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Inhibition of Human Methionine Adenosyltransferase 1A Transcription by Coding Region Methylation

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Abstract

Two genes (*MAT1A* and *MAT2A*) encode for the essential enzyme methionine adenosyltransferase (MAT). *MAT1A* is silenced in hepatocellular carcinoma (HCC), and absence of *MAT1A* leads to spontaneous development of HCC in mice. Previous report correlated promoter methylation to silencing of *MAT1A* but definitive proof was lacking. Here we investigated the role of methylation in regulating *MAT1A* expression. There are three *Msp*I/*Hpa*II sites from −1913 to +160 of the human *MAT1A* gene (numbered relative to the translational start site) at position −977, +10, and +88. Bisulfite treatment and DNA sequencing, and Southern blot analysis showed that methylation at +10 and +88, but not −977, correlated with lack of *MAT1A* expression. *MAT1A* promoter construct methylated at −977, +10 or +88 position has 0.7-fold, 3-fold, and 1.6-fold lower promoter activity, respectively. Methylation at −977 and +10 did not inhibit the promoter more than methylation at +10 alone; while methylation at +10 and +88 reduced promoter activity by 60%. Mutation of +10 and +88 sites also resulted in 40% reduction of promoter activity. Reactivation of *MAT1A* correlated with demethylation of +10 and +88. In vitro transcription assay showed that methylation or mutation of +10 and +88 sites reduced transcription. In conclusion, our data support the novel finding that methylation of the *MAT1A* coding region can inhibit gene transcription. This represents a key mechanism for decreased *MAT1A* expression in HCC and a target for therapy. To our knowledge, this is the first example of coding region methylation inhibiting transcription of a mammalian gene.

Keywords

methionine adenosyltransferase 1A; hepatocellular carcinoma; coding region methylation; in vitro transcription

INTRODUCTION

Methionine adenosyltransferase (MAT) is an essential cellular enzyme that catalyzes the formation of S-adenosylmethionine (AdoMet, also known as SAM and SAMe), the principal biological methyl donor and the ultimate source of the propylamine moiety used in polyamine biosynthesis (Mato et al., 1997; Finkelstein 1990). In mammals, two different genes, *MAT1A* and *MAT2A*, encode for two homologous MAT catalytic subunits, α1 and α2 (Horikawa and Tsukada, 1992; Alvarez et al., 1993; Kotb et al., 1997). *MAT1A* is expressed

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mostly in liver and it encodes the α1 subunit found in two native MAT isozymes, which are either a dimer (MAT III) or tetramer (MAT I) of this single subunit (Kotb et al., 1997). *MAT2A* encodes for a catalytic subunit (α2) found in a native MAT isozyme (MAT II), which is associated with a catalytically inactive regulatory subunit (β) in lymphocytes encoded by *MAT2B* (Kotb et al., 1997; Halim et al., 1999). *MAT2A* is widely distributed (Horikawa and Tsukada, 1992; Alvarez et al., 1993; Kotb et al., 1997). *MAT2A* also predominates in the fetal liver and is progressively replaced by *MAT1A* during liver development (Horikawa et al., 1993; Gil et al., 1996). In adult liver, a switch in MAT expression occurs when the liver undergoes rapid growth or de-differentiation (Cai et al., 1996; Huang et al., 1998, 1999). In human hepatocellular carcinoma (HCC), *MAT1A* is transcriptionally silenced while *MAT2A* is induced (Cai et al., 1996). This switch in MAT gene expression plays an important pathogenetic role as *MAT2A* expression facilitates cancer cell growth (Cai et al., 1998). The influence of MAT expression on liver growth and injury was further demonstrated using a *MAT1A* knockout mouse model (Lu et al., 2001; Martínez-Chantar et al., 2002). In this model, absence of hepatic *MAT1A* is compensated by induction of *MAT2A*. These animals exhibit chronic hepatic AdoMet deficiency, are prone to liver injury and develop spontaneous HCC (Lu et al., 2001; Martínez-Chantar et al., 2002).

Given the importance of MAT expression in liver disease and cancer, we have been interested in understanding transcriptional regulation of MAT genes. We characterized the promoter region of both human MAT genes (Mao et al., 1998; Zeng et al., 2000) and showed previously that *MAT1A* expression is regulated by histone acetylation and methylation (Torres et al., 2000). Specifically, *MAT1A* gene expression in HepG2 cells can be induced by treatment with a demethylating agent or inhibitor of histone deacetylase (Torres et al., 2000). Furthermore, in HepG2 cells and in human cirrhotic livers, decreased *MAT1A* expression is associated with hypermethylation of a *Hpa*II site at −977 position of the *MAT1A* promoter (Torres et al., 2000; Avila et al., 2000). However, these studies did not demonstrate directly that methylation at the −977 site of *MAT1A* can influence gene transcription. The aim of the current study was to examine the functional relationship between *MAT1A* gene methylation and expression. In the course of these studies, we have uncovered a highly novel finding, namely the ability of methylation of the coding region to influence the transcriptional activity of *MAT1A*. To our knowledge, this is the first example of coding region methylation affecting transcriptional activity of a mammalian gene.

MATERIALS AND METHODS

Materials

Cell culture media were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from Atlas Biologicals (Fort Collins, CO). The Luciferase Assay System and the β-Galactosidase Enzyme Assay System were obtained from Promega (Madison, WI). All restriction enzymes and methylases were purchased from New England Biolabs (Beverly, MA) and Invitrogen. [α-^{32}P] dCTP (10 μCi/μl) was purchased from Perkin Elmer (Wellesley, MA). pGL3-enhancer and pcDNA 3.1 vectors were purchased from Promega and Invitrogen, respectively. All other reagents were of analytical grade and were purchased from commercial sources.

Source of Normal and Cancerous Liver Tissue

Normal liver tissue was obtained from normal liver included in the resected liver specimens of patients with metastatic colon or breast carcinoma. Cancerous liver tissue was obtained from patients undergoing surgical resection for primary HCC. Written informed consent was obtained from each patient. The contamination of HCC samples with noncancerous tissue was less than 5% as determined by histopathology. These tissues were immediately frozen

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in liquid nitrogen for subsequent isolation of RNA, DNA, or nuclear proteins as described below.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by Keck School of Medicine University of Southern California's human research review committee.

Cell Culture

Primary cultures of human hepatocytes, Huh-7, HEK293, and HepG2 cells were obtained from the Cell Culture Core of the USC Liver Disease Research Center and grown according to instructions provided by the American Type Culture Collection (Rockville, MD) or as we previously described (Cai et al., 1996). Primary cultures of human hepatocytes were also obtained from CellzDirect (CellzDirect, Inc., Tucson, Az).

