Changes in methionine adenosyltransferase and S-adenosylmethionine homeostasis in alcoholic rat liver

SHELLY C. LU,1 ZONG-ZHI HUANG,1 HEPING YANG,1 JOSÉ M. MATO,2 MATIAS A. AVILA,2 AND HIDEKAZU TSUKAMOTO1
1Division of Gastrointestinal and Liver Diseases, University of Southern California-University of California, Los Angeles, Research Center for Alcoholic Liver and Pancreatic Diseases and University of Southern California Research Center for Liver Disease, University of Southern California School of Medicine, Los Angeles, California 90033; and 2Division of Hepatology and Gene Therapy, University of Navarra School of Medicine, 31008 Pamplona, Spain

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Lu, Shelly C., Zong-Zhi Huang, Heping Yang, José M. Mato, Matías A. Avila, and Hidekazu Tsukamoto. Changes in methionine adenosyltransferase and S-adenosylmethionine homeostasis in alcoholic rat liver. Am J Physiol Gastrointest Liver Physiol 279: G178–G185, 2000.—Liver-specific and non-liver-specific methionine adenosyltransferase (MAT) are products of two genes, MAT1A and MAT2A, respectively, that catalyze the formation of S-adenosylmethionine (SAM). We previously showed that MAT2A expression was associated with more rapid cell growth. Changes in MAT expression have not been examined in animal models of alcoholic liver injury, which is the focus of the current study. After rats were fed intragastrically with ethanol and high fat for 9 wk, the mRNA level of both MAT forms doubled but only the protein level of MAT2A increased. Although liver-specific MAT activity did not change, it was 32% lower after one and 68% lower after eight weekly enteral doses of lipopolysaccharide. Hepatic levels of methionine, SAM, and DNA methylation fell by ~40%. c-myc was hypomethylated, and its mRNA level increased. Genome-wide DNA strand break increased. Thus in the prefibrotic stage of alcoholic liver injury, there is already a switch in MAT expression, global DNA hypomethylation, increased c-myc expression, and genome-wide DNA strand break. These changes may be important in predisposing this liver disease to malignant degeneration.

deoxyribonucleic acid methylation; c-myc; deoxyribonucleic acid stability; methionine metabolism; liver cancer

METHIONINE ADENOSYLTRANSFERASE (MAT) is an essential cellular enzyme that catalyzes the only reaction that generates S-adenosylmethionine (SAM), the principal methyl donor and precursor for polyamine synthesis (38). In mammals, two different genes, MAT1A and MAT2A, encode for two homologous MAT catalytic subunits, α1 and α2 (1, 23, 30). MAT1A is expressed only in the liver, and it encodes the α1-subunit found in two native MAT isozymes, which are either a dimer (MAT III) or tetramer (MAT I) of this single subunit (30). MAT2A encodes for a catalytic subunit (α2) found in a native MAT isozyme (MAT II) that is widely distributed (23, 30). MAT2A and its gene product also predominate in the fetal liver and are progressively replaced by MAT1A during development (20, 22). Expression of MAT2A is associated with rapid growth of the liver. We and others (8, 48) have shown a switch in the gene expression from MAT1A to MAT2A in liver cancer. Using a cell line model that differs only in the type of MAT expressed, we (7) showed that cells expressing MAT1A exhibited the slowest rate of growth whereas the opposite was true for cells expressing MAT2A. MAT isozymes differ in kinetic parameters and in their regulatory properties (6, 32, 41, 42, 50). The Michaelis constant (Km) for methionine is lowest for MAT II (~4–10 μM), intermediate for MAT I (23 μM–1 mM), and highest for MAT III (215 μM–7 mM), with different values reported depending on the purification method used (6, 32, 41, 42, 50). The activity of MAT is also modulated by SAM. SAM strongly inhibits MAT II [IC50 = 60 μM, which is close to the normal intracellular SAM concentration (16)], whereas it minimally inhibits MAT I (IC50 = 400 μM) and stimulates MAT III (up to 8-fold at 500 μM SAM concentration) (50). Because of these differences in kinetic and regulatory properties, a switch in MAT expression is likely to affect the steady-state SAM level and methylation. Consistent with this, we found that cells expressing MAT1A have much higher levels of SAM and DNA methylation than cells expressing MAT2A (7). A switch in hepatic MAT expression from MAT1A to MAT2A also occurred early after two-thirds partial hepatectomy (25) and after thioacetamide treatment (26). Thus the relative expression of MAT isozymes in liver is likely to influence the rate of liver growth and possibly facilitates hepatocarcinogenesis.

