of nuclear factor binding site form nucleotide -1243 to +72 to a luciferase reporter gene. The luciferase activity of human MT-III promoter was increased in the human neuroblastoma cell line, SH-SY5Y, after the treatment of Trichostatin A (TSA) or cotransfected with the p300, which contains acetyltransferase activity. The activity of human MT-III promoter was dramatically increased after the cotransfection of p300 and TSA compared with p300 alone. Furthermore, the activity of human MT-III promoter was significantly increased in the cotransfection of SP-1 (stimulation protein-1). These results suggested that the regulation of human MT-III gene expression related to SP-1 transcriptional activity by p300 binding protein-mediated acetylation.

I. Introduction

Metallothioneins (MTs) are small cysteine-rich proteins that bind transition metal ions such as Cd, Zn, and Cu. All vertebrates examined contain two or more distinct human MTs that are grouped into four families, human MT-I to MT-IV. Whereas the human MT-I and MT-II genes are expressed in most tissues and are coordinately inducible by a variety of chemical and physical conditions that include metal ions and hormones, the human MT-III and MT-IV display tissue-specific expression. Human MT-III is genes expressed predominantly in sub-populations of neurons and astrocytes in the brain and spinal cord and in the organs of the reproductive system, whereas human MT-IV expression is restricted to certain stratified epithelia. Human MTs can protect cells and organisms against metal toxicity, and appear to play important roles in Zn and Cu homeostasis. Metal activation of human MT gene transcription is dependent on the presence of metal regulatory elements (MREs), and on the capacity of MRE binding transcription factor-1 (MTF-1) to bind to the MREs in the presence of zinc and induce transcription. Human MT-III, also known as growth inhibitory factor (GIF), was originally purified as a human brain protein inhibiting the survival and neurite outgrowth of cultured rat cortical neurons, a property not shared by other MT family members. Human MT-III possesses biological properties that differentiate it from other human MT isoforms and that may serve a specialized function. The mechanisms controlling human MT-III gene transcription also

appear to be distinct from those involved in the regulation of the human MT-I and MT-II genes. Indeed, the human MT-III gene displays a unique tissue-specific expression pattern, and is not inducible by any of the stimuli that normally increase human MT-I and MT-II gene transcription in liver and brain. In regards to regional distribution, human MT-III is expressed within astrocytes in many of the same CNS regions as human MT-I/-II, including cortex, brainstem and spinal cord [Choudhuri et al., 2003]. In regards to neuronal expression, human MT-III is most abundant within the granule cells of the dentate gyrus in the hippocampus and particularly those neurons that store zinc in their synaptic vesicles [Choudhuri et al., 1993; Masters et al., 1994].

Most sequence-specific transcription factors regulate gene expression by interacting with transcriptional coregulators, which influence chromatin structure. Acetyltransferases and chromatin facilitate remodelling complexes chromatin opening, while deacetylases (HDACs) and methyltransferases frequently contribute to gene silencing [Jenuwein and Allis, 1999; Narlikar et al., 2002]. The transcriptional co-regulators CBP and p300 possess histone acetyltransferase activity [Bannister and Kouzarides, 1996; Ogryzko et al., 1996] and are capable of interacting with a large variety of transcription factors playing central roles in a wide range of cellular processes including proliferation, differentiation and apoptosis [Goodman and Smolik, 2000; Chan and La Thangue, 2001].

In this study, the objectives of the present study was to investigated the transcription factor of strength and specificity of human MT-III promoter region. Increased of human MT-III promoter of TSA (Trichostatin A) or cotransfected with the p300 expression vector, which contains acetyltransferase activity in human neuroblastoma cells. Here, I try to demonstrate that the regulation of human MT-III gene expression relate to SP-1 transcriptional activity by p300 binding protein-mediated acetylation.



Fig. 1. Stereo drawing of the Metallothionein crystal structure and Electrostatic potential map of the surface of Metallothionein. Red: negative charged. Blue: positively charged [Zangger et al., 2002: Vasak et al., 2000].



Fig. 2. Structure of Trichostatin A



Fig. 3. Features of Repressed and Active chromatin. Activation is an absolute requirement for transcription from chromatin templates. Whereas the presence of nucleosomes exclude the bulky TFIID and RNA Pol II complexes, transcriptional activators are proteins with simple limited DNA binding sites which are able to intrude into the nucleosomal DNA. Histone acetyltransferase (HAT) activity is associated with several factors already discussed, including p300/CBP. Repression is mediated bv histone deacetylase, HDAC, associated with corepressors. Deacetylation restores the histones to their original state, excluding the transcriptional machinery.

