Decreased Methylation and Transcription Repressor Sp3
Up-regulated Human Monoamine Oxidase (MAO) B Expression
during Caco-2 Differentiation*

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Monoamine oxidase (MAO) A and B catalyze the oxidative deamination of neuroactive and dietary monoamines such as serotonin, tyramine, and phenylethylamine. Here we show that MAO B, but not MAO A, gene expression was induced during Caco-2 cell differentiation; thus this cell line was used as a model system to study the gene regulation unique for MAO B. Luciferase and gel shift assays showed that transcription factors Sp1 and Sp3 binding to \(-246\) and \(-99\) bp were responsible for the observed gene activation. Overexpression of Sp3 inhibited the induction of MAO B gene by Sp1, and the expression of Sp3 was decreased during Caco-2 cell differentiation. Computer analysis revealed a putative CpG island containing 22 potential CpG methylation sites between \(-261\) and \(-58\) bp. In vitro methylation of MAO B promoter with 5-aza-2'-deoxycytidine, a DNA methyltransferase inhibitor, up-regulated MAO B gene expression in both HeLa and Caco-2 cells. Sodium bisulfite sequencing showed a gradually reduced methylation of the CpG sites during Caco-2 cell differentiation. These results suggested that MAO B gene expression is selectively induced by a decreased Sp3/Sp1 ratio and reduced DNA methylation. This new information may provide insights on the tissue-specific expression of these two isoenzymes.

Monoamine oxidase (MAO); amine:oxygen oxidoreductase (deaminating, flavin-containing), EC 1.4.3.4) catalyzes the oxidative deamination of neuroactive, vasoactive, and dietary amines such as serotonin, norepinephrine, dopamine, tyramine, and phenylethylamine with the production of \(\text{H}_2\text{O}_2\) (for review see Refs. 1 and 2). MAO is present in two isoforms, MAO A and MAO B, which are encoded by two closely linked genes on the X chromosome (3, 4) and share 70% amino acid sequence identity (5). These two isoenzymes have distinct substrate specificity and inhibitor sensitivity. MAO A preferentially oxidizes serotonin and norepinephrine and is inhibited by low concentrations of clorgyline (6). MAO B has higher affinity for substrates phenylethylamine and benzylamine and is inhibited by low concentrations of deprenyl (7). Active sites of MAO A and B have been extensively studied (8–16). The studies of MAO A and MAO B knockout mice have clearly demonstrated that these isoenzymes have distinct functions in monoamine metabolism and play important roles in behavioral and neurological disorders (17, 18). The human brain MAOs have been implicated in a number of psychiatric and neurological disorders and targeted for drugs against depression and Parkinson’s disease (19, 20). MAO regulates the levels of serotonin in both the central and enteric nervous systems. It has been shown that serotonin plays important roles in the gut (21, 22) and is implicated for the management of irritable bowel syndrome (23). However, the mechanisms that govern the regulation of these two genes remain largely unknown.

MAO A and B are co-expressed in most human tissues and are most abundant in the intestine (24). However, fibroblasts and placenta express only MAO A (25, 26). In contrast, platelets and lymphocytes express only MAO B (27). In the human brain MAO A is found in catecholaminergic neurons; surprisingly MAO B, instead of MAO A, is in serotonergic neurons and astrocytes (28, 29). To understand the tissue-specific expression of MAO A and B, it is important to study the regulation of their gene expression.

We have previously characterized the promoter regions of MAO A and MAO B genes and shown that they have a different promoter organization. The MAO A core promoter consists of three binding sites for the transcription factor Sp1 in reversed orientations and lacks a TATA box. In contrast, the MAO B core promoter contains a TATA box and two clusters of Sp1 binding sites separated by a CACCC box (30, 31). Recently, we have shown that both transcription factors Sp1 and Sp3 interact with the two clusters of Sp1 binding sites within the MAO B core promoter. In addition, overexpression of Sp1 transactivates promoter activity through the proximal cluster of Sp1 binding sites (32). The promoter activation by Sp1 could be inhibited by overexpression of Sp3. Furthermore, we have demonstrated that MAO B, but not MAO A, gene expression is induced by the activation of protein kinase C and mitogen-activated protein kinase signaling pathway involving the transcription factors Sp1, Sp3, c-Jun, and Egr-1 (33).

