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Lack of Evidence for Hepatitis G Virus Replication in the Livers of Patients Coinfected with Hepatitis C and G Viruses

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The pathogenic implications of hepatitis G virus (HGV) infection are still unclear. We searched for the presence of HGV RNA and HCV RNA sequences in liver and serum samples from 10 patients with chronic liver disease, 9 of whom were coinfected with HCV. All livers were negative for the presence of the HGV RNA minus strand and only six were positive for the presence of the positive strand, albeit at low levels. In striking contrast, the HCV RNA positive strand was detectable in the liver samples from all nine HCV-positive patients in titers ranging from 10^2 to 10^8 genomic eq/μg of RNA, and the negative HCV RNA strand was present in all but two of these patients. However, the positive-strand RNA titers in serum for the two viruses had similar ranges. These findings imply that the liver is not the primary replication site for HGV, at least in the population of HCV/HGV-coinfected patients. Absence of replication in liver tissue may explain the reported lack of influence of HGV coinfection on the course of chronic hepatitis C.

Recently, two independent groups of investigators described a novel flavivirus, designated by one group as hepatitis G virus (HGV) and by the other as hepatitis GB virus C (10, 12). HGV infection was found to be common in those exposed to blood transfusions (4, 10, 16) and hemodialysis (11), intravenous drug addicts (10, 13), and patients with various forms of chronic hepatitis, being particularly prevalent in patients with chronic hepatitis C (2, 3, 10, 14). Although originally identified in patients experiencing hepatitis, it is now clear that in the absence of concomitant infection with hepatitis C virus (HCV) or hepatitis B virus (HBV), HGV is usually not associated with liver injury nor does it have any effect on the course of chronic hepatitis C both in immunocompetent and in immunosuppressed hosts (1, 2, 11, 14, 16). Although the clinical aspects of HGV infection have been extensively studied, so far no studies have addressed the issue of viral replication sites.

HGV genome organization was found to be similar to that of HCV, with a single open reading frame and 5′- and 3′-untranslated regions (10, 12). Analysis of the HGV predicted amino acid sequences indicated the presence of structural and nonstructural proteins, including a serine protease, helicase, and RNA-dependent RNA polymerase, as well as a number of putative proteolytic cleavage sites in the same relative positions as the corresponding sites in HCV (7). Considering the resemblance in genomic structure, it can be assumed, although it has not been formally shown, that HGV replicates similarly to HCV through negative-strand RNA, the presence of which could be regarded as direct evidence of viral replication.

However, strand-specific detection of RNA is fraught with problems, as it has been demonstrated that it is prone to false priming of the incorrect strand or self-priming related to RNA secondary structures (5). These mispriming events can to a large extent be avoided by conducting cDNA synthesis at high temperature with the thermostable enzyme Tth (5, 6). In the current study we employed this technique to search for HGV RNA and HCV RNA negative strands, the latter providing a convenient control for the integrity of viral RNA in the studied samples, in liver tissues from patients with chronic hepatitis C who were coinfected with HGV. At the same time we determined the titers of positive strands of HGV and HCV in liver and serum samples. The sensitivity and strand specificity of our assays were determined with synthetic RNA templates.

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MATERIALS AND METHODS

Biological samples. We studied the livers and sera from 10 HGV RNA-positive patients who received liver transplantation for end-stage liver disease between February and May 1995. All were hepatitis B surface antigen and anti-human immunodeficiency virus negative, and nine were anti-HCV positive. Explant liver tissues and sera, which were collected at the time of transplantation, were stored at −80°C until analysis. RNA was extracted from livers and sera by means of a modified guanidinium thiocyanate–phenol-chloroform technique by using commercially available kits (Ultraspec 2 and Ultraspec 3; Biotecx Laboratories, Houston, Tex.). RNA extracted from each liver (1 μg, as determined by spectrophotometry) was routinely used for reverse transcriptase PCR (RT-PCR); in the case of serum, RNA extracted from 20 μl of serum was loaded into each reaction mixture.

