L-Methionine Availability Regulates Expression of the Methionine Adenosyltransferase 2A Gene in Human Hepatocarcinoma Cells

ROLE OF S-ADENOSYLMETHIONINE*

Maria L. Martínez-Chantar‡§¶, M. Ujue Latasa‡§‖, Marta Varela-Rey¶, Shelly C. Lu,
Elena R. García-Trevijano‡, José M. Mato‡ ‡‡, and Matías A. Avila‡ ‡‡§§**

*From the ‡Laboratorio de Proteómica, Genómica y Bioinformática, and División de Hepatología y Terapia Génica, Universidad de Navarra, Facultad de Medicina, 31008 Pamplona, Spain and the **División of Gastroenterology and Liver Diseases, USC Research Center for Liver Diseases, USC-UCLA Research Center for Alcoholic Liver and Pancreatic Diseases, Keck School of Medicine, University of Southern California, Los Angeles, California 90033*

In mammals, methionine adenosyltransferase (MAT), the enzyme responsible for S-adenosylmethionine (AdoMet) synthesis, is encoded by two genes, *MAT1A* and *MAT2A*. In liver, *MAT1A* expression is associated with high AdoMet levels and a differentiated phenotype, whereas *MAT2A* expression is associated with lower AdoMet levels and a dedifferentiated phenotype. In the current study, we examined regulation of *MAT2A* gene expression by L-methionine availability using HepG2 cells. In L-methionine-deficient cells, *MAT2A* gene expression is rapidly induced, and methionine adenosyltransferase activity is increased. Restoration of L-methionine rapidly down-regulates *MAT2A* mRNA levels; for this effect, L-methionine needs to be converted into AdoMet. This novel action of AdoMet is not mediated through a methyl transfer reaction. *MAT2A* gene expression was also regulated by 5'-methylthioadenosine, but this was dependent on 5'-methylthioadenosine conversion to methionine through the salvage pathway. The transcription rate of the *MAT2A* gene remained unchanged during L-methionine starvation; however, its mRNA half-life was significantly increased (from 100 min to more than 3 h). The effect of L-methionine withdrawal on *MAT2A* mRNA stabilization requires both gene transcription and protein synthesis. We conclude that *MAT2A* gene expression is modulated as an adaptive response of the cell to L-methionine availability through its conversion to AdoMet.

Regulation of cellular functions by nutrients, including the control of gene expression, is well documented in prokaryotes and lower eukaryotes. In mammals, these responses have also been observed, and lipids and carbohydrates, among other dietary constituents, have been shown to be important regulators of gene expression (1–3). Mammalian cells can also adapt to dietary or pathological fluctuations in amino acid availability (4, 5). Amino acid limitation elicits a series of cellular responses, among which the up-regulation of amino acid transport systems was one of the first to be recognized (reviewed in Refs. 6 and 7). Subsequently, a number of genes have been shown to specifically change their expression levels following amino acid deprivation, although the underlying mechanisms are not completely known (4, 5). Under such limiting conditions, amino acids play a signaling role in the cell, conveying regulatory messages to the transcriptional machinery and affecting the turnover of specific mRNAs and proteins (5, 8). These processes are beginning to be understood with work carried out mainly in cultured cells, given the complexity of the response to amino acid deprivation in intact animals, which includes nutrient-induced changes in hormone levels.

L-Methionine is an essential amino acid required for protein synthesis and participates, together with ATP, in the formation of S-adenosylmethionine (AdoMet),[1] the principal biological methyl donor (9–11). Upon transfer of its activated methyl group to many different acceptor molecules, AdoMet is converted into S-adenosylhomocysteine (AdoHcy), a molecule that can be further hydrolyzed to adenosine and homocysteine in a reversible reaction (10). Homocysteine in turn may be converted into cystathionine or remethylated back to L-methionine, depending on the cellular needs for this amino acid (10). Mammalian cells have developed a series of adaptive mechanisms that respond to fluctuations in L-methionine availability, which have been best characterized in the liver and involve the modulation of enzymatic activities implicated in methionine metabolism (10, 12). Some of these mechanisms, including the capacity to remethylate homocysteine, are impaired in the transformed cell, giving rise to the condition of "methionine dependence" in cancer (reviewed in Ref. 13).

The first step in L-methionine metabolism, the synthesis of

---

[1] The abbreviations used are: AdoMet, S-adenosylmethionine; ActD, actinomycin D; AdoHcy, S-adenosylhomocysteine; C³-Ado, 3-deazaadenosine; C³-AdoHcy, S-3-deaza-adenosylhomocysteine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KRB, Krebs-Ringer bicarbonate; MAT, methionine adenosyltransferase; MEM, minimum Eagle's medium; MTA, 5'-methylthioadenosine; MTAP, 5'-methylthioadenosine phosphorylase.

S-Adenosylmethionine Regulates MAT2A mRNA Stability

AdoMet, is catalyzed by the enzyme methionine adenosyltransferase (MAT). In mammals, there are three isoenzymes for MAT (MAT I, MAT II, and MAT III); MAT I and MAT III are two oligomeric forms (a tetramer and dimer, respectively) of the protein coded by *MAT1A* gene, whereas MAT II is the product of the *MAT2A* gene (11, 14, 15). The expression of both genes is differentially regulated; *MAT1A* is expressed in the adult hepatocyte, whereas *MAT2A* is expressed in all other cells of the organism and in the fetal hepatocyte (11, 14). Interestingly, *MAT2A* expression is induced when the adult hepatocyte proliferates (as occurs during liver regeneration) and upon neoplastic transformation of the liver, where *MAT1A* expression is silenced (11, 16–18). Although both enzymes catalyze the same reaction, they display distinct catalytic and regulatory properties that impact on the intracellular concentrations of AdoMet (11). We have recently shown that in liver, AdoMet plays a pivotal role in the regulation of essential cellular functions, such as the control of cellular proliferation, differentiation, and apoptosis (18–24); consequently, the levels of this metabolite must be tightly controlled in this organ. In the present work, we describe how expression of the *MAT2A* gene is acutely regulated by the availability of L-methionine and investigate the mechanisms behind this response. Our observations indicate that fluctuations in L-methionine availability regulate *MAT2A* expression mainly at the level of mRNA turnover; additionally, we demonstrate an essential role for AdoMet in this response, further supporting the hypothesis of AdoMet as a signaling molecule in the liver (25).