The mechanism underlying the gene regulation of MAO A and MAO B appears to be distinct and complex. This has led us to further investigate the transcriptional regulation of these genes. In the present study, we have found that the Caco-2 cell line (human colon adenocarcinoma) expressed both MAO A and MAO B. However, the expression of MAO B, but not MAO A, was progressively increased when the cells were undergoing differentiation. The Caco-2 cells undergo cell cycle arrest and...
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differentiation after reaching confluence (12, 13). Promoter deletion and reporter gene expression studies have identified the MAO B core promoter region (~246/~99 bp) as crucial for the induced gene expression during Caco-2 cell differentiation. In addition, the transcriptional repressor Sp3 for MAO B promoter was significantly down-regulated during the activation of MAO B gene expression. Within this promoter region, we have identified a 204-bp CpG island and 22 potential CpG methylation sites, suggesting that cytosine methylation plays a role in the transcriptional activation of MAO B gene. Indeed, sodium bisulfite sequencing showed a progressively decreased methylation status of the CpG sites in MAO B core promoter during cell differentiation. Northern blot analysis showed that treatment with 5-aza-2'-deoxycytidine, a DNA methyltransferase inhibitor, up-regulated MAO B transcript levels in undifferentiated Caco-2 cells and HeLa cells. These results demonstrated that the up-regulation of MAO B gene expression is modulated by down-regulation of transcriptional repressor Sp3 and modifications of promoter methylation during Caco-2 cell differentiation. This is the first study demonstrating the regulation of MAO B gene expression by Sp3 level and DNA methylation downstream of signaling pathways.

MATERIALS AND METHODS

Cell lines and Reagents—The Caco-2 (human colonic adenocarcinoma) and HeLa (human cervical adenocarcinoma) cell lines were purchased from the American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 mM Hepes, 2 mM l-glutamine, 100 units/ml penicillin, 10 μg/ml streptomycin, and 10% fetal bovine serum. Sodium bisulfite, hydroquinone, and 5-aza-2'-deoxycytidine were obtained from Sigma (St. Louis, MO). Polyclonal antisera against Sp1 and Sp3 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Human MAO B Promoter-luciferase Reporter Constructs—The BamHI/BamHI MAO B promoter fragment (~2099/~99 bp) was cloned into the polylinker site (BglII) upstream of the luciferase gene (LUC) in the pGL2-Basic vector (Promega, Madison, WI). Serial deletion constructs were generated by restriction enzyme digestion using the ~2099/~99 LUC as a template followed by Kleenow fill-in and self-ligation. The following restriction enzymes were used to generate the deletion constructs: XhoI/AspI (pGLB-1313); XhoI/BglII (pGLB-1180); XhoI/SpeI (pGLB-988); XhoI/PstI (pGLB-425); XhoI/PstII (pGLB-246). DNA Methylation–luciferase Activity Assays—One microgram monodisperse LUC plasmids and total proteins were incubated with 100 μM 3H-labeled serotonin (5-hydroxytryptamine) for (MAO A) or 10 μM 3H-labeled PEA (for MAO B) and centrifuged at 4°C for 10 min. The organic phase containing the reaction product was extracted, and its radioactivity was obtained by liquid scintillation spectroscopy.

Northern Blot Analysis—Total RNA were purified using TRizol reagents (Invitrogen). Thirty micrograms of total RNA was loaded onto each gel lane. Electrophoresis, transfer onto BrightStar nylon membrane, and hybridization were carried out using NorthernMax according to the manufacturer's protocol (Ambion). The human MAO A and MAO B cDNA probes and an internal control probe encoding human β-actin were labeled by random-priming technique using the Multi-prime kit (Amersham Biosciences) following the manufacturer's instructions. Membrane hybridized with the MAO A or MAO B probe was autoradiographed for 72 h. When the β-actin control probe was used, membrane was autoradiographed for 1 h.

Western Blot Analysis—Cells were harvested and washed with phosphate-buffered saline. The protein concentration was determined by the Bradford protein assay (Bio-Rad). For MAO A and B detection, 100 μg of total proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After the transfer, membranes were blocked at room temperature for 2 h with 5% bovine serum albumin in TTBS (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20). The membranes were then incubated with anti-MAO A or anti-MAO B antibodies (1:1000) in 0.5% bovine serum albumin in TTBS overnight at room temperature. After incubation with the secondary antibody at room temperature for 2 h, the bands were visualized by horseradish peroxidase reaction DAB (Sigma). Nuclear extraction and Electrophoretic Mobility Gel Shift Assay—Cells were washed with cold phosphate-buffered saline, harvested by scraping, and pelleted. Cells were resuspended in a 5-ml volume of buffer A (10 mM KCl, 20 mM HEPES, 1 mM MgCl2, 0.5 mM DTT, and 0.5 μM phenylmethylsulfonylfluoride) incubated on ice for 10 min, and centrifuged for 10 min. Pellets were then resuspended in a 3-ml volume of buffer A plus 0.1% Nonidet P-40, incubated on ice for 10 min, and centrifuged for 10 min. The pellets were resuspended in buffer B (10 mM HEPES, 400 mM NaCl, 0.1 mM EDTA, 1 mM MgCl2, 1 mM DTT, 0.5 μM phenylmethylsulfonylfluoride, and 15% glycerol) and incubated on ice for 30 min with gentle shaking. Nuclear proteins were then centrifuged for 30 min at 4°C against 1 liter of buffer C (10 mM HEPES, 200 mM KCl, 1 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonylfluoride, and 15% glycerol). Protein extracts were cleared by centrifugation at 4°C for 15 min. Protein concentrations were determined by using a Bio-Rad protein assay.

