Liver-specific methionine adenosyltransferase *MAT1A* gene expression is associated with a specific pattern of promoter methylation and histone acetylation: implications for *MAT1A* silencing during transformation

LUIS TORRES,*,[1] MATÍAS A. ÁVILA,*,[1] M. VICTORIA CARRETERO,* M. UJUE LATASA,* JOAN CABALLERÍA,† GERARDO LÓPEZ-RODAS,‡ ABDELHALIM BOUKABA,‡ SHELLY C. LU,§ LUIS FRANCO,‡ AND JOSÉ M. MATO*,[2]

*División de Hepatología y Terapia Génica, Departamento de Medicina Interna, Facultad de Medicina, Universidad de Navarra, 31008 Pamplona, Spain; †Servicio de Hepatología, Hospital Clínico y Provincial, 08036 Barcelona, Spain; ‡Departamento de Bioquímica y Biología Molecular, Universidad de Valencia, Burjassot, 46100 València, Spain; and §Center for Liver Disease Research and Division of Gastrointestinal Liver Diseases, Department of Medicine, University of Southern California School of Medicine, Los Angeles, California 90033, USA

ABSTRACT **Methionine adenosyltransferase (MAT) is the enzyme that catalyzes the synthesis of S-adenosylmethionine (AdoMet), the main donor of methyl groups in the cell. In mammals MAT is the product of two genes, *MAT1A* and *MAT2A*. *MAT1A* is expressed only in the mature liver whereas fetal hepatocytes, extrahepatic tissues and liver cancer cells express *MAT2A*. The mechanisms behind the tissue and differentiation state specific *MAT1A* expression are not known. In the present work we examined *MAT1A* promoter methylation status by means of methylation sensitive restriction enzyme analysis. Our data indicate that *MAT1A* promoter is hypomethylated in liver and hypermethylated in kidney and fetal rat hepatocytes, indicating that this modification is tissue specific and developmentally regulated. Immunoprecipitation of mononucleosomes from liver and kidney tissues with antibodies mainly specific to acetylated histone H4 and subsequent Southern blot analysis with a *MAT1A* promoter probe demonstrated that *MAT1A* expression is linked to elevated levels of chromatin acetylation. Early changes in *MAT1A* methylation are already observed in the precancerous cirrhotic livers from rats, which show reduced *MAT1A* expression. Human hepatoma cell lines in which *MAT1A* is not expressed were also hypermethylated at this locus. Finally we demonstrate that *MAT1A* expression is reactivated in the human hepatoma cell line HepG2 treated with 5-aza-2′-deoxycytidine or the histone deacetylase inhibitor trichostatin, suggesting a role for DNA hypermethylation and histone deacetylation in *MAT1A* silencing.—Torres, L., Ávila, M. A., Carretero, M. V., Latasa, M. U., Caballería, J., López-Rodas, G., Boukaba, A., Lu, S. C., Franco, L., Mato, J. M. Liver-specific methionine adenosyltransferase *MAT1A* gene expression is associated with a specific pattern of promoter methylation and histone acetylation: implications for *MAT1A* silencing during transformation. *FASEB J.* 14, 95–102 (2000)**

Key Words: S-adenosylmethionine synthesis · DNA methylation · tissue-specific expression · liver cirrhosis · hepatocarcinoma

METHIONINE ADENOSYLTRANSFERASE (MAT) is an essential enzyme that catalyzes the formation of S-adenosylmethionine (AdoMet), the most important methyl donor in cellular metabolism (1, 2). MAT activity in mammals is the product of two genes, *MAT1A* and *MAT2A*, which display a tissue-specific pattern of expression. *MAT1A* is expressed only in the adult liver whereas *MAT2A* shows a wider distribution, being responsible for AdoMet synthesis in the extrahepatic tissues (reviewed in ref 2). In addition, *MAT2A* is expressed in the fetal liver, where it is replaced by *MAT1A* upon maturation of this organ (3). Similarly, in the transformed hepatocyte, a switch in MAT gene expression is observed and the dedifferentiated hepatoma cell mimics the fetal pattern by expressing *MAT2A* in place of the liver-specific *MAT1A* (4). This switch in MAT gene expression appears to confer a growth advantage to the tumor cell (5).

[1] Both authors made equal contribution to this work.

[2] Correspondence: División de Hepatología y Terapia Génica, Departamento de Medicina Interna. Facultad de Medicina. Universidad de Navarra. 31008, Pamplona, Navarra, Spain. Tel: 34–948-425678. Fax: 34–948-425677. E-mail: jmmato@unav.es

Although all this evidence clearly establishes that *MAT1A* is a tissue-specific gene silenced outside the liver and in situations such as development and malignant transformation, the mechanisms behind this tightly controlled pattern of expression are not known. CpG methylation in mammalian DNA has been linked for many years to the establishment of tissue-specific patterns of gene expression (6, 7). However, the molecular mechanisms underlying methylation-dependent repression of gene expression have only recently been elucidated. The recruitment of histone deacetylase, by means of methylated DNA binding proteins such as MeCP2, seems to be critical for assembly of the methylated sequences into a condensed state (8, 9). This situation renders the promoter regions inaccessible to the transcription machinery and thus can block transcription (10–12). In light of these recent developments, we have searched for the molecular mechanisms that may explain the tissue- and differentiation stage-specific expression of *MAT1A*.