II. Materials & Methods

II-1. Materials

Materials and cell culture materials used in this study were obtained from the following sources: Trichostatin A (TSA) and TNF-a purchased from Sigma Co. Human MT-I, MT-III, and MT-III promoter primer were purchased from Bioneer. LipofectAMINE Plus, Eagle's minimum essential Medium (EMEM), fetal bovine serum (FBS), and penicillin-streptomycin solution were purchased from invitrogen, Inc.

II-2. Cell culture.

The SH-SY5Y human neuroblastoma cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD), and grown in EMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine and antibiotics at 37°C in a 5% CO₂ humidified incubator.

Π -3. Plasmid construction.

To construct the luciferase (LUC) reporter plasmid human MT-I gene fragment containing 921 bp 5' flanking sequences and 69 bp 5' untranslated region (UTR), was cloned into the plasmid pGL3-Basic (Promega). To construct the plasmid -1243 human MT-III-LUC, a DNA fragment containing 1243 bp 5' flanking sequences and 78 bp 5'UTR from the human MT-III gene was first amplified by PCR, using a human MT-III genomic clone as the template, and cloned into pGL3-Basic. The promoter fragment was obtained form a subclone, and fragments form -921, -689, -419, -224, 176, -106 to +78 were isolated and designated -1243 human MT-III-LUC. Eukaryotic expression vector for p300, p300DN, HDAC1, HDAC11, SP-1 have been described previously (Fig. 3.)

II-4. Site-directed mutagenesis.

Site-directed mutagenesis of SP-1 binding site formed using a LAPCR *in vitro* Mutagenesis Kit (TAKARA SHUZO Ltd.). The sequence of binding site form 5`-GG<u>GGGGGGGGGG3`</u> 5`-GG<u>AA</u>CGGG-3` and designated human MT-III-419. For the mutants, correct insertions and mutations were confirmed by sequencing.

II-5. Isolation of RNA and RT-PCR.

The total cellular RNA was isolated using an acidic phenol extraction procedure. For the human MT-I and MT-III primers, PCR consisted of initial denaturation at 94°C for 2 min each cycle consisted of 94°C for 2 min, 55°C for 1 min and 72°C for 1 min. After 28 cycles, another 72°C cycle was run for 5 min. PCR products were visualized by ethidium bromide staining of 2 % agarose gels. Integrity of RNA and cDNA synthesis were monitored by amplification of GAPDH mRNA.

II-6. Transfection, luciferase and β -galactosidase assays.

The SH-SY5Y cells were plated in 48-well plates in EMEM supplemented with 10% FBS. After 12 h, the cells were

- 8 -

transfected using LipofectAMINE Plus. Subsequently, the cells were co-transfected with 0.5 µg of pCMV- β -gal, 0.5 µg of the human MT-I-921, human MT-III-1243, -921, -689, -419, -224, 176, -106, human MT-III SP-1 mtuant-419, and p300 regulated luciferase reporter gene. Four hours after transfection, a fresh EMEM medium containing 10% FBS was added to the cells, which were treated with the vehicle, TSA and TNF- α as indicated in the figures. Following 18 h exposure, the cells were washed once with 2 ml of PBS and lysed. The lysed cell preparations were then centrifuged, and the supernatant was assayed for both luciferase and β -galactosidase activity. Luciferase activity was determined using the luciferase assay system and used according to the manufacturer's instructions using a luminometer. The β -galactosidase assay was carried out in 250 µl of assay buffer containing 0.12 M Na₂HPO₄, 0.08 M Na₂HPO₄, 0.02 M KCl, 0.002 M MgCl₂, 0.1 M β -mercaptoethanol, 50µg of ρ -nitrophenyl- β -galactoside, and 100µg of the cell extract. The luciferase activity was normalized using the β -galactosidase activity, and is expressed as a proportion of the activity detected with the vehicle control.

Π -7. Statistical analysis.

All experiments were repeated at least three times to ensure reproducibility. The results are reported as means \pm S.D.. ANOVA was used to evaluate differences between multiple groups.

Table 1. Nucleotide sequence homology of human MT-I and human MT-III. The translated sequences and primer binding sequences are written in bold, respectively.

