Methionine Adenosyltransferase II β Subunit Gene Expression Provides a Proliferative Advantage in Human Hepatoma

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Background & Aims: **Of the 2 genes (*MAT1A, MAT2A*) encoding methionine adenosyltransferase, the enzyme that synthesizes S-adenosylmethionine, *MAT1A*, is expressed in liver, whereas *MAT2A* is expressed in extrahepatic tissues. In liver, *MAT2A* expression associates with growth, dedifferentiation, and cancer. Here, we identified the β subunit as a regulator of proliferation in human hepatoma cell lines. The β subunit has been cloned and shown to lower the K_m of methionine adenosyltransferase II α2 (the *MAT2A* product) for methionine and to render the enzyme more susceptible to S-adenosylmethionine inhibition.** *Methods:* **Methionine adenosyltransferase II α2 and β subunit expression was analyzed in human and rat liver and hepatoma cell lines and their interaction studied in HuH7 cells. β Subunit expression was up- and down-regulated in human hepatoma cell lines and the effect on DNA synthesis determined.** *Results:* **We found that β subunit is expressed in rat extrahepatic tissues but not in normal liver. In human liver, β subunit expression associates with cirrhosis and hepatoma. β Subunit is expressed in most (HepG2, PLC, and Hep3B) but not all (HuH7) hepatoma cell lines. Transfection of β subunit reduced S-adenosylmethionine content and stimulated DNA synthesis in HuH7 cells, whereas down-regulation of β subunit expression diminished DNA synthesis in HepG2. The interaction between methionine adenosyltransferase II α2 and β subunit was demonstrated in HuH7 cells.** *Conclusions:* **Our findings indicate that β subunit associates with cirrhosis and cancer providing a proliferative advantage in hepatoma cells through its interaction with methionine adenosyltransferase II α2 and down-regulation of S-adenosylmethionine levels.**

Methionine adenosyltransferase enzymes (MAT I, MAT II, and MAT III) have recently been implicated as playing a role in cirrhosis and cancer.[1–3] In mammals, 2 different genes, *MAT1A* and *MAT2A*, encode for 2 homologous MAT catalytic subunits, α1 and α2.[4–6] *MAT1A* is expressed mostly in the liver, and it encodes the α1 subunit found in 2 native MAT isoenzymes, which are either a dimer (MAT III) or tetramer (MAT I) of this single subunit.[6] *MAT2A* encodes for a catalytic subunit (α2) found in a native MAT isoenzyme (MAT II), which is widely distributed.[4,6] *MAT2A* and its gene product also predominate in the fetal liver and are progressively replaced by *MAT1A* during development.[7,8] *MAT1A* gene is specifically silenced by hypermethylation in human cirrhosis,[9] which leads to a marked reduction of S-adenosylmethionine synthesis.[10,11] In adult liver, increased expression of *MAT2A* is associated with rapid growth or dedifferentiation of the liver. Thus, a switch in the gene expression from *MAT1A* to *MAT2A* has been found in liver cancer,[9,12,13] from 12 to 24 hours after partial hepatectomy in the rat,[14] and after treatment with thioacetamide.[15] Using a cell line model that differs only in the type of MAT expressed, it has been demonstrated that the type of MAT expressed by the cell significantly influences the rate of cell growth.[16] The mechanism is likely via a change in the steady state S-adenosylmethinone content. This is because MAT isoenzymes differ in kinetic parameters and regulatory properties,[2] so a switch in MAT expression can affect the steady state S-adenosylmethionine content. MAT II has the lowest K_m for methionine but is tightly regulated by S-adenosylmethionine with an IC_{50} of 60 μmol/L, which is close to the normal intracellular hepatic S-adenosylmethionine concentration.[17–19] In contrast, S-adenosylmethionine has a minimal inhibitory effect on MAT I and stimulates MAT III.[19] Thus, the S-adenosylmethionine content in cells

that express only the MAT II isoform is relatively unaffected by fluctuations in methionine availability because of the negative feedback inhibition. In contrast, the rate of S-adenosylmethionine synthesis and S-adenosylmethionine level increased with increasing methionine availability in cells that express mostly MAT I/III.[18–20] Consistent with this, cells that express *MAT1A* have much higher levels of S-adenosylmethionine but lower rates of cell growth than cells that express *MAT2A.*[16] A caveat to this is the recently described regulatory β subunit that is associated with MAT II in lymphocytes.[21–23] The β subunit was shown to lower the K_m of MAT II for methionine and render the enzyme more susceptible to feedback inhibition by S-adenosylmethionine.[22] Whether the β subunit regulates MAT II similarly in other cells is unknown. The biologic function of β subunit is also unknown.