Synthetic HGV RNA. To generate synthetic positive and negative HGV and HCV RNA strands, PCR products encompassing the 5′-untranslated regions of both viruses were cloned into a plasmid vector (pGEM-3Z; Promega) and, after plasmid linearization, subsequently transcribed with T7 polymerase (Riboprobe Transcription System; Promega). Orientation of the insert was checked by sequencing of the plasmid directly. The template was removed by digestion with DNase I (1 U/μg of DNA for 60 min at 37°C), and the absence of significant amounts of residual DNA was ascertained by routine inclusion of control PCR without the RT step.

RT-PCR with MMLV RT. For the Moloney murine leukemia virus (MMLV) RT-based detection of HGV RNA, extracted RNA was incubated for 20 min at 42°C in 30 μl of reaction mixture containing 100 pM positive-sense primer (5′ AATCCCGGTCAC/TCC/TTGGTAGCCACT 3′, nucleotides [nt] 146 to 170 [for the detection of the negative strand]) or antisense primer (5′ CCCCACT-GGTCC/TTTGC/TCAACTC 3′, nt 401 to 380 [for the detection of the positive strand]), 1× PCR buffer II (Perkin Elmer), 5 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM dNTP, 20 U of RNase inhibitor (RNasin; Promega), and 20 U of MMLV RT (Gibco BRL). After heating to 99°C for 10 min, the other primer (100 pM), 7 μl of 10× PCR buffer II (Perkin Elmer), and 5 U of *Taq* DNA polymerase (Perkin Elmer) were added and the volume was adjusted to 100 μl. Amplification was performed with a DNA thermal cycler 480 (Perkin Elmer) as follows: initial denaturing at 94°C for 4 min, followed by 50 cycles at 94°C for 1 min and 58°C for 1 min and then a final extension at 72°C for 7 min. Twenty microliters of the final product was analyzed by agarose gel electrophoresis and Southern hybrid-

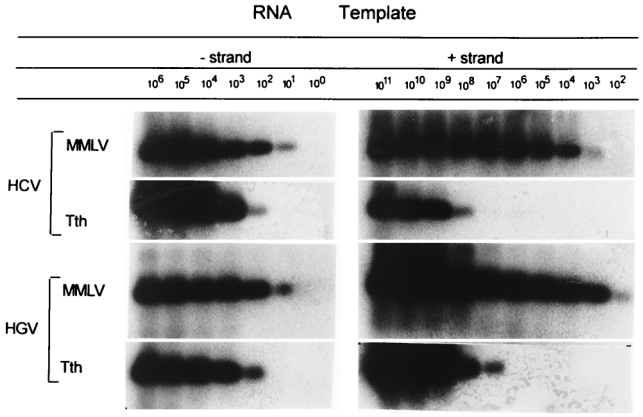

FIG. 1. The sensitivity and specificity of RT-PCR with MMLV RT and Tth for the detection of negative RNA strands of HGV and HCV. A positive-sense primer was present during cDNA synthesis, after which the enzyme was inactivated, either by heating for 10 min at 99°C (MMLV RT) or by chelating with Mn^{2+} (Tth), and negative-sense primer was added. Samples were amplified for 50 cycles with either *Taq* polymerase (MMLV RT-based assay) or Tth (Tth-based assay) as described in the text. Synthetic positive and negative strands were generated by in vitro "run off" transcription with T7 RNA polymerase from a vector (pGEM-3Z) containing the 5′-untranslated sequences of both viruses and serially diluted in water. The number of target template copies was calculated from optical density readings and results of gel electrophoresis. Samples were analyzed by agarose gel electrophoresis and Southern hybridization with a ^{32}P-labeled probe internal to the amplification primers. When 1 μg of total cellular RNA extracted from normal human livers was added, the sensitivity of the reactions was lowered by 1/2 log unit.

ization with a ^{32}P-labeled internal oligoprobe, 5′ CACGGTCCACAGGTGTTG GCCCTACCGG 3′, nt 227 to 254. The primers used for HGV RNA amplification matched all the major strains deposited in the GenBank database and were found to be efficient in an extensive epidemiological study conducted on American patients with chronic liver disease (15).

