ONOMY Science

International Journal of Pancreatology & Liver Disorders

Research Article Open Access

Expression of Hepatitis C: Replicating and Circulating Hepatitis C Virus

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Citation: Expression of Hepatitis C: Replicating and Circulating Hepatitis C Virus. Inte J Panc & Liv Dis. 2019; 1(1): 001-008.

Submitted: 18 May 2019; **Approved:** 24 May 2019; **Published:** 26 May 2019

Abstract

In previous cross-sectional studies, we demonstrated that, in most patients with chronic hepatitis C, the composition and complexity of the circulating hepatitis C virus (HCV) population do not coincide with those of the virus replicating in the liver. In the subgroup of patients with similar complexities in both compartments, the ratio of quasispecies complexity in the liver to that in serum (liver/serum complexity ratio) of paired samples correlated with disease stage. In the present study we investigated the dynamic behavior of viral population parameters in consecutive paired liver and serum samples, obtained 3 to 6 years apart, from four chronic hepatitis C patients with persistently normal transaminases and stable liver histology. We sequenced 359 clones of a genomic fragment encompassing the E2(p7)-NS2 junction, in two consecutive liver-serum sample pairs from the four patients and in four intermediate serum samples from one of the patients. The results show that the liver/serum complexity ratio is not stable but rather fluctuates widely over time. Hence, the liver/serum complexity ratio does not identify a particular group of patients but a particular state of the infecting quasispecies. Phylogenetic analysis and signature mutation patterns showed that virtually all circulating sequences originated from sequences present in the liver specimens. The overall behavior of the circulating viral quasispecies appears to originate from changes in the relative replication kinetics of the large mutant spectrum present in the infected liver.

INTRODUCTION

Hepatitis C virus (HCV) is one of the leading causes of chronic liver disease worldwide (22). The quasispecies nature of the single-stranded RNA genome of HCV is thought to play a central role in maintaining and modulating viral replication (10, 29). In general, the natural history of HCV infection does not follow a defined pattern, but the rates of progression of the disease (from minimal changes of the liver to cirrhosis and hepatocarcinoma) vary greatly in different individuals (22). The cytopathic potential of the virus and the characteristics of the host's immune response have both been postulated to explain the observed differences in disease progression (6, 17, 18, 45).

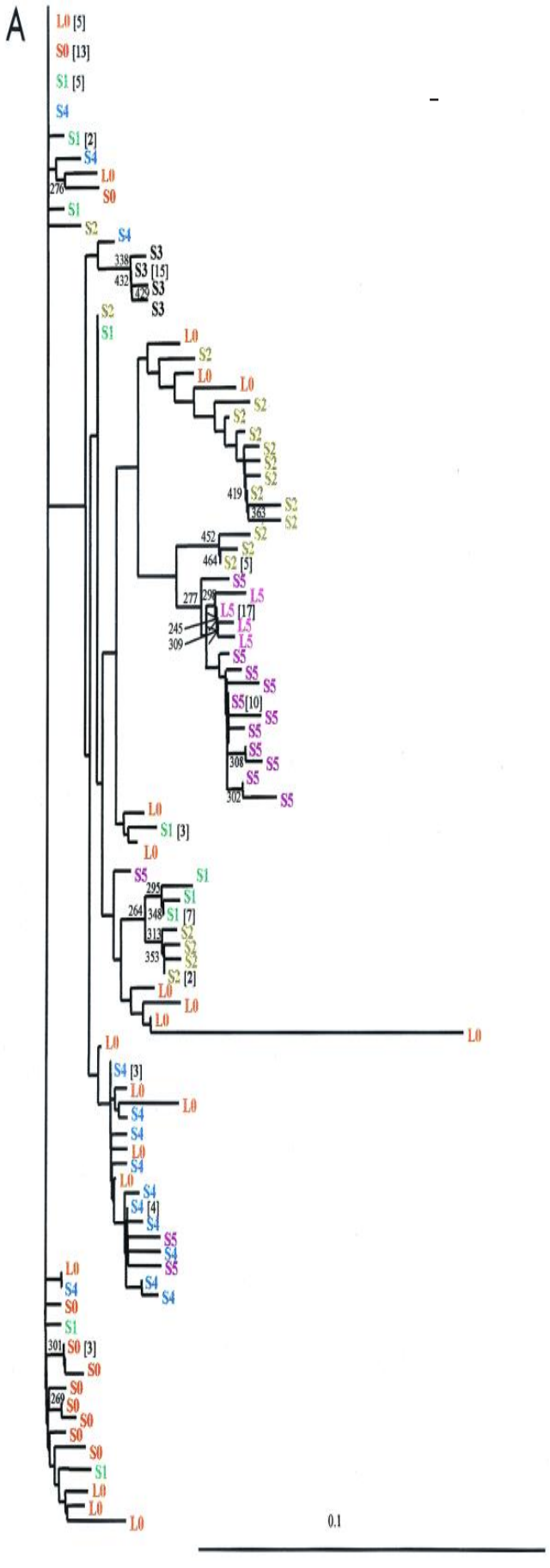
In the absence of an appropriate animal model or culture system and in the context of the extreme heterogeneity of HCV, it has not been possible to associate particular sequences with distinct cytopathic potential. Similarly, attempts to correlate the process of quasispecies diversification with disease progression have yielded controversial results. While in cross-sectional studies some authors found a correlation between quasispecies complexity and extent of liver damage (20, 20, 21, 25, 48), others did not (19, 27, 33, 41). In longitudinal studies, the nucleotide complexity of the hypervariable domain of the HCV E2 region (HVR1) did not increase cumulatively over time but fluctuated in consecutive serum samples (2). Similarly, at more conserved regions of the genome, an oscillatory pattern of quasispecies complexity over time has been observed in long-term studies with frequent serum sampling (7). The factors that drive these fluctuations and their relative contributions are not defined, and, to further complicate the interpretation of the biological meaning of circulating quasispecies complexity, it has been proven that within an infected patient

