S-Adenosylmethionine: a control switch that regulates liver function

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ABSTRACT **Genome sequence analysis reveals that all organisms synthesize S-adenosylmethionine (AdoMet) and that a large fraction of all genes is AdoMet-dependent methyltransferases. AdoMet-dependent methylation has been shown to be central to many biological processes. Up to 85% of all methylation reactions and as much as 48% of methionine metabolism occur in the liver, which indicates the crucial importance of this organ in the regulation of blood methionine. Of the two mammalian genes (*MAT1A*, *MAT2A*) that encode methionine adenosyltransferase (MAT, the enzyme that makes AdoMet), *MAT1A* is specifically expressed in adult liver. It now appears that growth factors, cytokines, and hormones regulate liver MAT mRNA levels and enzyme activity and that AdoMet should not be viewed only as an intermediate metabolite in methionine catabolism, but also as an intracellular control switch that regulates essential hepatic functions such as regeneration, differentiation, and the sensitivity of this organ to injury. The aim of this review is to integrate these recent findings linking AdoMet with liver growth, differentiation, and injury into a comprehensive model. With the availability of AdoMet as a nutritional supplement and evidence of its beneficial role in various liver diseases, this review offers insight into its mechanism of action.—Mato, J. M., Corrales, F. J., Lu, S. C., Avila, M. A. S-Adenosylmethionine: a control switch that regulates liver function. *FASEB J.* 16, 15–26 (2002)**

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THE IDENTIFICATION BY Cantoni in 1951 of S-adenosylmethionine (AdoMet, also abbreviated SAM) was based on the earlier demonstration by du Vigneaud of the transfer of the methyl group of methionine to creatine (1). In 1958, Tabor's group demonstrated that after decarboxylation, AdoMet could donate the propylamino moiety attached to the sulfonium ion to putrescine to form spermidine and methylthioadenosine and to spermidine in order to form spermine and a second molecule of methylthioadenosine (2). Du Vigneaud and others demonstrated in the late 1930s that methionine is a metabolic precursor of homocysteine and, in the late 1950s, the methylation of homocysteine to form methionine, an integrated view combining the transmethylation and transsulfuration pathways was finally provided by Laster's group in 1964 (**Fig. 1**) (3). This seminal work of du Vigneaud, Cantoni, and others provided a perspective of methionine metabolism where AdoMet could be viewed as the main biological methyl donor. Since then, AdoMet-dependent methylation has been shown to be central to many biological processes from metal detoxification through biosynthesis to gene regulation via DNA methylation. Moreover, analysis of genome sequences from archaea, eubacteria, fungi, plants, and animals reveals that all organisms synthesize AdoMet and that a surprisingly large fraction of all genes are AdoMet-dependent methyltransferases.

The crucial importance of the liver in the regulation of blood methionine concentration was first established by Kinsell et al. in 1947 (4), who showed a marked impairment of methionine metabolism in patients with liver cirrhosis. Later work, mainly by the laboratories of Finkelstein and Mudd, demonstrated that under normal conditions, both in humans and rats, $< 85\%$ of all methylation reactions and $\sim 50\%$ of methionine metabolism occur in the liver (5, 6). These authors estimated that the half-life of AdoMet in the liver is ~ 5 min. In normal liver, the majority of AdoMet is used in methylation reactions, since the AdoMet decarboxylation pathway accounts for less than 10% of the available AdoMet. In humans, the fraction of available homocysteine converted to cystathionine during each cycle is 53% and this percent drops to $\sim 20\%$ when the dietary content of methyl groups is restricted. A central role of AdoMet in the regulation of hepatic methionine metabolism was first proposed by Finkelstein based on his findings that AdoMet activated cystathionine β-synthase (CBS) and inactivated betaine-homocysteine methyltransferase (7–9), and on the earlier observation of Kutzbach and Stokstad that AdoMet inhibited methylenetetrahydrofolate reductase (10, 11). Today, when the discovery of AdoMet reaches its 50 anniversary, this

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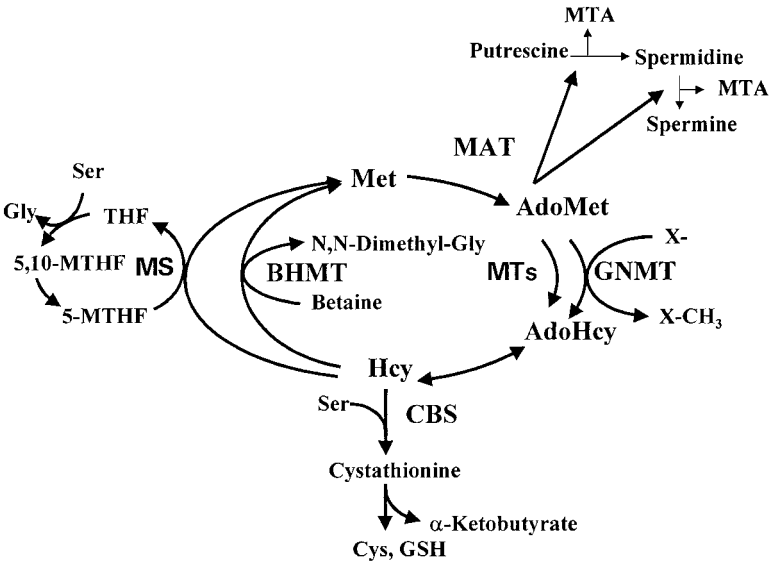

Figure 1. Hepatic methionine metabolism. Methionine (Met) is converted into homocysteine (Hcy) via S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy). The conversion of Met into AdoMet is catalyzed by the enzyme methionine adenosyltransferase (MAT). After decarboxylation, AdoMet can donate the remaining propylamino moiety attached to its sulfonium ion to putrescine to form spermidine and methylthioadenosine (MTA) and to spermidine to form spermine and a second molecule of MTA. AdoMet donates its methyl group in a large variety of reactions catalyzed by dozens of methyltransferase (MTs), the most abundant in the liver being glycine-N-methyltransferase (GNMT). The AdoHcy thus generated is hydrolyzed to form Hcy and adenosine through a reversible reaction catalyzed by the enzyme AdoHcy hydrolase. The methylation of Hcy to form methionine is catalyzed by two enzymes: methionine synthase (MS) and betaine methyltransferase (BHMT). Hcy, in the presence of serine, forms cystathionine in a reaction catalyzed by the enzyme cystathionine β-synthetase (CBS). Cystathionine is then hydrolyzed to form cysteine, a precursor of the synthesis of glutathione (GSH). THF, tetrahydrofolate; 5,10-MTHF, methylenetetrahydrofolate; 5-MTHF, methyltetrahydrofolate; Ser, serine; Gly, glycine; X, methyl acceptor molecule; X-CH_3, methylated molecule.

molecule is enjoying increased attention and promises to contribute significantly to our understanding of liver function. It now appears that growth factors, cytokines, and hormones regulate hepatic AdoMet synthesis and that AdoMet should not be viewed only as the principal biological methyl donor and a regulator of methionine metabolism, but also as an intracellular signal that controls essential hepatic functions such as hepatocyte growth and differentiation as well as sensitivity to liver injury.