It has long been realized that patients with cirrhosis of different causes, including alcohol, often have hypermethioninemia and delayed plasma clearance of me-
thionine after intravenous injection (24, 29). Subsequent studies attributed this to a 50–60% decrease in the activity of liver-specific MAT (5, 14) without a change in MAT1A mRNA level (1). Although liver-specific MAT is inactivated in end-stage alcoholic liver injury in humans, changes in MAT and SAM in earlier stages of alcoholic liver disease and in animal models of ethanol-induced liver injury are far from clear. In rats given 50% ethanol by gavage and a low-protein diet for up to 10 days, Finkelstein et al. (17) showed induction of hepatic MAT activity. No other study has examined changes in MAT. Changes in SAM level have been variable. Despite a 50% fall in MAT activity, cirrhotic patients did not have a lower hepatic SAM level (5). In the baboon model, both decreased SAM and GSH levels occurred and SAM treatment ameliorated alcohol-induced liver injury (33). In rats fed the Lieber-DeCarli ethanol liquid diet, Barak et al. (3) showed that although SAM level was unchanged at 4 wk, it fell with more prolonged feeding (2 mo). Using the same diet, Trimble et al. (51) showed decreased hepatic SAM level after 3 wk of ethanol feeding. However, micropigs fed chronic ethanol (up to 12 mo) exhibited no change in the hepatic SAM level (21). The reasons for these discrepancies are unclear. In the current study, we used the Tsukamoto-French model of intragastric ethanol infusion [which has the advantage of absolute control over nutrient and ethanol intake, resulting in excellent reproducibility (52, 53)] to examine changes in MAT and SAM homeostasis and address possible consequences during the early stages of ethanol-induced liver injury.

**EXPERIMENTAL PROCEDURES**

**Materials.** SAM, S-adenosylhomocysteine (SAH), 5-methylcytosine (5-mC), cytosine, and HEPS were purchased from Sigma Chemical (St. Louis, MO). [32P]dCTP (3,000 Ci/mmol), L-[methyl-3H]methionine (214 mCi/mmol), and d-L-[1-14C]ornithine (53.7 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Mspl, Hpa II, and a total RNA isolation kit were obtained from Promega (Madison, WI). All other reagents were of analytic grade and were obtained from commercial sources.

**Animal experiments.** The rat model of alcoholic liver disease we used has been previously described in detail (18, 28, 53). Briefly, male Wistar rats weighing 350–400 g were implanted with long-term gastrostomy catheters to enable continuous intragastric infusion of the high-fat diet plus ethanol. Ethanol was gradually increased from 8 to 13.5 g · kg⁻¹ · day⁻¹ during a 9-wk experimental period. At week 9, the caloric percentages for intake of macronutrients and ethanol were 20.2% protein, 6.6% carbohydrate, 28.7% fat, and 44.5% ethanol. All required vitamins and minerals were included at the recommended concentrations by using AIN-76A vitamin and salt mixtures (Dyets, Bethlehem, PA). Controls received intragastric infusion of a high-fat diet plus isocaloric dextrose solution. This regimen was continued for 4 or 9 wk, the durations that result in fatty liver only or fatty liver, focal centrilobular steatonecrosis, mononuclear cell infiltration, and activation of Kupffer cells, respectively (28). The animal protocol used was approved by the Institutional Animal Care and Use Committee of the University of Southern California, and all animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (DHHS publication no. (NIH) 86-23, revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205).

To assess the effect of acute and chronic lipopolysaccharide (LPS) treatment on ethanol-induced liver injury, animals were treated for 9 wk with ethanol plus high fat or high fat alone. LPS (5 mg/kg as a bolus) was given intragastrically 16 h before death (acute effect) or weekly starting at week 2 (chronic effect) to both groups.

Animals were killed after 4 or 9 wk of feeding. Liver specimens were snap frozen in liquid nitrogen for subsequent measurement of SAM, SAH, and percent 5-mC levels, MAT activity, MAT1A and MAT2A steady-state mRNA, and protein levels. Liver specimens were also processed for routine histological examination, DNA extraction, and measurement of genome-wide strand break accumulation as described below. Blood was obtained in representative animals at the time of death for determination of plasma alanine aminotransferase and aspartate aminotransferase levels using an autoanalyzer (model 704, Hitachi Instruments, San Jose, CA).