MATERIALS AND METHODS

General procedures

Fetal rat hepatocytes (gestational day 19) were prepared by collagenase digestion of pooled fetal livers as reported previously (3). The human hepatoma cell line HepG2 was grown in DMEM medium supplemented with 10% fetal calf serum, glutamine, and antibiotics. Treatments were performed on exponentially growing cultures. Total RNA from cells and tissues was obtained by the guanidinium thiocyanate method. Aliquots (20 μg) of total RNA were size fractioned by electrophoresis in agarose gels under denaturing conditions, blotted, and fixed on Nytran (Schleicher and Schuell, Keene, N.H.) membranes using standard procedures. Blots were prehybridized and hybridized as described (13). Human and rat *MAT1A* cDNAs probes (14, 15) were labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech, Amersham, U.K.) by random priming. Hybridization with a probe for 18S rRNA was performed as loading control. Membranes were then exposed to X-ray films. Genomic DNA was prepared as described previously (16). Aliquots of high molecular weight DNA (10 μg) were digested to completion with restriction enzymes (Boehringer Mannheim, Mannheim, Germany), loaded on agarose gels, electrophoresed, and blotted onto Nytran membranes as described (17). Hybridizations were carried out with a 1.4 kb *Ssp*I-*Sma*I probe derived from the rat *MAT1A* promoter (probe 1) (18) or with the human *MAT1A* full-length cDNA (15) labeled with [α-^{32}P]dCTP. Membranes were then exposed to X-ray films.

Induction of cirrhosis

Male Wistar rats (250 g) were fed *ad libitum* a standard laboratory diet and received humane care according to our institution's guidelines for the use of laboratory animals in research. Cirrhosis was induced by intraperitoneal injections of CCl_4 (0.5 ml administered twice a week) as described previously (19). The establishment of cirrhosis was monitored by histological examination of liver sections from treated animals (19). Rats were killed after 9 wk of treatment by cardiac puncture and exsanguination.

Nuclei isolation

Liver and kidney from Wistar rats were placed in buffer A (0.25M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 0.2% Triton X-100, 2 mM *p*-aminobenzamidine, 1 mM PMSF, 2.5 μg/ml leupeptin, 1.5 μg/ml chymostatin, 50 mM Tris-HCl, pH 7.5) at a ratio of 5 ml buffer per gram of tissue. All the steps were carried out at 4°C. Tissues were homogenized on ice; after filtration of the homogenates through 200 and 100 μM nylon filters, nuclei were collected by centrifugation at 1500 × *g* for 5 min and the sediment was washed twice in buffer B (buffer A without Triton and EDTA). Nuclear pellets were resuspended in the same buffer and placed on a discontinuous sucrose gradient (0.75 M/1.75 M) in buffer B. After centrifugation at 10,000 × *g* for 30 min, nuclei were collected from the 1.75 M sucrose cushion and washed twice with buffer B.

Micrococcal nuclease digestion

Nuclei were resuspended in digestion buffer (0.25 M sucrose, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM sodium butyrate, 2 mM *p*-aminobenzamidine, 1 mM PMSF, 2.5 μg/ml leupeptin, 1.5 μg/ml chymostatin, 50 mM Tris-HCl, pH 7.5) at a concentration of 5 mg DNA/milliliter. Nuclei from liver were digested with micrococcal nuclease (Worthington, Freehold, N.J.) at 15 u per mg DNA and nuclei from kidney with 20 u per mg DNA for 10 min at 37°C. The digestion was stopped by adding EDTA to a final concentration of 5 mM and the samples were cooled on ice and centrifuged at 12,000 × *g* for 10 min. The supernatant was saved and the pellet was resuspended in lysis buffer (0.25 mM EDTA, 10 mM sodium butyrate, 2 mM *p*-aminobenzamidine, 1 mM PMSF, 2.5 μg/ml leupeptin, 1.5 μg/ml chymostatin, 50 mM Tris-HCl, pH 7.5) incubated 10 min on ice and recentrifuged as above. The first and second supernatants were pooled and analyzed on agarose gels to determine the extent of the micrococcal digestion.

Production of antisera to hyperacetylated histone H4 and antibody purification

Polyclonal antisera to hyperacetylated H4 were raised by immunization with a synthetic peptide corresponding to residues 2–19 of histone H4 and acetylated in the ε-amino groups of lysines 5, 8, 12, and 16. The acetylated peptide was custom-synthesized by Genosys and coupled to keyhole limpet hemocyanin (20). The immunoglobulin (IgG) fraction was purified by HiTrap protein-A FPLC chromatography according to the manufacturer (Amersham Pharmacia Biotech).

Immunofractionation of nucleosomes

Immunofractionation of nucleosomes obtained by micrococcal nuclease digestion of nuclei were performed essentially as described previously (21). FPLC-purified antibody (500 μg of IgG) dissolved in fractionation buffer (50 mM NaCl, 10 mM sodium butyrate, 1 mM EDTA, 0.2 mM *p*-aminobenzamidine, 0.1 mM PMSF, 2.5 μg/ml leupeptin, 1.5 μg/ml chymostatin, 10 mM Tris-HCl, pH 7.5) was incubated with 100 mg of protein A Sepharose prewashed with fractionation buffer for 3 h at 4°C under gentle rotation. The protein A Sepharose-bound antibody was mixed with 2 mg of soluble chromatin in a final volume of 3 ml of fractionation buffer and incubated

overnight with gentle rotation at 4°C. The immunocomplexes were collected by centrifugation (6500 × *g*, 1 min) on a 0.65 μM Ultrafree filter unit (Millipore) and the filtered through fraction was removed and stored on ice. Protein A Sepharose beads were washed eight times with 500 μl of fractionation buffer as before and filtrates were pooled together (unbound fraction). Antibody-bound fraction was eluted from protein A Sepharose by addition of 400 μl of 1.5% sodium dodecyl sulfate (SDS) in fractionation buffer and rotated for 15 min at room temperature. After centrifugation as before, the eluate was saved and protein A Sepharose was reincubated for another 15 min period with 400 μl of 0.5% SDS in fractionation buffer. The two eluates (bound fraction) were pooled. DNA from all chromatin fractions (input, unbound, and bound) was purified and quantitated by fluorescence with PicoGreen dye (Molecular Probes, Eugene, Oreg.).