For the detection of HCV sequences the primers were 5′ A/GAC/TCACTCC CCTGTGAGGAAC 3′, nt 35 to 55 (sense), and 5′ TGA/GTGCACGGTCTA CGAGACCTC 3′, nt 342 to 320 (antisense), while the probe was 5′ ACTGTCT TCACGCAGAAAGCGTC 3′, nt 57 to 79. The RT-PCR was performed as described above.

RT-PCR with Tth. For Tth-based RT-PCR detection of the negative strand, cDNA was generated in 20 μl of reaction mixture containing 50 pM sense primer, 1× RT buffer (Perkin Elmer), 1 mM $MnCl_2$, 200 μM (each) dNTP, and 5 U of Tth (Perkin Elmer). After 20 min at 65°C, Mn^{2+} was chelated with 8 μl of 10× EGTA chelating buffer (Perkin Elmer), 50 pM antisense primer was added, the volume was adjusted to 100 μl, and the $MgCl_2$ concentration was adjusted to 2.2 mM. The amplification was performed in a Perkin Elmer GenAmp PCR System 9600 thermocycler as follows: initial denaturing for 1 min at 94°C, and then 50 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s followed by a final extension at 72°C for 7 min. PCR products were analyzed as described above.

To increase the specificity and sensitivity of our assays, wax beads (Ampliwax; Perkin Elmer) were routinely employed for "hot start" of all PCRs after the RT step. All RT-PCR runs included positive controls, consisting of end point dilutions of the respective RNA strands, and negative controls, including normal livers and normal sera. The titers were determined by analyzing 10-fold serial dilutions of the template.

To prevent contamination, pre-PCR and post-PCR steps were carried out in separate rooms. To detect carryover contamination, negative controls were included in all reaction series: one negative sample was processed for every 3 to 4 tested specimens, and nontarget controls were included in each run. Under these conditions, none of the negative samples or controls was positive.

RESULTS AND DISCUSSION

Sensitivity and strand specificity of negative-strand RT-PCR. The results of analysis of serial dilutions (1:10) of synthetic RNA are presented in Fig. 1. MMLV RT-PCR assays for HGV and HCV negative strands were capable of detecting 10 genomic equivalent molecules (eq) of the respective templates. However, they also unspecifically detected $\geq 10^2$ and $\geq 10^3$ genomic eq of the positive strands, respectively. When Tth was used at the 70°C RT step, the specificity of the reaction was increased by 8 to 9 log units since the incorrect strand was now detected only at 10^{11} genomic eq; however, the sensitivity was lowered 100-fold (not shown). Conducting the RT step at 65°C improved sensitivity while lowering strand specificity; the assays were now capable of detecting 100 genomic eq of the correct strand while unspecifically detecting 10^7 to 10^8 genomic eq of the incorrect strand (Fig. 1). To imitate the conditions encountered in biological sample amplification, 1 μg of RNA extracted from uninfected human liver was added to each reaction mixture. This slightly lowered the sensitivity of our assays by approximately one-half log unit (not shown). The sensitivity of our assays for the detection of the positive strand was identical to that for the detection of the negative strand.

The sensitivity and specificity of our Tth protocol were even higher than those described by Lanford et al. (6) for the detection of the HCV negative strand, probably reflecting such factors as the simplified hot start procedure with wax beads and the short ramp times achievable in the Perkin Elmer GenAmp PCR System 9600, which are likely to lower unspecific amplification and increase sensitivity. Consequently, we were unable to achieve similar results with a DNA thermal cycler 480 (Perkin Elmer).