MAO B promoter DNA fragment (~246/~99 bp) was radiolabeled with [32P]dCTP by Klenow fill-in and purified by gel electrophoresis (5% polyacrylamide) and eluted in TE. For DNA-protein binding, 5 μg of nuclear extracts were diluted in binding buffer (40 mM Hepes (pH 8.0), 50 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, 10 μg/ml of poly(dI-dC) (Sigma)) with a total volume of 20 μl. Antibodies against Sp1 or Sp3 were added (when required), and the mixture was incubated for 20 min at room temperature. Labeled probes (0.2 μg) were added to the mix and incubated for additional 20 min at room temperature. The samples were then run on a 5% non-denaturing polyacrylamide gel in 1× Tris borate/EDTA at 150 V for 3 h. Gels were dried and visualized by autoradiography.

Sodium Bisulfite Genomic Sequencing—10–μg aliquots of EcoRl-digested genomic DNA from cells were denatured at 0.3 M NaOH at 37°C for 15 min in a total volume of 50 μl. Sulfonation and hydrolytic deamination reactions were then carried out by adding 450 μl of 2.5 M sodium bisulfite (Na2SO3;10 μM hydroquinone solution (pH 5.0) to) the digested genomic DNA and incubating at 50°C for 4 h in the dark. Bisulfite-converted DNA was purified using a QAQuick spin purification kit (Qiagen) and eluted with TE according to the manufacturer's protocol. Desulfonation reactions were then performed in 0.3 M NaOH at 37°C for 15 min, and DNA was precipitated with 3 mM sodium acetate/ethanol and resuspended in 100 μl of water. PCR amplification was performed in 50 μl of reaction containing 5 μl of bisulfite-treated genomic DNA, 0.5 μl primers, 100 μl dNTPs, 2 μM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 2.5 units of Taq polymerase (Invitrogen). The PCR conditions were: 97°C for 4 min (1 cycle) and 95°C for 1 min, 56°C for 1 min, 72°C for 2 min (30 cycles). The following primers were used: 5'-GCCCTCTCCAGCCTTAACAC-3' (MAO B, forward −752) and 5'-CCCTCGATCCGTCCTGCGCC-3' (MAO B, reverse −555). Amplified DNA was cloned into vector pcR2.1 using a TOPO-TA cloning kit (Invitrogen) and sequenced.

Transient Transfection and Luciferase Assay—Transfections were performed using SuperFect transfection reagent (Qiagen) following the manufacturer's instructions. Exponentially growing cells were plated at a density of 4 × 104 cells/well in 6-well plates (Costar, Cambridge, MA) in 2 ml of DMEM, 10% fetal bovine serum and grown until 80% confluent (24–36 h). Plasmids were mixed with 100 μl of serum-free, and antibiotic-free medium, and 10 μl of SuperFect reagent. After a 15-min incubation at room temperature, 600 μl of DMEM (10% fetal bovine serum and antibiotics) were added to the DNA-SuperFect complex. The cells were washed once with phosphate-buffered saline and incubated with DNA-SuperFect complexes. After 2 h incubation, the cells were washed with phosphate-buffered saline and cultured with DMEM (10% fetal bovine serum and antibiotics). Cells were harvested 48 h later with Luciferase Assay lysis buffer (Promega). The cell lysates were then assayed for luciferase using the Promega dual luciferase assay system following the manufacturer's instructions.