Slot-blot and hybridization analysis

DNA samples were denatured in 0.5 M NaOH, 1.5 M NaCl for 10 min at 37°C, followed by 1 min at 95°C and then kept on ice. 150 ng of DNA from each fraction were loaded through a slot-blot manifold (Bio-Rad, Hercules, Calif.) on a Biodyne B membrane (Pall) prewetted in 2× SSC. The filters were immersed in denaturation solution for 5 min, neutralized in 1.5 M NaCl, 1 mM EDTA, 0.5 M Tris-HCl, pH 7.5 for 30 s and the membranes were dried by baking at 80°C for 30 min. Hybridizations were carried out using a 750 bp *Msp*I-*Sma*I probe derived from *MAT1A* promoter (probe 2). The probe was labeled by random priming. Filters were prehybridized for 2 h and hybridized overnight using Quick Hyb solution (Stratagene, San Diego, Calif.) at 65°C in the presence of 200 μg of heat-denatured salmon sperm DNA. Filters were rinsed in 2× SSC, 0.1% SDS and washed sequentially twice with 2× SSC, 0.1% SDS at 65°C for 5 min, once with 2× SSC, 0.1% SDS at 65°C for 30 min, and once with 0.2× SSC, 0.1% SDS at 65°C for 20 min. The radioactivity present in the filters was initially measured with an InstantImager (Packard, Downers Grove, Ill.) and the filters were then autoradiographed.

RESULTS

As mentioned, *MAT1A* is expressed only in liver but is silenced in all other tissues. We first evaluated whether this pattern of expression correlated with the methylation status of *MAT1A* promoter in liver and extrahepatic tissues such as the kidney. For this purpose, we used the methylation-sensitive restriction isoschizomers *Msp*I and *Hpa*II. Both enzymes recognize the sequence CCGG, but *Hpa*II cannot cleave if the internal C is methylated whereas methylation at the external C is inhibitory for both enzymes (22). Two *Msp*I/*Hpa*II restriction sites are present in the reported 5′-flanking region of rat *MAT1A* (18) (**Fig. 1*A***). Genomic DNA samples from rat liver and kidney were sequentially digested with the methylation-insensitive enzyme *Ssp*I and either *Msp*I or *Hpa*II. Southern blot analysis was then carried out using a 1.4 kb *Ssp*I-*Sma*I probe from rat *MAT1A* promoter (probe 1 in Fig. 1*A*). DNA from liver was unmethylated as indicated by the presence of two fragments of 0.64 and 0.75 kb, respectively, consistent with cleavage at the *Msp*I/*Hpa*II sites by both enzymes (Fig. 1*B*). In the case of kidney DNA, *Msp*I treatment resulted in the same pattern obtained in liver, but *Hpa*II could not cleave with the same efficiency observed in hepatic tissue and generated higher molecular weight bands (Fig. 1*B*). These results are consistent with hypomethylation of the cytosines located at positions −13 and −755 of *MAT1A* promoter in liver and enhanced levels of methylation of these two sites in kidney DNA. Other tissues examined, such as spleen and heart, generated the same restriction pattern observed in the kidney (data not shown).

The mechanism of gene silencing by means of cytosine methylation has been recently worked out through the implication of histone deacetylation (8, 9). We wanted to ascertain the degree of acetylation displayed *in vivo* by the histones associated with *MAT1A* promoter in the liver and kidney and whether this covalent modification correlated with the degree of promoter methylation. Mononucleosomes obtained from both tissues were immunoprecipitated with an antibody mainly specific to acetylated histone H4, as described in Materials and Methods. DNA was extracted from the input, unbound, and bound fractions; equal amounts from each fraction were immobilized onto nylon membranes by slot-blot and hybridized with a 750 bp probe derived from the 5′-flanking region of rat liver *MAT1A* gene, close to the transcription initiation site (probe 2 in Fig. 1*A*). The intensity of signal from the antibody-bound slot relative to the intensity from the input slot gives the enrichment generated by the antibody selection. Our data show a strong enrichment (10- to 15-fold) in the bound fraction compared to the input fraction in liver, where the gene is actively transcribed. However, no preferential selection by the antibody was observed in the kidney, indicating the absence of hyperacetylation in this region of *MAT1A* in a tissue where the gene is not expressed.

We have also analyzed *MAT1A* promoter methylation in developing rat hepatocytes. Genomic DNA was isolated from fetal rat hepatocytes corresponding to gestational day 19, when *MAT1A* expression is negligible (**Fig. 2**) (3), and then digested with *Ssp*I and *Msp*I or *Hpa*II. The restriction pattern obtained with probe 1 indicated that in the developing hepatocyte the *MAT1A* promoter is hypermethylated, as evidenced by the appearance of high molecular weight bands in the *Hpa*II-treated samples (Fig. 2). This pattern was identical to that obtained in extrahepatic tissues, where *MAT1A* is not expressed.