EXPERIMENTAL PROCEDURES

Chemicals—AdoMet, in the stable form of sulfate-*p*-toluenesulfonate salt produced by Knoll (Milan, Italy) was from Europharma (Madrid, Spain). 5′-Methylthioadenosine (MTA) was generously provided by Knoll (Milan, Italy). All other reagents were of analytical grade, and unless otherwise stated, they were purchased form Sigma.

Cell Culture and Treatment Conditions—Human HepG2 hepatoma cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA), and HuH7 human hepatoma cells were from our tissue culture facility at the University of Navarra. Cells were maintained in minimum Eagle's medium (MEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen) at 37 °C in a humid atmosphere of 5% CO_2 in air. To test *MAT2A* expression, cells were cultured in L-methionine-deficient MEM supplemented with 5% fetal calf serum and varying concentrations of L-methionine (Sigma). It has been estimated that L-methionine concentration in bovine serum is around 15 μM, so the final concentration of L-methionine in our experiments would be around 0.75 μM; in this work, this is referred to as L-methionine restriction. In some experiments, cells were kept in Krebs-Ringer bicarbonate (KRB) and varying concentrations of L-methionine. No significant differences in cell viability, as determined by trypan blue exclusion test, were observed under the different treatments.

Determination of MAT Activity and AdoMet Levels—Cells (3×10^6) were lysed by freeze thawing in 300 μl of 10 mM Tris-HCl (pH 7.5) containing 0.3 mM sucrose, 0.1% β-mercaptoethanol, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged for 30 min at $10,000 \times g$, and MAT activity was assayed in the supernatant as described previously (27). For the determination of AdoMet concentrations, cells were lysed and deproteinized in 0.4 M perchloric acid and centrifuged at $12,000 \times g$ for 15 min at 4 °C. Supernatants were filtered and analyzed by high performance liquid chromatography as described previously (27).

RNA Isolation and Northern Blot Analysis—Total RNA was isolated by the guanidinium thiocyanate method as described (28, 29). 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 (27, 29). Northern hybridization analysis was performed on total RNA using standard procedures as described (27, 29). All probes (human *MAT2A* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs) were labeled with [^{32}P]dCTP using a random primer kit (RediPrime DNA Labeling System; Amersham Bisociences). To ensure equal loading of RNA samples, membranes were also hybrid-
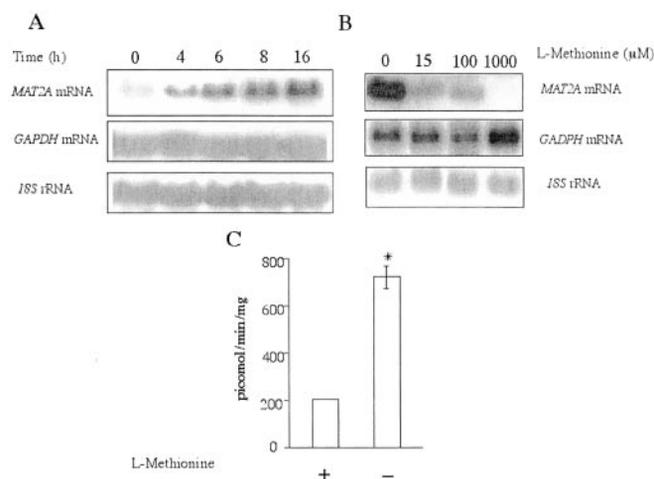

FIG. 1. **L-Methionine depletion induces MAT2A mRNA levels and MAT activity in HepG2 cells.** *A*, HepG2 cells were incubated in MEM without L-methionine for different periods of time, and *MAT2A* mRNA levels were analyzed by Northern blotting. The expression of GAPDH and the 18 S rRNA genes was also examined. *B*, HepG2 cells were cultured for 24 h in MEM with increasing concentrations of L-methionine, and the expression of *MAT2A*, GAPDH, and 18 S rRNA genes was assessed by Northern blotting. Representative blots of three different experiments performed in duplicate are shown. *C*, specific MAT II activity in HepG2 cells cultured for 24 h in MEM supplemented with L-methionine (100 μM) (+) or without L-methionine (−). Data are means ± S.E. of three experiments performed in triplicates (*asterisk* indicates $p < 0.05$).

ized with ^{32}P-labeled 18 S rRNA DNA probe. Quantitation was performed by scanning densitometry of the x-ray films, using the Molecular Analyst software (Bio-Rad), and values were normalized to 18 S rRNA.

Nuclear Run-on Transcription Assay—Nuclei from 3×10^7 HepG2 cells kept in MEM without L-methionine for different durations were isolated as described previously (27). Transcription reactions were performed for 30 min at 37 °C with 60 μl of the isolated nuclei in a final volume of 200 μl in the presence of 100 μCi of [α-^{32}P]uridine triphosphate (800 Ci/mmol; Amersham Biosciences) and 0.5 mM unlabeled nucleotides essentially as described (27). Elongated RNA transcripts were purified by phenol extraction as described above, denatured, and hybridized (at equivalent amounts of radioactivity (*i.e.* 1–2 × 10^6 cpm)) to 5 μg of the corresponding cDNAs and immobilized on Nytran filters in hybridization buffer (0.2 M NaPO$_4$ (pH 7.2), 1 mM EDTA, 7% SDS, 45% formamide, and 250 mg/ml *Escherichia coli* transfer RNA as carrier) for 48 h at 42 °C. The DNAs used were the human *MAT2A*, GAPDH, MTAP, *S*-adenosylhomocysteine hydrolase, and 18 S cDNAs; pUC18 was used for background control. The filters were washed and exposed to x-ray films. Quantitation was performed by scanning densitometry of the x-ray films, using the Molecular Analyst software (Bio-Rad). Data were normalized to the transcription of the *GAPDH* gene, the steady-state mRNA levels of which were not affected by short term L-methionine depletion.