For DNA methylation studies, promoter fragments for MAO B (~246/~99 bp) were digested with EcoRI and HindIII, ligated, and then in vitro methylated with SssI methylase (New England Biolabs) in the presence of 160 μM S-adenosylmethionine according to manufacturer's protocol. The procedure for mock methylation except no methylase was included. Complete methylation of the fragments was confirmed by the HpaII and HhaI methylation-sensitive restriction enzymes. The promoter fragment was digested with PstI and HindIII and ligated with an equimolar combination of methylated or unmethylated promoter fragments. One microgram of methylated or unmethylated MAO B promoter-reporter constructs was co-transfected into the cells with 20 ng of pRL-TK.
For the co-transfection experiments, the Sp1 expression plasmid (pCMV-Sp1) was kindly provided by Dr. Robert Tjian, and the Sp3 expression plasmid (pCMV-Sp3) was a generous gift from Dr. Guntram Suske. The total amount of DNA for each transfection was kept constant by the addition of the empty expression vector pCMV.

CpG Analysis—The presence of a CpG island was analyzed with the EMBOSS program CpGplot (available at www.hgmp.mrc.ac.uk/Software/EMBOSS/Apps/cpgplot.html). This program detects regions of genomic sequences that are rich in the CpG pattern known as CpG islands. The program defines a CpG island as a region where the moving average of percentage of G/C nucleotides is greater than 50 and the moving average of observed/expected CpG is greater than 0.6 within a minimum of 200 bases.

Statistical Analysis—All values were presented as means ± S.E. Student t test was used for statistical analysis, and differences were considered significant when p < 0.05.

RESULTS

Increased MAO B but Not MAO A Gene Expression during Caco-2 Cell Differentiation—To determine the expression of MAO A and MAO B during Caco-2 cell differentiation, we cultured the Caco-2 cells at various growth stages (pre-confluence; confluence, day 0; post-confluence, days 2, 7, and 14). Radiochemical and enzymatic assays using enzyme-specific 14C-labeled substrates serotonin (5-hydroxytryptamine) and phenylethylamine for MAO A and MAO B, respectively, indicated that both MAO A and MAO B were expressed as early as 2 days before cells reached confluence (Fig. 1A). Interestingly, MAO B enzymatic activity increased when the cells reached confluence (day 0) and continued to increase progressively after confluence during cellular differentiation (days 7 and 14). In contrast, the enzymatic activity of MAO A remained relatively unchanged during the course of differentiation. These enzymatic activities were specific to MAO A and MAO B as indicated by the selective inhibition of these enzymes by clorgyline and deprenyl, respectively (data not shown).

Northern and Western blot analyses revealed that the specific increase of MAO B enzymatic activity correlated with the increased level of MAO B mRNA transcript and protein (Fig. 1, B and C) at confluent and post-confluent states. In contrast, the levels of MAO A transcript and protein remained relatively unchanged. The major MAO A transcripts of 5 and 2 kb, and MAO B transcript of 3 kb were detected with their specific probes as early as 2 days before confluence, indicating that both MAO genes were constitutively expressed in the proliferating Caco-2 cells. The induced level of MAO B mRNA transcript was followed by the increased level of MAO B protein. These data suggested that MAO B, but not MAO A, gene expression was selectively induced during Caco-2 cell differentiation.

Identification of Essential Regulatory Region for MAO B Gene Activation—Promoter deletion and reporter gene assay were performed to map the regulatory region responsible for the increased MAO B gene expression during Caco-2 cell dif-
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Characterization of Nuclear Factors Binding to MAO B Core Promoter during Differentiation—EMSA. Coupled with supershift assays, was performed to characterize the transcription factors that interact with the MAO B core promoter region. The nuclear proteins from pre-confluent (PC), confluent (day 0), and post-confluent (days 7 and 14) Caco-2 cells were extracted and analyzed. As shown in Fig. 3C, the expression levels of Sp1 and Sp3 were determined in duplicates and was normalized with activity of internal control pRL-TK. The pGL2-Basic was the promoter-less luciferase reporter gene, as an internal control. Luciferase (LUC) activity was determined in duplicates and was normalized with activity of internal control pRL-TK. The pGL2-Basic was the promoter-less luciferase gene construct used as a negative control. Data were the mean ± S.D. from three independent experiments with duplicates for each experiment.

Fig. 2. Identification of promoter region responsible for MAO B gene induction. MAO B promoter activity in Caco-2 cells at three growth stages: 2-day pre-confluent (PC), day 7, and day 14 after confluence. Two micrograms of each deletion MAO B promoter construct and 20 ng of the pRL-TK vector (herpes simplex virus thymidine kinase promoter upstream of Renilla luciferase reporter gene, as an internal control) were co-transfected into Caco-2 cells. Luciferase (LUC) activity was determined in duplicates and was normalized with activity of internal control pRL-TK. The pGL2-Basic was the promoter-less luciferase gene construct used as a negative control. Data were the mean ± S.D. from three independent experiments with duplicates for each experiment.