1. BIOSYNTHESIS OF S-ADENOSYLMETHIONINE

AdoMet is synthesized from L-methionine and ATP in a two-step reaction where the complete tripolyphosphate (PPPi) moiety is cleaved from ATP as AdoMet is being formed. This unusual reaction is catalyzed by the enzyme methionine adenosyltransferase (MAT, EC 2.5.6.1.; also abbreviated SAM synthetase) (12). The biosynthesis of AdoMet has been studied by the groups of Mudd (13) and Markham (14) using MAT purified from *Escherichia coli* and mammals, respectively. The cloning and sequencing of the structural genes or cDNAS encoding for a large variety of MATs, including *Mycoplasma genitalium, E. coli, Saccharomyces cerevisiae, Arabidopsis thaliana*, mouse, rat, and human liver, and mouse, rat, and human kidney, have revealed that MAT is an exceptionally well-conserved enzyme through evolution (15). MAT from *E. coli* and humans share 59% sequence identity (16).

MAT from *E. coli* has been crystallized and the structure solved with a resolution of 2.8 Å (17). The active enzyme exists as a tetramer of a single subunit of 383 amino acids. Each subunit consists of three structural domains related to each other by a pseudo threefold symmetry. Pairs of subunits form dimers, and each dimer is a tight complex, with a wide interface area between subunits that accommodates two active sites. The active site of the enzyme is made by both subunits. The crystal structure of the tetrameric form of MAT (MAT I) from rat liver has been solved with a resolution of 2.7 Å and the geometry of the binding site of methionine identified (18). Each MAT I subunit consists of 396 amino acids and, as expected, the tetramer structure is essentially the same as that of the *E. coli* enzyme with all of the polar active site residues conserved.

Replacement of arginine 264 by histidine (R265H) of MAT I results in a monomeric MAT with undetectable AdoMet synthetic activity (19). The tripolyphosphatase activity of the R265H mutant was similar to that found in the wild-type MAT. These data suggest that the active site of MAT has two coordinated subsites: a synthetic site, configured by amino acid residues from both subunits (17), that is responsible for the binding of methionine and ATP and performs the AdoMet synthetic reaction; and a hydrolytic site, configured by amino acid residues from one single subunit, which accounts for the binding of PPPi and performs the PPPi hydrolytic reaction.

In the crystal structure of *E. coli* MAT crystallized at 26°C, residues 102 to 107, which are located over the active site, are not visible in the electron density maps and have been suggested to form a 'flexible loop' (17). When the crystallization temperature was decreased (4°C), the amino acid residues forming the loop were in a fixed conformation sealing the active site and preventing the binding of substrates (20). It has been proposed that when a substrate/product binds in the active site, the loop becomes flexible and acts as a 'mobile flap' in the catalytic reaction (20). In the crystal structure of rat liver MAT I, the region comprised by residues 118 to 128 (located over the active site and

corresponding to the flexible loop in *E. coli*) is disordered. However, in contrast to the results in *E. coli*, the conformation of the flexible loop in MAT I was not temperature dependent, suggesting that the mobility of this loop might be a consequence of its direct involvement in catalysis (18). Whereas the exact function of this loop remains unclear, the finding that S-nitrosylation of cysteine 121 completely inactivates MAT I (21) clearly indicates that the flexible loop plays an important role in enzyme activity. Flexible loops have been identified in many protein structures and, in several cases, have been located over the active sites and found to play important roles in enzyme activity. An alignment of 11 MAT sequences in the region of the flexible loop indicates that this region is relatively poorly conserved compared with other parts of the sequence and that the aligned sequences vary in length by two to three residues in this region (20). It has been suggested that the differences in kinetic properties of different MATs may be due in part to differences in the composition of the flexible loop (20).

2. MAMMALIAN MATS

Three distinct forms of MAT (MAT I, MAT II, and MAT III) have been identified in mammalian tissues that are the products of two different genes (*MAT1A* and *MAT2A*) (13). Kinetic and regulatory properties of the different MAT isoforms in mammalian tissues are summarized in **Table 1**. The gene *MAT2A* encodes a 396 amino acid catalytic subunit (α_2) expressed in all mammalian tissues that have been examined including erythrocytes, lymphocytes, brain, kidney and fetal liver, and to a lesser extent in adult liver (12, 15, 22, 23). The structural and kinetic properties of MAT II have been studied by the group of Kotb. MAT II consists of α_2 and β subunits (22).The exact way in which the α_2 and β subunits interact is not yet known. The β subunit is a noncatalytic 334 amino acid protein that has no sequence homology with the α_2 subunit, but has a similarity to enzymes that catalyze the reduction of dTDP-linked sugars (24). Although this reaction has been best studied in bacteria (25), a BLAST search reveals the occurrence of a cDNA from mammalian cells encoding a protein with high similarity to the enzymes that catalyze the reduction of dTDP-linked sugars (26). Whether the β subunit has dTDP reductase activity is not yet known. The fact that the expression of the α_2 subunit in *E. coli* yields an active enzyme indicates that the β subunit is not necessary for MAT II activity (27).The function of the β subunit is to regulate MAT II activity by lowering its K_m for L-methionine and by increasing the sensitivity of the enzyme to feedback inhibition by AdoMet (27). Therefore, regulation of the expression of the β subunit may be a mechanism to regulate the intracellular content of AdoMet. This has been shown in T cells where superantigen stimulation leads to a marked reduction in the expression of the β subunit and a fivefold increase in the cellular AdoMet content (28).