Gene	Primer sequence(sense, antisence)	size(bp)	Tm(℃)	cycles
GAPDH	5`-gatgaattetgaaggtegga gteaacggatttggt-3`(sense) 5`-gataagetteatgtggg ecatgagteeaceac-3`(antisence)	983	54	18
human MT-III	5`-tgagacctgccctgccctt-3`(sense) 5`-tcactggcagcagctgcact-3`(antisence)	192	60	25
human MT-I	5`-caactgctcctgcgccactg-3`(sence) 5`-tcaggcacagcagctgcact-3`(antisence)	162	58	23

Table 2. Primers and reaction conditions for amplication of humanMT-I and MT-III.



Fig. 4. Human MT-III promoter luciferase vector construction used in transfection experiments.



Fig. 5. Construction of p300, HDAC 1 and HDAC11 expression vector used in transfection experiments.

III. Results

III-1. Up-regulation of human MT-III expression by TSA in SH-SY5Y cells.

The effects of several chemicals such as, TSA, ZnCl₂ or TNF-a, on the expression of human MT-I and MT-III were examined in SH-SY5Y cells. Human MT-I is well known to be a stress protein whose expression is increased in response to multiple inducers but human MT-III expression is well unknown still. In order to compared the expression of endogenous human MT-I and MT-III mRNA, SH-SY5Y cells were treated with TSA for 16 h and total RNA was prepared. As show in Fig. 6, treatment of SH-SY5Y cells with ZnCl₂, TNF-a, or TSA for 16 h was found to increase the level of human MT-I mRNA. However, the level of human MT-III mRNA is increased by only the TSA. These results indicated that TSA altered the expression of human MT-III.



Fig. 6. Human MT-III up-regulation by TSA in SH-SY5Y cells. Total RNAs were extracted from the cells after the TSA, ZnCl, TNF-a treatment. Cells were lysed and total RNA was prepared for the RT-PCR analysis of gene expression. PCR was carried out using the specific primers for the amplification of human MT-I and MT-III. The PCR amplification products were electrophoresed in 2% agarose gel and stained with ethidium bromide. One of three representative experiments is show.

III-2. Effects of $ZnCl_2$ and TSA on human MT-I and MT-III promoter activities.

To examine the effect of TSA or/and ZnCl₂ on human MT-I, MT-III transcription, SH-SY5Y cells were transiently transfected with a luciferase reporter construct containing the 5' regulatory region of the human MT-I and MT-III gene. Acetylation of core histones is known to modulate gene expressionby altering chromatin structure. Treatment of cells with inhibitors of HDAC increases acetylation of histones, which, in turn, could alter chromatin structure and induce gene expression. As shown in Fig. 7, the promoter activity of human MT-I was increased after TSA. However, the activity of human MT-III promoter was dramatically increased by TSA.



Fig. 7. Effects of Zn and TSA on human MT-I and II promoter luciferase gene expression. SH-SY5Y cells were transiently co-transfected with pGL3-human MT-I-Luc or pGL3-human MT-III-Luc and pCMV- β -gal. After 18 h, cells were treated with the indicated concentrations of ZnCl₂ (100 μ M) or/and TSA(100 μ M) for 12 h. Each bar shows the means \pm S.D. of three independent experiments, performed in triplicate.

III-3. Effects of ZnCl₂ and p300 on human MT promoter activities.

To examine the effects of p300 and ZnCl₂ on human MT-I and MT-III transcription, SH-SY5Y cells were transiently transfected with a luciferase reporter construct containing the 5' regulatory region of the human MT-I and MT-III gene. The p300 histone acetylase was described initially as a transcriptional coactivator interacting wide that functions by with а variety of enhancer-binding proteins. As shown in Fig. 8, the promoter activities of human MT-I and MT-III are increased by the cotransfection with p300 expression vector. And the increment is more significant in human MT-III gene expression.



Fig. 8. Effects of Zn and p300 on human MT promoter luciferase gene expression. SH-SY5Y cells were transiently co-transfected with pGL3-human MT-I-Luc, pGL3-human MT-III-Luc, p300 expression vector and pCMV- β -gal. After 18 h, cells were treated with ZnCl₂ (100 μ M) for 12 h. Each bar shows the means \pm S.D. of three independent experiments, performed in triplicate.

III-4. Effects of HDAC 1, HDAC 11, and p300 on human MT-III promoter *activities*.