Characterization of Nuclear Factors Binding to MAO B Core Promoter during Differentiation—EMSA. Coupled with supershift assays, was performed to characterize the transcription factors that interact with the MAO B core promoter region. The nuclear proteins from pre-confluent (PC), confluent (day 0), and post-confluent (days 7 and 14) Caco-2 cells were extracted and analyzed. As shown in Fig. 2, increased MAO B promoter activity was found to correlated with the states of differentiation except the promoter construct pB–246/H11002 that lacked the core promoter. This promoter construct had similar activity to that of the control promoter-less vector (pGL2-Basic), indicating that the promoter region between –246 and –99 was essential for the induced MAO B promoter activity during Caco-2 cell differentiation. A similar correlation between MAO B promoter activity and level of gene expression was also found in HeLa (human cervical adenocarcinoma), 1242-MG (human glioma), and HepG2 (human hepatocarcinoma) cell lines (data not shown). These data indicated that Caco-2 cell is a suitable model system to study the MAO B gene regulation. Interestingly, the pattern of progressively increasing promoter activity was increased progressively from pre-confluence to days 7 and 14, similar to increasing levels of MAO B catalytic activity in RNA and protein levels (Fig. 1). This result indicates that MAO B promoter activity was correlated with the differentiation states of Caco-2 cells, and the regulatory region between –246 and –99 was essential for the MAO B gene up-regulation.

The gradually decreased intensity of complexes I and III suggested that the expression of Sp3 was reduced during Caco-2 cell differentiation. Western blot analysis was performed to determine the expression levels of Sp1 and Sp3 in Caco-2 cells. The nuclear proteins from pre-confluent (PC), confluent (day 0), and post-confluent (days 7 and 14) Caco-2 cells were extracted and analyzed. As shown in Fig. 3C, although the expression level of Sp1 decreased when cells reach confluence, its expression level appeared relatively the same during differentiation from day 0 to day 14. In contrast, the expression level of Sp3 gradually decreased throughout the course of differentiation. This decreased expression level of Sp3 protein may explain the reduced intensity of complexes I and III in EMSA.

Functional Analysis of Sp1 and Sp3 on MAO B Promoter Activity in Caco-2 Cells. The decreased expression of transcription factor Sp3 correlated inversely with the increased MAO B gene expression during Caco-2 cell differentiation. The MAO B core promoter consisted of two clusters of overlapping binding sites for transcription factors Sp1 or Sp3. To determine the functional roles of Sp1 and Sp3 in the up-regulation of MAO B gene expression in Caco-2 cells, the core promoter-luciferase reporter gene construct (pB–246/H11002) was transiently co-transfected with increasing amounts of cDNA plasmids expressing the human Sp1 or Sp3 in pre-confluent Caco-2 cells. Overexpression of Sp1 enhanced MAO B promoter activity in a dose-dependent manner (Fig. 4). In contrast, overexpression of Sp3 resulted in a slight decrease of promoter activity. Because the binding sites were overlapped, competition between Sp1 and Sp3 for binding to these sites may influence the overall MAO B promoter activity. To determine whether an interplay exists between Sp1 and Sp3 on promoter activity, transient co-transfection experiments were performed using a constant amount of Sp1 expression plasmid along with gradually increasing amounts of Sp3 expression plasmids. As expected, expression of Sp1 enhanced MAO B promoter activity. However, the Sp1-mediated promoter activation was gradually reduced by increasing amounts of Sp3 expression (Fig. 4). The result of Sp3 suppression of MAO B promoter was found in other cell lines such as 1243-MG (human glioma), HepG2 (human hepatocytoma), and HeLa (human cervical adenocarcinoma) (32). These findings suggested that variation in the ratio of Sp1 and Sp3 expression might influence the regulation of MAO B gene expression.