the composition of the circulating viral population does not necessarily reflect that of the hepatic population (4, 15, 28, 34, 38).

Recently, we observed a significant correlation between quasispecies complexity at the amino acid level, in both serum and liver quasispecies, and the extent of liver fibrosis, albeit only in those patients with a similar levels of complexity in both compartments (5). It is currently unknown whether the ratio of quasispecies complexity in the liver to that in serum (liver/serum complexity ratio) is a stable parameter, so that patients may be characterized by their ratios, or if it fluctuates over time.

We have investigated the behavior of viral population parameters over time in consecutive liver biopsy and serum samples obtained from four patients with nonprogressive chronic hepatitis C.of the body (1, 17). Isolated hypermethioninemia has been considered a benign inborn error of metabolism, because patients with mutations in the MAT1A gene are generally asymptomatic (17). However, the observation that MAT I/III activity is impaired in patients with liver cirrhosis (18, 19) and the demonstration that AdoMet treatment increases survival in patients with alcoholic liver cirrhosis (3, 10, 20) suggest that impaired synthesis of AdoMet may be a necessary but not sufficient component of the pathogenesis of cirrhosis in some patients with this syndrome.

In adult liver, increased expression of MAT2A is associated with rapid growth or dedifferentiation of the liver. We showed a switch in gene expression from MAT1A to MAT2A in human liver cancer (21), from 12 to 24 h after partial hepatectomy in the rat (22) and after treatment with thioacetamide (23). Using a cell line model that differs only in the type of MAT expressed, we showed that cells expressing MAT1A exhibited the slowest rate of growth, whereas the opposite was true for cells expressing MAT2A (24). Because of the differences in kinetic and regulatory properties, a switch in MAT expression is likely to affect the steady-state AdoMet level, methylation reactions, and consequently gene expression. Consistent with this, we found that cells that express MAT1A have much higher levels of AdoMet than cells that express MAT2A (24). Thus, the relative expression of MAT isoenzymes in liver is likely to influence the rate of liver growth and possibly facilitates the development of liver damage and hepatocarcinogenesis. To address many of these possibilities, we have developed a MAT1A knockout mouse model by using the targeted gene disruption approach.



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DNA from offspring of a heterozygous mating.

The targeting vector (30 μg) was linearized with EcoRI and introduced into the embryonic stem cells (129/Sv-derived) by electroporation (performed by Genome Systems, St. Louis). Genomic DNA of resistant clones were screened for recombination events by Southern blot analysis by using a 0.85-kb NcoI fragment probe (Fig. (Fig.11A). A targeted clone was used for injection into the blastocysts of C57BL/6J mice and transferred into pseudopregnant female mice as described (27). Chimeric male offspring were bred to C57BL/6J females, and agouti F1 offspring were tested for transmission of the disrupted allele by Southern blot analysis of EcoRI-SalI-digested genomic DNA by using the NcoI fragment probe. Heterozygous matings of the F1 mice were carried out to produce homozygous F2 mutant mice.