The gene *MAT1A* encodes a 395 amino acid catalytic subunit (α_1) that organizes into dimers, MAT III, and tetramers, MAT I (12, 15). The α_1 and α_2 subunits share an amino acid sequence identity of 84% (12). *MAT1A* is expressed only in adult liver (23), predominantly in the parenchymal cells, although liver endothelial and Kupffer cells have been recently reported to express this gene (29). In developing rat liver, *MAT1A* expression increases progressively from day 20 of gestation, increases 10-fold immediately after birth, and reaches a peak at 10 days of age, decreasing slightly by adulthood (23). Conversely, *MAT2A* expression decreases toward birth, increases threefold in the newborn, and decreases further in the postnatal life, reaching a minimum in the adult (23). Consequently, under normal conditions the contribution of MAT II to the hepatic metabolism of methionine is probably negligible due to the small amount of this enzyme expressed in normal liver compared to MAT I and MAT III. It is obvious that the developmental pattern of *MAT1A* and *MAT2A* are closely related to those of albumin and α-fetoprotein, respectively, suggesting that *MAT1A* is a marker for hepatocyte differentiation (23).

For many years, it has been intriguing why there are two different MAT isoforms in the liver. The majority of methionine is metabolized by this organ and therefore the existence of two different MAT isoforms might be an adaptation to cope with the special metabolic requirements of the liver. In contrast to other mammalian enzymes, methionine has recently been shown to switch MAT III, the predominant liver form, to a higher specific activity conformation, a phenomenon known as hysteretic behavior (30). This has been demonstrated both in vitro with the purified enzyme and in vivo in isolated rat hepatocytes exposed to various physiologi-

TABLE 1. *Summary of properties of MAT isoforms in mammalian tissues*[a]

MAT isoform	Gene	Catalytic subunit	Regulatory subunit	Subunit arrangement	Tissue localization	K_m (Met) μM	K_m (ATP) mM	Regulation by ROS and NO	Inhibition by AdoMet
MAT I	*MAT1A*	α1	No	$(\alpha1)_4$	Adult liver	20; 600	0.2	+	No
MAT III	*MAT1A*	α1	No	$(\alpha1)_2$	Adult liver	120	1	+	No
MAT II	*MAT2A*	α2	β	$(\alpha2)_x\beta_y$	Extrahepatic tissues, fetal liver, HCC.	80	0.03	No	+

[a] See text for a detailed description of the regulatory and kinetic properties of the different MAT isoforms in mammalian tissues.

cal concentrations of methionine (30). The kinetic behavior of MAT I has also been studied by several laboratories, and different K_m values for ATP and methionine have been reported (12). Highly purified rat liver MAT I was found to have a sigmoidal kinetics for methionine that could be divided into two components: one with a K_m of ~600 μM and the second with a K_m of ~20 μM (31). The K_m for ATP was ~200 μM (31).

It seems that the availability of methionine is probably the determining factor that regulates MAT III activity. The concentration of methionine in rat hepatocytes is between 50 and 80 μM (6). Under these conditions, MAT III will be predominantly in the low specific activity conformation and the enzyme responsible for AdoMet synthesis will be MAT I. However, after an increase in methionine concentration, i.e., after a protein-rich meal, conversion to the high activity MAT III form would occur and methionine excess will be rapidly eliminated. Such a mechanism would be very sensitive to changes in methionine concentration. The liver also contains glycine N-methyltransferase (GNMT), a liver-specific enzyme that accounts for ~1% of the cytosolic protein in this organ, shows positive cooperativity for AdoMet, and whose main function seems to be to remove excess AdoMet synthesized during a methionine load (32). In this way, when large quantities of AdoMet are synthesized by the liver, the excess of this molecule is not used to hypermethylate DNA or proteins or for the synthesis of polyamines, which may be harmful to the liver, but is converted rapidly into S-adenosylhomocysteine by GNMT (Fig. 1). Human subjects with GNMT deficiency have an enormous increase in plasma AdoMet and methionine levels (33). Whereas the increase in plasma AdoMet can easily be explained by the reduction in GNMT activity, the relationship between GNMT deficiency and hypermethioninemia is less clear.

3. POST-TRANSLATIONAL REGULATION OF MAT ACTIVITY

The energy required to catabolize methionine is high. For each molecule of AdoMet synthesized, the three high-energy phosphodiester bonds of ATP are hydrolyzed. This may be one reason why *MAT1A* expression is limited to the liver, an organ with a high capacity to synthesize ATP. Conditions that impair the hepatic capacity to maintain normal ATP levels, as during septic shock or episodes of hypoxia, induce liver MAT inactivation (34, 35). This may be a compensatory mechanism to spare ATP and prevent hepatocytes from becoming vulnerable to ATP depletion when energy needs increase acutely. The results showing that expression of *MAT1A* in nonhepatic cells, such as CHO cells, led to a marked reduction of ATP content and higher sensitivity to cell injury induced by oxidative stress (36) support this concept.

Nitric oxide (NO) and reactive oxygen substances (ROS) have been shown to switch MAT I and MAT III to an inactive conformation through S-nitrosylation (formation of a −SNO group) and oxidation (formation of a −SOH group) of a single cysteine residue in position 121 (C121), respectively. The α_1 subunit from which MAT I and MAT III are made contains 10 cysteine residues. It has been shown that only replacement of cysteine 121 by serine (C121S) yields an active enzyme that is resistant to NO and ROS inactivation (34, 37). Cysteine 121 is located over the active site of the enzyme in the flexible loop described in section 1 of this review, suggesting that the covalent modification of this thiol group switches MAT I/III to an inactive conformation (21). Protein S-nitrosylation involves an acid base-catalyzed nitrosothiol SNO/SH exchange reaction where the target cysteine is surrounded by basic and acidic amino acids that reduce the pK_a of its thiol group and make it more nucleophilic (38). Accordingly, MAT S-nitrosylation has been shown to be regulated by the basic (Arg 357, Arg 363) and acidic (Asp 355) amino acids surrounding the target thiol; replacement of any of these three residues by serine markedly reduces the capacity of NO to S-nitrosylate and inactivate liver MAT (21). In MAT II, Arg 357, Arg 363, and Asp 355 are conserved but the equivalent position to C121 in MAT I/III is occupied by a residue of glycine (G120). MAT II is not inactivated by NO, but replacement of G120 by cysteine (G12°C) yields an active enzyme that, in the presence of NO donors, is S-nitrosylated and inactivated (39). An alignment of a large variety of MATs cloned from bacteria, yeast, plants, *Drosophila*, mouse, rat, and human liver, and mouse, rat, and human kidney has revealed that the presence of a cysteine residue in position 121 is characteristic of the liver enzymes and that all other MATs have a different amino acid in this position, most often a residue of glycine.