Statistics—Unless otherwise stated, the data are means ± S.E. of at least three independent experiments performed in duplicate. Statistical significance was estimated with Student's *t* test. A *p* value of <0.05 was considered significant.

RESULTS

L-Methionine Restriction Induces MAT2A mRNA Levels and MAT Activity in HepG2 Cells—To evaluate the effect of L-methionine levels on *MAT2A* gene expression, HepG2 cells were cultured for different periods of time in L-methionine-deficient MEM with 5% fetal calf serum, and *MAT2A* expression was assessed by Northern blotting. As shown in Fig. 1*A*, *MAT2A* mRNA levels were dramatically induced by L-methionine restriction in HepG2 cells in a time-dependent fashion. The intracellular levels of AdoMet in HepG2 cells before L-methionine restriction ($t = 0$) were 1.2 ± 0.10 nmol/mg of protein and were reduced to 0.28 ± 0.02 nmol/mg of protein after 4 h of incubation in L-methionine-deficient medium. The magnitude of *MAT2A* mRNA accumulation was also dependent
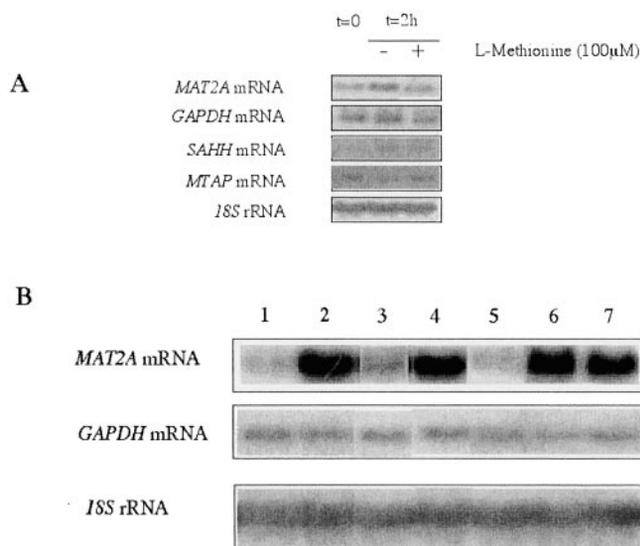
t=0   t=2h

—   +   L-Methionine (100μM)

A
MAT2A mRNA
GAPDH mRNA
SAHH mRNA
MTAP mRNA
18S rRNA

B

1   2   3   4   5   6   7

MAT2A mRNA

GAPDH mRNA

18S rRNA

FIG. 2. **Specificity of *MAT2A* gene response to L-methionine depletion.** *A*, HepG2 cells were grown on L-methionine (100 μM)-supplemented MEM until 70% confluence, and then medium was changed to KRB buffer for 2 h in the presence (+) or absence (−) of L-methionine (100 μM). *MAT2A*, MTAP, *S*-adenosylhomocysteine hydrolase, GAPDH, and 18 S rRNA gene expression was assessed by Northern blotting; blots representative of three experiments performed in duplicate are shown. *B*, HepG2 cells were cultured for 24 h in the presence of 100 μM L-methionine (*lane 1*); 0 μM L-methionine (*lane 2*); 4 mM AdoMet (*lane 3*); 100 μM D-methionine (*lane 4*); 500 μM MTA (*lane 5*); 200 μM homocysteine, 100 μM folinic acid, and 15 μM cyanocobalamine (*lane 6*); or 2 mM betaine and 200 μM homocysteine (*lane 7*). *MAT2A*, GAPDH, and 18 S rRNA gene expression were examined by Northern blotting; blots representative of three experiments performed in duplicate are shown.

FIG. 3. **L-Methionine and AdoMet metabolic pathways.** L-Methionine recycling and salvage pathways are presented.

on the levels of L-methionine in the culture medium, as observed when HepG2 cells were cultured for 24 h in MEM supplemented with increasing concentrations of this amino acid (Fig. 1*B*). The intracellular levels of AdoMet in cells cultured during 24 h in MEM supplemented with 0, 100, or 1000 μM L-methionine were 0.16 $\pm$ 0.02, 1.04 $\pm$ 0.06, and 1.52 $\pm$ 0.01 nmol/mg of protein, respectively. The levels of *GAPDH* mRNA and 18 S ribosomal RNA were also measured, and no significant changes were observed (Fig. 1, *A* and *B*). The induction of *MAT2A* gene expression correlated with the up-regulation of MAT II activity when measured 24 h after culture in L-methionine-restricted MEM (Fig. 1*C*). The same observations were made in HuH7 cells (data not shown).

Specificity of MAT2A Response to L-Methionine Depletion—To determine whether the observed up-regulation of *MAT2A* mRNA levels was limited to L-methionine restriction in culture medium, HepG2 cells were grown in complete MEM (100 μM L-methionine) until 70% confluence and subsequently cultured in KRB buffer for 2 h in the presence of 0 or 100 μM L-methionine. As shown in Fig. 2*A*, *MAT2A* gene expression was induced in methionine-deficient KRB medium, whereas the addition of L-methionine prevented this response. The expression of other genes related to AdoMet metabolic pathways, such as *S*-adenosylhomocysteine hydrolase and 5′-methylthioadenosine phosphorylase, was also tested in HepG2 cells under the above mentioned conditions, and no significant changes were observed (Fig. 2*A*).