Animal Experiments

Mice were fed a standard diet (Harlan Teklad irradiated mouse diet 7912), unless indicated otherwise. Three- and eight-month-old male homozygous and wild-type littermates were killed for routine histologic examination of the liver. In addition, liver specimens were snap frozen for subsequent Northern and Western blot analyses, determination of MAT activity as described under saturating concentrations of the substrates, 5 mM methionine, and 5 mM ATP (28); levels of AdoMet, S-adenosylhomocysteine (AdoHcy), and methylthioadenosine (MTA), as determined by HPLC after the procedure described by Fell et al. (29) and modified by Miller et al. (30); and glutathione (GSH) and oxidized GSH, as described by Reed et al. (31). MAT activity was also determined in the kidney homogenates of these animals. At time of death, serum samples were collected and deproteinized by ultrafiltration, and methionine levels were analyzed by using a Beckman 6300-aa analyzer, following the procedure recommended by the manufacturer for physiological sample analysis.

In one series of experiments, 3-month-old male knockout mice and wild-type littermates were fed the amino acid-defined low methionine (0.17%) choline-free diet, Lombardi choline-deficient (CD) diet (Dyets, Bethlehem, PA), or control diet (0.4% methionine, 0.2% choline, Harlan Teklad) for 6 days. Four groups of animals were established (five mice per group): wild-type and MAT1A knockout mice on the control diet and wild-type and knockout mice on the CD diet. At the end of the treatment, animals were killed, and livers were removed. Liver samples were formalin fixed and embedded in paraffin for general histology.

Liver Histology

Sections from formalin-fixed liver tissue from 3- and 8-month old MAT1A knockout and wild-type littermates, fed either a CD diet or a control diet for 6 days, or a normal diet for 3 or 8 months, were examined by two pathologists blinded to the animals' identity and treatment. All sections were stained with Masson's trichrome or hematoxylin and eosin. Liver tissues from four knockout and four wild-type animals were examined and compared.

Nucleic Acids Extraction and Northern Hybridization Analysis

RNA was isolated from frozen liver specimens according to the method of Chomczynski and Sacchi (32). RNA concentration was determined spectrophotometrically before use, and integrity was checked by electrophoresis with subsequent ethidium bromide staining. Electrophoresis of RNA and gel blotting were carried out as described (33).

MAT1A and MAT2A cDNA probes were as described (21–23). The probes (cDNA fragments) for betaine homocysteine methyltransfease (BHMT), AdoHcy hydrolase (SAHH), and phosphatidylethanolamine N-methyltransferase 2 (PEMT2) were cloned by reverse transcriptase–PCR (34–36). The probes for glycine N-methyltransferase (GNMT), cystathionine β-synthase (CBS), and α fetoprotein (AFP) were the generous gifts of C. Wagner (Vanderbilt University, Nashville, TN), J. T. Brosnan (Memorial University, Newfoundland, Canada), and J. L. Danan, (Centre de Recherche sur l'Endocrinologie Moleculaire, Meudon, France), respectively.

Northern hybridization analysis was performed on total RNA by using standard procedures, as described (21–23, 33), with specific cDNA probes for MAT1A, MAT2A, GNMT, SAHH, PEMT2, CBS, BHMT, and AFP. All probes were labeled with [32P]dCTP by using a random-primer kit (Primer-It II Kit, Stratagene) or the Megaprime DNA labeling system (Amersham Pharmacia). To ensure equal loading of RNA samples and transfer in each of the lanes, before hybridization, membranes were rinsed with ethidium bromide and photographed, and the same membranes were also rehybridized with 32P-labeled 18S rRNA cDNA probe (22). 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 the Northern blot analysis were normalized to 18S rRNA.

Western Blot Analysis

The steady-state levels of liver-specific MAT and proliferating cell nuclear antigen (PCNA) were measured as described (23). Anti-PCNA antibodies were obtained from Zymed.

DNA Methylation Status

Genomic DNA was isolated from livers of MAT1A

knockout and wild-type mice, as described (37). DNA methylation at the CpG site assay was carried out essentially as described

Affymetrix genechip Analysis

The RNA samples were processed as recommended by Affymetrix (Santa Clara, CA). Briefly, after column cleanup of the RNA [Qiagen RNeasy (Chatsworth, CA)], cDNA was synthesized by using an oligo-dT primer attached to a sequence of the T7 promoter region. This cDNA was then used to perform in vitro transcription by using a bacteriophage T7 RNA polymerase promoter and incorporating biotin-labeled nucleotides [Enzo BioArray kit (Enzo Biochem)]. The in vitro transcription reaction product was purified and analyzed via agarose gel electrophoresis to confirm a size distribution ranging from 500 to 1,200 bp. cRNA was fragmented and hybridized at 45°C overnight to an Affymetrix murine U74A array. The arrays were washed, stained, and scanned by using a Fluidics Station 400 and Hewlett–Packard scanner, components of the Affymetrix genechip Instrument System. Finally, the results were analyzed by using genechip analysis suite software (Ver. 3.3, produced by Affymetrix) and the fold change between the hybridization intensity of the MAT1A homozygous deletion and wild-type samples obtained.