Recently, we showed the importance of *MAT1A* in maintaining a normal liver phenotype using the *MAT1A* null mice.[1] Three-month-old MATO mice express *MAT2A,* have reduced hepatic S-adenosylmethionine and GSH levels, hyperplasia, spontaneous oxidative stress, increased cytochrome P4502E1 and uncoupling protein 2 expression, are prone to fatty liver induced by a choline-deficient diet, and are more susceptible to carbon tetrachloride (CCl_4)-induced liver injury.[1,3] On a normal diet, MATO mice develop non-alcoholic steatohepatitis by 8 months and hepatocellular carcinoma (HCC) by 18 months,[1,3] suggesting that *MAT1A* can function as a tumor suppressor gene in the liver. Our recent studies have also revealed that S-adenosylmethionine plays a central role in regulating hepatocyte cell growth, differentiation, and death. S-adenosylmethionine blocks the mitogenic effect of hepatocyte growth factor and inhibits cell-cycle progression.[24] S-adenosylmethionine is antiapoptotic in normal hepatocytes but proapoptotic in hepatoma cells.[25] Taken together, these data suggest that a normal hepatic S-adenosylmethionine level favors the differentiated state of hepatocytes, blocks apoptosis, and prevents cell proliferation. A fall in hepatic S-adenosylmethionine level facilitates liver regeneration but, if this conditions persists, predisposes to liver injury, steatohepatitis, and, finally, to the development of HCC.

In this study, we evaluated the β subunit as a candidate regulator of growth in human hepatoma cell lines through its interaction with MAT II and the downregulation of S-adenosylmethionine levels. We report here that the β subunit gene was frequently expressed in human cirrhotic livers and HCC and in some (HepG2, PLC, and Hep3B) but not all (HuH7) human hepatoma cell lines. In addition, transfection of the β subunit reduced S-adenosylmethionine content and stimulated DNA synthesis in HuH7 cells, whereas inhibition of β subunit expression in HepG2 cells had the reversed phenotype.

Materials and Methods

Cloning and Expression of MAT II α2 and β Subunits

Total human resting peripheral blood lymphocyte RNA was isolated by the guanidinium thiocyanate method[26] and transcribed to cDNA using the Superscript preamplification system (Invitrogen, Barcelona, Spain). The cDNA was amplified using Taq Long Plus enzyme (Stratagene, La Joya, CA) and the β sense 5′-CAGCAAATGGGTATGGT-GGGGCGGGAGAAAG-3′ and antisense 5′-CTAATGAAA-GACCGTTTGTCTCCATC-3′ primers, derived from the human MAT II β subunit cDNA sequence.[23] A T7 sense 5′-GGATGGCTAGCATGACTGGTGGACAGCAAATGGG-3′ primer was used to incorporate a T7 tag in the 5′ of the MAT II β subunit. The MAT II α2 subunit was cloned from the same source using a 5′-AGCATGGGCCACCATCACCAT-CACCATATGAACGGACAGCTCAAC-3′ sense primer, which incorporated the His tag epitope, and an antisense 5′-TCAATATTTAAGCTTTTTGGGCA-3′ primer derived from the human MAT II α2 subunit cDNA.[4] The PCR products were purified using QIAquick Gel Extraction kit (Qiagen, Valencia, CA) and ligated into the expression vector pCR3.1-Uni, according to the supplier's instructions (Eukaryotic TA cloning Kit Unidirectional, Invitrogen). The cloned products were sequenced and their identities confirmed as the full length MAT II α2 and β subunit coding sequences.

Cell Culture and Transfections

The human HepG2, PLC, HuH7, and Hep3B HCC-derived cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were mantained in Eagle's minimum essential medium supplemented with 5% fetal calf serum, 2 mmol/L glutamine, 100 U/mL penicilin, and 100 μg/mL streptomycin (all from Life Technologies/Invitrogen) at 37°C in a humid atmosphere of 5% CO_2 in air.

Plasmids PCR3.1 MAT IIα2 and PCR3.1 MAT II β, containing the MAT II α2 and β subunits, respectively, or the empty vector (PCR3.1 CAT) were independently prepared in large quantities by using the Qiagen plasmid Kit (Qiagen) according to the supplier's instructions. Twelve μg of each plasmid were used to transfect HuH7 cells grown at 70% confluency in 60-mm tissue culture plates using a Lipofectamin Reagent Transfection System (Life Technologies/Invitrogen). Transfection efficiency was always around 50%, as determined by immunofluorescent staining of transfected HuH7 cells (see below).

HepG2 cells were seeded at a density of 10^4 cells/well in 96-well plates in Dulbecco's modified Eagle's medium

(DMEM) supplemented with 10% fetal calf serum and grown for 24 hours. Transfections were performed with the following oligonucleotides: 5′-ACCATGCCCGCCGTCTTCAC-3′ (antisense to β subunit mRNA), and a second oligonucleotide containing the same bases but in a random order ("scrambled" oligonucleotide) was used for control experiments (5′-GCTC-CATCCGACCTGATCC-3′). These oligonucleotides were synthesized by Sigma-Genosys, and the first 2, 5′ and 3′ internucleotide linkages were modified as phosphorothioates. The transfection mixture contained 200 nmol/L (final concentration) of the above described oligonucleotides and 0.8 μL of Oligofectamine (Invitrogen) in Opti-MEM I medium (Invitrogen) (100 μL final volume). After 4 hours incubation in this mixture, 50 μL of DMEM supplemented with 15% fetal calf serum were added per well, and incubations were continued for another 24 hours before DNA synthesis was measured as described below.