Nucleic Acids Extraction

RNA was isolated from frozen liver specimens, Huh-7, and HEK293 cells according to the method of Chomczynski and Sacchi (1987). Genomic DNA was isolated from frozen human liver specimens, primary cultures of human hepatocytes, Huh-7, and HEK293 cells as we described previously (Cai et al., 1996) and used for Southern blot analysis and sodium bisulfite genomic DNA sequencing (see below).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Aliquots of 2μg total RNA were reverse transcribed using MMLV (Invitrogen, Carlsbad, CA) and DecaPrime II decamers (Ambion, Austin, TX) by a 1 h incubation at 42ºC followed by 10 min at 90ºC to stop the reaction. Multiplex PCR using previously described primers (Avila et al., 2000) designed to flank intronic sequences of *MAT1A* and yield a 167 bp product and Alternate 18S internal standards (Ambion) to simultaneously amplify *MAT1A* and an 18S internal control was performed using Platinum Pfx DNA polymerase (Invitrogen). The PCR reaction parameters included an initial 20 sec incubation at 95ºC, followed by 28 cycles of incubation at 94ºC for 1 min, 54ºC for 1 min, 72ºC for 1 min, and a final extension time of 3 min at 72ºC. PCR products were visualized by ethidium bromide staining under UV light on a 2% agarose gel.

Sodium Bisulfite Genomic DNA Sequencing

In order to compare the methylation status of 15 CG sites in human *MAT1A* promoter from −1902 to −25 between the genomic DNAs of normal liver and the genomic DNAs of liver cancer cells, bisulfite-mediated genomic DNA sequencing was performed following the procedure of Paulin et. al. (1998). Basically, 2 μg of normal human liver genomic DNAs or liver cancer cell line Huh-7 genomic DNAs were digested by restriction enzymes to release four small areas of genomic DNAs that span *MAT1A* promoter from −1686 to −1398, −1228 to −778, −792 to −454 and −392 to −84. There is no CG site from −1902 to −1686, −1398 to −1228, −454 to −392, nor −84 to −25. Digested genomic DNAs were denatured, modified by bisulfite, purified, and desulphonated. The top strand (for example, −1686 as 5′ and −1398 as 3′) DNA was PCR using four sets of primers that complement to the bisulfite-modified DNA fragments. The first set of primers were: for the upper strand, 5′-GGGTTAGTAATGTTGAAATAGTT (annealed to modified *MAT1A* promoter from −1686 to −1663); for the lower strand, 5′-TAACTAATAACCAAACATCATCC (−1420 to −1398). The second set primers were: forward, 5′-ATTTTTTATTGTATTTTAGTGGGAA (−1228 to −1204); reverse, 5′-CCTAACCTATCAACTTCTCTAAAAA (−802 to −778). The third set of primers were: forward, 5′-AGTTGATAGGTTAGGTGGTTTT (−792 to −771); reverse, 5′-TCCTTCAACTCTTAACCAATCTT (−476 to −454). The fourth set of

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primers were: forward, 5′-GGTGTTTAGAGTTTGGAAAGTT (−392 to −371); reverse, 5′-CTTCTTCTTCTTCTTTCAACC (−104 to −84). PCR reaction was set up as follows: forward primer (5 μM) 0.8 μl, reverse primer (5 μM) 0.8 μl, 4dNTPs (10 mM) 0.15 μl, bisulfite treated genomic DNAs (normal liver or Huh-7) (1 mg/ml) 1 μl, and water 7.55 μl. The mixture was placed in the PCR machine and heated to 95ºC for 5 min. 3.95 μl of Taq polymerase mixture containing 1.25 μl 10X Taq buffer and 0.15 μl Taq polymerase were mixed with the heated sample. The cycling condition was as follows: 94ºC, 40 sec, 55ºC, 45 sec, 72ºC, 50 sec for 40 cycles, 72ºC for 10 mins, then 4ºC for at least 10 mins. PCR products were cloned into TOPO TA vector (Invitrogen). As many as 12 positive colonies with the insert from each PCR reaction were sequenced using the automated ABI Prism dRhodamine Terminator Cycle Sequencer (Sequencing Core Facility, Norris Cancer Center, Keck School of Medicine USC).

In a separate experiment, bisulfite-mediated genomic DNA sequencing was performed using genomic DNA from primary cultures of human hepatocytes (to avoid contamination from non-hepatocytes) and Huh-7 cells to compare the methylation pattern from −61 to +251. Following bisulfite treatment as described above, DNA was PCR using the primer pair: forward 5′-GGTAAAGTTATAGTTTTAAAATTGTTT-3′ (−61 to −35) and reverse 5′-AATACTCATATTATAAATTACTAATTTAA-3′ (+223 to +251). The cycling condition was as follows: 95ºC for 3 min, 94ºC, 40 sec for 40 cycles, 57ºC for 50 sec, 72ºC for 1 min, 72 C for 2 min. PCR products were cloned into TOPO TA vector (Invitrogen). Sixteen positive colonies were sequenced using the automated LI-COR 4200 Sequencer (LI-COR, Inc., Lincoln, NE).

Restriction Enzyme Digestion and Southern Blot Analysis

*Msp*I and *Hpa*II restriction endonucleases were used as described (Yang et al., 2001). *Msp*I and *Hpa*II can distinguish between the unmethylated and methylated cytoine in the nucleotide sequence 5′-CCGG. *Msp*I is insensitive to the methylation status whereas *Hpa*II will digest only if the internal cytosine is unmethylated. Genomic DNA (30 μg) was completely digested with *Eco*RI only, *Eco*RI and *Msp*I, or *Eco*RI and *Hpa*II overnight at 37ºC. The digested DNAs were separated on 1% agarose gels, Southern transferred and hybridized with [α-^{32}P]dCTP-labeled −1666 to −1515 fragment of the human *MAT1A* promoter [16]. Autoradiography was performed by exposure the membranes to Kodak BioMax MR film at −80ºC. Southern blot of genomic DNA digested with *Hind*III only, *Hind*III and *Msp*I, or *Hind*III and *Hpa*II was probed with fragment −612 to −134 of the human *MAT1A* promoter.

In Vitro Methylation Reactions

To examine the effect of CG methylation at the three *Hpa*II sites (−977, +10, and +88) on *MAT1A* promoter activity, different regions of human *MAT1A* (−1111 to +30, −839 to +30, and −839 to +112) were PCR amplified and inserted between *Kpn*I and *Xho*I sites of pGL3-enhancer vector to generate pGL3-1111/+30, pGL3-839/+30 and pGL3-839/+112. The human *MAT1A* promoter fragments were isolated by *Xho*I and *Kpn*I double digestion and purified for methylation experiments. These fragments were either mock methylated (incubated with *Hpa*II methylase in the absence of AdoMet), or methylated by *Hpa*II methylase. The status of methylation was determined by digestion with *Hpa*II restriction enzyme. Methylated and mock-methylated *MAT1A* promoter fragments were then ligated into *Xho*I and *Kpn*I sites of pGL3-enhancer vector. The ligated pGL3-839/+30 plasmid (mock-methylated or HpaII methylated) was isolated from 1% agarose gel using Gel Purification Kit from Qiagen. The ligated pGL3-1111/+30 (mock-methylated or *Hpa*II methylated) plasmids, and the ligated pGL3-839/+112 (mock-methylated or *Hpa*II methylated) plasmids were also isolated and purified using the same protocol as described

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above for the ligated pGL3-839/+30 plasmid. Concentration of each purified ligated plasmid was measured by spectrophotometer to ensure equal amount of ligated DNA was used for cell transfection. This was further verified by electrophoresis on 1% agarose gel.