**Nucleic acid extraction.** RNA isolated from frozen liver specimens according to the method of Chomczynski and Sacchi (10). RNA concentration was determined spectrophotometrically before use, and the integrity was checked by electrophoresis with subsequent ethidium bromide staining. Poly(A) RNA (mRNA) was obtained using oligo(dT) cellulose columns according to the protocol provided by Life Technologies (Grand Island, NY). Genomic DNA was isolated from frozen liver specimens according to standard procedures (46) and used for Southern blot analysis (see below).

**Northern hybridization analysis.** Northern hybridization analysis was performed on total or poly(A) RNA using standard procedures as described previously using specific MAT1A and MAT2A probes (26). c-myc cDNA probe was obtained from American Type Culture Collection (Manassas, VA). All probes were labeled with [32P]dCTP using a random-primer kit ( Primer-It II kit, Stratagene, La Jolla, CA). To ensure equal loading of RNA samples and transfer in each of the lanes, the same membranes were also rehybridized with 32P-labeled 18S or β-actin cDNA probes as described previously (8, 26). Autoradiography and densitometry (gel documentation system, Scientific Technologies, Carlsbad, CA and NIH Image 1.60 software program) were used to quantitate relative RNA. Results of Northern blot analysis were normalized to 18S or β-actin.

**Western blot analysis.** The steady-state protein level of liver-specific and non-liver-specific MAT was determined by Western blot analysis as described previously (26).

**Methionine and ornithine decarboxylase activities.** MAT activity was measured in liver cytosols as described previously using either 20 μM or 5 mM methionine (25, 26). In some experiments, all reducing agents were removed from the homogenizing buffer and MAT activity assay buffer. MAT activity is reported in nanomoles of SAM formed per milligram of protein per 40 min. Ornithine decarboxylase activity was measured as described previously (15).

**DNA methylation.** Hepatic 5-mC DNA content was measured as described previously (26). The percentage of methylation was calculated by the ratio of nanomoles of 5-mC to nanomoles of 5-mC plus nanomoles of cytosine multiplied by 100.

**Methionine, SAM, and SAH measurement.** Hepatic methionine level was determined as described previously (27) by the Amino Acid Analysis Laboratory in the Department of Molecular Biosciences at the University of California, Davis.
I and Msp I of protein to adjust for changes in milligrams of protein per (26). All metabolites are reported in nanomoles per milligram SAM and SAH levels were measured as described previously in EXPERIMENTAL PROCEDURES. The same membrane was then sequentially rehybridized with 32P-labeled MAT2A and 18S cDNA probes. A representative Northern blot is shown. MAT, methionine adenosyltransferase.

SAM and SAH levels were measured as described previously (26). All metabolites are reported in nanomoles per milligram of protein to adjust for changes in milligrams of protein per gram of liver due to ethanol feeding.

Restriction enzyme digestion and Southern blot analysis. Msp I and Hpa II restriction endonucleases were used as described previously with modifications (19). Msp I and Hpa II can distinguish between the unmethylated and methylated cytosine in the nucleotide sequence 5’-CCGG. Msp I is insensitive to the methylation status whereas Hpa II will digest only if the internal cytosine is unmethylated (19). Limit digests were obtained by digesting 15 μg of DNA for 6 h at 37°C with 45 U of enzymes. The digested DNA was separated on 1% agarose, transferred onto nylon filters by 20 min and hybridized for 3–4 h at 65°C in the same buffer with rapid-hybridization buffer (Amersham Life Sciences) for 20 min in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), and cross-linked in an ultraviolet cross-linker (FS-UVXL-1000, Fisher Biotech). The filters were prehybridized at 65°C in rapid-hybridization buffer (Amersham Life Sciences) for 20 min and hybridized for 3–4 h at 65°C in the same buffer with a c-myc genomic kindly provided by Clifford J. Steer (31). The blots were washed twice at room temperature for 20 min in 2× SSC containing 0.1% SDS and at 65°C for 30 min in 0.1× SSC containing 0.1% SDS. Autoradiography was performed by exposure to Kodak BioMax MR film at ~80°C.