We next examined the mechanisms through which L-methionine could mediate this acute effect on *MAT2A* gene expression. As previously stated, besides its involvement in protein synthesis, intracellular L-methionine is metabolized into AdoMet by the action of MAT (Fig. 3 summarizes AdoMet metabolic pathways). We tested whether AdoMet could mimic the effect of L-methionine on *MAT2A* gene expression. For this
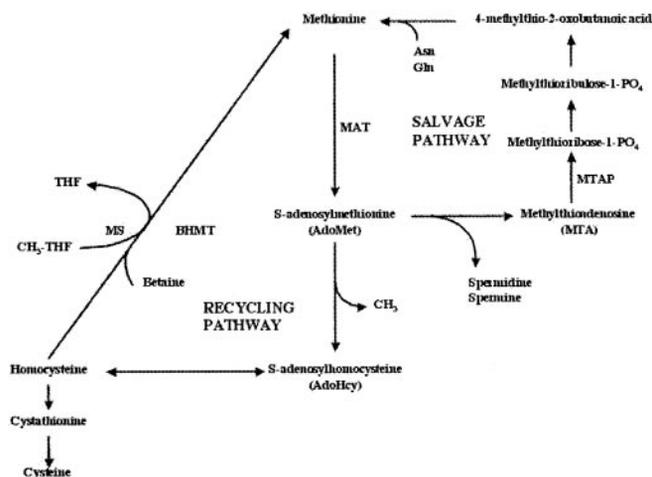
purpose, HepG2 cells were cultured in methionine-restricted MEM for 24 h in the presence of 4 mM AdoMet, and *MAT2A* mRNA levels were subsequently analyzed by Northern blotting. Intracellular AdoMet concentrations were measured in control and AdoMet-treated cells 8 h after the addition of this compound. These concentrations were 0.20 $\pm$ 0.01 and 5.02 $\pm$ 0.2 nmol/mg of protein in control and AdoMet-treated cells, respectively. As shown in Fig. 2*B*, the potent induction of *MAT2A* gene expression that occurs in the absence of L-methionine (*lane 2*) was not observed to such an extent when AdoMet was added to the culture medium (Fig. 2*B*, compare *lanes 2* and *3*). However, under the same conditions, the nonmetabolizable D-isomer of methionine (at 100 μM) had no effect on *MAT2A* gene expression (Fig. 2*B*, *lane 4*). Interestingly, MTA (at 500 μM), a product of AdoMet metabolism generated in the polyamine biosynthetic pathway and the first molecule in the methionine salvage route (30–32) (Fig. 3), was very effective in preventing the induction of *MAT2A* mRNA levels by methionine restriction (Fig. 2*B*, *lane 5*). In liver cells, homocysteine can be remethylated and converted back to methionine through two alternative pathways that involve the enzyme methionine synthase or the enzyme betaine-homocysteine methyltransferase (10, 11) (Fig. 3). Methionine synthase is a cobalamin-dependent enzyme that requires 5-methyltetrahydrofolate to convert homocysteine into methionine (33). We incubated HepG2 cells with homocysteine (200 μM), folinic acid (100 μM, a precursor of 5-methyltetrahydrofolate), and cyanocobalamin (15 μM) for 24 h in the absence of L-methionine. As shown in Fig. 2*B*, *lane 6*, this treatment had no effect on *MAT2A* mRNA levels that were strongly induced after 24 h in a methionine-deficient medium. Similarly, we explored the pathway catalyzed by betaine-homocysteine methyltransferase for the conversion of homocysteine into methionine by incubating cells with betaine (2 mM) and homocysteine (200 μM) for 24 h in the absence of the L-methionine addition. This treatment was also unable to modulate the induction of *MAT2A* mRNA in response to L-methionine restriction (Fig. 2*B*, *lane 7*).

The induction of *MAT2A* mRNA elicited by methionine restriction could be due either to an increase in transcription of the gene or to increased mRNA stability. Consequently, we have measured the effect of L-methionine restriction on the transcription rate of the *MAT2A* gene in HepG2 cells by performing nuclear run-on studies. We observed that the transcription rate of the *MAT2A* gene remained constant at different time points after L-methionine withdrawal for up to 4 h; similarly, the transcription rate of *GAPDH* was not affected under this condition (Fig. 4*A*).
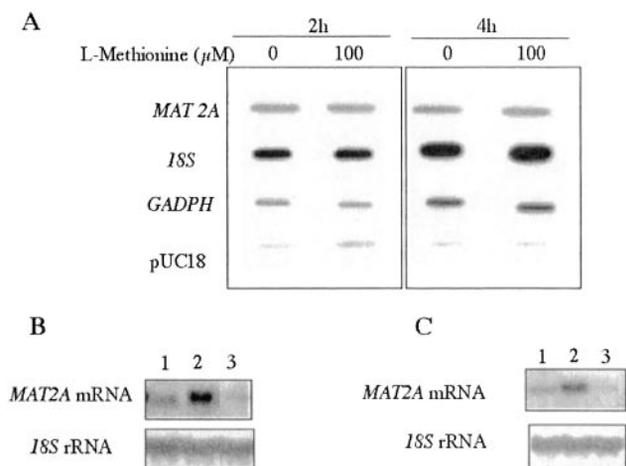
FIG. 4. **Effect of L-methionine levels on MAT2A gene transcription in HepG2 cells.** *A*, nuclear run-on assay of *MAT2A* gene transcription performed in nuclei isolated from HepG2 cells maintained for either 2 or 4 h in 0 or 100 μM L-methionine. Labeled RNAs were synthesized by isolated nuclei and hybridized against *MAT2A*, GAPDH, and 18 S rRNA cDNAs on slot blots (pUC18 plasmid DNA was used for background control). Experiments were performed two times in duplicate, and a representative blot is shown. *B*, Northern blot analysis of *MAT2A* mRNA levels from HepG2 cells kept in MEM with 100 μM L-methionine (*lane 1*), 0 μM L-methionine for 3 h (*lane 2*), or 0 μM L-methionine plus 5 μg/ml ActD for 3 h. *C*, Northern blot analysis of *MAT2A* mRNA levels from HepG2 cells kept in MEM with 100 μM L-methionine (*lane 1*), 0 μM L-methionine for 3 h (*lane 2*), or 0 μM L-methionine plus 0.1 mM cycloheximide for 3 h. Analysis was performed also with an 18 S rRNA cDNA probe for loading control. Representative blots from three experiments performed in duplicate are shown.