RNA Isolation and Northern Hybridization Analysis

Total RNA was isolated by the guanidinium thyocianate method as described.[9,26] RNA concentration was determined spectrophotometrically before use, and the integrity was checked by electrophoresis with subsequent ethidium bromide staining. Electrophoresis of RNA and gel blotting were carried out as described.[24] Northern hybridization analysis was performed on total RNA using standard procedures as described by Avila et al.[27] All probes were labeled with [^{32}P]dCTP using a random primer kit (RediPrime DNA Labeling System, Amersham Bisociences, Uppsala, Sweden). To ensure equal loading of RNA samples, membranes were also hybridized with ^{32}P-labeled 18S rRNA cDNA probe.

Immunoblot Analysis

Transfected cells were scraped and lysed by sonication in phosphate-buffered saline solution containing 1% of protease inhibitor cocktail (Sigma, St. Louis, MO). Equal amounts of protein (20 μg) were subjected to 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred to nitrocellulose membranes as described.[27] Blots were incubated with mouse anti-T7 tag (Novagen, Madison, WI) or anti-His tag antibodies (Invitrogen) diluted 1:500 for 1 hour. The blots were developed with secondary anti-mouse antibodies conjugated to horseradish peroxidase (Invitrogen) and the luminol-chemiluminescence reagent (ECL, Amersham Biosciences). The processed blots were exposed to x-ray film, and the autoradiograms were analyzed.

MAT II α2 and β Subunits Interaction

The association between the His-tagged α2 subunit and the T7-tagged β subunit was analyzed by Protein A immunoprecipitation (Seize X Protein A Immunoprecipitation Kit; Pierce, Rockford, IL). HuH7 cells were transfected with PCR3.1 MAT II α2, PCR3.1 MAT II β, or both; controls were transfected with PCR3.1 CAT. Twenty-four hours after trans-

fection, cells (5 × 10⁶) were lysed in 500 microliters of lysis buffer (M-PER Reagent, Pierce). The samples were centrifuged at 13,000 rpm for 10 minutes, and the supernatants were diluted 1:1 with binding buffer and loaded onto protein A-agarose columns cross-linked with anti-His antibodies (Seize X Protein A Immmunoprecipitation Kit, Pierce). The columns were washed with quenching/wash buffer 4 times, and the proteins were eluted with elution buffer, pH 2.8, following manufacturer's instructions. Proteins were analyzed by SDS-PAGE as described above. After blotting onto nitrocellulose membranes, blots were probed with anti-His tag and anti-T7 tag antibodies.

Isolation and Culture of Rat Hepatocytes

Liver cells were isolated from male Wistar rats (200–250 g) by collagenase (Invitrogen) perfusion as described previously.[27] Cells were plated onto 60-mm collagen-coated culture dishes (type I collagen from rat tail; Collaborative Biomedical Products, Bedford, MA) at a density of 3 × 10⁶ cells per dish. Cultures were maintained in minimal essential medium (Invitrogen), supplemented with 10% fetal calf serum (Invitrogen), essential amino acids (Invitrogen), 2 mmol/L glutamine, 50 mmol/L penicillin, and 50 mg/mL streptomycin sulfate (Invitrogen). After 2-hour incubation, the culture medium was removed, and cultures were again given the same medium with 2.5% fetal calf serum. Cell viability was measured by trypan blue exclusion, and no significant differences were observed at any time between controls and any of the different treatments performed in this study. Animals were treated humanely, and this study was approved by our institution's Committee for Animal Experimentation.

Assay of DNA Synthesis

For DNA synthesis experiments, HuH7 were plated at a density of 5 × 10⁴ cells/well in a 96-well plate and transfected with PCR3.1 MAT II β or PCR3.1 CAT as described above. Twenty-four hours posttransfection, the medium was replaced by fresh minimal essential medium without fetal bovine serum, containing increasing concentrations of L-methionine. A 6-hour pulse of [³H]thymidine (25 Ci/mmol, Amersham Biosciences) (1 μCi/well) started 6 hours after L-methionine addition. Cells were harvested, and thymidine incorporation was determined in a scintillation counter. HepG2 cells were transfected with the antisense oligonucleotide to β subunit mRNA and control oligonucleotide as described above, and DNA synthesis was assayed following the same protocol used for HuH7 cells.

Determination of S-Adenosylmethionine and S-Adenosylethionine Content in HuH7 Cells

For the determination of S-adenosylmethionine and S-adenosylethionine (SAE) in HuH7, cultures were washed with phosphate-buffered saline, and, subsequently cells (5 × 10⁶) were lysed and deproteinized as described.[24] S-adenosyl-

methionine and SAE contents were determined by reverse-phase, high-performance liquid chromatography as described.[27]

Cell Staining

The HuH7 cells were transfected with PCR3.1 MAT II β, and 24 hours after transfection, cells were fixed in iced-methanol as described.[28] The anti-T7 antibody was used at 1:50 dilution, the secondary antibody (anti-mouse IgG fluorescein-conjugated; Sigma) at 1:200 dilution.