A significant number of cirrhotic patients develop hepatocellular carcinoma, thus, the cirrhotic liver could be considered to be in a precancerous condition (23, 24). This notion along with our previous observation of *MAT1A* silencing in transformed liver

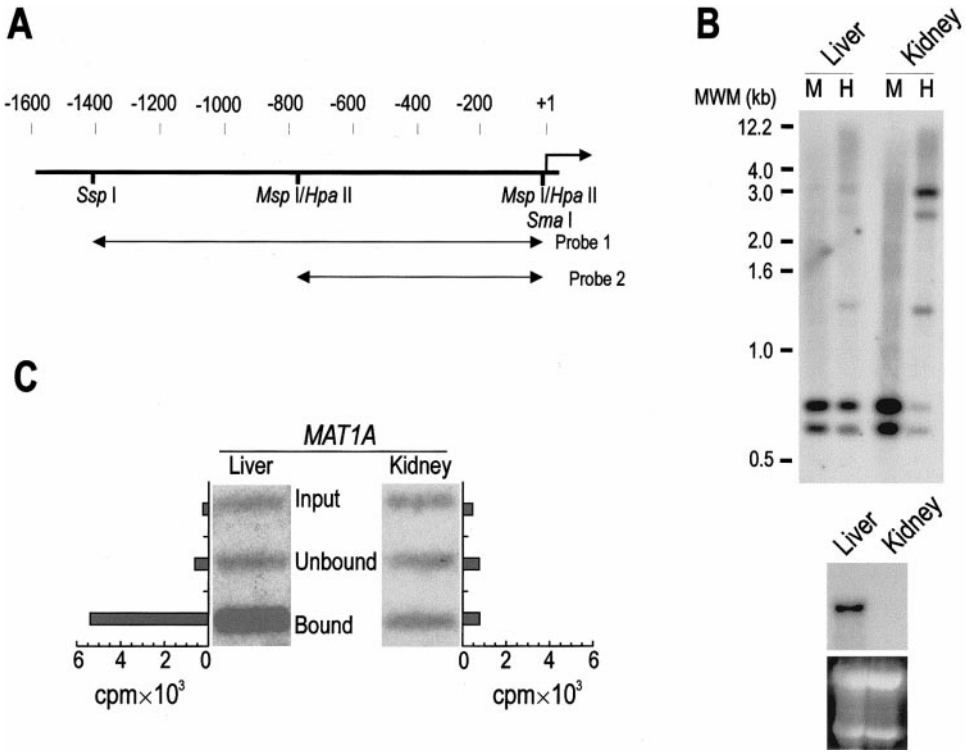

Figure 1. Analysis of the methylation and histone acetylation status of the 5′ region of *MAT1A* gene in rat liver and kidney. *A)* Restriction map of *MAT1A* promoter indicating the location of *Msp*I/*Hpa*II restriction sites and the probes used in the experiments represented here and in subsequent figures. Probe 1 is a 1.4 kb *Ssp*I-*Msp*I fragment and probe 2 is a 0.75 kb *Msp*I-*Sma*I fragment, both derived from rat *MAT1A* promoter. *B)* Southern blot analysis of rat liver and kidney genomic DNA after digestion with *Ssp*I and *Msp*I (M) or *Hpa*II (H), performed with probe 1 derived from *MAT1A* promoter. The migration of molecular weight markers is indicated (MWM). Lower panel shows the levels of *MAT1A* expression in liver and kidney as assayed by Northern blotting with a rat cDNA probe. Ethidium bromide staining of the gel is shown as loading control. *C)* Mononucleosomes from liver and kidney were prepared and immunoprecipitated with antibodies specific mainly to acetylated histone H4, as described in Materials and Methods. DNA was extracted from input, unbound, and bound fractions; equal amounts were loaded and analyzed by slot-blot hybridization with probe 2 from *MAT1A* promoter. Quantitation of the radioactivity incorporated in each slot is also shown. Representative autoradiograms are shown.

(4) led us to evaluate *MAT1A* expression in a rat model of CCl_4-induced cirrhosis. In agreement with our previous results (19), no large changes were observed in *MAT1A* mRNA levels, although a significant trend toward a reduction in the hepatic content of this messenger was observed (**Fig. 3*A***). *MAT1A* promoter methylation was evaluated in control and cirrhotic livers using the same strategy described above. High molecular weight bands were detected in the *Hpa*II-digested samples from cirrhotic rat liver, which is consistent with enhanced methylation of the *Msp*I/*Hpa*II sites in *MAT1A* promoter (Fig. 3*B*).

As mentioned before, transformed hepatic cells lose *MAT1A* expression (4). We have tested whether the silencing of *MAT1A* in this condition is accompanied by changes in *MAT1A* methylation. Since the human *MAT1A* promoter has not been cloned, we performed *Msp*I/*Hpa*II restriction analysis using a full-length human *MAT1A* cDNA clone as a probe (15). As observed in Fig. 3*A*, *Hpa*II treatment of normal human liver yielded low molecular weight bands that were absent in the transformed hepatic cells. This result indicates hypermethylation at the internal C of CCGG sites of *MAT1A* in HepG2 cells. Similar results consistent with hypermethylation of *MAT1A* were obtained in Hep3B cells, another human hepatoma cell line in which *MAT1A* is also silenced (data not shown). To test whether this epigenetic modification could play a role in *MAT1A* silencing, we tried to modify the methylation status of this cell line by treatment with the demethylating agent 5-aza-2′-deoxycytidine (5-Aza-CdR) (25). For this purpose, HepG2 cells were incubated with 0.1 μM 5-Aza-CdR for 24 or 48 h and *MAT1A* expression was assessed by Northern blotting. Under these conditions, *MAT1A* expression was induced after 48 h of treatment (**Fig. 4*B***). It has recently been established that methylation-dependent gene silencing involves histone deacetylation (8, 9). These observations led us to evaluate the effect of the histone deacetylase inhibitor trichostatin A (26) on *MAT1A* expression in HepG2 cells. In this case, 24 h of treatment with 3 μM trichostatin A was sufficient to induce *MAT1A* mRNA as detected by Northern blotting (Fig. 4*B*).