Genome-wide DNA strand break accumulation. Quantitation of genome-wide DNA strand break accumulation was performed as previously described (45). Briefly, 3’-OH DNA fragments present in the high-molecular-weight DNA extract are separated first into single strands by a denaturation step. After reassociation, these DNA fragments serve as primer and the excess of high-molecular-weight DNA serves as template in a reaction with DNA polymerase. The [32P]dCTP incorporation initiated by Klenow fragment of DNA polymerase I under strictly defined conditions is proportional to the number of 3’-OH breaks present. The data are expressed as picomoles of dCTP incorporated per microgram of DNA.

Statistical analysis. Two-tailed nonpaired Student’s t-tests were used for comparisons between two groups. For changes in mRNA and protein levels, ratios of MAT1A, MAT2A, and c-myc to housekeeping control or Coomassie blue staining densitometric values were compared by two-tailed nonpaired Student’s t-test. Unless otherwise stated, results represent means ± SE. Significance was defined by P < 0.05.

RESULTS

We (36) have recently reported the effect of intragastric ethanol plus high-fat feeding on weight gain, plasma transaminase levels, and histology. Briefly, both ethanol-fed and control animals had similar body weight gains, and ethanol-fed animals exhibited a three- to fivefold increase in plasma transaminase levels (36). Histological examination revealed moderate to severe centrilobular micro- and macrovesicular steatosis in 100% of ethanol-fed animals, centrilobular spotty necrosis in 50%, and mononuclear cell infiltration in 80% (36). In contrast, high-fat fed controls exhibited no histological abnormalities (36). Addition of one intragastric dose of LPS the night before death accentuated sinusoidal congestion and mononuclear infiltration and induced coagulative necrosis in some animals (P. Mathurin, A. Keshavarzian, S. Chaudery, E. Holmes, S. W. French, and H. Tsukamoto, unpublished data).

Chronic LPS administration to high-fat fed control animals did not result in any liver injury but greatly potentiated ethanol-induced liver injury, including development of centrilobular fibrosis, which was not observed in ethanol-fed animals without LPS at this stage (Ref. 37; unpublished results).

Effect of chronic ethanol feeding on hepatic MAT expression. After 9 wk of an ethanol and high-fat diet, the steady-state hepatic mRNA levels of both MAT1A and MAT2A nearly doubled (MAT1A = 186 ± 13% of control, MAT2A = 194 ± 14% of control; n = 9 in each group; P < 0.05) (Fig. 1). A doubling of both MAT mRNA levels was already present by 4 wk after ethanol feeding (data not shown). Despite a similar increase in the mRNA level, only the protein level of non-liver-specific MAT was markedly increased (1,680 ± 228% of control; n = 4 in each group). Liver-specific MAT protein level increased no more than 30% (126 ± 6% of control; n = 4 in each group) (Fig. 2).

Effect of chronic ethanol feeding on MAT activity. Liver-specific MAT exhibits a much higher K_m for methionine (mM range) than non-liver-specific MAT (~10

Fig. 1. Effect of chronic ethanol ingestion on hepatic steady-state MAT1A and MAT2A mRNA levels. Liver RNA (30 μg/lane) samples obtained from 3 control rats fed a high-fat diet and 3 rats fed an ethanol + high-fat diet for 9 wk were analyzed by Northern blot hybridization with a 32P-labeled MAT1A cDNA probe as described in EXPERIMENTAL PROCEDURES. The same membrane was then sequentially rehybridized with 32P-labeled MAT2A and 18S cDNA probes. A representative Northern blot is shown. MAT, methionine adenosyltransferase.

Fig. 2. Effect of chronic ethanol ingestion on hepatic steady-state liver-specific (top) and non-liver specific (bottom) MAT protein levels. Liver cytosol (50 μg/lane) obtained from 3 control rats fed a high-fat diet and 3 rats fed an ethanol + high-fat diet for 9 wk were analyzed by Western blot analysis using anti-rat liver-specific or non-liver-specific MAT antibodies as described in EXPERIMENTAL PROCEDURES. Equivalent protein loading was assured by Coomassie blue staining of gels after transblotting (not shown). A representative Western blot analysis is shown.
Table 1. Effect of chronic ethanol plus high-fat feeding on hepatic MAT activity