Reduced Promoter Methylation during Caco-2 Cell Differentiation—MAO B promoter region between –246 and –99 bp was GC-rich. We analyzed the CpG dinucleotide distribution and found a potential CpG island within the middle of this region using the program CpGPlot (Fig. 5A). Twenty-two CpG sites were located in this promoter region. The effect of in vitro methylation on the promoter activity of MAO B gene was tested in a transient expression assay using the pB–246/H11002 promoter-reporter gene construct. After methylation with SssI methylase, which methylated cytosine residues of CpG dinucleotides, the methylated and unmethylated reporter constructs were transiently transfected into pre- and post-confluent Caco-2 cells and assayed for promoter activity. As shown in Fig. 5B, in vitro methylation almost completely suppressed MAO B promoter activity compared with the unmethylated promoter construct. Similar results were obtained with experiments using HeLa and HepG2 cell line (data not shown). Computer analysis of the MAO B promoter revealed a putative 204-bp region of Sp1 antibody (lane 2), whereas the complexes I and III were supershifted by the addition of Sp3 antibody (lane 3) (Fig. 3B). Addition of both Sp1 and Sp3 antibodies supershifted complexes I–III.
CpG island located between −261 and −58 bp, within which the core promoter (−246/−99) is situated (Fig. 5A). Detailed analysis showed that MAO B core promoter consisted of 22 CpG sites that could be potentially methylated (Fig. 5B). To determine the methylation patterns of MAO B core promoter during Caco-2 cell differentiation, we performed bisulfite genomic sequencing of DNA from pre-confluent, confluent, and post-confluent Caco-2 cells. Genomic DNA was extracted and treated with sodium bisulfite, which converted cytosines to uracils while methylated cytosines remained unmodified. We found that the methylation at the 22 CpG sites was correlated inversely with degree of differentiation. The highest methylation state was found in pre-confluent cells ranging from 42 to 83%. In contrast, methylation in post-confluent cells ranged from 16.7 to 42% in day 7 cells and from 0 to 25% in day 14 cells (Fig. 5C). These results indicated a strong inverse correlation between methylation states of MAO B promoter and its gene expression during Caco-2 cell differentiation. To investigate the effect of in vitro methylation on the binding of transcription factors to the MAO B promoter, the MAO B core promoter fragment (−246/−99 bp) was treated with (+) or without (−) SssI methylase and incubated with nuclear proteins extracted from pre-confluent Caco-2 cells. The protein-DNA complexes were then analyzed in EMSA. As shown in Fig. 5D, methylation drastically reduced the binding of transcription factors Sp1, Sp3 to the MAO B promoter. Furthermore, in vitro methylation by methylase SssI significantly reduced the MAO B core promoter activity in transient transfection experiments (Fig. 5E). Same results were obtained in HeLa cells (data not shown).

Treatment of 5-Aza-deoxycytidine Up-regulate MAO B Expression—To test the potential role of DNA methylation in the regulation of MAO B gene expression, −4 days pre-confluent Caco-2 cells were treated with the methyltransferase inhibitor 5-azacytidine (a demethylating agent) for 3 days. Northern blot analysis revealed that treatment of 5-azacytidine induced the
level of MAO B mRNA transcript 2- to 3-fold. In contrast, no significant increase of the level of MAO A mRNA transcript was found (Fig. 6A). Furthermore, the induced MAO B transcription by 5-azacytidine was followed by a 2-fold increased MAO B enzymatic activity. These results showed that inhibition of methylation activated MAO B promoter activity and gene expression, suggesting that methylation was responsible for MAO B gene repression in the undifferentiated Caco-2 cells.

**DISCUSSION**

The human Caco-2 cells differentiate into an enterocyte-like phenotype after reaching confluence. The growth of Caco-2 cells reaches a plateau 3 days after cell confluence and begins to differentiate 6 days after confluence (34, 35). In the present study, we showed that MAO B, but not MAO A, enzymatic activity was increased during Caco-2 cell differentiation thus Caco-2 cell was a good model system to study the MAO B gene regulation. Because serotonin has been shown to play important roles in the physiology of the bowel (21) and the development of the enteric nervous system (22), the use of this model system for studying MAO genes is particularly important. This increased MAO B activity was directly resulted from induced gene expression as shown by increased level of MAO B mRNA transcript and increased level of MAO B protein (Fig. 1, A–C). Interestingly, the gene expression of MAO A remained constant throughout the course of cell differentiation, indicating a distinct regulation of transcription between MAO A and MAO B genes. Indeed, our previous studies of MAO A and MAO B regulatory regions had shown that the core promoters of these two genes had different promoter organizations. The MAO A core promoter consisted of three separated Sp1 binding sites that were in reversed orientations (30). In contrast, the MAO B core promoter contained a functional TATA box, and five Sp1 and one Egr-1 binding sites that were overlapped and grouped into two clusters that were separated by a CACCC box (32). These different promoter organizations may explain the distinct regulation of transcription between MAO A and MAO B genes and consequently provide the basis of their different tissue- and cell-specific expression.