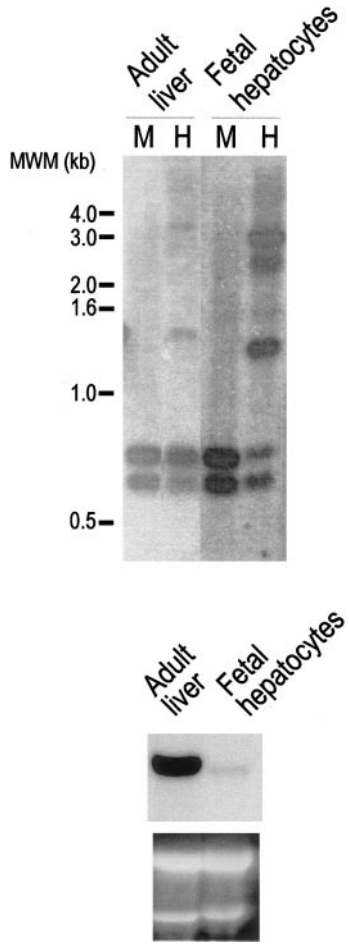

Figure 2. Methylation status of *MAT1A* promoter in adult and fetal rat hepatocytes. Genomic DNA was extracted from adult liver and fetal hepatocytes isolated from embryonic liver (corresponding to gestational day 19). DNA samples were digested with *Ssp*I and either *Msp*I (M) or *Hpa*II (H) and subsequently analyzed by Southern blotting using probe 1 described in Fig. 1. The migration of molecular weight markers (MWM) is indicated. Lower panel shows the expression of *MAT1A* in adult rat liver and fetal hepatocytes obtained from gestational day 19 embryos as analyzed by Northern blotting with a rat *MAT1A* cDNA probe. Ethidium bromide staining of the gel is shown as loading control.

DISCUSSION

Mammalian liver plays a central role in the metabolism of methionine (27). This function is met in part by the presence of a liver-specific MAT enzyme, which is the product of the *MAT1A* gene expressed only in this organ (2). We have recently reported the cloning and functional analysis of rat liver *MAT1A* promoter (18). We observed that this promoter was able to drive the expression of a reporter gene not only in liver-type cells such as HepG2, but also in the nonhepatic cell line CHO (18). These observations raised the possibility that the silencing of *MAT1A* outside the liver could be the consequence of situations known to repress transcription, such as DNA methylation and chromatin compaction (7, 25), rather than to the lack of liver-specific transcription factors.

In the present work we have first demonstrated that *MAT1A* promoter methylation is tissue specific, being hypomethylated in liver but hypermethylated in nonexpressing tissues such as the kidney. We have observed that *MAT1A* promoter is also hypermethylated in the developing hepatocyte, in which we had previously demonstrated that this gene is not expressed and MAT activity derives from the expression of the extrahepatic *MAT2A* gene (3). Thus, cytosine methylation in the 5′ region of *MAT1A* conforms to a pattern of hypermethylation, where the gene is inactive, and hypomethylation, where the gene is active. DNA methylation has been generally associated to an inactive chromatin conformation; however, the mechanisms behind the transcriptional repression dictated by methyl groups in the 5-position of cytosine residues had not been exposed until recently. The recruitment of histone deacetylases to methylated 5′CpG dinucleotides through methylcytosine binding proteins such as MeCP2 can lead to a condensed and inactive chromatin (8, 9). These observations linking DNA methylation to histone deacetylation led us to evaluate the degree of acetylation of histones associated with *MAT1A* promoter. Immunoprecipitation of mononucleosomes with antibodies specific to acetylated histone H4 indicated

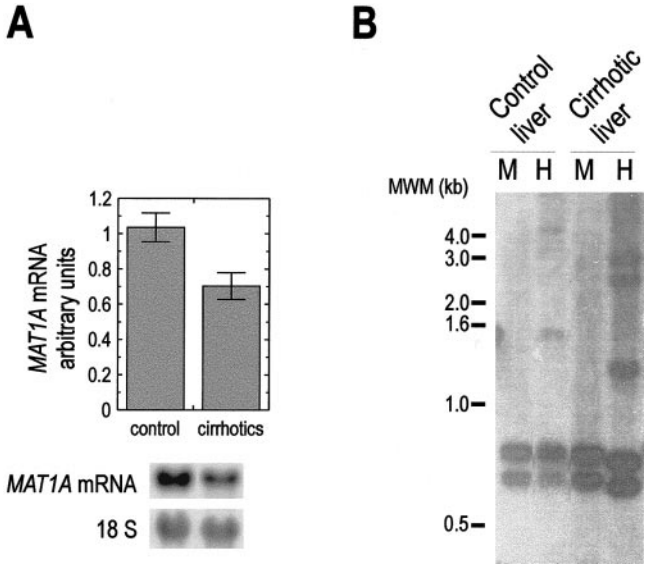

Figure 3. Expression and methylation status of *MAT1A* promoter in liver from control and CCl_4-treated cirrhotic rats. *A)* Levels of *MAT1A* mRNA in liver of control and cirrhotic rats as assayed by Northern blotting. Data are the average of four animals per group. Signal was normalized to 18S ribosomal RNA in each lane. Differences between control and cirrhotic groups were statistically significant ($P<0.05$). Representative blots are shown. *B)* Genomic DNA samples from livers of control and cirrhotic animals were digested with *Ssp*I and *Msp*I (M) or *Hpa*II (H) and analyzed by Southern blotting using probe 1 corresponding to *MAT1A* promoter as described in Fig. 1. A representative blot is shown.