We next tested whether ongoing transcription or translation was necessary for the observed induction of *MAT2A* mRNA levels in response to L-methionine restriction. For this purpose, cells were grown in complete MEM until 70% confluence and then transferred to L-methionine-restricted MEM in the presence of either actinomycin D (ActD) (5 μg/ml) or cycloheximide (0.1 mM) for 3 h. As shown in Fig. 4, *B* and *C*, both treatments completely abolished the induction of *MAT2A* mRNA levels. These observations indicate that both transcription and protein synthesis are required for the up-regulation of *MAT2A* gene expression during L-methionine depletion.

MAT2A mRNA Is Stabilized in L-Methionine-depleted Cells—Taken together, our data suggest that L-methionine restriction increases *MAT2A* gene expression by stabilizing the mRNA. To support this hypothesis, we determined *MAT2A* mRNA half-life in complete and in L-methionine-depleted MEM. HepG2 cells growing in complete MEM were incubated for different periods of time in the presence or absence of ActD (5 μg/ml), and *MAT2A* mRNA levels were measured by Northern blotting. Under this condition, we could estimate that *MAT2A* mRNA decayed with a half-life of ~100 min in L-methionine-supplemented medium (Fig. 5*A*). In order to determine *MAT2A* mRNA half-life in L-methionine-restricted medium, cells were cultured in the absence of this amino acid for 4 h and then treated with ActD (5 μg/ml) for up to 3 h in the same medium. As shown in Fig. 5B, the level of *MAT2A* mRNA remained constant in L-methionine-depleted cells, suggesting an mRNA half-life much longer than 3 h.

We wanted to further explore the mechanisms through which L-methionine could mediate this acute regulation of *MAT2A* mRNA levels. We first determined whether AdoMet and MTA were acting on *MAT2A* gene expression also at the level of mRNA stability. For this purpose, HepG2 cells were incubated for 4 h in L-methionine-deficient MEM, and then AdoMet (4 mM) or MTA (500 μM) was added to the cultures in
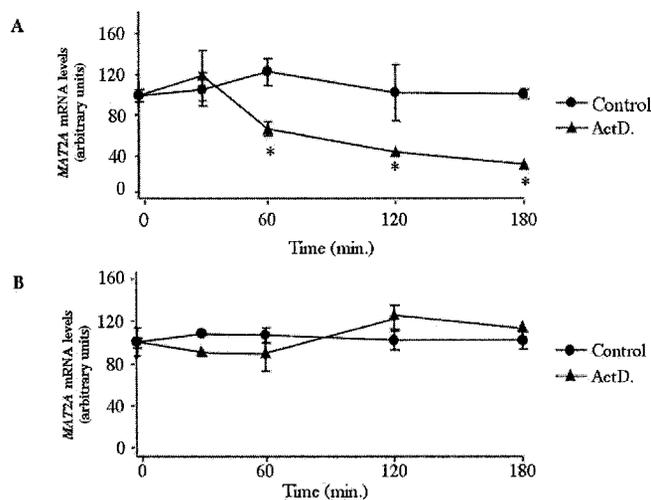

FIG. 5. **Effect of L-methionine depletion on the half-life of MAT2A mRNA in HepG2 cells.** *A*, quantitation of MAT2A mRNA levels in HepG2 cells kept in MEM supplemented with 100 μM L-methionine in the absence (*closed circles*) or presence (*closed triangles*) of 5 μg/ml ActD for different periods of time. *B*, quantitation of *MAT2A* mRNA levels in HepG2 cells that were kept for 4 h in L-methionine-depleted MEM and then (time 0) further treated for up to 3 h in the absence (*closed circles*) or presence (*closed triangles*) of 5 μg/ml ActD. Data are means + S.E. of three experiments performed in duplicate (*asterisk* indicates $p < 0.05$ with respect controls without ActD). Values were normalized to 18 S rRNA levels.