<table>
<thead>
<tr>
<th>Condition</th>
<th>MAT Activity, nmol SAM formed/mg protein(^{-1}) 40 min(^{-1})</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>With reducing agents</td>
</tr>
<tr>
<td>Control</td>
<td>0.029 ± 0.003</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.035 ± 0.003*</td>
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Values are means ± SE from 4-6 animals for each condition. Animals received either ethanol + high-fat or isocaloric high-fat diets intragastrically for 9 wk, and hepatic methionine adenosyltransferase (MAT) activity was determined in the presence or absence of reducing agents as described in EXPERIMENTAL PROCEDURES. Some animals also received an intragastric dose (5 mg/kg) of lipopolysaccharide (LPS) administered 16 h before death (acute LPS) or weekly starting at week 2 (chronic LPS). SAM, S-adenosylmethionine. *P < 0.05 vs. respective control by unpaired Student’s t-test.

Table 2. Effect of chronic ethanol plus high-fat feeding on hepatic methionine, SAM, SAH, and DNA methylation levels

<table>
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<th>Condition</th>
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<td>Control</td>
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<td>Ethanol</td>
<td>0.27 ± 0.05*</td>
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Values are means ± SE from 5–6 animals for each condition. Animals received either ethanol + high-fat or isocaloric high-fat diets intragastrically for 9 wk, and hepatic methionine, SAM, S-adenosylhomocysteine (SAH), and DNA methylation levels were determined as described in EXPERIMENTAL PROCEDURES. *P < 0.05, †P < 0.005 vs. control by unpaired Student’s t-test.

DISCUSSION

In mammals, the liver plays a central role in methionine metabolism because nearly one-half of the daily intake of methionine is metabolized there (38). Under normal conditions, SAM can be decarboxylated to be used as a precursor for the synthesis of polyamines, but most of it is used in transmethylation reactions in which methyl groups are added to compounds and expression of the MAT isozymes. Because chronic ethanol treatment resulted in a relative increase in the hepatic expression of non-liver-specific MAT, we determined how this change influenced hepatic SAM, SAH, and DNA methylation. Table 2 shows that after 9 wk of ethanol feeding the hepatic SAM level fell by 43% while the SAH level remained unchanged. The SAM-to-SA ratio also fell in parallel and as expected, global DNA methylation was significantly lower in the ethanol-fed group.

Effect of chronic ethanol feeding on hepatic methionine and ornithine decarboxylase activity. Although a switch in MAT expression can influence SAM level (7), decreased SAM level can also result from increased utilization for polyamine synthesis or decreased availability of methionine. Consistent with previous reports (13), ornithine decarboxylase activity was significantly lower in the ethanol-fed rats (control = 26.7 ± 1.1 pmol ^14CO\(_2\) · h\(^{-1}\) · mg protein\(^{-1}\) vs. ethanol fed = 17.3 ± 3.2 pmol ^14CO\(_2\) · h\(^{-1}\) · mg protein\(^{-1}\); 3 determinations each; P < 0.05 by unpaired t-test). Hepatic methionine level was 41% lower in the ethanol-fed rats (Table 2).

Possible biological consequences. Hypomethylation and increased expression of protooncogenes such as c-myc have been well described in chemical-induced hepatocarcinogenesis (12, 43, 44, 49, 54). We next examined whether c-myc is hypomethylated in the chronic ethanol fed livers. Figure 3 shows that c-myc is hypomethylated at the genomic DNA level and hepatic c-myc mRNA level doubled in the ethanol-fed animals (190 ± 10% of control; n = 3 in each group) (Fig. 4). Hypomethylation has also been shown to result in increased genome-wide strand break accumulation (45). Consistent with this, there is increased genomewide DNA strand break accumulation in the livers of animals fed chronic ethanol (control = 1.31 ± 0.14 pmol dCTP incorporated/µg DNA, ethanol fed = 2.29 ± 0.42 pmol dCTP incorporated/µg DNA; n = 7 in each group; P < 0.05 by unpaired t-test).

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Values are means ± SE from 4-6 animals for each condition. Animals received either ethanol + high-fat or isocaloric high-fat diets intragastrically for 9 wk, and hepatic methionine adenosyltransferase (MAT) activity was determined in the presence or absence of reducing agents as described in EXPERIMENTAL PROCEDURES. Some animals also received an intragastric dose (5 mg/kg) of lipopolysaccharide (LPS) administered 16 h before death (acute LPS) or weekly starting at week 2 (chronic LPS). SAM, S-adenosylmethionine. *P < 0.05 vs. respective control by unpaired Student’s t-test.