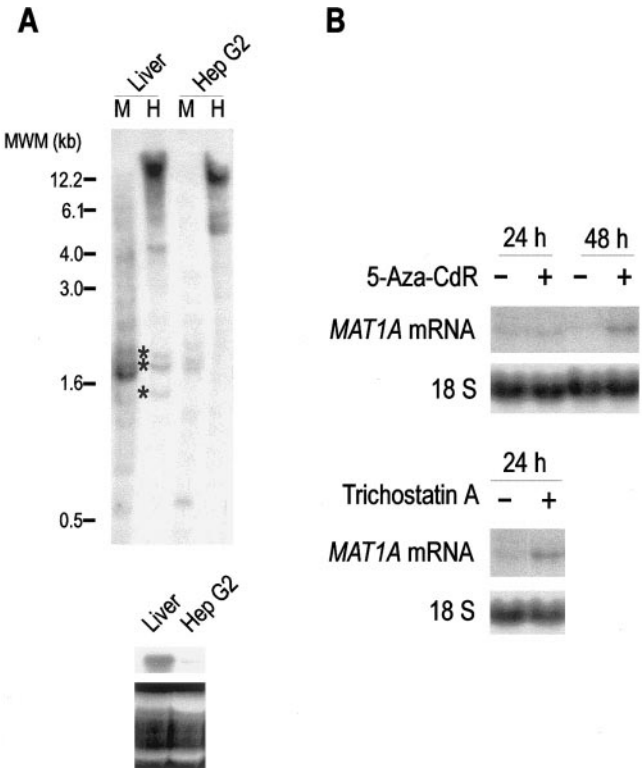

Figure 4. *A)* Methylation status of *MAT1A* locus in healthy human liver and the human hepatoma cell line HepG2. Genomic DNA extracted from control human liver and the human transformed cell line HepG2 was digested with *Msp*I (M) or *Hpa*II (H) and analyzed by Southern blotting using a full-length human *MAT1A* cDNA as a probe. Asterisks indicate bands present in *Hpa*II-digested DNA from control human liver that are absent in the *Hpa*II-digested DNA from HepG2 cells, indicating hypermethylation of CCGG sites. Lower panel shows *MAT1A* expression in control human liver and HepG2 cells as assayed by Northern blotting with the same probe. Ethidium bromide staining of the gel is shown as loading control. *B)* Effects of 5-Aza-CdR or trichostatin A on *MAT1A* expression in HepG2 cells. Upper panel shows the effect of 5-Aza-CdR treatment (0.1 μM) for 24 or 48 h on *MAT1A* expression as analyzed by Northern blotting. Lower panel shows the effect of 3 μM trichostatin A on *MAT1A* mRNA levels after 24 h of treatment. Hybridization with a probe specific for 18S ribosomal RNA was carried out as loading control. Representative blots are shown.

that in liver *MAT1A* is enriched in the highly acetylated mononucleosome fraction, whereas in the kidney *MAT1A* is not preferentially associated with acetylated histones. The histone acetylation pattern of *MAT1A* promoter in liver corresponds to that of an actively transcribing gene; however, in the kidney this pattern is that of a silent gene. Similar patterns of histone acetylation have been reported for active and silent genes, respectively (28). *MAT1A* promoter methylation profile correlates with the local acetylation status of histones. Thus, in liver, where *MAT1A* promoter is hypomethylated, elevated levels of histone acetylation would result in a chromatin structure accessible to transcription factors whereas in the kidney we find the opposite situation, which may be consistent with a condensed and inactive chromatin state (8, 9, 29). These observations may be relevant to the mechanisms by which tissue-specific *MAT1A* expression is achieved *in vivo*.

Hepatic methionine metabolism is altered in various chronic liver disorders as well as in the transformed liver (2, 30). We wanted to know whether the *MAT1A* methylation pattern would be preserved in situations of hepatocellular damage or abnormal proliferation. We first examined the methylation status of *MAT1A* in an experimental model of liver cirrhosis, trying to reproduce a pathological condition that in many cases precedes the neoplastic conversion of the liver (23, 24). Our data indicate that enhanced levels of *MAT1A* promoter methylation can be detected in the liver of cirrhotic rats. Although no marked changes in liver *MAT1A* expression occur in this model, a significant reduction (30% compared to controls) in steady-state *MAT1A* mRNA levels was detected. Although global DNA hypomethylation has been observed in this model of cirrhosis (31), it is known that DNA methyltransferase expression is higher in liver tissues that show chronic hepatitis or cirrhosis (32). In this regard, *de novo* methylation and reduced gene expression in cirrhosis have been reported for certain loci such as the D17S5 locus, in which the candidate tumor suppressor *HIC-1* was identified (33). Our present findings indicate that *MAT1A* expression in diseased liver is compromised and that hypermethylation of its promoter region could participate in this process.