To examine the effect of each specific site, site-directed mutagenesis was performed with the Site-directed Mutagenesis kit from Stratagene (Cedar Creek, TX). Site-directed mutagenesis was carried out on pGL3-839/+112 to mutate +10 *Hpa*II site, +88 *Hpa*II site, or both. In every mutation, the 5′CCGG *Hpa*II site was mutated to 5′CCAG. Each mutation was verified by sequencing using the automated ABI Prism dRhodamine Terminator Cycle Sequencer performed by the Sequencing Core Facility, Norris Cancer Center, Keck School of Medicine USC. The mutated *MAT1A* plasmids were subjected to the same methylation and ligation treatments as described above.

Transfection Assays

To study the effect of methylation on relative transcriptional activities of the *MAT1A* promoter fragments, HepG2 cells (5×10^5 cells in 1 ml medium) were transiently transfected with 400–500 ng of unmethylated or methylated *MAT1A* promoter-pGL-3 enhancer constructs and 500 ng of a β-galactosidase expression plasmid (as an internal standard of transfection efficiency) using the Superfect Transfection Reagent (Qiagen, Valencia, CA) as we described (Yang et al., 2002). After 48 hours, cells were harvested and lysed in 100 μl of reporter lysis buffer (Luciferase Assay System, Promega). The luciferase activity was measured using a TD-20/20 Luminometer (Promega). The β-galactosidase assay was done according to the supplier's instructions (β-Galactosidase Enzyme Assay System, Promega). The luciferase activity of each transfection was expressed as luciferase activity/β-galactosidase activity.

In vitro Transcription

RNA transcripts were synthesized in vitro with T7 RNA polymerase using the TranscriptAid™ T7 High Yield Transcription Kit (Fermentas, Glen Burnie, Maryland) from 1 μg templates consisting of the −839/+112 region of *MAT1A* promoter contained in pcDNA3.1 vector linearized with *Xho*I. RNA synthesized *in vitro* was digested with RNase-free DNase I, precipitated with isopropanol, washed in ethanol, and transcription reaction products were analyzed by gel electrophoresis.

Statistical Analysis

Data are given as mean±SEM. Statistical analysis was performed using ANOVA followed by Fisher's test for multiple comparisons. Significance was defined by $p<0.05$.

RESULTS

MAT1A Expression and Bisulfite-Mediated Genomic DNA Sequencing

We first examined whether there is a correlation between *MAT1A* expression and the specific site of promoter methylation. RT-PCR indicates that while *MAT1A* is expressed in normal liver, it is not expressed in Huh-7 and 293 cells, or in HCC (Fig. 1A). Sodium bisulfite genomic sequencing was carried out to identify the site(s) of methylation. Figure 1B shows that there is an increase in overall methylation of the *MAT1A* promoter in Huh-7 cells, which do not express *MAT1A*. The five CpG sites within nucleotides −257 to −132 showed the most dramatic methylation increases in Huh-7 cells while the CpG sites more upstream showed less methylation differences to the normal liver control.

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MAT1A Expression and Methylation at HpaII Sites

The *Hpa*II site at −977 was previously reported to be methylated in cirrhotic liver specimens with decreased *MAT1A* expression (Avila et al., 2000). To further examine this site, Southern Blot Analysis was carried out in HCC, normal liver and Huh-7 cells. Figure 2 shows that the site at −977 is partially methylated in all three specimens. There is an additional band around 1.923 kb that is present only in normal liver DNA treated with *Eco*RI and *Hpa*II. This suggests the *Hpa*II site at +10 is partly unmethylated in normal liver but methylated in both HCC and Huh-7 cells.

Using a separate probe designed to investigate the +10 site more carefully, both Huh-7 and 293 cells have methylation at +10 and +88 sites, since *Hpa*II was unable to digest the DNA and results in a band similar to *Hind*III digestion alone. However, primary cultures of human hepatocytes (to avoid any contamination from other cell types in human liver specimens) have the +10 site fully unmethylated (Fig. 3).

To confirm the results of the Southern blots, bisulfite-mediated genomic DNA sequencing was also carried out to compare methylation pattern spanning the region from −61 to +207 of *MAT1A* in primary cultures of human hepatocytes to Huh-7 cells. There are six methylation sites in this region at position −23, +10, +65, +88, +123, and +207. Human hepatocytes are completely unmethylated at all of these sites (16 out of 16 clones) while Huh-7 cells have all of these sites methylated (16 out of 16 clones) with the exception of the +88 site where 11 out of 16 clones were methylated.

HpaII Site Methylation and MAT1A Promoter Activity

To assess directly the influence of methylation of each of the *Hpa*II sites (−977, +10 and +88) on *MAT1A* promoter activity, DNA fragments containing these sites were *in vitro* methylated by *Hpa*II and re-ligated back to the pGL-3 enhancer vector for transient transfection analysis in HepG2 cells. Figure 4 shows the results of these experiments using constructs −1111/+30-LUC and −839/+30-LUC. When either of the constructs is *in vitro* methylated by *Hpa*II, the promoter activity is decreased by 3-fold (Fig. 4B).

To assess the importance of the *Hpa*II sites at +10 and +88, *MAT1A* construct −839/+112 was used (Fig. 5). When both sites are methylated, there is a 60% decrease in luciferase activity. Mutation of either +10 (labeled as −839/+112 mut +10) or +88 (labeled as −839/+112 mut +88) or both (labeled as −839/+112 mut +10/+88) also reduced basal promoter activity of this construct by about 40%. With either the +10 or +88 site mutated, *Hpa*II methylation reduced the promoter activity by about 40%. However, if both sites are mutated, *Hpa*II methylation no longer inhibited the promoter activity.

To confirm that methylation status of +10 and +88 correlates with *MAT1A* expression, Huh-7 cells were treated with an inhibitor of DNA methylation, 5′aza-2′-deoxycytidine (5-Aza-CdR, 0.1 μM for 24 or 48 hrs). 5-Aza-CdR induced the expression of *MAT1A* after 48 hrs (Fig. 6A) and Southern blot analysis using the probe designed to examine methylation status of +10 and +88 (see Fig. 3) shows that these sites became partially demethylated as *MAT1A* was reactivated (Fig. 6B).