The deletion analysis of the 5’-flanking MAO B promoter region demonstrated that the region between −246 and −99 bp was necessary for the induced MAO B promoter activity as deletion of this region abolished promoter activation in post-confluent Caco-2 cells. In addition, deletion of this region drastically reduced transcriptional activity with promoter activity similar to the control promoter-less vector, suggesting this region was essential for the transcriptional activity of MAO B gene (Fig. 2). This finding was consistent with our previous results in HeLa, 1242-MG, and HepG2 cell lines showing that the regulatory region between −246 and −99 bp provided the core promoter activity of MAO B gene (32).

The electrophoretic mobility gel shift assays confirmed the functional importance of the −246/−99 bp regulatory region. The formation of two DNA-protein complexes was gradually reduced as Caco-2 cells undergoing differentiation (Fig. 3A). Supershift assays clearly demonstrated that these proteins within the two complexes belonged to the transcription factor Sp3 (Fig. 3B). The two differentially migrating bands of Sp3 may correspond to the two Sp3 isoforms that arise from Sp3 mRNA via translational initiation at two internal sites located within the trans-activation domain. Although the internally initiated Sp3 could bind to Sp1 binding sites, it was shown to be unable to stimulate the transcription of Sp1 regulated genes (36). The identities of complexes IV and V remained to be determined. Because the −246/−99 bp region contained multiple Sp1 binding sites, these complexes may belong to other members of Sp family.

We showed that the protein expression of Sp3 was drastically reduced, whereas protein level of Sp1 remained constant during Caco-2 cell differentiation (Fig. 3C). This finding explained the gradually reduced Sp3 binding in the electrophoretic mobility gel shift assays and suggested that the reduced Sp3/Sp1 ratio may be responsible for the progressively increased MAO B gene expression during Caco-2 cell differentiation. The transcription factors Sp1 and Sp3 are members of the Sp family encoding proteins with similar structural features and highly conserved zinc finger DNA binding domain that can recognize the GC box (GGGCGCGGC) and/or the GT motif (GGGTGT-GGC) with similar affinities (37). The presence of proteins with DNA-binding specificity similar to Sp1 indicates that gene regulation by Sp1 is complex. The −246/−99 bp regulatory region consists of overlapping Sp1 sites that can be recognized by Sp1 and Sp3 based on their conserved DNA binding domains. Previous studies have demonstrated that the binding of two adjacent Sp1 molecules to a DNA sequence requires at least 10 bp between the central C of the two Sp1 elements (GGGCGGGG) (38). Because the Sp1 binding sites in the MAO B core promoter are overlapped, only one Sp1 can bind to each cluster of Sp1 binding sites at a time. Because only one of these factors can bind to each cluster at one time, alterations of the Sp3/Sp1 ratio and/or their DNA binding activities, and the competition between these factors for binding to the same cluster may account in part for the differential regulation of MAO B gene expression during Caco-2 cell differentiation. Indeed, functional studies of altered Sp3/Sp1 ratio in Caco-2 cells showed that overexpression of Sp3 prevented promoter induction of MAO B gene by Sp1. This finding clearly demonstrated the functional significance of Sp3/Sp1 ratio on the regulation of MAO B expression in Caco-2 cells. This was similar to our previous results of overexpression studies of Sp1 and Sp3 in 1242-MG cells.

The presence of a CpG island suggested that MAO B gene may be regulated through changes in methylation status. We showed a CpG pattern of gradually decreased methylation status associated with the progressively increased MAO B gene expression during Caco-2 cell differentiation (Fig. 5C). This was supported by the in vitro methylation studies showing that methylation decreased the binding of Sp1 and Sp3 to this promoter region (Fig. 5D) and drastically reduced promoter activity (Fig. 5E). Methylation of the Sp1 binding site has been