Patients

We have studied a group of 25 patients (16 males and 9 females; mean age, 54 ± 8.5 years) with cirrhosis of different etiology (12 hepatitis C virus [HCV]-induced cirrhosis, 12 alcoholic cirrhosis, 1 hepatitis B virus [HBV]-induced cirrhosis). The control group for the cirrhotic patients consisted of 16 subjects in whom a cholecystectomy was performed for the treatment of a symptomatic cholelithiasis and who consented to a liver biopsy during the surgical procedure. Liver samples were immediately frozen and kept at $-80°C$ until processed. In the control group, both the liver function tests and the liver biopsy were normal. Cancerous liver tissues were obtained from 16 liver patients undergoing surgical resection for primary HCC. The contamination of HCC samples with noncancerous tissue was less than 5% as determined by histopathology. These tissues were immediately frozen in liquid nitrogen for subsequent isolation of RNA. Written informed consent was obtained from each patient. This study was approved by the Human Research Review Committee of the University of Navarra, Hospital Clínic of Barcelona, and Hospital Príncipe de Asturias, Alcalá de Henares (Spain).

RT-PCR Studies

Total RNA was extracted from liver biopsy specimens as previously described.[9] Aliquots of 2 μg of RNA were reverse transcribed using the M-MLV Reverse Transcriptase (Invitrogen) and subsequently amplified by PCR using the BioTaq DNA Polymerase (Bioline, London, United Kingdom). The primers used for the MAT II β subunit were 5′-TGGAGGAGGAAGTTAACATCCC-3′ and 5′-GGCCA-CATCTTTGACATGTGTG-3′. To avoid amplification of genomic DNA, which could contaminate our RNA preparations, these primers were designed flanking intronic sequences. Eighteen S rRNA was simultaneously amplified using the Alternate 18S Internal Standards primers set (Ambion, Austin, TX) and served as an internal loading control. Reactions were resolved in a 2% agarose gel, stained with ethidium bromide, and quantitated using the Molecular Analyst software (Bio-Rad, Hercules, CA).

Statistics

Data are the means $\pm$ SEM of at least 3 independent experiments. Statistical significance was estimated with Student t test. A P value of < 0.05 was considered significant.

Results

β Subunit Is Expressed in Extrahepatic Tissues but Not in Normal Adult Liver

Because the β subunit has been purified in association with MAT II α2,[21–23] we first determined the expression of β subunit in several rat tissues that express only *MAT2A* spleen, heart, and lung and in normal adult rat liver, which expresses *MAT1A* and also small amounts of *MAT2A*. As shown in Figure 1, β subunit gene was expressed in all extrahepatic tissues examined, but, in normal adult liver, its expression was very weak or undetectable.

β Subunit Is Frequently Expressed in Cirrhosis, in Hepatocellular Carcinoma, in Hepatoma Cell Lines, and in Culture Hepatocytes

We have previously demonstrated that *MAT1A* promoter is methylated and silenced both in cirrhosis and in HCC[9,29] and that *MAT2A* expression is markedly induced in HCC.[9,16] We therefore examined the expression of β subunit in 16 normal and 25 cirrhotic human livers and in 16 human HCC. According to the expression levels of β subunit, samples were distributed in 3 groups: group 1: undetectable levels, group 2: low levels, and group 3: high levels of expression. Only 4 normal livers (25%) showed small but significant expression of β subunit. In cirrhosis, 21 of 25 liver samples (84%) expressed β subunit (48% in group 2 and 36% in group 3). No correlation could be established between the different etiologies of cirrhosis and the expression of β subunit. All HCC samples expressed β subunit (Figure 2*A*). As a rule, expression of β subunit was more intense in HCC than in cirrhosis (Figure 2*A*). Expression of β
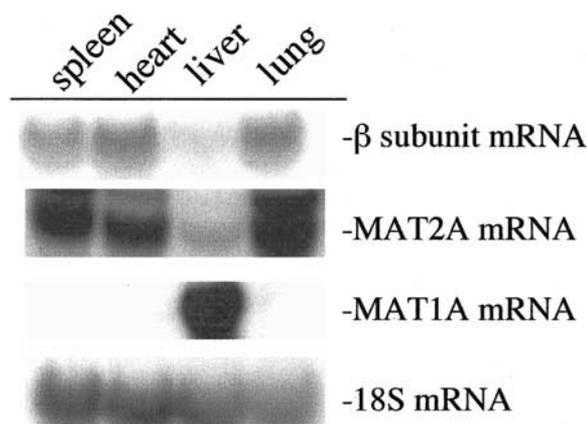

Figure 1. Expression of β subunit, *MAT2A* and *MAT1A* in rat tissues as analyzed by Northern blotting. Hybridization with an 18S rRNA probe was carried out as loading control.

subunit was also observed in diethylnitrosamine-induced HCC in rats (not shown). Moreover, most (HepG2, PLC, and H3B) but not all (HuH7) human hepatoma cell lines examined expressed β subunit (Figure 2B). Because *MAT2A* is induced in culture hepatocytes and *MAT1A* mRNA levels decrease,[30] a situation reminiscent of that found in fetal and regenerating liver and in HCC, we also examined β subunit expression in hepatocytes in culture. As shown in Figure 3, *MAT2A* expression in culture hepatocytes is accompanied by a marked increase in β subunit expression. Thus, the β subunit is frequently expressed in human cirrhosis, in HCC, in hepatoma cell lines, and in culture hepatocytes.