the absence or presence of ActD (5 μg/ml) and incubations continued up to 6 h more. Analysis of *MAT2A* gene expression under these treatments indicated that both AdoMet and MTA shared with L-methionine its ability to down-regulate *MAT2A* mRNA in the absence of ongoing transcription (Fig. 6). Interestingly, MTA needed to be metabolized through the methionine salvage pathway to exert its effect on *MAT2A* mRNA levels, since the concomitant addition of adenine (1 mM), an inhibitor of MTAP, the first and rate-limiting enzyme in this pathway (33), completely blocked its effect (Fig. 6). Of particular relevance is the observation that L-methionine has to be metabolized and converted into AdoMet to mediate its effect on *MAT2A* mRNA levels, as evidenced when L-methionine treatment was performed in the presence of cycloleucine (20 mM), a competitive inhibitor of MAT activity (34) (Fig. 6). If under these conditions (treatment with L-methionine and cycloleucine) exogenous AdoMet (4 mM) was added to the cells, *MAT2A* mRNA levels were down-regulated (Fig. 6). The addition of either adenine or cycloleucine to L-methionine-starved cells had no effect on *MAT2A* mRNA levels (data not shown). As previously mentioned, an essential role of L-methionine, through its conversion into AdoMet, is to provide the cell with the necessary activated methyl groups for a vast number of transmethylation reactions. Hence, it was important to know if a transmethylation reaction(s) could be involved in the regulation of *MAT2A* mRNA levels. For this purpose, L-methionine-deprived cells were treated with L-methionine in the presence of the adenosine analog 3-deazaadenosine (C³-Ado) (30 μM, 30 min prior to L-methionine addition) (37). C³-Ado is an inhibitor of *S*-adenosylhomocysteine hydrolase and leads to a significant increase in the intracellular levels of AdoHcy; additionally, it can be converted into the more stable 3-deaza-derivative of AdoHcy (*S*-3-deaza-adenosylhomocysteine (C³-AdoHcy)) (37). AdoHcy and C³-AdoHcy are strong inhibitors of methylation reactions (14, 37). We observed that the effect of L-methionine was preserved in the presence of C³-Ado (Fig. 6), suggesting that methylation reactions were not involved in the regulation of *MAT2A* gene expression by this amino acid.
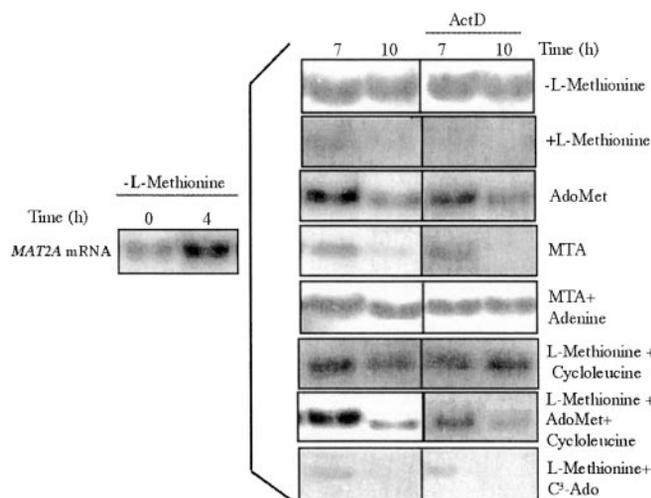
FIG. 6. **Mechanism of L-methionine regulation of MAT2A mRNA stability.** MAT2A mRNA levels were determined by Northern blotting in HepG2 cells that were kept in L-methionine-depleted MEM for 4 h and then further incubated for 3 or 6 h more in the absence or presence of 5 μg/ml ActD and the indicated treatments: without L-methionine, 100 μM L-methionine, 4 mM AdoMet, 500 μM MTA, 500 μM MTA plus 1 mM adenine, 100 μM L-methionine plus 30 μM C^3-Ado, 100 μM L-methionine plus 20 mM cycloleucine, 100 μM L-methionine, and 4 mM AdoMet plus 20 mM cycloleucine. Representative blots of three experiments performed in duplicate are shown.

DISCUSSION

In this work, we have provided evidence for the acute regulation of *MAT2A* gene expression in response to the availability of L-methionine. Incubation of human hepatoma HepG2 cells (and HuH7 cells; not shown) in medium restricted in this amino acid resulted in the rapid induction of *MAT2A* mRNA levels in a time- and concentration-dependent manner. A parallel increase in MAT activity was observed in cells cultured under reduced L-methionine availability, suggesting that translation of *MAT2A* mRNA was taking place even in the presence of limiting amounts of L-methionine. This observation is in agreement with other reports showing the induction of specific proteins under limited availability of L-methionine (38, 39). *MAT2A* gene response seemed to be mainly due to changes in L-methionine levels and not to fluctuations in other amino acids or nutrients. This may be inferred by our experiments in which HepG2 cells were incubated in KRB buffer and induction of *MAT2A* gene expression was modulated by the sole addition of L-methionine.

As mentioned previously, besides its role in protein synthesis, L-methionine enters a metabolic pathway in which it is converted, together with ATP, into AdoMet, the main methyl donor compound in the cell. It was important to know if the observed response of *MAT2A* gene to fluctuations in L-methionine levels could be related to this metabolic pathway. We observed that AdoMet addition to L-methionine-starved cultures was effective in down-regulating the expression of *MAT2A* gene. This observation, together with the finding that the nonmetabolizable D-isomer of methionine had no effect on *MAT2A* mRNA levels, provided the first evidence supporting the concept that L-methionine mediates its effect on *MAT2A* gene expression through its conversion into AdoMet. We also tested the effect of other molecules that are subsequent metabolic products of L-methionine and AdoMet, such as homocysteine and MTA. Homocysteine treatment had no effect on *MAT2A* mRNA levels, suggesting that this metabolite was not involved in the cellular signaling of L-methionine limitation. Homocysteine can serve as a precursor of L-methionine in all cells; however, transformed cells, including hepatoma cells, are unable to grow if homocysteine is used in place of methionine in culture medium (13, 40, 41). Our present data further support this notion. With regard to MTA, a product of AdoMet metabolism in the polyamine biosynthetic pathway and a precursor of L-methionine in the so-called methionine salvage pathway, we observed that it was able to modulate *MAT2A* mRNA levels in L-methionine-starved cultures. As we later observed, MTA effect was dependent on its metabolism through the methionine salvage pathway and therefore probably due to its ultimate conversion into this amino acid and subsequently into AdoMet. Adenine, a product of MTA metabolism and an inhibitor of MTAP, the first and rate-limiting enzyme in the methionine salvage pathway (30–32, 35), blocked MTA effect. Methionine dependence of tumor cells has been associated with defects in MTAP expression leading to the suppression of this salvage pathway (13, 41, 42); however, this is not the case for HepG2, where we found that MTAP expression was normal (data not shown). Additionally, these data attest to the importance of the methionine salvage pathway in methionine conservation under limited supply of this amino acid and further support the relevance of this metabolic route as a target in antineoplastic therapy.

We have provided evidence showing that *MAT2A* gene transcription remained constant during L-methionine starvation at least up to 4 h after the removal of this amino acid from the culture medium. These data suggested that the potent effect of L-methionine on *MAT2A* gene expression might take place at the post-transcriptional level. In the regulation of gene expression by amino acids, both types of mechanisms, namely the regulation of gene transcription and of mRNA stability, have been described. For instance, transcription of the asparagine synthetase gene and the stability of the high affinity cationic amino acid transporter-1 mRNA are known to be modulated by amino acid starvation (43, 44). In the case of certain genes, such as cationic amino acid transporter-1, changes in its mRNA turnover rate alone account for the dramatic induction on the expression of this gene in amino acid-depleted medium (44, 46).