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Fig. 3. Effect of chronic ethanol ingestion on c-myc methylation pattern. Hepatic DNA from 3 control and 3 ethanol-fed samples was digested with Msp I, which is insensitive to methylation of the internal cytosine residue of CCGG sequences, or Hpa II, which is inhibited if the internal cytosine is methylated, and Southern blot analysis was performed using a c-myc genomic probe as described in EXPERIMENTAL PROCEDURES. Note that digestion of control or ethanol-fed DNA samples with Msp I resulted in the same number and size of bands. However, digestion of DNA with Hpa II resulted in high-molecular-weight bands in normal liver (see arrows at right) that are not present in the ethanol-treated livers.

SAM is converted to SAH (16, 38). SAH is a potent competitive inhibitor of transmethylation reactions; both an increase in SAH level as well as a decrease in the SAM-to-SAH ratio are known to inhibit transmethylation reactions (38). For this reason, the removal of SAH is essential. The reaction that converts SAH to homocysteine and adenosine is reversible and catalyzed by SAH hydrolase (16, 38). In fact, the thermodynamics favor synthesis of SAH (16). In vivo, the reaction proceeds in the direction of hydrolysis only if the products, adenosine and homocysteine, are rapidly removed (16, 38). In liver, there are three pathways that metabolize homocysteine. One is the transsulfuration pathway, which converts homocysteine to cysteine (35). Two other enzymatic reactions resynthesize methionine from homocysteine; one is catalyzed by methionine synthase and the other by betaine-homocysteine methyltransferase (16, 38).

Several abnormalities in methionine metabolism are well described in ethanol-induced liver injury. First is inactivation of liver-specific MAT in end-stage cirrhosis (5, 14). The mechanism was shown to be posttranslational, as modification of critical cysteine residues inactivated the enzyme by either directly interfering with the substrate-binding site(s) or causing dissociation of the oligomers (2, 38, 39, 47). This can occur under conditions of increased oxidative stress, nitric oxide formation, or decreased intracellular GSH level, which have been described in ethanol-induced liver injury (27, 33, 36). However, Finkelstein et al. (17) showed induction in MAT activity after 10 days of ethanol feeding. Thus changes in MAT may depend on the stage of the liver disease or the model used, and whether non-liver-specific MAT was induced was not examined. Other abnormalities include decreased methionine synthase activity (3, 21, 51) and increased betaine-homocysteine methyltransferase activity in an attempt to conserve the methionine pool at the expense of betaine (3, 51). Changes in SAM and SAH have not been consistent, as some studies have shown decreased SAM (3, 33, 51) and increased SAH (21, 51), but others have not (5). The reasons for this discrepancy are unclear and may be partially related to the model used and different duration of ethanol treatment.

Using the Tsukamoto-French intragastric ethanol infusion model (52, 53), we observed an early induction in the mRNA level of both MAT1A and MAT2A to comparable levels. This occurred as early as after 4 wk of feeding when the liver exhibits predominantly macrovesicular steatosis. The induction was maintained at the same level after 9 wk of feeding when the liver exhibits in addition to steatosis, focal centrilobular necrosis (50%) and mononuclear cell infiltration (80%). Interestingly, although the mRNA level of both MAT forms increased comparably, only the protein level of non-liver-specific MAT was markedly induced. This differential effect of ethanol on MAT mRNA and protein expression suggests that either the translational efficiency of liver-specific MAT decreased or the protein was degraded more rapidly. We (26) have observed a dissociation in liver-specific MAT mRNA and protein levels previously with thioacetamide treatment. We are not able to differentiate between these possible mechanisms at present because the anti-liver-specific MAT antibody currently available was not able to immunoprecipitate the protein after overnight radiolabeling (unpublished observations).