Transfection of β Subunit Reduced S-Adenosylmethionine Content and Stimulated DNA Synthesis in HuH7 Cells

The high frequency of β subunit expression observed in HCC and hepatoma cell lines suggested that expression of β subunit might confer a selective advantage. Because a variety of data indicate that S-adenosylmethionine inhibits the rate of growth of HCC[31,32] and hepatoma cell lines,[16] and the β subunit has been shown to render MAT II more susceptible to feedback inhibition by S-adenosylmethionine,[22] we examined the effect
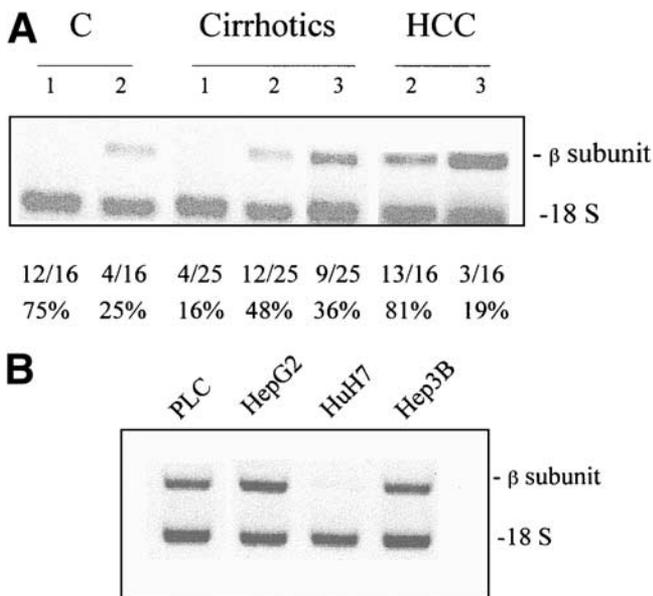

Figure 2. (*A*) Expression of β subunit in the liver of control (C), cirrhotic patients, and in hepatocarcinoma (HCC) samples. Subjects were divided into 3 groups according to the levels of β subunit expression: group 1, subjects with very low or nondetectable expression; group 2, subjects with a moderate expression; group 3, subjects with high expression. The number of subjects per group is indicated for each group analyzed. Representative RT-PCR reactions are shown. 18S ribosomal RNA was used as internal control. (*B*) Expression of β subunit gene in human hepatocarcinoma cell lines as analyzed by quantitative RT-PCR. 18S ribosomal RNA was used as internal control.
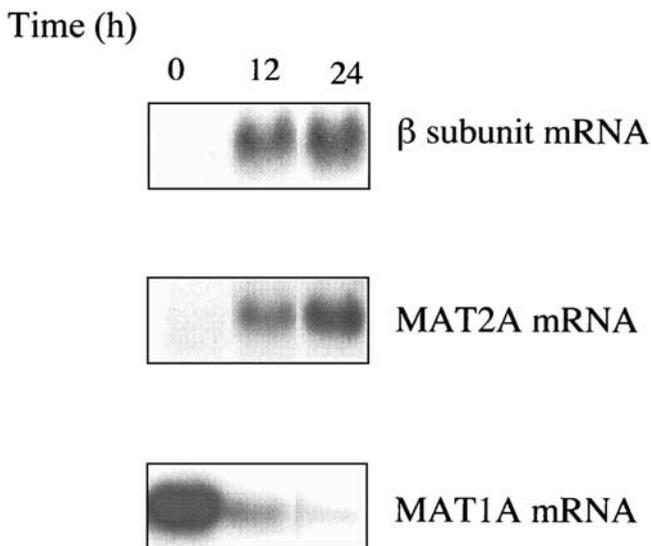

Figure 3. β Subunits *MAT2A* and *MAT1A* expression levels in cultured rat hepatocytes. Rat hepatocytes were cultured as described in Materials and Methods. β subunits *MAT2A* and *MAT1A* mRNA levels were evaluated by Northern blotting at the onset of cultures (time = 0) and after 12 and 24 hours of incubation. Representative blots are shown.