As we previously mentioned, *MAT1A* expression is lost upon neoplastic transformation of the hepatocyte (4). *MAT1A* silencing is accompanied by the induction of *MAT2A*, a situation that reproduces the pattern of expression found in the fetal hepatocyte (3). In addition, *MAT2A* is also induced under conditions of active proliferation in the liver parenchyma as happens during liver regeneration after partial hepatectomy (34). Thus, it appears that proliferating cells may find an advantage in switching MAT genes (5) whose products behave differently from a kinetic and regulatory point of view (2). Although global hypomethylation is a characteristic of many transformed cells, certain genomic sequences such as that of tumor suppressor and tissue-specific genes appear to be hypermethylated and repressed in cancer (7). We next tested *MAT1A* methylation status in the human transformed hepatic cell line HepG2, in which *MAT1A* is silenced. Our data indicate that hypermethylation of *MAT1A* gene accompanies loss of expression. This cellular model has allowed us to directly assess the role of cytosine methylation and histone acetylation on *MAT1A* expression regulation. We therefore treated HepG2 cells with 5-Aza-CdR, a demethylating agent well known for its ability to reactivate the expression

of genes silenced by *de novo* methylation (7, 35). In the presence of 5-Aza-CdR, *MAT1A* expression was induced after 48 h of treatment; this observation suggests that methylation may indeed play a role in the transcriptional regulation of this gene. A slower rate of cell growth accompanied 5-Aza-CdR-mediated induction of *MAT1A* expression (data not shown). Growth arrest of tumor cells has previously been observed in response to 5-Aza-CdR, and the reactivation of genes such as the cell cycle regulatory *p16* gene, among others, may explain the effect of this drug (35). However, it is worth mentioning that when *MAT1A* is transfected into HepG2 cells, slower rates of cell growth and DNA synthesis are observed (5). As mentioned above, recent findings have demonstrated that transcriptional repression associated with cytosine methylation relies to a significant extent on histone deacetylation (8, 9). These observations prompted us to test whether this mechanism could also participate in the silencing of *MAT1A* in the transformed HepG2 cells. Our findings indicate that treatment with the histone deacetylase inhibitor trichostatin A (26) for 24 h restored *MAT1A* expression in HepG2 cells. Taken together, these observations suggest that *MAT1A* silencing during transformation is mediated by *de novo* cytosine methylation and the recruitment of histone deacetylase, a situation in which a condensed and inactive chromatin state is favored. These results also support a role for *MAT1A* promoter methylation and histone acetylation in the maintenance of a tissue-specific expression profile, which may be progressively lost as the transition from a healthy to a diseased and, finally, neoplasic liver proceeds. FJ

This work was supported by grants SAF 98/0132, SAF 99/0038 from CICYT (Spain), Europharma and Knoll to J.M.M., grant PB97–1368 from DGICYT (Spain) to G.L.R. and L.F., grant 923/1998 from Gobierno de Navarra (Spain) to M.A.A., and NIH grant DK 51719 to S.C.L. L.T. was supported by the Generalitat Valenciana. M.V.C. is a fellow of the Universidad de Navarra. M.U.L. is a fellow of Fundación Renal Íñigo Álvarez de Toledo. We thank Estefanía Fernández for technical help and Dr. Luis Álvarez for critical reading of the manuscript.