Inactivation of MAT I and MAT III by NO or ROS is reversed by physiological (millimolar) concentrations of glutathione (GSH) (34, 37, 40). In isolated rat hepatocytes, incubation with NO donors or inhibition of GSH synthesis induces the S-nitrosylation and inactivation of MAT and a reduction in AdoMet content (41). Removal of the NO donor from the incubation media or restoration of the cellular GSH levels leads to the denitrosylation and reactivation of liver MAT and to the rapid recovery of AdoMet content (41). Intraperitoneal (i.p.) injection of bacterial lipopolysaccharide (LPS) to rats results in S-nitrosylation and inactivation of hepatic MAT (40). Similarly, inhibition in rats of GSH synthesis by i.p. injection of buthionine sulfoximine leads to the S-nitrosylation and inactivation of hepatic MAT (41).

Liver MAT I/III activity seems to be regulated in a minute-to-minute basis by NO and ROS (which maintain the enzyme in an inactive conformation) and by GSH (which reactivates the enzyme). This may be the mechanism by which a variety of conditions that induce oxidative stress (septic shock, ethanol, hepatitis B- and

hepatitic C-induced liver cirrhosis, CCl_4) or increase NO synthesis (hypoxia, liver regeneration, cytokines) inactivate hepatic MAT I/III (15, 34, 35, 42–44). Moreover, these results suggest that liver MAT I/III inactivation may be a key signal to respond to injury.

4. REGULATION OF *MAT1A* AND *MAT2A* EXPRESSION

MAT1A, *MAT2A,* and *MAT2B* promoters have been cloned and sequenced (45–49). The murine and rat *MAT1A* promoters share limited similarities to the human promoter. The rat and human *MAT1A* promoters contain several consensus binding sites for C/EBP (CAAT enhancer binding protein), NF-1 (nuclear factor 1), and HNF (hepatocyte-enriched nuclear factor), transcriptional factors important in liver-specific gene expression (47–49) (**Fig. 2**). Two regions spanning nucleotides −1251 to −958 and −197 to +65 were found to be crucial for promoter efficiency in the rat promoter (47). Eight protein binding sites have been characterized in both regions by DNase I footprinting analysis, and two of these regions were found to be essential for the regulation of promoter activity. One region contains a NF1 site, suggesting that NF1, which usually acts as a positive transcription factor, plays a key role in directing *MAT1A* expression. The second region appears to interact with a member of the HNF-3 family when nuclear extracts from rat liver and H35 hepatoma cells were used, but an additional binding activity to an HNF-1-like protein was obtained with the hepatoma cell extracts (47). It has been suggested that this differential binding may contribute to the negative regulation of *MAT1A* in human hepatocellular carcinoma (HCC; 47). The *MAT1A* promoter contains several GRE (glucocorticoid response element) consensus binding sites (47). Consistently, glucocorticoid treatment has been shown to increase rat and human *MAT1A* expression and promoter activity in a dose- and time-dependent manner (49, 50).

Functional analysis by transient transfection has shown that the rat *MAT1A* promoter is active not only in hepatocytes and liver-type cells such as the human hepatoma cell line HepG2, but also in a nonhepatic cell such as CHO cells (47). These findings suggest that the liver-restricted expression of the endogenous gene is not mediated by the action of tissue-specific factors. It was then found that *MAT1A* promoter is hypermethylated at two CpG sites in extrahepatic tissues and fetal liver but unmethylated in adult liver, where the gene is actively transcribed (51, 52). It has been demonstrated that elevated levels of histone acetylation are critical to maintain a decondensed and active state of the chromatin and the underlying pattern of CpG methylation modulates histone acetylation (53, 54). Accordingly, the degree of acetylation of histones (H4) associated with *MAT1A* promoter in the liver is ~15-fold higher than in the kidney (51, 52). In rat and human-derived hepatoma cell lines and HCC, *MAT1A* is expressed at reduced or undetectable levels whereas *MAT2A* expression is markedly induced (55, 56). It has been found that *MAT1A* is hypermethylated in HepG2 cells and that treatment of these cells with the demethylating agent 5-aza-2′-deoxycytidine or the histone deacetylase inhibitor trichostatin A results in the induction of *MAT1A* expression (51). Taken together, these observations indicate that mechanisms involving DNA methylation and histone deacetylation may be the main factors responsible for liver-restricted expression of *MAT1A* as well as its silencing in neoplastic transformation.

Independent of the etiology (alcohol, hepatitis B, hepatitis C, etc.), *MAT1A* is expressed at reduced and, in some cases, undetectable levels in human liver cirrhosis whereas the expression of *MAT2A* remains low (56). It has been shown that *MAT1A* promoter is hypermethylated in the liver of cirrhotic patients (56), suggesting that this and the increased production of ROS and NO (see section 3 of this review) may be responsible for the impaired hepatic MAT activity (42, 55), increased serum levels of methionine (56, 57), and abnormal clearance of this amino acid (4, 42, 57) in human liver cirrhosis. Understanding why *MAT1A* be-

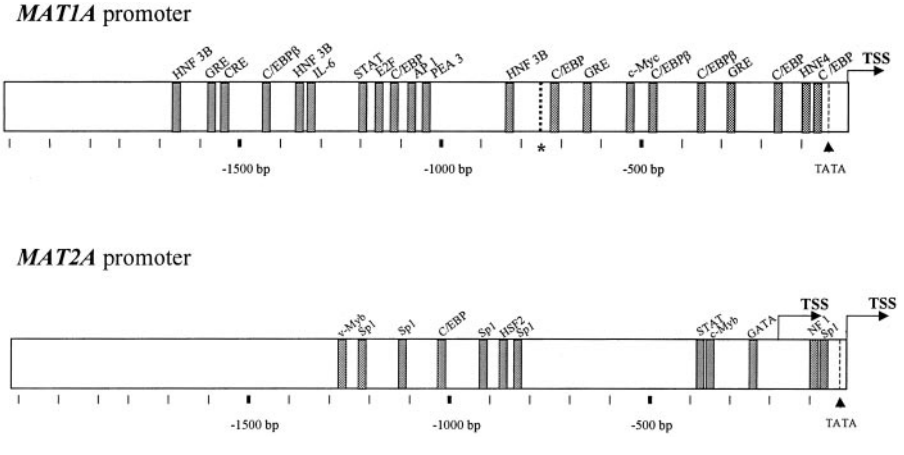

Figure 2. Schematic representation of putative transcription factor binding sites in human MAT1A and MAT2A promoters. HNF-3B (hepatocyte-enriched nuclear factor 3B), GRE (glucocorticoid response element), CRE (cyclic AMP response element), C/EBP (CAAT enhancer binding protein), IL-6 (interleukin-6 response element), STAT (signal transducer and activator of transcription), AP-1 (activator protein 1), PEA3 (polyomavirus enhancer A binding protein 3), HSF2 (heat shock factor 2), TSS (transcription start site). The protein binding sites characterized in detail are described in the text. Asterisk in MAT1A promoter indicates the location of the CpG dinucleotide (position −737) hypermethylated in cirrhotic human liver.

comes hypermethylated may have applications in the treatment of this disease.