of β subunit transfection on S-adenosylmethionine content and DNA synthesis in HuH7 cells. Transient transfection showed that HuH7 cells were well able to express exogenous α2 and β subunits, as determined by Western blot (Figure 4A and B). Immunofluorescent detection showed that the expression of β subunit was cytosolic (Figure 4C). S-adenosylmethionine content increased less with increasing methionine availability in HuH7 cells that express β subunit than in control cells (Figure 5A). To exclude the possibility that reduced S-adenosylmethionine content in HuH 7 cells transfected with β subunit was due to an increased utilization of S-adenosylmethionine, cells were incubated with various concentrations of ethionine and the accumulation of SAE determined. Ethionine is an analog of methionine that is utilized by MAT to form SAE, a nonmetabolizable analog of S-adenosylmethionine that accumulates into the cells.[20] SAE content increased less with increasing ethionine availability in HuH7 cells that express β subunit than in control cells (Figure 5B). Finally, transfection with β subunit in HuH7 cells enhanced serum-induced DNA synthesis in a methionine-dependent manner (Figure 5C). This effect was evident at 1000 μmol/L L-methionine, a concentration at which β subunit-expressing HuH7 cells had significantly reduced S-adenosylmethionine levels as compared with untransfected controls. The significance of this effect (1.5-fold increase with respect controls) is more meaningful when we consider that the average transfection efficiency was about 50% of the cells.
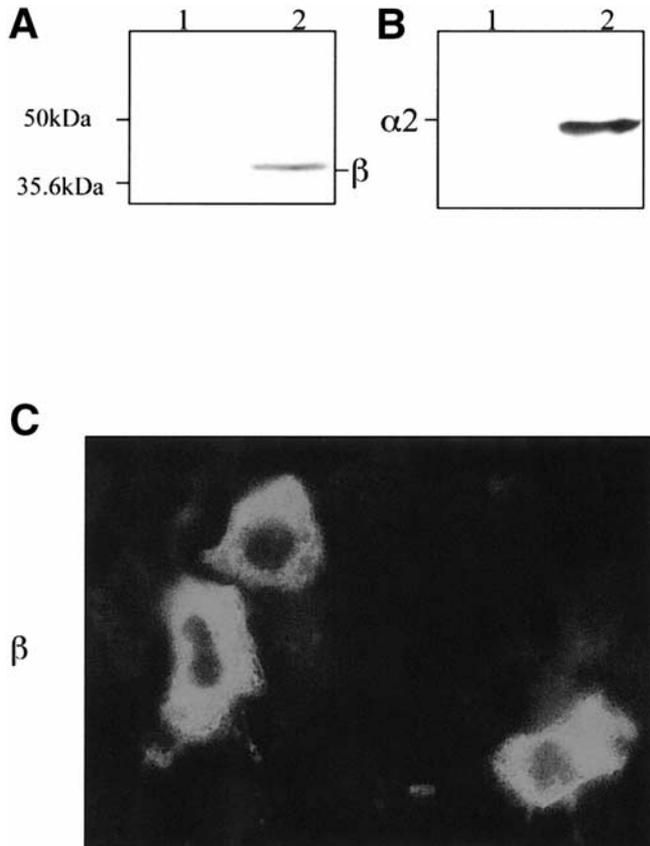
Figure 4. Expression of MAT II α2 and β subunits after transient transfection of HuH7 cells. (*A*) Western blot of total lysates from HuH7 cells that were transfected with the PCR3.1 CAT vector (*lane 1*) or with the PCR3.1 MAT II β vector harboring the β subunit cDNA with a T7 tag (*lane 2*). (*B*) Western blot of total lysates from HuH7 cells that were transfected with the PCR3.1 CAT vector (*lane 1*) or with the PCR3.1 MAT II α2 vector harboring the MAT II α2 subunit cDNA with a poly-His tag (*lane 2*). (*C*) Immunofluorescent detection of β subunit in transiently transfected HuH7 cells. Twenty-four hours after transfection, cells were fixed with ice-cold methanol, permeabilized, and labeled with anti-T7 tag mouse monoclonal antibodies. Immune complexes were detected by incubation with a fluorescein-conjugated anti-mouse IgG (original magnification 400×).

Figure 5. Effect of β subunit expression on S-adenosylmethionine levels and SAE and serum-induced DNA synthesis in transiently transfected HuH7 cells. (*A*) HuH7 cells were transfected with PCR3.1 CAT (*controls, open bars*) or PCR3.1 MAT II β (*solid bars*), and S-adenosylmethionine levels were determined after 6 hours incubation in the presence of different concentrations of L-methionine. *$P < 0.05$ with respect to control cells incubated with the same concentration of L-methionine. (*B*) The same experiment as above was performed, but L-methionine was substituted by L-ethionine. *$P < 0.05$ with respect to control cells incubated with the same concentrations of L-ethionine. (*C*) HuH7 cells were transfected with either PCR3.1 CAT or PCR3.1 MAT II β. Twenty-four hours after transfection, cells were incubated for 6 hours in serum-free medium with increasing concentrations of L-methionine; subsequently, cultures were given a 6-hour pulse with [³H]thymidine and then harvested, and thymidine incorporation was determined in a scintillation counter. Values represent fold-change vs. their respective controls (PCR3.1 CAT-transfected cells) for each L-methionine concentration. *$P < 0.05$ with respect to control (PCR3.1 CAT) transfections. All experiments were performed at least 3 times in duplicate.