REFERENCES

1. Mudd, S. H., and Cantoni, G. L. (1958) Activation of methionine for transmethylation. III. The methionine-activating enzyme of Baker's yeast. *J. Biol. Chem.* **231,** 481–49
2. Mato, J. M., Alvarez, L., Ortiz, P., and Pajares, M. A. (1997) S-Adenosylmethonine synthesis: molecular mechanisms and clinical implications. *Pharmacol. Ther.* **73,** 265–280
3. Gil, B., Casado, M., Pajares, M. A., Boscá, L., Mato, J. M., Martín-Sanz, P., and Alvarez, L. (1996) Differential expression pattern of S-adenosylmethionine synthetase isoenzymes during rat liver development. *Hepatology* **24,** 876–881
4. Cai, J., Sun, W., Hwang, J., Stain, S. C., and Lu, S. C. (1996) Changes in S-adenosylmethionine synthetase in human liver cancer: molecular characterization and significance. *Hepatology* **24,** 1090–1097
5. Cai, J., Mao, Z., Hwang, J., and Lu, S. C. (1998) Differential expression of methionine adenosyltransferase genes influences the rate of growth of human hepatocellular carcinoma cells. *Cancer Res.* **58,** 1444–1450
6. Razin, A., and Riggs, A. D. (1980) DNA methylation and gene function. *Science* **210,** 604–610
7. Szyf, M. (1996) The DNA methylation machinery as a target for anticancer therapy. *Pharmacol. Ther.* **70,** 1–37
8. Jones, P. L., Veenstra, G. J. C., Wade, P. A., Vermaak, D, Kass, S. U., Landsberger, N., Strouboulis, J., and Wolffe, A. P. (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nature (London) Genetics* **19,** 187–191
9. Nan, X., Ng, H .H., Johnson, C.A., Laherty, C. D., Turner, B. M., Eisenman, R. N., and Bird, A. (1998) Transcriptional repression by the methyl CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature (London)* **393,** 386–389
10. Ura, K., Kurumizaka, H., Dimitrov, S., Almouzni, G., and Wolffe, A. P. (1997) Histone acetylation: influence on transcription, nucleosome mobility and positioning and linker histone-dependent transcriptional repression. *EMBO J.* **16,** 2096–2107
11. Razin, A. (1998) CpG methylation, chromatin structure and gene silencing-a three-way connection. *EMBO J.* **17,** 4905–4908
12. Bestor, T. H. (1998) Methylation meets acetylation.*Nature (London)* 393, 311–312
13. Avila, M. A., Carretero, M. V., Rodriguez, E. N., and Mato, J. M. (1998) Regulation by hypoxia of methionine adenosyltransferase activity and gene expression in rat hepatocytes. *Gastroenterology* **114,** 364–371
14. Alvarez, L., Asunción, M., Corrales, F., Pajares, M. A., and Mato, J. M. (1991) Analysis of the 5′ noncoding region of the rat liver S-adenosylmethionine synthetase mRNA and comparison of the M_r deduced from the cDNA sequence and the purified enzyme. *FEBS Lett.* **290,** 142–146
15. Alvarez, L., Corrales, F., Martín-Duce, A., and Mato, J. M. (1993) Characterization of a full-length cDNA encoding human liver S-adenosylmethionine synthetase. Tissue-specific gene expression and mRNA levels in hepatopathies. *Biochem. J* **293,** 481–486
16. Velasco, J. A., Castro, R., Avila, M. A., Laborda, J., DiPaolo, J. A., Cansado, J., and Notario, V. (1994) *cph*, a novel oncogene which cooperates with H-ras in the transformation of NIH 3T3 fibroblasts. *Oncogene* **9**, 2065–2069
17. Carretero, M. V., Torres, L., Latasa, M. U., García-Trevijano, E. R., Prieto, J., Mato, J. M., and Avila, M. A. (1998) Transformed but not normal hepatocytes express UCP2. *FEBS Lett.* **439,** 55–58
18. Alvarez, L., Sánchez-Góngora, E., Mingorance, J., Pajares, M. A., and Mato, J. M. (1997) Characterization of rat liver-specific methionine adenosyltransferase gene promoter. *J. Biol. Chem.* **272,** 22875–22883
19. Corrales, F. J., Giménez, A., Alvarez, L., Caballería, J., Pajares, M. A., Andreu, H., Pares, A., Mato, J. M., and Rodés, J. (1992) S-Adenosylmethionine treatment prevents CCl_4-induced s-adenosylmethionine synthetase inactivation and attenuates liver injury. *Hepatology* **16,** 1022–1027
20. Tsao, J., Lin, H., Lackland, H., Tous, G., Wu, Y., and Stein, S. (1991) Internally standardized amino acid analysis for determining peptide/carrier protein coupling ratio. *Anal. Biochem.* **197,** 137–142
21. Crane-Robinson, C., Hebbes, T. R., Clayton, A. L., and Thorne, A. W. (1997) Chromosomal mapping of core histone acetylation by immunoselection. *Methods* **12,** 48–56
22. Counts, J. L., McClain, R. M., and Goodman, J. I. (1997) Comparison of effect of tumor promoter treatments on DNA methylation status and gene expression in B6C3F1 and C57BL/6 mouse liver and in B6C3F1 mouse liver tumors. *Mol. Carcinog.* **18,** 97–106
23. Tsuda, H., Hirohashi, S., Shimosato, Y., Terada, M., and Hasegawa, H. (1988) Clonal origin of atypical adenomatous hyperplasia of the liver and clonal identity with hepatocellular carcinoma. *Gastroenterology* **95,** 1664–1666
24. Yasui, H., Hino, O., Ohtake, K., Machinami, R., and Kitagawa, T. (1992) Clonal growth of hepatitis B virus-integrated hepatocytes in cirrhotic liver nodule. *Cancer Res.* **52,** 6810–6814
25. Zingg, J. M., and Jones, P. A. (1997) Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. *Carcinogenesis* **18,** 869–882

26. Yoshida, M., Kijima, M., Akita, M., and Beppu, T. (1990) Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. *J. Biol. Chem.* **265,** 17174–17179
27. Finkelstein, J. D. (1990) Methionine metabolism in mammals. *J. Nutr. Biochem.* **1,** 228–236
28. Clayton, A. L., Hebbes, T. R., Thorne, A. W., and Crane-Robinson, C. (1993) Histone acetylation and gene induction in human cells. *FEBS Lett.* **336,** 23–26
29. Eden, S., Hashimshony, T., Keshet, I., Cedar, H., and Thorne, A. W. (1998) DNA methylation models histone acetylation. *Nature (London)* **394,** 842
30. Hoffman, R. M. (1984) Altered methionine metabolism, DNA methylation and oncogene expression in carcinogenesis. *Biochim. Biophys. Acta.* **738,** 49–87
31. Varela-Moreiras, G., Alonso-Aperte, E., Rubio, M., Gassó, M., Deulofeu, R., Alvarez, L., Caballería, J., Rodés, J., and Mato, J. M. (1995) Carbon tetrachloride-induced hepatic injury is associated with global DNA hypomethylation and homocysteinemia: effect of S-adenosyl-L-methionine treatment. *Hepatology* **22,** 1310–1315
32. Sun, L., Hui, A. M., Kanai, Y., Sakamoto, M., and Hirohashi, S. (1997) Increased DNA methyltransferase expression is associated with an early stage of human hepatocarcinogenesis. *Jpn. J. Cancer Res.* **88,** 1165–1170
33. Kanai, Y., Hui, A., Sun, L., Ushijima, S., Sakamoto, M., Tsuda, H., and Hirohashi, S. (1999) DNA hypermethylation at the D17S5 locus and reduced *HIC-1* mRNA expression are associated with hepatocarcinogenesis. *Hepatology* **29,** 703–709
34. Huang, Z. Z., Mao, Z., Cai, J., and Lu, S. C. (1998) Changes in methionine adenosyltransferase during liver regeneration in the rat. *Am. J. Physiol.* **275,** G14–G21
35. Bender, C. M., Pao, M. M., and Jones, P. A. (1998) Inhibition of DNA methylation by 5-aza-2′-deoxycytidine suppresses the growth of human tumor cell lines. *Cancer Res.* **58,** 95–101

Received for publication April 20, 1999.
Revised for publication August 2, 1999.