When the methylation pattern of *MAT2A* promoter was analyzed, no differences were observed between the liver and other tissues where the gene is actively transcribed, such as kidney and spleen (58). Moreover, this analysis showed the same degree of methylation in all three tissues. However, differences in the degree of methylation have been observed between normal liver and in human liver cancer; the human *MAT2A* promoter is hypomethylated in HCC but hypermethylated in normal liver (59). The reason for these differences is not clear, and the exact role of promoter methylation in *MAT2A* expression remains to be established. Histones associated with *MAT2A* promoter are hyperacetylated in kidney and hypoacetylated in liver (60), suggesting that the low activity of *MAT2A* in liver results from the inaccessibility of transcription factors to binding sites within the condensed chromatin.

In the human MAT2A promoter, two major transcriptional start sites have been identified by primer extension assay: one located within 10 nucleotides downstream of the TATA box and another one 158 nucleotides upstream from the TATA box (48) (Fig. 2). The *MAT2A* promoter is highly rich in GC (75%) in the first 300 base pairs and contains several Sp-1 binding sites, a C/EBP, a HSF2 (heat shock transcription factor 2), a STAT (signal transducer and activator of transcription), a c-Myb, v-Myb, and GATA consensus binding sites (48, 61) (Fig. 2). Two protein binding sites have been characterized by DNase I footprinting analysis (61). These protected sites (−354 to −312 and −73 to −28) contain consensus binding sites for c-Myb and Sp1. Mutation of any of these two sites markedly reduces *MAT2A* promoter activity (61), suggesting that c-Myb and Sp1 may contribute to the up-regulation of *MAT2A* transcription in HCC. In support of this view, it has been observed that the mRNA levels of c-Myb and Sp1 are induced in HCC and that overexpression of these two factors increases *MAT2A* promoter expression (61). Treatment of Jurkat cells with interleukin 2 (IL-2) increases *MAT2A* mRNA levels and stimulates *MAT2A* promoter activity (62). This effect of IL-2 on *MAT2A* expression seems to be mediated by c-Myb (62). Thus, treatment of Jurkat cells with IL-2 increases c-Myb mRNA content, and the overexpression in these cells of c-Myb increases *MAT2A* promoter activity. Moreover, mutation of the *MAT2A* c-Myb site blocked the stimulatory of IL-2 on c-Myb nuclear binding and *MAT2A* promoter activities in Jurkat cells. Taken together, these observations indicate that mechanisms involving the acetylation of histones associated with the gene and the interaction of its promoter with c-Myb and Sp1 may be among the main factors responsible for the increased expression of *MAT2A* during liver regeneration and neoplastic transformation.

Liver regeneration is a fundamental feature of the response of this organ to injury. Caused by hepatotoxins or partial hepatectomy, liver mass loss initiates a cellular response involving a vast number of growth factors, cytokines, and transcription factors that has positive and negative effects on liver until the restoration of normal hepatic architecture and function (63). In regenerating liver after partial hepatectomy, a switch in MAT gene expression has been observed: *MAT2A* is induced and *MAT1A* mRNA levels decrease (60, 64). The induction of *MAT2A* expression is accompanied by a marked increase in the acetylation of the histones (H4) associated with the promoter of this gene (60). *MAT2A* is induced in culture hepatocytes and *MAT1A* mRNA levels decrease (65), a situation reminiscent of that found in fetal and regenerating liver and in HCC. Addition of hepatocyte growth factor (HGF, a potent mitogen for hepatocytes that plays a key role in liver regeneration) (63) to culture hepatocytes markedly stimulates the acetylation of histones associated with *MAT2A* promoter and expression of the gene (60). The effect of HGF on *MAT2A* expression is prevented by AdoMet (60) through a mechanism that involves inhibition of growth factor-induced acetylation of histones (H4) associated with the gene promoter (A. Avila, L. Torres, L., and J. M. Mato, unpublished results). On the other hand, the reduction of *MAT1A* expression in hepatocytes in culture is prevented by AdoMet through a mechanism that remains unclear, but may involve protein methylation (65). In HuH-7 cells differing only in the type of MAT gene that is expressed, *MAT2A* expression associates with more rapid cell growth whereas the opposite is observed for *MAT1A* (66). Cells expressing *MAT2A* had lower AdoMet levels than cells expressing *MAT1A* and treatment of HuH-7 cells with AdoMet led to reduced cell growth (66). These data suggest AdoMet has a growth modulatory effect. In fact, addition of AdoMet to culture hepatocytes has been found to markedly inhibit the mitogenic activity of HGF (65). Although AdoMet has no effect on the activation of extracellular signal-regulated protein kinase (ERK) (67) by HGF, this molecule strongly inhibits HGF-dependent induction of cyclin D1 and D2 expression, indicating that AdoMet suppresses HGF-induced signals between ERK activation and cyclin D1 and D2, resulting in the inhibition of DNA synthesis (E. R. Garcia-Trevijano, J. M. Mato, and M. A. Avila, unpublished results). Downstream of ERK activation, the up-regulation of D-type cyclins collectively control cell cycle progression by activating the cell cycling kinases CDK4 and CDK6, which leads to phosphorylation of the retinoblastoma protein and to acceleration of the G_1 phase of the cell division cycle (68). Cyclin D1 is induced in rodents after partial hepatectomy and in regenerating human liver (69); moreover, its overexpression promotes mitogen-independent cell cycle progression in rat hepatocytes (70). Genes encoding D-type cyclins are themselves proto-oncogenes and, in several types of tissues, can be turned into oncogenes by viruses and overexpression (71). AdoMet therapy has been shown to be effective in preventing the growth of rat HCC (72, 73). These findings provide a new and unexpected view of AdoMet as a modulator of cell cycle.