The relevance of β subunit expression in the proliferation of human hepatoma cells was also demonstrated in HepG2 cells. In this cell line, which as shown in Figure 2B expresses β subunit, we have inhibited the expression of this gene using an antisense oligonucleotide (60% reduction in β subunit mRNA levels; Figure 6A) and measured serum-induced DNA synthesis under different concentrations of L-methionine in the culture medium. As shown in Figure 6B, the specific inhibition of β subunit expression in HepG2 cells resulted in reduced DNA synthesis when cells were incubated under increasing concentrations of L-methionine. Taken together, these findings indicate that β subunit expression indeed provides a growth advantage to human hepatoma cells.
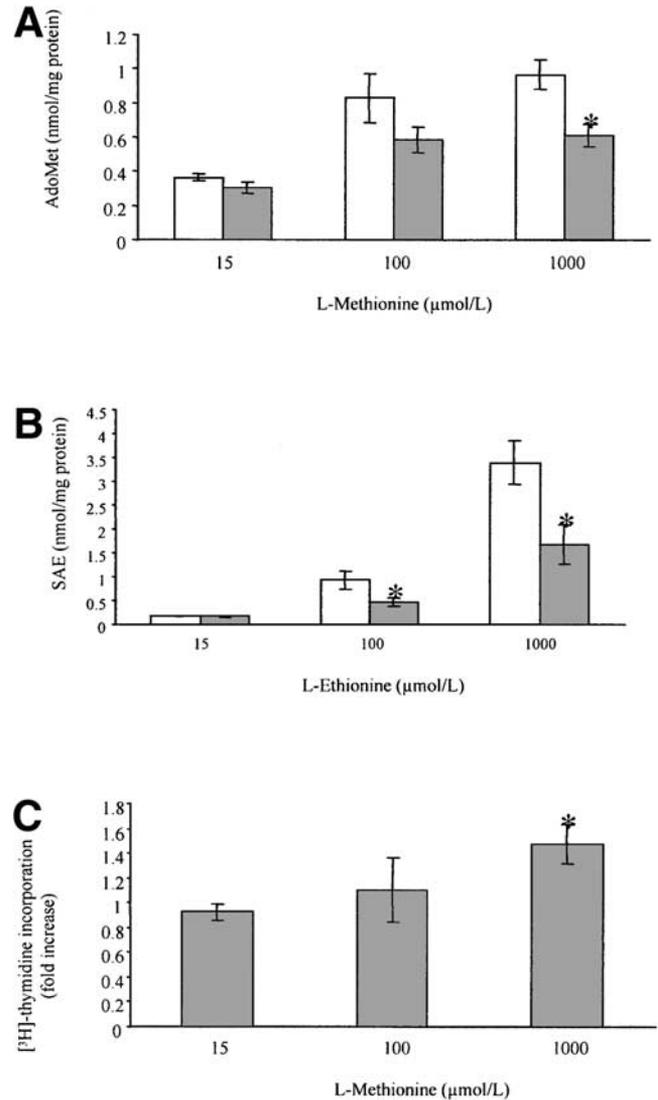
β Subunit Interacts With the α2 Subunit of MAT II in HuH7

To demonstrate that the α2 subunit of MAT II and the β subunit interact, HuH7 cells were cotransfected with MAT II α2 and β subunits. After transfection, cells were disrupted, and the cell extract passed through a column containing a covalently cross-linked antibody that recognized a poly-His epitope tag attached to the α2 subunit in the expression vector. After washing, the proteins retained in the column were eluted and the presence of the β subunit associated to the α2 subunit determined by Western analysis for the T7 epitope tag attached to the β subunit in the expression vector (Figure 7). In agreement with previous findings in COS cells,[22,23] these results indicate that the α2 subunit of MAT II and the β subunit interact in HuH7 cells.
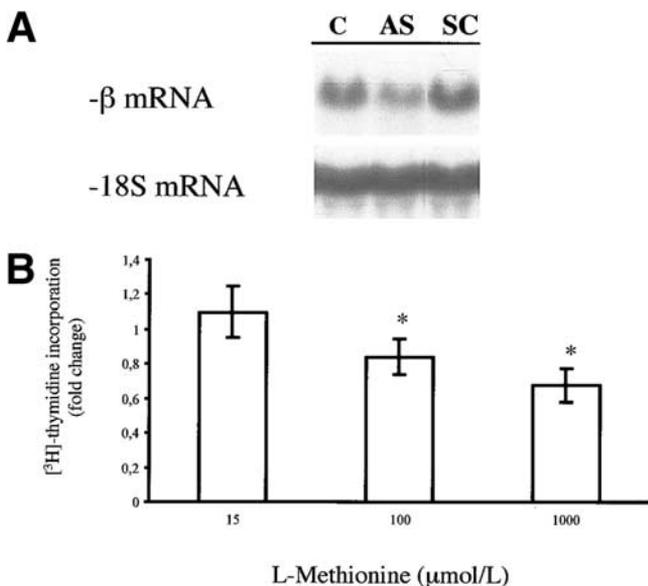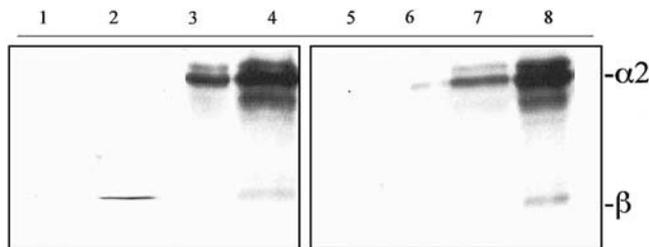
Figure 7. MAT II α2 and β subunits interaction. Cellular protein extracts were loaded onto a protein A agarose column cross-linked with anti-His antibody. The proteins were eluted with elution buffer pH 2.8. Samples were analyzed by SDS-PAGE and transblotted onto nitrocellulose membranes. Blots were probed with anti-His tag antibodies (for MAT II α2) and anti-T7 tag antibodies (for MAT IIβ). (*Left panel*) Total cellular protein extracts from PCR3.1 CAT (*lane 1*), PCR 3.1 MAT II β (*lane 2*), PCR3.1 MAT II α2 (*lane 3*), and α2/β transfected HuH7 cells (*lane 4*) simultaneously probed with anti-His and anti-T7 tags antibodies. (*Right panel*) Proteins eluted from the protein A agarose column cross-linked with anti-His tag antibodies from the following: PCR 3.1CAT (*lane 5*), PCR 3.1 MAT II β (*lane 6*), PCR3.1 MAT II α2 (*lane 7*), and α2/β (*lane 8*) transfected HuH7 cells. Blot was probed with anti-His and anti-T7 tags antibodies simultaneously. This experiment was performed 3 times with equivalent results; representative blots are shown. The higher amount of α2 in lanes 4 and 8 could represent an increased stability of the protein when bound to β.