Methylation or Mutation at +10 and +88 Sites Decrease Transcription of MAT1A

To verify that methylation or mutation of +10 and +88 sites in the *MAT1A* coding region can inhibit transcription, we employed an *in vitro* transcription assay system. Figure 7 shows that similar to what we observed with reporter activity driven by these constructs, methylation of +10 and +88 sites as well as mutation of these sites all reduced the amount of

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a single transcript from *MAT1A* promoter, with methylation causing a greater reduction than mutation.

DISCUSSION

MAT is a critical cellular enzyme because it catalyzes the only reaction that generates AdoMet. The MAT gene is one of 482 genes absolutely required for survival of an organism (Mato et al., 1997). In mammals, two distinct genes encode for MAT (Horikawa and Tsukada, 1992; Alvarez et al., 1993; Kotb et al., 1997). *MAT1A* is expressed in the liver shortly before birth and becomes the major form of MAT as the liver matures (Gil et al., 1996). It is a marker for differentiated or mature liver phenotype. In contrast, *MAT2A* is expressed in all non-hepatic tissues as well as during periods of rapid liver growth and de-differentiation (Mato et al., 2002). Although the two MAT genes are highly homologous, the enzymes they encode for are different in the kinetic profiles and regulatory property (Mato et al., 2002). Due to these differences, the type of MAT expressed can influence the steady state AdoMet level in the cell. Using a cell line model that differs only in the type of MAT expressed, cells that expressed *MAT1A* had the highest intracellular AdoMet level whereas cells that expressed *MAT2A* had the opposite (Cai et al., 1998). Interestingly, cells that expressed *MAT2A* grew faster than cells that expressed *MAT1A* (Cai et al., 1998). Thus, the switch in MAT expression in liver cancer is pathogenetically important since it offered the cancerous cell a growth advantage.

Decreased hepatic MAT activity is well known in patients with liver cirrhosis (Mato et al., 1997, 2002; Avila et al., 2000). Both decreased *MAT1A* mRNA levels and inactivation of MAT I/III isoenzymes occur with the consequence of decreased AdoMet biosynthesis (Avila et al., 2000; Mato et al., 2002). The importance of MAT expression on AdoMet level and liver phenotype was also confirmed in the *MAT1A* knockout mouse model where replacement of *MAT1A* with *MAT2A* resulted in chronic hepatic AdoMet depletion, predisposition to liver injury, and eventual development of HCC (Lu et al., 2001; Martínez-Chantar et al., 2002). Given the importance of maintaining *MAT1A* expression, it is important to understand how its expression is regulated. We had previously reported that *MAT1A* expression is regulated by histone acetylation and methylation (Torres et al., 2000). However, in those studies we did not directly demonstrate that methylation of *MAT1A* can influence *MAT1A* expression. We also did not examine sites other than 977. The current work investigated in detail whether methylation of *MAT1A* can influence its transcriptional activity and if so, how.

Using a combination of sodium bisulfite treatment genomic sequencing and Southern blot analysis, we found that methylation at the −977 site of the *MAT1A* promoter does not correlate with *MAT1A* expression, as both Huh-7 cells and normal liver exhibit partial methylation of this site. There is better correlation with methylation closer to the transcriptional start site, and near perfect correlation with methylation at both +10 and +88 sites and the lack of *MAT1A* expression. Promoter methylation has been extensively studied and is well known to inhibit gene expression (Razin, 1998). However, methylation of the coding region has not been reported to influence mammalian gene expression. Methylation of the coding region has been reported to influence luciferase gene expression on an episome (Irvine et al., 2002). Methylation of the coding region of the herpes simplex virus *tk* gene was shown to inhibit *tk* expression (Graessmann et al., 1994). Interestingly, methylation of single *Hpa*II sites, as far as 571 downstream from the transcription start site, blocked expression of the *tk* gene (Graessmann et al., 1994). This prompted us to examine in more detail whether methylation at the +10 and +88 of *MAT1A* can influence its expression.

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To examine the effect of methylation on *MAT1A* promoter activity, *MAT1A* promoter constructs were methylated *in vitro* by *Hpa*II methylase and subsequently used for transfection experiments. Using the constructs −839/+30-Luc and −1111/+30-Luc, we found that methylation at the +10 site led to a 3-fold reduction in promoter activity, while methylation at both the +10 and −977 site reduced the promoter activity by an additional 4% or so (Fig. 4B). This suggests it is the +10 site that is more critical for reporter activity. This prompted us to further examine the role of the two coding region methylation at +10 and +88. Methylation of both of these sites reduced the promoter activity by 60%, while mutation of these sites reduced the activity by 40% (Fig. 5B). These results clearly establish the ability of methylation of these two *Hpa*II sites in the coding region to inhibit *MAT1A* transcription. Furthermore, the *in vivo* significance is demonstrated by the reactivation of *MAT1A* as these sites became demethylated.

Methylation of the promoter region of genes is thought to inhibit gene expression by either directly blocking the binding of transcription factors to important enhancer elements, or indirectly by recruiting proteins that bind to methylated cytosines. These proteins are thought to modify chromatin structure by recruiting histone deacetylase, leading to a condensed and inactive chromatin (Razin, 1998; Jones et al., 1998). Coding region methylation in the herpes simplex virus *tk* gene is thought to block at the level of transcription elongation (Graessmann et al, 1994). We next examined whether methylation and/or mutation of +10 and +88 sites inhibit either transcription initiation and/or elongation using an *in vitro* T7 transcription system. We found that either methylation or mutation reduced the amount of a single transcript suggesting that they are more likely to influence transcription initiation and not elongation, as transcripts of shorter sizes would have been expected. However, we cannot exclude the possibility that elongation very close to the initiation site was inhibited.

Mutation at the +10/+88 sites also exert an inhibitory effect on *MAT1A* transcription, but to a lesser extent than methylation. We speculate that methylation of these sites may affect the binding of factor(s) that are important for *MAT1A* transcription, whereas a single mutation may not completely abrogate the binding of such factor(s).

Our findings suggest that coding region methylation is a key mechanism for reduced *MAT1A* expression in HCC. Recently we demonstrated that forced expression of *MAT1A* reduced *in vivo* HCC growth and angiogenesis (Li et al., 2010). Understanding how the +10/+88 sites of *MAT1A* become hypermethylated in HCC and whether this can be reversed may lead to novel strategy for HCC treatment.

In summary, we have uncovered a highly novel finding, the ability of coding region methylation of a human gene to regulate gene transcription. This has not been previously reported to our knowledge and adds another layer of complexity to how methylation can regulate gene expression.