After 9 wk of ethanol feeding, liver-specific MAT activity did not change but non-liver-specific MAT activity was increased, consistent with changes in the respective protein levels. However, this result differs from the observation that liver-specific MAT activity was inactivated in patients with alcoholic cirrhosis (5, 14). Part of the reason may be related to the stage of the liver disease. Another factor may be endotoxemia, which is well recognized in chronic alcoholics (4, 37). Because chronic ethanol feeding is known to increase gut permeability to endotoxin (4, 37) and LPS has been
shown to inactivate liver-specific MAT (2), we investigated the effect of LPS on MAT activity in this model. Interestingly, acute low-dose LPS treatment reduced liver-specific MAT activity by 32% while chronic LPS treatment reduced it by 68%. These doses of LPS exerted no toxic effect in high-fat fed control animals. Thus our results raise the possibility that the inactivation of liver-specific MAT observed in alcoholic liver disease may be more related to endotoxemia.

Chronic ethanol treatment resulted in a significant fall in hepatic SAM level, SAM-to-SAH ratio, and DNA hypomethylation. The fall in SAM level can be partly explained by the change in the relative expression of the MAT isozymes we (7) observed with our cell line model. We excluded the possibility of increased SAM utilization for polyamine synthesis because the activity of the rate-limiting enzyme for polyamine synthesis, ornithine decarboxylase, was actually decreased. Our result is consistent with that of others (13) in terms of the effect of chronic ethanol on polyamine synthesis. However, another major reason is decrease (41%) in hepatic methionine availability. The effect of ethanol feeding on hepatic methionine level depends on the severity of ethanol-induced liver injury. Short-term feeding produced either no change (51) or even an increase in methionine level (27). Our (27) previous study using a lower amount of fat (25%) combined with intragastric ethanol feeding found increased hepatic methionine level after 5 wk but 50% reduction in methionine level after 16 wk. The diet regimen used in the current study contains additional fat, which is known to result in accentuated ethanol-induced liver injury (40). The most likely explanation for the fall in hepatic methionine level is decreased resynthesis of methionine from homocysteine. Methionine synthase has already been shown (3, 21, 51) to be inactivated. It is likely that the induction in betaine-homocysteine methyltransferase was not sufficient to maintain the methionine pool. Another possibility that remains to be examined is decreased methionine uptake as ethanol-induced liver injury progresses.

The question is whether these changes are of pathogenic importance. Reduced SAM level and methylation affect gene expression, membrane fluidity, and GSH level in liver (33, 38). Effects on membrane fluidity (11, 34, 38) and GSH level (33, 38) have been well studied. It is unclear how changes in DNA methylation affect ethanol-induced liver injury. Alcoholic liver disease is associated with increased risk of liver cancer, but the molecular mechanism is unclear. DNA methylation is related to mammalian gene activities, somatic inheritance, and cellular differentiation. Activation of some genes has been ascribed to the demethylation of critical mCpG loci, and silencing of some genes may be related to the methylation of specific CpG loci (9). In this regard, several studies (43, 44, 49) reported a fall in liver SAM level, SAM-to-SAH ratio, and overall DNA methylation and increased expression of protooncogenes such as c-myc, c-Ha-ras, and c-Ki-ras during the early stages of rat liver carcinogenesis. All of these changes were prevented with exogenous SAM treatment (44, 49). The protective effect of SAM appears to be related to DNA methylation because the protection was reversed by an inhibitor of DNA methylation, 5-azacytidine (43). A common hypothesis is that hypomethylation of growth-promoting protooncogenes and/or hypermethylation of tumor suppressor genes will alter transcription factor binding and expression of these genes to promote a selective growth advantage for the initiated cell (45). Alternatively, DNA hypomethylation may promote malignant transformation by inducing regional alterations in DNA conformation and chromatin structure, rendering affected regions more accessible to DNA-damaging agents (45). Similar to the findings in chemical-induced hepatocarcinogenesis, we found hypomethylation and increased expression of c-myc in the ethanol-fed livers. Furthermore, we also detected increased genome-wide DNA strand break accumulation. Thus even at the prefibrotic stage of ethanol-induced liver injury there are already changes that may predispose the liver to malignant degeneration.

In summary, we have shown, for the first time in the prefibrotic stage of ethanol-induced liver injury, a relative switch in MAT expression, global DNA hypomethylation, increased c-myc expression, and genome-wide DNA strand break accumulation. These changes may have important implications in predisposing this chronic liver disease to malignant degeneration.

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REFERENCES
7. Cai J, Mao Z, Hwang JJ, and Lu SC. Differential expression of methionine adenosyltransferase genes influences the rate of