Because the *MAT2A* gene transcription rate was not significantly altered by L-methionine starvation, we measured *MAT2A* mRNA half-life in L-methionine-fed and -starved cultures. We observed a significant increase in *MAT2A* mRNA half-life in response to L-methionine starvation. Interestingly, this effect was completely dependent on *de novo* protein synthesis. This observation suggests that a protein factor(s) may be synthesized that binds and stabilizes *MAT2A* mRNA when cellular L-methionine levels are reduced. This kind of mechanism has been reported for the regulation of mRNAs encoding proteins such as transferrin receptor (45), the cationic amino acid transporter-1 (46), and others (47).

Once we established that *MAT2A* gene expression was regulated by L-methionine at the level of mRNA turnover, we confirmed that the observed effects of AdoMet and MTA on *MAT2A* steady state mRNA levels were also mediated at this same level. In these experiments, it was also clearly established that L-methionine *per se* was not sufficient to convey the cellular signal(s) that modulated *MAT2A* mRNA turnover. Conversion of L-methionine into AdoMet was absolutely necessary, given the fact that cycloleucine, a competitive inhibitor of MAT II activity (34), completely abolished L-methionine action. In line with this is the observation that when exogenous AdoMet was added, the blockade imposed by cycloleucine on L-methionine effect was overcome. Taken together, these findings provide an explanation for our previous observations made in *MAT1A* knockout mice. These animals display dramatically elevated levels of circulating L-methionine (around 8-fold increase compared with wild type mice), and at the same time the

expression of the *MAT2A* gene is induced in their liver (21). These seemingly contradictory observations may be explained by the fact that the hepatic concentration of AdoMet in these knockout mice is reduced to about 25% of that found in wild type animals (21).

Another interesting finding was the observation that treatment with C^3-Ado, a compound that increases the intracellular concentration of AdoHcy, and C^3-AdoHcy, two potent inhibitors of methylation reactions, did not interfere with the effect of L-methionine on *MAT2A* mRNA levels. This finding suggests that a methylation reaction is not involved in the effect of L-methionine (and consequently of AdoMet) on *MAT2A* gene expression, although we cannot exclude a methyl transfer reaction that would be resistant to AdoHcy or C^3-AdoHcy inhibition. We have previously described that AdoMet is able to induce the transcription of the *MAT1A* gene in cultured rat hepatocytes (24, 25) and that in this effect a methylation reaction is probably involved (24). Now we report on a novel effect of AdoMet on gene expression that occurs at the posttranscriptional level and in which a different mechanism of action is seemingly involved. An alternative mode of action for AdoMet could be its direct binding to a cellular target structure. Such interaction of AdoMet is known to occur in eukaryotic cells, and the best characterized target is the enzyme cystathionine β-synthase, which is allosterically regulated by AdoMet binding to a specific domain termed the cystathionine β-synthase domain (49). Interestingly, this cystathionine β-synthase domain is a widespread structure that was originally described in archaebacteria and is well conserved throughout evolution (50).

Given the central role played by AdoMet in cellular metabolism, the sharp induction in *MAT2A* gene expression in response to reduced L-methionine and AdoMet availability could be regarded as an adaptive mechanism of the cell aimed at preserving the intracellular levels of AdoMet. As we have shown, this is a finely tuned response, and we can envision different potential explanations for this to be so. In the first place, for each AdoMet molecule synthesized, one ATP molecule and one L-methionine molecule are consumed at the expense of the homeostasis of cellular energetics and protein synthesis (10, 11). Additionally, recent observations suggest that AdoMet plays a role in the cell that transcends its purely metabolic function as a methyl donor and metabolic intermediate (reviewed in Ref. 26). Changes in AdoMet levels modulate proliferation, apoptosis, and differentiation, so its synthesis needs to be carefully controlled. In this regard, the inhibitory effect of AdoMet on MAT II activity has been well characterized (12, 13, 48). Our present observations represent a novel action of AdoMet in the regulation of cellular physiology, this time modulating its own production also through the regulation of *MAT2A* mRNA stability.