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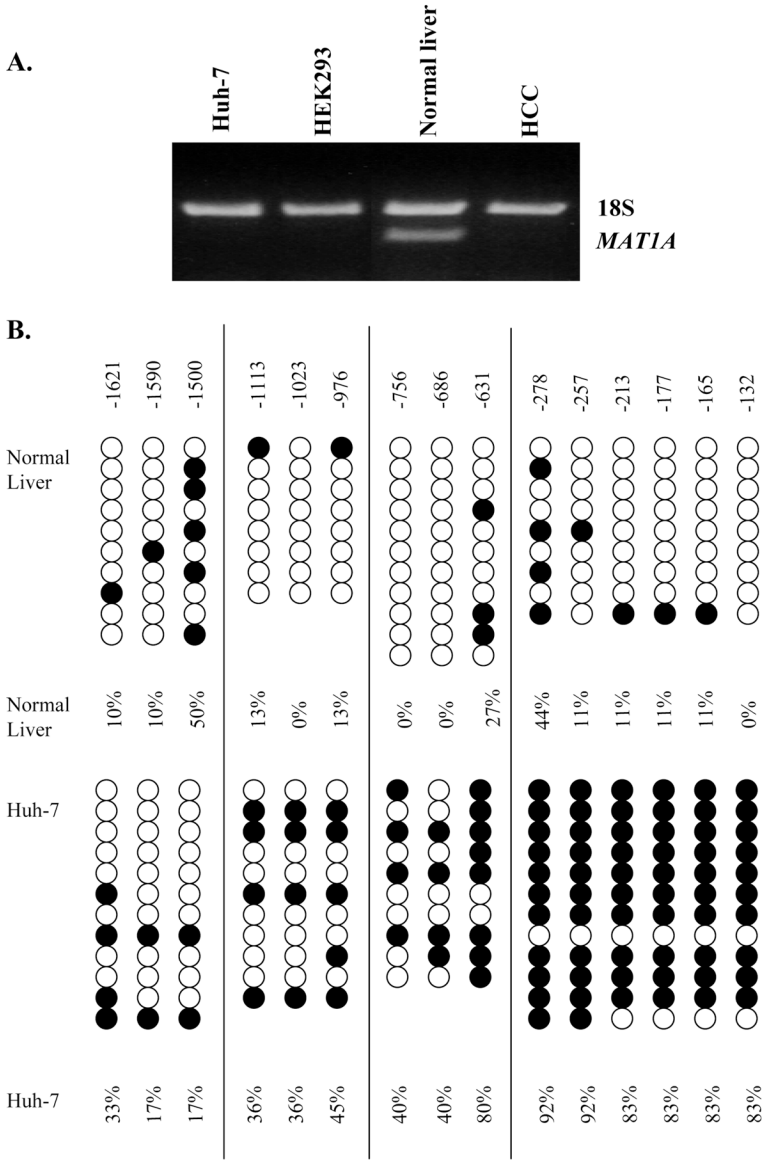

Fig 1.

A. *MAT1A* expression in Huh-7, HEK293 cells, normal liver and HCC. *MAT1A* expression was assessed by RT-PCR as described in Methods. B. Sodium bisulfite genomic DNA sequencing. Genomic DNA from normal liver and Huh-7 cells were extracted and subjected to bisulfite treatment and DNA sequencing as described in Methods. Cytosine residues are shown from −1621 to −132. Open circle is unmethylated and closed circle is methylated.

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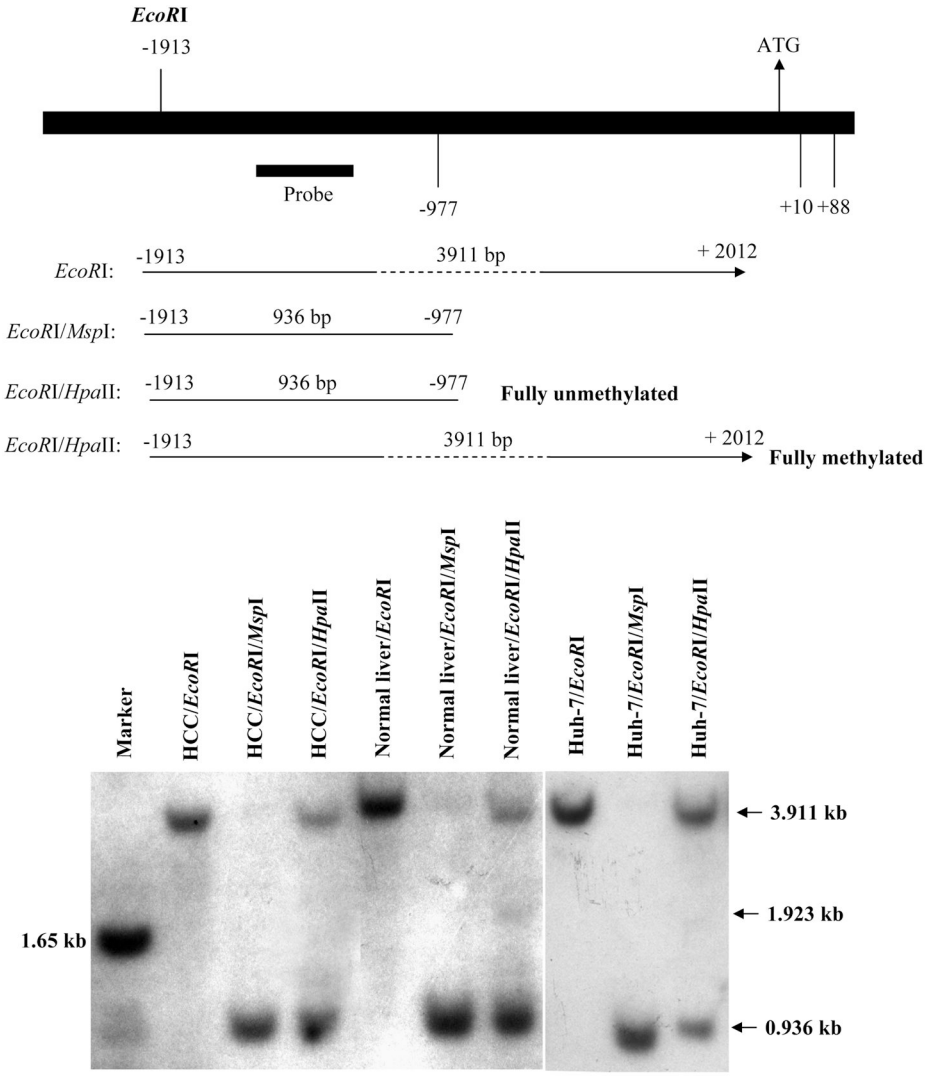

Fig 2.