Figure 6. Inhibition of β subunit gene expression in HepG2 cells results in reduced DNA synthesis. (*A*) HepG2 cells were cultured in 96-well plates and transfected with 200 nmol/L of an antisense oligonucleotide to β subunit mRNA or a control oligonucleotide as described in the Materials and Methods section. β subunit mRNA expression was examined 24 hours after the transfections by Northern blotting in nontransfected control cells (C), antisense-transfected cells (AS), and cells transfected with a control "scrambled" oligonucleotide (SC). Blots were probed with an 18S rRNA probe as loading control. This is a representative blot of 3 independent experiments. (*B*) HepG2 cells were transfected with an antisense oligonucleotide to β subunit mRNA or a control "scrambled" oligonucleotide. Twenty-four hours after transfection, cells were incubated for 6 hours in serum-free medium with increasing concentrations of L-methionine; subsequently, cultures were given a 6-hour pulse with [^{3}H]thymidine and then harvested, and thymidine incorporation was determined in a scintillation counter. Values represent fold-change vs. their respective controls ("scrambled" oligonucleotide-transfected cells) for each L-methionine concentration. *$P < 0.05$ with respect to control ("scrambled" oligonucleotide) transfections. All experiments were performed at least 3 times in duplicate.

Discussion

In this study, we have identified MAT II β subunit as a protein that confers a growth advantage to human hepatoma cells. β subunit was expressed in all rat extrahepatic tissues examined, but, in normal adult rat liver, its expression was very weak or undetectable. β Subunit expression was detected in all primary HCC, suggesting that β subunit expression is a common event in liver cancer. Consistently, β subunit expression was detected in 3 of 4 human hepatoma cell lines. β subunit expression was also detected in most human cirrhosis (84%) but only in 4 of 16 human normal adult livers (25%), indicating that induced expression of hepatic β subunit is often associated with human liver dysfunction. Expression of β subunit was also induced in hepatocytes in culture, suggesting that expression of this gene is associated with liver dedifferentiation and growth. Moreover, transfection with β subunit enhanced serum-stimulated DNA synthesis in HuH7 cells. The involvement of β subunit in the control of DNA synthesis was further demonstrated in the human hepatoma cell line HepG2, in which the β subunit gene is expressed. In this cell line, we observed that, when the expression of the β subunit gene was down-regulated using an antisense oligonucleotide, DNA synthesis was reduced at increasing concentrations of L-methionine, as compared with cells transfected with a control oligonucleotide.

We have also demonstrated that the β subunit is a cytosolic protein that interacts with the α2 subunit of MAT II in HuH7 hepatoma cells. The β subunit has been shown to lower the K_m of purified MAT II α2 for methionine and renders the enzyme more susceptible to feedback inhibition by S-adenosylmethionine.[22] Our results indicate that transfection with β subunit reduced the cellular content of S-adenosylmethionine in HuH7 cells, which agrees with the negative feedback inhibition that the β subunit exerts on MAT II α2 activity. This effect of β subunit expression on S-adenosylmethionine levels in HuH7 cells was accompanied by increased DNA synthesis when cells were induced to proliferate by serum treatment. There is abundant evidence indicating that S-adenosylmethionine has a growth modulatory effect in liver, HCC, and hepatoma cell lines. This has been observed in rat liver after partial hepatectomy (PH), in which S-adenosylmethionine levels drastically decrease shortly after the intervention, coinciding with the onset of DNA synthesis and the induction of early response genes.[14] When this decrease in S-adenosylmethionine after PH was prevented by the addition of S-adenosylmethionine, hepatocyte DNA synthesis was inhibited.[33,34] Similarly, addition of S-adenosylmethionine inhibits the mitogenic activity of hepatocyte growth factor (HGF) in hepatocytes in culture[30,35] and prevents the growth of rat HCC[31,32] and of hepatoma cell lines.[16] In HuH7 cells differing only in the type of MAT gene that is expressed, *MAT2A* expression associates with more rapid cell growth (about 1.5-fold increase) and lower S-adenosylmethionine content (about 2-fold reduction), whereas the opposite is observed for *MAT1A.*[16] Moreover, disruption of *MAT1A* in mice induces the expression of hepatic *MAT2A* and of a variety of genes involved in cell proliferation, a reduction in S-adenosylmethionine content, liver hyperplasia, and, finally, HCC.[1,3] In hepatocytes, S-adenosylmethionine inhibits HGF-dependent induction of cyclin D1 and D2 expression without affecting the activation of extracellular signal-regulated protein kinase (ERK) by HGF,[24] suggesting that S-adenosylmethionine is a negative modulator of cell cycle progression. Our findings indicate that the β subunit provides a proliferative advantage in human hepatoma cell lines and that this effect could be mediated through its interaction with MAT II α2 and the downregulation of S-adenosylmethionine levels. This is the first identification of a candidate biologic function for this protein. Whether the β subunit regulates DNA synthesis similarly in other tumor and nontumor cells is unknown. Future work may establish the presumptive value as a prognosis marker that we suggest for the β subunit in HCC.

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