**Fig. 4. The interplay between transcription factors Sp1 and Sp3 on MAO B promoter activity.** One microgram of the pB−246/−99Luc was co-transfected with variable amounts (500 ng) of the expression plasmids for Sp1 and Sp3 along with 20 ng of internal control plasmid pRL-TK into 2-day pre-confluent Caco-2 cells. For the Sp1 and Sp3 co-transfection experiments, 500 ng of the expression plasmid of Sp1 was co-transfected with increasing concentration of expression plasmid for Sp3 into 2-day pre-confluent Caco-2 cells. The amount of transfected plasmids was kept constant (2 μg) using vector construct (pCMV). Luciferase activity was normalized with activity of internal control pRL-TK. Data were the mean ± S.D. from three independent experiments with duplicates for each experiment.
FIG. 5. Analysis of methylation status in MAO B promoter. A, location of the CpG island at the MAO B promoter. The CpG island is marked by a box, numbers on the x-axis indicated the nucleotide positions relative to the translational start site. B, CpG sites within human MAO B core promoters. The 5′-flanking sequence of MAO B gene was shown with core promoter regions indicated in boldface. The potential CpG sites for methylation were indicated with black-filled boxes and numbered. Twenty-two CpG sites were present within the MAO B core promoter. The numbering scheme is based on one in which +1 is assigned to the A of ATG condon of translation starting site. C, genomic DNA from pre-confluent or 7-day post-confluent or 14-day post-confluent Caco-2 cells was modified with sodium bisulfite, PCR-amplified, and subsequently cloned and sequenced. Twelve clones (n = 12) from each growth stage were sequenced. The frequency of methylation at every CpG site within the human MAO B core promoters was shown. D, effect of in vitro methylation on protein-DNA complex formation. The MAO B core promoter fragment (-246/−99 bp) was treated with (+) or without (−) SssI methylase and incubated with nuclear proteins extracted from pre-confluent Caco-2 cells. The protein-DNA complexes were then analyzed in EMSA. E, effect of in vitro methylation on MAO B core promoter activity. The MAO B (-246/−99) core promoter-luciferase reporter construct was transiently transfected into Caco-2 cells. The transcriptional activities of promoter-less vector (pGL2-Basic) and MAO B core promoter (with or without SssI methylation) are represented by the reporter gene luciferase activity normalized by the internal control (pRL-TK). Luciferase activity was measured 48 h after transfection. Data are the mean ± S.D. from three independent experiments with duplicates for each experiment. p < 0.001.
shown to cause gene silencing. All of the CpG sites within the overlapping Sp1 binding sites appeared to be methylated, and the degree of methylation correlated with the level of MAO B gene expression. Treatment with 5-aza-2'-deoxycytidine was able to induce MAO B mRNA expression in Caco-2 cells in a time-dependent manner (Fig. 6). A similar increase in mRNA level after 5-aza-2'-deoxycytidine treatment was also observed in HeLa cells (Fig. 6).

Fig. 6. Effect of 5-aza-deoxycytidine on MAO B gene expression. A, Northern blot. Thirty micrograms of total RNA from HeLa or pre-confluent Caco-2 cells treated for various times (0, 48, and 72 h) with 10 μM 5-aza-deoxycytidine were hybridized to human MAO A or MAO B cDNA probe. The positions of the MAO A and MAO B transcripts are indicated. The same membrane was re-probed with a β-actin probe as an internal control. Results are representative of three independent experiments. B, autoradiography scanning of the Northern blot shown in A. Relative amounts (corrected by β-actin expression) of MAO A (5 kb) and B (3 kb) transcripts in 5-azacytidine-treated cells were compared with untreated control cells. C, MAO A and MAO B catalytic activities from HeLa or Caco-2 cells treated with 5-aza-deoxycytidine for various times. Data were the mean ± S.D. from three independent experiments. *p < 0.05 versus untreated cells.
Confluence-induced alteration of CpG methylation was shown to occur in the promoters of estrogen receptor, E-cadherin, and O6-methylguanine-DNA methyltransferase in cultured normal human fibroblasts (39). Our data showed that MAO B core promoter was highly methylated in proliferating cells compared with the differentiated cells. It was suggested that cells grown at high density may be nutrient deprived and severely stressed, which may activate or deactivate various mechanisms controlling CpG methylation (39). Further studies will be needed to elucidate the molecular mechanism regulating the methylation states of MAO B promoter in Caco-2 cells.

In conclusion, this is the first study demonstrating that the gene expression of MAO B, but not MAO A, is selectively induced during Caco-2 cell differentiation. The promoter region between −246 and −99 bp was responsible for the induced gene expression. We have shown that the expression of Sp3 was gradually down-regulated during differentiation and the ratio of Sp3/Sp1 correlated with the level of MAO B gene expression. Moreover, we showed that a pattern of gradually decreased methylation was associated with the progressively increased MAO B gene expression during Caco-2 cell differentiation, and treatment with methyltransferase inhibitor induced MAO B mRNA expression. This study provided novel information on the molecular mechanism of MAO B gene regulation. Given the importance of Sp3/Sp1 ratio and methylation on MAO B gene expression, it will be of interest for the future studies to determine whether alteration of these factors is involved in the MAO B-related disorders and whether manipulation of these factors can restore its normal expression. A further understanding of the molecular basis of MAO B regulation will provide insights into the pathophysiology of MAO B-related disorders and may ultimately lead to design of new therapeutics.

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