REFERENCES

1. Pégorier, J. P. (1998) *Curr. Opin. Clin. Nutr. Metab. Care.* **1,** 329–334
2. Vaulont, S., and Kahn, A. (1994) *FASEB J.* **8,** 28–35
3. Foufelle, F., Girard, J., and Ferre, P. (1998) *Curr. Opin. Clin. Nutr. Metab. Care* **1,** 323–328
4. Kilberg, M. S., Hutson, R. G., and Laine, R. O. (1994) *FASEB J.* **8,** 13–19
5. Fafournoux, P., Bruhat, A., and Jousse, C. (2000) *Biochem. J.* **351,** 1–12
6. Kilberg, M. S., Stevens, B. R., and Novak, D. A. (1993) *Annu. Rev. Nutr.* **13,** 137–165
7. Palacin, M., Estevez, B. J., and Zorzano, A. (1998) *Physiol. Rev.* **78,** 969–1054
8. Kilberg, M. S., and Barbosa-Tessmann, I. P. (2002) *J. Nutr.* **132,** 1801–1804
9. Cantoni, G. L. (1975) *Annu. Rev. Biochem.* **44,** 435–441
10. Finkelstein, J. D. (1990) *J. Nutr. Biochem.* **1,** 228–236
11. Mato, J. M., Avila, M. A., and Corrales, F. J. (2001) *Homocysteine in Health and Disease*, pp. 47–62, Cambridge University Press, Cambridge, UK
12. Finkelstein, J. D., and Martin, J. J. (1986) *J. Biol. Chem.* **261,** 1582–1587
13. Hoffman R. M. (1985) *Anticancer Res.* **5,** 1–30
14. Mato, J. M., Alvarez, L., Ortiz, P., and Pajares, M. A. (1997) *Pharmacol. Ther.* **73,** 265–280
15. Kotb, M., Mudd, H. S., Mato, J. M., Geller, M. A., Kredich, N. M., Chou, J. Y., and Cantoni, G. L. (1997) *Trends Genet.* **13,** 51–52
16. Cai, J., Sun, W-M., Hwang, J-J., Stain, S. C., and Lu, S. C. (1996) *Hepatology* **24,** 1090–1097
17. Huang, Z. Z., Mao, Z., Cai, J., and Lu, S. C. (1999) *Am. J. Physiol.* **275,** G14–G21
18. Latasa, M. U., Boukaba, A., García-Trevijano, E. R., Torres, L., Rodriguez, J. L., Caballería, J., Lu, S. C., Lopez-Rodas, G., Franco, L., Mato, J. M., and Avila, M. A. (2001) *FASEB J.* **15,** 1248–1250
19. Cai, J., Mao, Z., Hwang, J., and Lu, S. C. (1998) *Cancer Res.* **58,** 1444–1450
20. García-Trevijano, E. R., Martínez-Chantar, M. L., Latasa, M. U., Mato, J. M., and Avila, M. A. (2002) *Gastroenterology* **122,** 1355–1363
21. Lu, S. C., Alvarez, L., Huang, Z. Z., Chen, L., An, W., Corrales, F. J., Avila, M. A., Kanel, G., and Mato, J. M. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 5560–5565
22. Martínez-Chantar, M. L., Corrales, F. J., Martínez-Cruz, L. A., García-Trevijano, E. R., Huang, Z. Z., Chen, L., Kanel, G., Avila, M. A., Mato, J. M., and Lu, S. C. (2002) *FASEB J.* **16,** 1292–1294
23. Ansorena, E., García-Trevijano, E. R., Martínez-Chantar, M. L., Huang, Z. Z., Chen, L., Mato, J. M., Iraburu, M., Lu, S. C., and Avila, M. A. (2002) *Hepatology* **35,** 274–280
24. Carretero, M. V., Latasa, M. U., García-Trevijano, E. R., Corrales, F. J., Wagner, C., Mato, J. M., and Avila, M. A. (2001) *Biochem. Pharmacol.* **61,** 1119–1128
25. García-Trevijano, E. R., Latasa, M. U., Carretero, M. V., Mato, J. M., and Avila, M. A. (2000) *FASEB J.* **14,** 2511–2518
26. Mato, J. M., Corrales, F. J., Lu, S. C., and Avila, M. A. (2002) *FASEB J.* **16,** 15–26
27. Avila, M. A., Carretero, M. V., Rodriguez, E. N., and Mato, J. M. (1998) *Gastroenterology* **114,** 364–371
28. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
29. Avila, M. A., Velasco, J. A., Cho, C., Lupu, R., Wen, D., and Notario, V. (1995) *Oncogene* **10,** 963–971
30. Tabor, H., and Tabor C. W. (1964) *Pharmacol. Rev.* **16,** 245–300
31. Williams-Ashman, H. G. (1982) *Biochem. Pharmacol.* **31,** 277–288
32. Pegg, A. E. (1988) *Cancer Res.* **48,** 759–774
33. Olteanu, H., and Banerjee, R. (2001) *Homocysteine in Health and Disease*, pp. 135–144, Cambridge University Press, Cambridge, UK
34. Garrow, T. (2001) *Homocysteine in Health and Disease*, pp. 145–152, Cambridge University Press, Cambridge, UK
35. White, M. W., Vandenbark, A. A., Barney, C. L., and Ferro, A. J. (1982) *Biochem. Pharmacol.* **31,** 503–507
36. Lombardini, J. B., and Talalay, P. (1971) *Adv. Enzyme Regul.* **9,** 349–384
37. Chiang, P. K. (1998) *Pharmacol. Ther.* **77,** 115–134
38. Bruhat, A., Jousse, C., Wang, X. Z., Ron, D., Ferrara, M., and Fafournoux, P. (1997) *J. Biol. Chem.* **272,** 17588–17593
39. Lu, S., and Epner, D. E. (2000) *Nutr. Cancer* **38,** 123–130
40. Avila, M. A., Berasain, C., Torres, L., Martín-Duce, A., Corrales, F. J., Yang, H., Prieto, J., Lu, S. C., Caballería, J., Rodés, J., and Mato, J. M. (2000) *J. Hepatol.* **33,** 907–914
41. Tang, B., Li, Y. N., and Kruger, W. D. (2000) *Cancer Res.* **60,** 5543–5547
42. Dumontet, C., Roch, A. M., and Quash, G. (1996) *Oncol. Res.* **8,** 469–471
43. Van Leung-Pineda, and Kilberg, M. S. (2002) *J. Biol. Chem.* **277,** 16585–16591
44. Aulak, K. S., Mishra, R., Zhou, L., Hyatt, S. L., De Jonge, W., Lamers, W., Snider, and Hatzoglou, M. (1999) *J. Biol. Chem.* **274,** 30424–30432
45. Klausner, R. D., and Harford, J. B. (1989) *Science* **246,** 870–872
46. Yaman, I., Fernández, J., Sarkar, B., Schneider, R. J., Snider, M. D., Nagy, L. E., and Hatzoglou, M. (2002) *J. Biol. Chem.* **277,** 41539–41546
47. Chen, F. Y., Amara, F. M., and Wright, J. A. (1993) *EMBO J.* **12,** 3977–3986
48. Sullivan, D. M., and Hoffman, J. L. (1983) *Biochemistry* **22,** 1636–1641
49. Janosik, M., Kery, V., Gaustadnes, M., Maclean, K. N., and Kraus, J. P. (2002) *J. Cell. Physiol.* **192,** 81–92
50. Bateman, A. (1997) *Trends. Biochem. Sci.* **22,** 12–13