Methylation pattern at −977 and +10 sites of *MAT1A* in HCC, normal liver and Huh-7 cells. DNA from HCC, normal liver and Huh-7 cells were first digested with *Eco*RI, which cleaves at −1913 and +2012 of *MAT1A* as depicted on top. The DNA is then digested with *Msp*I, which is insensitive to methylation of the internal cytosine residue of CCGG sequences, or *Hpa*II, which is inhibited if the internal cytosine is methylated and Southern blot hybridization was performed using the labeled human *MAT1A* probe as shown on top. Note that digestion of all three samples with *Hpa*II results in both a low molecular weight (0.936 kb) and a high molecular weight (3.911 kb) band, consistent with the site at −977 being partially methylated. In normal liver but not in HCC or Huh-7 cells, *Hpa*II digestion also result in a band of about 1.923 kb, consistent with the notion that +10 is partially unmethylated.

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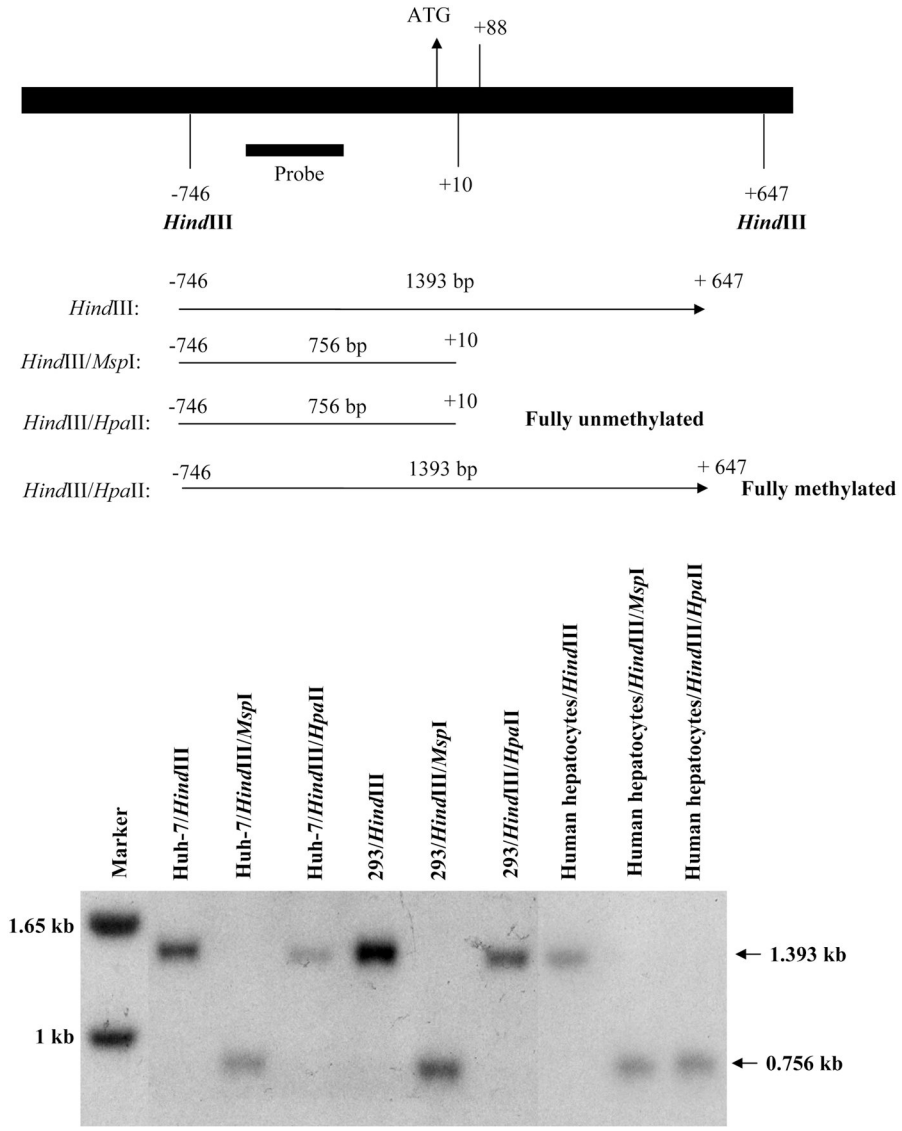

Fig 3.

Methylation pattern at +10 and +88 sites of *MAT1A* in Huh-7, HEK293 (293), and primary human hepatocytes. DNA from Huh-7, 293 cells, and human hepatocytes were first digested with *Hind*III, which cleaves at −746 and +647 of *MAT1A* as depicted on top. The DNA is then digested with *Msp*I or *Hpa*II and Southern blot hybridization was performed using the labeled human *MAT1A* probe as shown on top. Note that digestion of Huh-7 and 293 cells with *Hpa*II results in only a high molecular weight (1.393 kb) band, the same size as with *Hind*III alone, which is consistent with the sites at +10 and +88 being fully methylated. In contrast, *Hpa*II digestion of human hepatocytes results only in a low molecular weight band (0.756 kb), the same size as with *Msp*I which is consistent with the notion that +10 is unmethylated.

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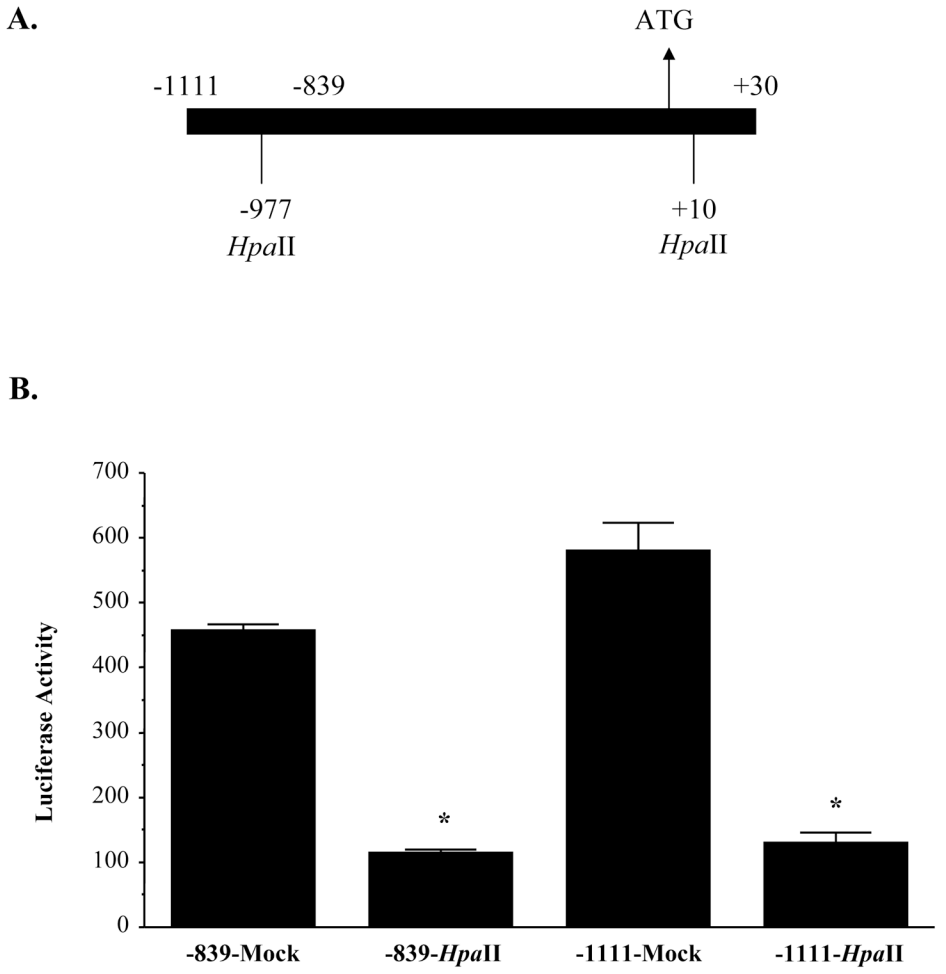