To examine the effect of HDAC on human MT-III transcription, SH-SY5Y cells were transiently transfected with a luciferase reporter construct containing the 5' regulatory region of the human MT-III gene. Human MT-III regulation of promoter activity can be repressed by HDAC, specifically HDAC11. As shown in Fig. 9, increased promoter activity of human MT-III by p300 is decreased by the cotransfection with HDAC-11 expression vector.



Fig. 9. Effects of HDAC 1, HDAC 11 and p300 on human MT-III promoter luciferase gene expression. SH-SY5Y cells were transiently co-transfected with pGL3-human MT-III-Luc, expression vector p300, HDAC 1, HDAC 11 and pCMV- β -gal. Each bar shows the means \pm S.D. of three independent experiments, performed in triplicate.

III-5. Effect of P300 on human MT-III deletion promoter luciferase gene expression.

To examine the regulation of the human MT-III gene by p300 was analyzed by comparing the transcriptional activity of different promoter 5' regulatory region in SH-SY5Y cells with/without p300 the-1,243/+78, -921/+78, Several overexpression vector. -698/+78, and -419/+78 fusion constructs promoted high levels of luciferase expression. In contrast, 5'-deletions of various lengths from -224 to +78 resulted in a reduction of promoter activity to about 60-70% that of the -419/+78 construct p300 stimulation of the activity. These results clearly indicate that a positive regulatory element(s) is located between -419 and -224 and that this region is essential for a high level expression of the human MT-III gene. This finding suggests that the distal SP-1 binding site is required for the regulation of human MT-III expression by p300. Therefore, human MT-III promoter regulation of acetyltransferase activity and SP-1 transcription factor (Fig. 11).



Fig. 10. The putative binding sites for transcription factor of human MT-I and MT-III promoter and the cloned regions.



Fig. 11. Effects of P300 on human MT-III deletion Promoter luciferase gene expression. SH-SY5Y cells were transiently co-transfected with 1µg of series of human MT-III promoter deletion constructs ligated to luciferase (-1243/+78, -921/+78, 689/+78, -419/+78, -176/+78) and 0.5µg of pCMV- β -gal. Each bar shows the means \pm S.D. of three independent experiments, performed in triplicate.

III-6. Effect of SP-1 on human MT-III-419 promoter luciferase gene expression.

To examine the effect of SP-1 on the promoter activity of luciferase gene expression of human MT-III -419/+78 was examined in SH-SY5Y cells. To further determine the location of the transcription binding sites increased by SP-1 of human MT-III -419/+78 gene expression. This results indicated that SP-1 binding site of transcription factor binding site located in the human MT-III promoter (Fig. 12).



Fig. 12. Effects of SP-1 on human MT-III-419 promoter luciferase gene expression. SH-SY5Y cells were transiently co-transfected with SP-1, pGL3-human MT-III-Luc and pCMV- β -gal. After 18 h, cells were treated with TSA(100 μ M) for 12 h. Each bar shows the means \pm S.D. of three independent experiments, performed in triplicate

III-7. Effects of SP-1 and p300 on human MT-III promoter or human MT-III point mutation promoter activities.

To examine the effect of SP-1 on human MT-III transcription, SH-SY5Y cells were transiently transfected with a luciferase reporter construct containing the 5' regulatory region of the human MT-III gene or point mutation human MT-III gene. To examine SP-1 DNA binding motifs could participate in the regulation of human MT-III promoter activity, especially because SP-1 is known to modulate the expression of other keratins. In order to examine the role of these SP-1 sites mutated the GGGGCGG motif to GGTTCGG in the context of the human MT-III -419/+78 promoter. This approach preserves the complete promoter sequence and focuses on the role of a putative binding site by mutation of only two nucleotides. Using a PCR-based site-directed mutagenesis approach, the mutated SP-1 element at positions -231(Fig. 13). In reporter gene assay reveals that there is significant decrease in the activity after the transient transfection using the human MT-III promoter contain of the point mutation of SP-1 binding site (Fig. 14).



Fig. 13. Mutation of the Sp-1 site present in the human MT-lll promoter. The SP-1 binding site of PGL3-419/+78 was mutated by site-directed mutagenesis, and the mutant promoter plasmid designated as PGL3-419/+78.