Statistical Analysis

Data are given as mean ± SD. For changes in metabolites, methylation, MAT activity, and liver weight, statistical analysis was performed by using the Kruskall–Wallis test (STATWORKS). For changes in mRNA and protein levels, ratios of the respective mRNA or protein to 18S or Coomassie blue staining densitometric values were compared between knockouts and wild-type littermates by Student's t test. Significance was defined as $P < 0.05$.

RESULTS

Generation of MAT1A Knockout Mice

A sequence-replacement gene-targeting vector (Fig. (Fig.11A), designed to interrupt the coding sequences of the murine MAT1A gene in exon 3, was used for electroporation of embryonic stem cells. After homologous recombination, the EcoRI-SalI fragment increased from 7.2 to 9.0 kb, which can be easily differentiated from the wild-type allele on Southern blot analysis (Fig. (Fig.11B). One targeted clone of about 300 was identified by Southern blot analysis, used for injection into the blastocysts of C57BL/6J mice, and transferred into pseudopregnant female mice for generation of chimeric mice. Chimeric males were bred with C57BL/6J females, and the agouti F1 offspring were tested for transmission of the disrupted allele by Southern blot analysis (Fig. (Fig.11B). Heterozygous matings of the F1 mice were carried out to produce homozygous F2 mutant mice. From 271 offspring, 86 wild-type mice, 111 heterozygotes, and 74 knockout homozygotes were identified.

MAT1A Is Not Expressed in MAT1A Knockouts, but MAT2A and AFP Are Induced

Absence of MAT1A expression is confirmed on both Northern (Fig. (Fig.22A) and Western blot analyses (Fig. (Fig.22B). In heterozygotes, the MAT1A mRNA and protein levels are reduced by 50% (results not shown). In the MAT1A knockouts, the steady-state mRNA level of MAT2A is induced by 3-fold ($P < 0.05$ vs. wild type). Because MAT2A expression is associated with rapid growth and dedifferentiation of the liver, we examined the expression of AFP. In the knockouts, AFP mRNA levels are also markedly increased (Fig. (Fig.2A).

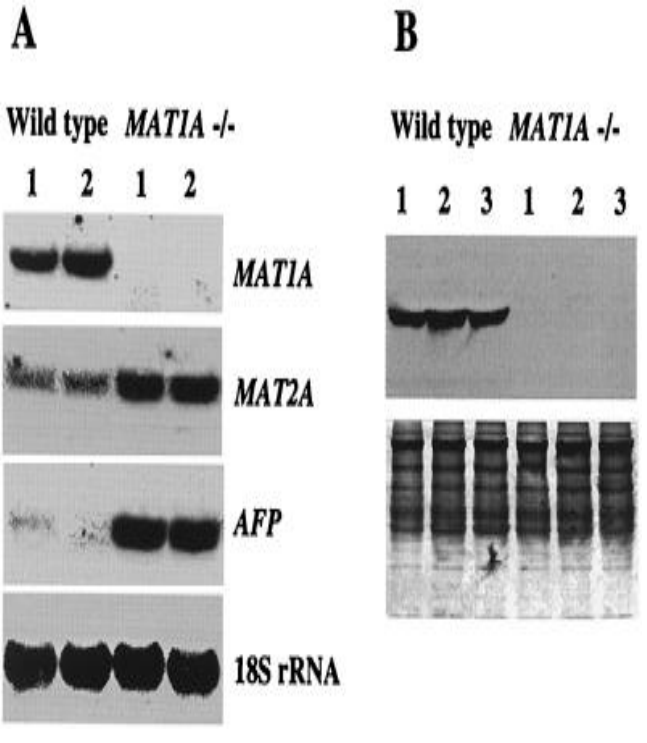

Figure 2: (A) Northern blot analysis of MAT1A, MAT2A, and AFP in MAT1A knockout and wild-type littermates. Liver RNA (30 μg each lane) samples obtained from two wild-type and two MAT1A knockout mice were analyzed by Northern blot hybridization with a 32P-labeled MAT1A cDNA probe, as described in Methods. A representative Northern blot is shown. (B) Hepatic steady-state liver-specific MAT protein level in MAT1A knockout and wild-type littermates. Liver cytosol (50 μg/lane) obtained from three wild-type and three MAT1A knockout mice was analyzed by Western blot analysis by using anti-rat liver-specific MAT antibodies, as described in Methods. Equivalent protein loading was assured by Coomassie-blue staining of gels after transblotting (Lower).

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