Fig 4.

Effect of methylation at the −977 and +10 sites of *MAT1A* on luciferase expression driven by the *MAT1A* promoter. Human *MAT1A* DNA fragments −1111 to +30 and −839 to +30 were *Hpa*II methylated or mock methylated *in vitro* and re-ligated back to the promoterless luciferase pGL-3 enhancer vector as described in Methods. Numbering is relative to the translational start site (Zeng et al., 2000). Part A shows the *MAT1A* constructs used and the *Hpa*II sites. Part B shows the effect of *Hpa*II methylation of the constructs on luciferase activity. Results represent mean±SEM from three independent experiments performed in triplicates. The luciferase activity of each transfection was expressed as luciferase activity/β-galactosidase activity. Data are expressed as relative luciferase activity to that of pGL-3 enhancer vector control, which is assigned a value of 1.0. *$p<0.05$ vs. its own mock methylated control.

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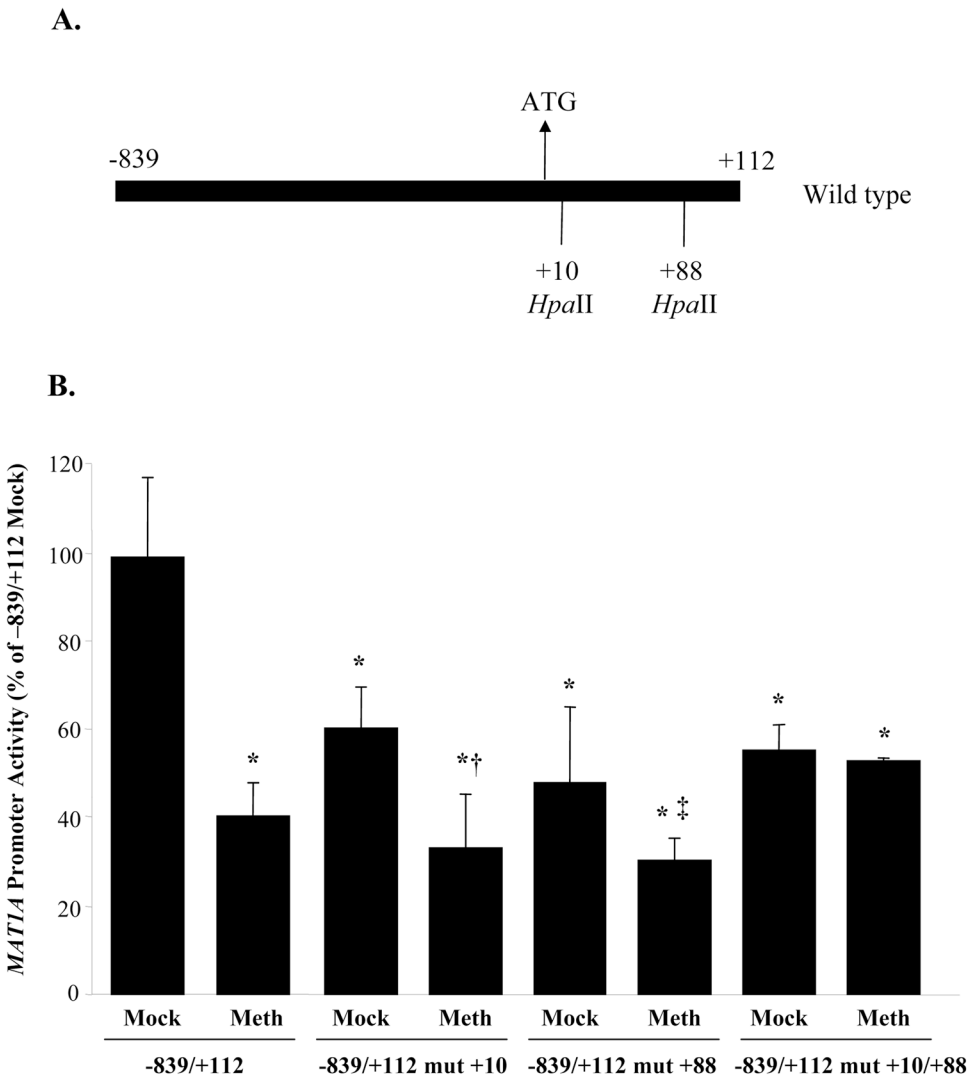

Fig 5.

Effect of methylation at the +10 and +88 sites of *MAT1A* on luciferase expression driven by the *MAT1A* promoter. Human *MAT1A* DNA fragments −839 to +112, either wild type or mutated at the +10 site (mut +10), +88 site (mut +88), or both (mut +10/+88) were generated, *Hpa*II methylated or mock methylated *in vitro* and re-ligated back to the promoterless luciferase pGL-3 enhancer vector as described in Methods. Results represent mean±SEM from three independent experiments performed in triplicates. The luciferase activity of each transfection was expressed as luciferase activity/β-galactosidase activity. Data are expressed as % of −839/+112 Mock control. *p<0.001 vs. −839/+112 Mock, †p<0.02 vs. −839/+112 Mock mut+10, ‡p<0.04 vs. −839/+112 Mock mut+88.