Fig. 14. Effects of SP-1 and p300 on human MT-III promoter or MT-III point mutation promoter luciferase gene expression. SH-SY5Y cells were transiently co-transfected with pGL3-human MT-III-419 Luc, pGL3-human MT-III-419 Luc(SP-1 mutation) and pCMV- β -gal. Each bar shows the means \pm S.D. of three independent experiments, performed in triplicate.

IV. Discussion

In this study, the objectives of the present study was to investigated that the expression of human MT-III could be regulated by physical interaction of SP-1 with p300 in SH-SY5Y neuroblastoma cells. $ZnCl_2$, TNF-a, or TSA for 16 h was found to increase the level of human MT-I mRNA. However, the level of human MT-III mRNA is increased by only the TSA. Human MT-I is well known to stress protein whose expression is increased in response to multiple inducers. In addition to MTF-1, several other transcription factor, such as SP-1 and STAT3, may participate in regulation of the expression of human MT-I through binding to the human MT-I promoter or by interaction with MTF-1. However, gene expression of human MT-III are not well understood. Human MT-III expression may be regulated by transcriptional factors other than classical human MT inducer, $ZnCl_2$, TNF-a, and this difference in inducibility is useful for identifying these factors such as, methylation, phosphorylation, and acetylation. These factors are among the post-translational modifications known to occur on the N-terminal tails of histones. These modifications have been correlated with a number of biological events. In the case of acetylation, hyperacetylation of lysine residues correlates with expression, while gene hypoacetylation correlates with silencing gene and heterochromatic chromosomal regions. The balance between HAT and histone deacetylase activities is important in maintaining the steady-state levels of histone acetylation. Histone acetylation is associated with transcriptional activity in eukaryotic cells. Acetylation occurs at lysine residues on the amino-terminal tails of histones, resulting in alteration of nucleosomal conformation, thus increasing the accessibility of transcriptional regulatory proteins to chromatin templates, and subsequent transcription. Histone acetyltransferase promotes transcription, while HDAC should act as a repressor. Generally speaking, inhibition of HDAC results in acetylation of histone protein. Furthermore, inhibition of HDAC is known to affect a variety of biological processes, such as the induction of differentiation, cell cycle arrest, and apoptotic cell death.

An increase in the level of histone acetylation and subsequent relaxation of chromatin at the sites of active transcription is thought to be one mechanism by which HDAC inhibitor activates gene expression. To showed that TSA and p300 can induce human MT-III expression. This conclusion is supported by the following results: an increase in histone acetylation is observed after treatment with TSA, a HDAC inhibitor; deletion of the SP1-binding site from a human MT-III promoter luciferase construct abolishes p300 induced promoter activity; mutation of the SP1-binding site in human MT promoter abolishes activity, and finally the SP-1 family of transcription factors specifically bind to the SP-1-binding site present in the human MT-III promoter. These conclusions are consistent with the effects of TSA on the activation of other genes. Recent studies have reported that the GC box in the promoter region of $p21^{\text{WAF1/Cip1}}$ is important for basal and TSA-induced promoter activity and that

SP-1 and SP-3 are activators of this GC-box. Other studies have reported that TSA activate transcription from the G₁₂ gene promoter by SP-1 binding in differentiating K562 cells. HDAC inhibitors release an inhibitory constraint on SP-1, which results in association with accessory protein to effect gene transcription. These findings support the hypothesis that Sp1-binding sites are involved in the transcriptional activation of genes in response to TSA. SP-1 plays a key role in the activation of a large number of genes, including housekeeping and cell cycle-regulated genes, containing upstream "GC Box" promoter elements. Furthermore, SP-1 is implicated in important regulatory functions during cell development, differentiation, and apoptosis, and contributes to the induction of several genes such as interleukin-1, p15^{INK4B}, and TNFR-II. The 5'-flanking region of the human MT-III gene has multiple transcriptional start sites, does not posses a TATA or CAAT box, and is GC rich. The SP-1-binding sites of human MT-III promoter region activate the basal human MT-III gene transcription. This data also indicated that this SP-1 site is involved in human MT-III transcription in SH-SY5Y cells.

In conclusion, the results presented that human MT-III expression may be increased by the acetylation of some transcription factor(s) or by coactivator(s) those could be related directly or indirectly to the function of p300 but hardly by the histone acetylation, since the expression of reporter gene existed in the transiently transfected vectors might not be affected by chromatin assembly and histone acetylation. Expression of human MT-III could be regulated by physical interaction of SP-1 with p300.

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