As expected, *MAT1A* null (MATO) mice have increased serum methionine levels and reduced hepatic AdoMet content (~fourfold reduction) (74). MATO mice display a phenotype resembling that observed in liver injury or stress with a vast array of growth, dedifferentiation, and acute-phase response genes (such as proliferating cell nuclear antigen, α-fetoprotein, and orosomucoid) up-regulated (74). Although histologically normal, 3-month-old MATO mice have hepatic hyperplasia, are more prone to develop liver injury, and at 8 months of age develop nonalcoholic steatohepatitis (NASH) (74). Since hepatic hyperplasia may precede the appearance of liver cancer (75), it will be important to know whether the risk for HCC is increased in MATO mice.

The available data indicate that *1)* in hepatocytes AdoMet content regulates the type of MAT gene that is expressed and *2)* the type of MAT gene expressed strongly influences the rate of cell growth and DNA synthesis. The main difference between *MAT1A* and *MAT2A* is that MAT I/III, the enzymes encoded by *MAT1A*, can maintain higher intracellular AdoMet concentrations than MAT II, the enzyme encoded by *MAT2A* (see section 1). Under normal conditions, MAT I/III are the main enzymes present in the liver and the hepatic AdoMet content is maintained at a high level. Consequently, *MAT1A* expression is high and *MAT2A* mRNA levels remain very low. Liver mass loss, caused by hepatotoxins or partial hepatectomy, initiates a cellular response involving the generation of NO and ROS (63, 76) **(Fig. 3).** This leads to the inactivation of MAT I/III and a concomitant reduction of AdoMet content, which in turn produces a reduction of *MAT1A* expression and a further decrease in MAT I/III activity and AdoMet synthesis. The reduction in AdoMet content releases the inhibition that this molecule exerts on the mitogenic activity of HGF (through its blocking effect on HGF-induced cyclin D1 and D2 expression) as well as on the expression of *MAT2A*. In response to liver injury, MAT I/III (both expression and activity) is switched off and MAT II expression is switched on; hepatic content of AdoMet changes to a new lower steady-state level that facilitates the progression of the cell division cycle and hepatocyte growth. During restoration of the original liver mass, the inhibitory effect that NO and ROS exert on MAT I/III activity is released, *MAT1A* expression is induced, AdoMet content increases, and *MAT2A* expression is again switched off, which may help the liver to recover its normal nonproliferative metabolic phenotype.

5. MATO MOUSE, A NOVEL MODEL THAT ILLUSTRATES THE IMPORTANCE OF A DEFICIENCY IN MAT I/III AND ADOMET IN THE PATHOGENESIS OF LIVER INJURY

It is known that when rats and mice are fed a diet deficient in lipotropes (choline, methionine, folate, and vitamin B12) the liver develops steatosis within a few days (77). If the diet continues, the liver develops NASH, fibrosis, and cirrhosis, with some animals developing HCC (78). Numerous nutritional studies have shown that dietary methyl insufficiency causes a decrease in hepatic levels of AdoMet and an increase in AdoHcy content (79, 80). Is lipotrope deficiency exerting its pathogenic effect in the liver through a decreased availability of AdoMet? The observation that MATO mice have hepatic hyperplasia, are more prone to develop liver injury (e.g., in response to a choline deficient diet), and spontaneously develop NASH (74) strongly suggests that AdoMet deficiency may be a key component of the mechanism by which lipotrope deficiency causes hepatic lesions. The question then is, How does a decreased availability of AdoMet lead to NASH?

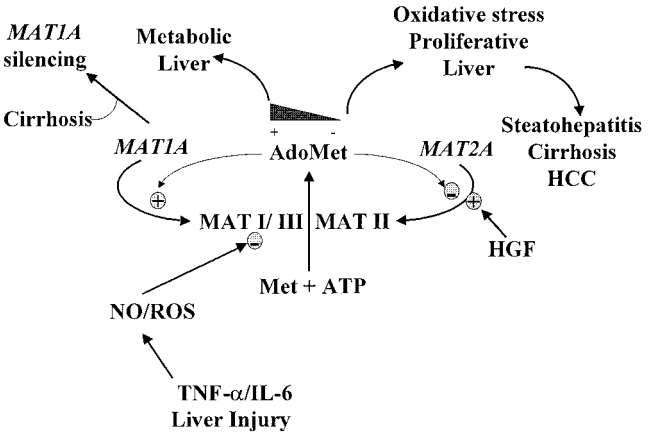

Figure 3. Regulation of liver function by AdoMet. Under normal conditions two main mechanisms operate in the liver that maintain the high cellular concentration of AdoMet: *1)* up-regulation by AdoMet of *MAT1A* expression, with the concomitant increase in MAT I/III activity; and *2)* the high capacity of MAT I/III to convert dietary methionine into AdoMet. Since AdoMet down-regulates *MAT2A* expression and inhibits MAT II activity, the contribution of this enzyme to the net synthesis of hepatic AdoMet under normal conditions is small. Liver injury caused by hepatotoxins or partial hepatectomy initiates a cellular response that involves a vast number of growth factors and cytokines (such as HGF, TNF-α, and IL-6) and generation of oxidative stress (NO and ROS). The increased production of NO and ROS leads to the inactivation of MAT I/III and a reduction of hepatic AdoMet content. This reduction in AdoMet content induces an increase in *MAT2A* expression and MAT II activity (which is not inactivated by NO or ROS). As a result, a new, lower steady-state level of AdoMet is reached. The reduced content of hepatic AdoMet releases the inhibitory effect that this molecule exerts on the proliferative activity of HGF, which facilitates liver regeneration. On restoration of the original liver mass, the inhibitory effect that NO and ROS exert on MAT I/III is released, *MAT1A* expression is induced, AdoMet content returns to its original value, and *MAT2A* expression is again down-regulated. However, if the conditions leading to oxidative stress persist (e.g., chronic exposure to alcohol, HBV, HCV), the hepatic levels of AdoMet are maintained continuously low, which predisposes the liver to injury and to develop steatohepatitis, cirrhosis, and ultimately HCC. In the cirrhotic liver, *MAT1A* expression is progressively silenced by a mechanism that involves the methylation of the gene promoter and its association with hypoacetylated histones.