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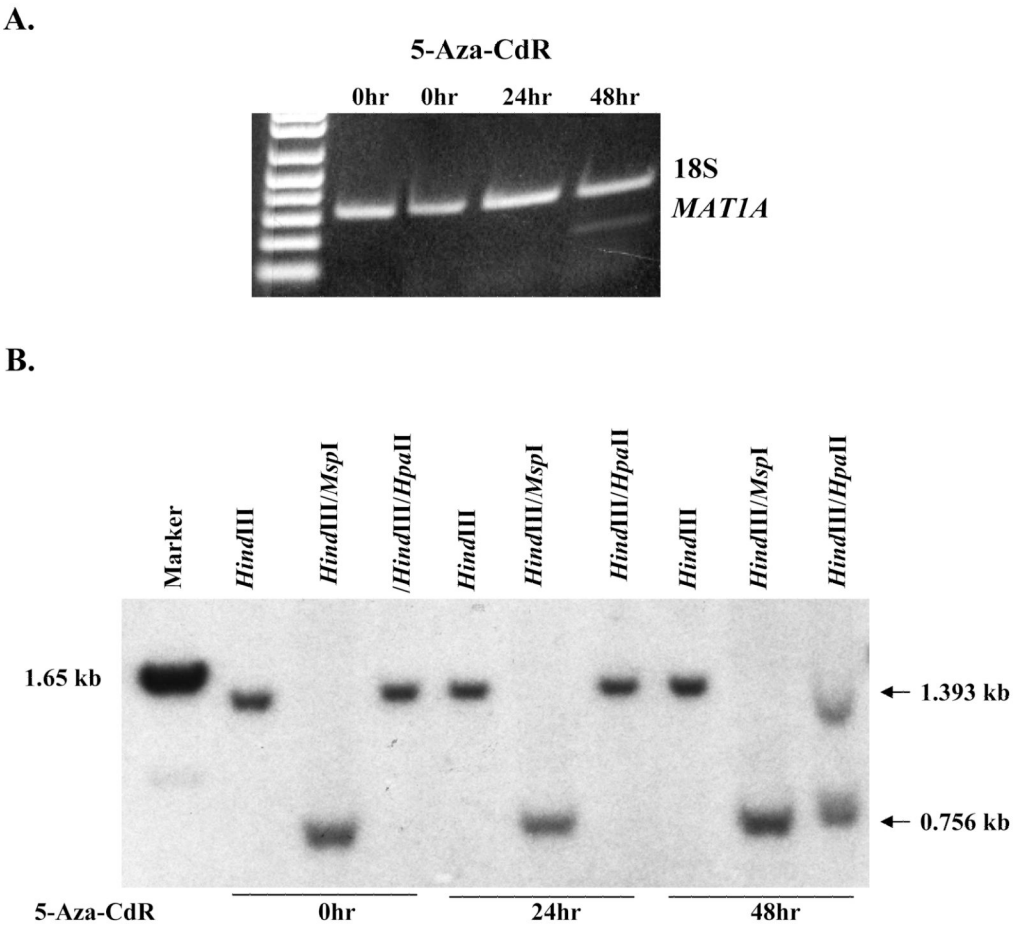

Fig 6.

Effect of 5′aza-2′-deoxycytidine (5-Aza-CdR) on *MAT1A* expression (part A), and methylation pattern at +10 and +88 (part B). Huh-7 cells were treated with 5-Aza-CdR (0.1 μM for 24 or 48 hrs). RNA and DNA were extracted for determination of *MAT1A* expression by RT-PCR or methylation pattern at +10 and +88 by Southern blot analysis as described in Methods. *MAT1A* is reactivated 48 hrs following 5-Aza-CdR treatment (part A). Southern blot analysis showed that at 0hr, digestion with *Hind*III/*Hpa*II results in only a high molecular weight (1.393 kb) band, the same size as with *Hind*III alone which is consistent with the sites at +10 and +88 being fully methylated. After 48 hrs of treatment with 5-Aza-CdR, *Hind*III/*Hpa*II digestion results in two low molecular weight bands and one high molecular weight band, consistent with the notion that +10 and +88 are partially unmethylated (part B).

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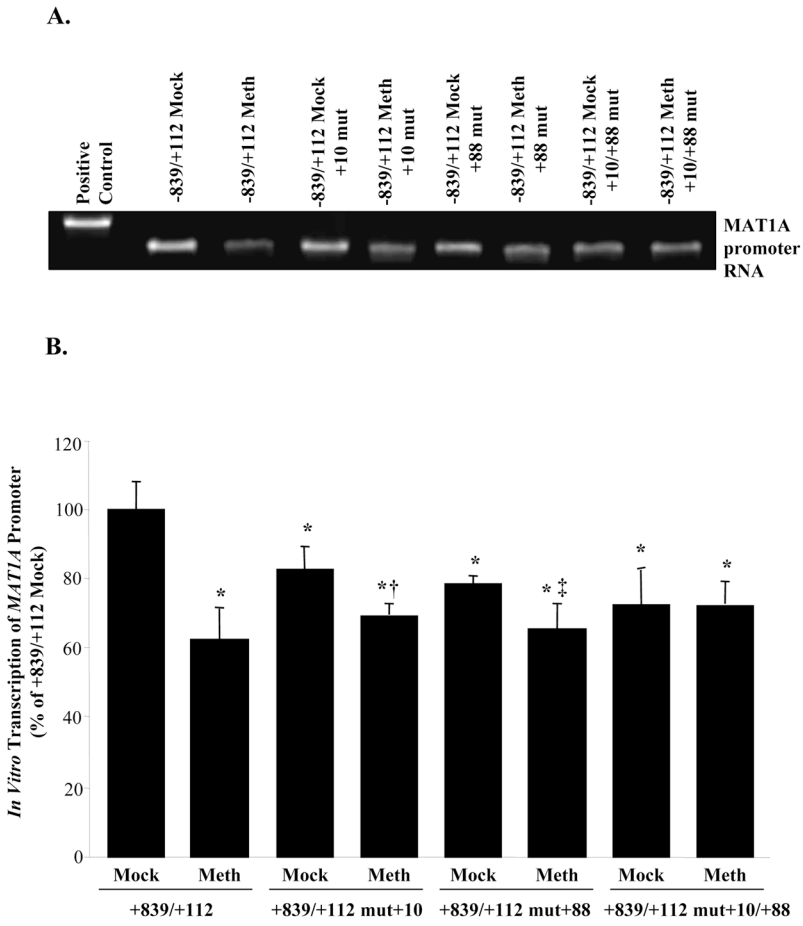

Fig 7.

Effect of methylation at the +10 and +88 sites of *MAT1A* on *in vitro* transcription. Human *MAT1A* DNA fragments −839 to +112, either wild type or mutated at the +10 site (mut +10), +88 site (mut +88), or both (mut +10/+88) were generated, *Hpa*II methylated or mock methylated *in vitro* and re-ligated back to the T7 promoter for *in vitro* transcription assay as described in Methods. Amount of *MAT1A* promoter RNA transcript was visualized by gel electrophoresis and subjected to densitometric analysis. Part A shows a representative gel and part B shows the densitometric summary of four independent experiments expressed as mean±SEM % of −839/+112 Mock control. *p<0.02 vs. −839/+112 Mock, †p<0.05 vs. −839/+112 Mock mut+10, ‡p<0.04 vs. −839/+112 Mock mut+88.