The similarity of hepatic lesions between NASH and alcoholic steatohepatitis (ASH) suggests the possibility that similar mechanisms may be involved in their pathogenesis. Numerous studies support the view that alcohol contributes to the development of liver disease by generating oxidative stress through its induction of the microsomal enzyme cytochrome P4502E1 (CYP2E1) (81–83). In the alcoholic, CYP2E1 is induced by ethanol as a substrate; in the nonalcoholic, CYP2E1 is induced by ketones and a high-fat diet (84). CYP2E1 catalyzes the formation of ROS and lipid peroxidation derivatives (81–83). CYP2E1 metabolizes ethanol and other endogenous (e.g., fatty acids, ketones) and xenobiotic (e.g., acetaminophen) compounds to reactive metabolites and ROS, which can injure cell membranes and promote hepatocyte death (81–83). Formation of ROS can occur even in the absence of added substrates, as indicated by the finding that the generation of ROS from microsomes of CYP2E1-expressing cells was not altered by addition of substrates and ligands of CYP2E1 (85). The important role of CYP2E1 in ethanol-induced ROS production, GSH depletion, and cell death has been confirmed using cell lines transfected with the microsomal enzyme (86). Moreover, there is a close correlation between induction of CYP2E1 activity and alcoholic liver disease (81, 85), and inhibitors of CYP2E1 ameliorate alcoholic liver injury (87). CYP2E1 knockout mice are, however, just as susceptible to alcoholic liver injury as the wild-type animals (88), which questions the essential role of this microsomal enzyme in the development of ASH. Similarly, CYP2E1 knockout mice are not protected from the development of NASH induced by a methionine/choline-deficient (MCD) diet (89). In CYP2E1 knockout mice that developed NASH, but not in the wild-type mice, CYP4A10 and CYP4A14 are induced, suggesting that members of the CYP4A family may substitute for CYP2E1 as catalysts of hepatic lipid peroxidation (89). Whether CYP4A enzymes are up-regulated in CYP2E1 mice after ethanol-induced liver injury is not known. The finding that hepatic CYP2E1 activity is induced in patients with NASH (90) and in rats and mice where NASH is induced by feeding a MCD diet (84) also suggests a role for this microsomal enzyme in its pathogenesis. However, Zucker rats and ob/ob mice, which develop obesity, diabetes, and hepatic steatosis, do not exhibit CYP2E1 induction but display constitutive up-regulation of CYP4A (91). It appears that induction of microsomal enzymes (mainly CYP2E1 and CYP4A) may be necessary but not sufficient to develop ASH and NASH. Other factors essential for the development of ASH and NASH (e.g., the magnitude of the oxidative stress imposed to the liver or the underlying immune status of the host) remain to be investigated.

In MATO mice, the levels of liver CYP2E1 mRNA and enzyme activity are markedly induced whereas mRNA levels of CYP4A10 and CYP4A14 are decreased (74; M. A. Avila, F. J. Corrales, and J. M., Mato, unpublished results), indicating that a decrease in AdoMet content generates oxidative stress. This conclusion is further supported by the following: *1)* the hepatic content of uncoupling protein 2 (UCP2) mRNA, an anion carrier that uncouples the respiratory chain from oxidative phosphorylation and influences generation of ROS and ATP (and hence might enhance the vulnerability of hepatocytes to necrosis) (84), is also induced in MATO mice with respect to wild-type animals (M. A. Avila, F. J. Corrales, and J. M. Mato, unpublished results); *2)* the hepatic mRNA levels of several key enzymes of the trans-sulfuration pathway involved in cysteine and GSH synthesis—CBS, γ-glutamylcysteine synthetase heavy (GCS-HS) and light (GCS-LS) subunits, and GSH synthetase—are increased in MATO mice (74; S. C. Lu, unpublished results); and *3)* the hepatic content of GSH, a critical factor for preserving normal cellular redox balance and protecting hepatocytes against oxidative stress, is reduced by ~50% in MATO mice (74). Whereas the induction of CYP2E1 expression and depletion of GSH content generate oxidative stress, the increase in CBS, GCS-HS, GCS-LS, and GSH synthetase is probably an adaptation of the liver to this stress (92, 93). Since AdoMet is a precursor of hepatic GSH, it seems obvious why a deficiency in MAT activity may lead to GSH depletion. The exact mechanism by which a reduction in AdoMet increases the hepatic content of CYP2E1 mRNA is unclear and needs to be investigated. The answer may lie in whether AdoMet, through the action of specific methyltransferases or AdoMet binding proteins, is involved in the down-regulation of CYP2E1. Releasing this inhibition by lowering hepatic AdoMet content may lead to a progressive induction of CYP2E1 expression by endogenous physiological substrates of the microsomal enzyme, making the liver more susceptible to develop injury. This may be part of the mechanism by which a diet deficient in choline or folates induces CYP2E1 in the liver. A deficiency in these lipotropes causes homocysteine accumulation through the inhibition of its conversion to methionine and, consequently, an increase in S-adenosylhomocysteine (AdoHcy) levels (note in Fig. 1 that AdoHcy-hydrolase, the enzyme that converts AdoHcy into homocysteine, is the only reversible enzyme of the cycle) (15, 79, 80). Since AdoHcy is a potent competitive inhibitor of many transmethylation reactions, its accumulation may inhibit a key AdoMet-dependent methylation reaction or AdoMet binding protein involved in the down-regulation of CYP2E1 and release the control that AdoMet exerts on hepatic oxidative stress. Identifying these target proteins will be challenging, since the liver contains dozens of methyltransferases and probably as many AdoMet binding proteins.

Consistent with the concept of oxidative stress caused by a deficiency in AdoMet, MATO mice are more prone to develop liver injury. Thus, feeding a choline deficient diet for only 6 days in 3-month-old MATO mice induced severe macrovesicular steatosis, whereas in wild-type animals it caused little histological change or only mild steatosis (74). Macrovesicular steatosis of both alcohol- and nonalcohol-related etiologies is associated with the development of steatohepatitis, fibrosis,

and cirrhosis (75). At 8 months of age, MATO mice spontaneously developed NASH (74). MATO mice are more likely than wild-type animals to develop liver injury in response to CCl_4. After CCl_4 administration the activity of transaminases and the serum concentration of thiobarbituric acid-reactive substances were much higher in MATO mice than in wild-type animals (F. J. Corrales, M. A. Avila, and J. M. Mato, unpublished results). The histological liver injury caused by CCl_4 was much more severe in MATO mice than in wild-type animals. To establish the role of CYP2E1 in CCl_4-induced liver injury, MATO mice and wild-type animals were treated with diallylsulfide (DAS), an effective inhibitor of CYP2E1. The addition of DAS prevented CCl_4-induced liver injury in both MATO mice and wild-type animals. These findings strongly suggest that AdoMet deficiency may be a key factor in the development of NASH and ASH.

In contrast to these findings in MATO mice, human MAT I/III deficiency has not been associated with hepatic pathology (94). However, the number of individuals identified with mutations in the *MAT1A* gene is small (11 patients and 3 pedigrees) and, with the exception of a 43-year-old individual, are young (under 14 years) (94). It is then possible that AdoMet deficiency may be necessary but not sufficient to develop liver pathology. Therefore, the possibility that these patients may be at risk for developing liver pathology at a later age cannot be excluded.

A variety of hepatotoxic agents (e.g., CCl_4, ethanol, LPS) are known to induce the inactivation of hepatic MAT and AdoMet depletion (15). The complete prevention by DAS of CCl_4-induced hepatic MAT inactivation and AdoMet depletion in mice (F. J. Corrales, M. A. Avila, and J. M. Mato, unpublished results) suggests that CYP2E1-derived ROS may play a key role in the inactivation of this enzyme during liver injury (see section 3). Conversely, in MATO mice the addition of CCl_4 has no effect on hepatic MAT activity (F. J. Corrales, M. A. Avila, and J. M. Mato, unpublished results). These results agree with the observation made in vitro that MAT I/III but not MAT II is inactivated by ROS and NO (see section 3). This linkage between CYP2E1 oxidative stress and hepatic MAT inactivation may sensitize the liver to oxidative injury and sets up a vicious cycle since, as previously seen, AdoMet depletion causes oxidative stress through the up-regulation of CYP2E1 and GSH depletion, which in turn may cause a further reduction in MAT activity and AdoMet content. Consistent with this model, GSH depletion by buthionine sulfoximine treatment caused hepatic MAT inactivation; the addition of the ethyl ester of GSH (a permeable derivative of GSH) prevented this effect (15).

6. USE OF AdoMet AS A THERAPEUTIC AGENT FOR LIVER DISEASE

In cirrhotic patients, the progressive silencing of MAT I/III (expression and activity) caused by this condition may impose the liver to additional oxidative stress (through the induction of CYP2E1 and depletion of GSH) that will contribute to progression and complications of the disease. Preventing AdoMet deficiency may then be a major therapeutic target for the treatment of human liver diseases. As reviewed elsewhere (15, 95), a variety of clinical studies indicate that AdoMet treatment, given either orally or parenterally, is beneficial in intrahepatic cholestasis. Thus, in a multicenter double-blind, placebo-controlled clinical trial performed in 220 patients with chronic liver disease, AdoMet treatment significantly improved serum markers and subjective symptoms of cholestasis (95). Similar findings have been obtained in a variety of blind and open studies, and a metanalysis of the results of these studies confirmed the efficacy of the drug (15, 95). AdoMet treatment has been shown to be beneficial in severe cholestasis of pregnancy (15, 95). Moreover, in patients with alcoholic liver disease, oral administration of 1.2 g/day of AdoMet for 6 months resulted in a significant increase in hepatic GSH (15); in animal models of alcoholic liver disease and CCl_4 hepatotoxicity, exogenous administration of AdoMet prevented the depletion of AdoMet and GSH levels and significantly ameliorated liver injury, including fibrosis (15, 95).

Based on these experimental and clinical results, a study was designed to investigate the effects of AdoMet treatment (1.2 g/day, orally) in 123 patients (106 men and 17 women) in a double-bind, randomized, placebo-controlled multicenter over a 24 month period (96). All patients had alcoholic liver cirrhosis and histological confirmation of the diagnosis was available in 84% of cases. Seventy-five patients were in Child class A, 40 in class B, and 8 in class C. Efficacy of the treatment was analyzed in terms of mortality from any cause or liver transplantation during a period of < 2 years. The overall mortality/liver transplantation at the end of the trial decreased from 30% in the placebo group to 16% in the AdoMet group, although the difference was not statistically significant ($P=0.077$). When patients with more advanced cirrhosis (Child C) were excluded from the analysis, the overall mortality/liver transplantation was significantly greater in the placebo group than in the AdoMet group (29% vs. 12%, $P=0.025$); differences between the two groups in the 2-year survival curves (defined as the time to death or liver transplantation) were statistically significant ($P=0.046$). These results indicate that long-term treatment with AdoMet may improve survival or delay liver transplantation in patients with alcoholic liver cirrhosis, especially those with less advanced liver disease. Furthermore, this study has shown that treatment with AdoMet is safe and is free of secondary effects.

How does AdoMet exert its therapeutic effects in the liver? Since MAT activity is markedly decreased in liver diseases, it has been assumed that the consequences of this enzymatic defect can be alleviated by supplementing AdoMet, the product of the reaction. However, due to the presence of a sulfonium ion in its structure, AdoMet bioavailability is poor. Therefore, the amount

of AdoMet that may enter the liver after its administration is small and its half-life short (around 5 min) (5, 6). When 1.2 g/day of AdoMet is administered orally, probably not more than half reaches the liver after crossing the intestinal barrier, of which (again) only a small fraction will cross the plasma membrane and enter the hepatocytes. Since a normal liver makes and consumes around 5 g/day of AdoMet, supplementation with 1.2 g/day orally or 200 mg/day i.v. of AdoMet will not contribute significantly to the daily hepatic requirement of this compound. On the other hand, AdoMet treatment provides the liver with a fast and transient increase in the content of this molecule that will be rapidly consumed in methylation reactions and ultimately used for the synthesis of GSH. This transient increase in hepatic AdoMet content may stimulate *MAT1A* expression (Fig. 3) and, through its conversion into GSH, reactivate MAT I/III subunits that may have been inactivated by NO or ROS, leading to increased synthesis of AdoMet. In this way, the biological effect of exogenous AdoMet will be amplified, and this may explain its therapeutic activity in liver diseases. This model can also explain why AdoMet treatment had no effect in patients with more advanced cirrhosis (Child C) (96): in these patients, *MAT1A* has been found to be silenced by methylation of the promoter (56); consequently, AdoMet supplementation will not be able to stimulate its transcription. FJ

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