During optimization of the MMLV RT-based assay we found that hot start of the PCR step increased sensitivity 3 to 4 log units (data not shown). This effect could be related to the strong secondary structure of the template in the 5′-untranslated region, as we did not observe it with other RNA templates which did not possess strong hairpin structures (unpublished data). The strand specificity of MMLV RT-PCR could not be improved; the 1-h boiling of cDNA after the RT step, recommended by some authors (8) to remove any trace activity of reverse transcriptase, did not have any effect on strand specificity while it lowered sensitivity by one-half log unit.

To exclude significant cross-reactivity between HGV and HCV assays, 10^{11} genomic eq of the synthetic HGV and HCV template was amplified by HCV and HGV RT-PCR, respectively. All reactions were negative (data not shown).

Detection of HGV and HCV positive and negative RNA strands. Livers from all 10 patients were negative for the presence of the HGV RNA minus strand when tested by Tth-based RT-PCR, and only 6 were positive for the presence of the positive strand, as determined by MMLV RT assay. The latter strand, however, was detectable at very low levels, ranging from 10 to no more than 10^3 genomic eq/μg of RNA (Table 1). To maximally increase the sensitivity of HGV RNA negative-strand detection, the reactions were repeated, loading 4 to 5 μg of total liver-extracted RNA into the reaction mixtures. Again, all reactions were negative.

In striking contrast, HCV RNA positive strand was detectable in the livers from all nine HCV-infected patients, with titers from 10^2 up to 10^8 genomic eq/μg of RNA, and negative HCV RNA strand, as determined by Tth assay, was present in the liver samples from all but two patients, those for whom the positive-strand titers were the lowest. However, the positive-strand RNA titers in serum for the two viruses were quite similar (Table 1). No negative-strand viral RNA sequences were detected in any of the serum samples.

In the present work we provide two lines of evidence that the liver is an unlikely site for primary HGV replication in HCV/HGV-coinfected patients. Firstly, we did not detect HGV RNA negative strand, a presumably viral replicative form, in the livers by assays which were rigorously tested for sensitivity and strand specificity on synthetic templates. With similar techniques HCV RNA negative strand was almost uniformly detected in the same livers, suggesting that the integrity of RNA

TABLE 1. Titers of positive and negative strands of HCV RNA and HGV RNA in liver tissues and sera from 10 patients with end-stage chronic liver disease[a]

Patient no.	Titer in liver (genomic eq/μg of RNA)				Titer in serum (genomic eq/ml)			
	HCV		HGV		HCV		HGV	
	+RNA	−RNA	+RNA	−RNA	+RNA	−RNA	+RNA	−RNA
1	10^2	Neg	10^3	Neg	5×10^5	Neg	5×10^5	Neg
2	10^2	Neg	Neg	Neg	5×10^4	Neg	5×10^7	Neg
3	10^5	10^4	10^1	Neg	5×10^3	Neg	5×10^5	Neg
4	10^6	10^5	Neg	Neg	5×10^5	Neg	5×10^3	Neg
5	10^6	10^4	10^1	Neg	5×10^4	Neg	5×10^3	Neg
6	10^6	10^4	Neg	Neg	5×10^7	Neg	5×10^4	Neg
7	10^6	10^5	10^1	Neg	5×10^3	Neg	5×10^5	Neg
8	10^6	10^3	Neg	Neg	5×10^4	Neg	5×10^6	Neg
9	10^8	10^6	10^1	Neg	5×10^6	Neg	5×10^3	Neg
10	Neg	Neg	10^1	Neg	Neg	Neg	5×10^3	Neg

[a] The positive-strand (tRNA) titers were determined by an MMLV RT-based assay, while the negative-strand (−RNA) titers were determined by a Tth-based assay. Neg, negative result.

in the studied material was not compromised. Secondly, positive-strand HGV RNA sequences were detected only in 6 of 10 livers and in titers so low that they most likely reflected nothing more than serum contamination. In contrast, levels of HCV RNA in liver were high. Taking into account the fact that the titers for the two viruses in serum were similar (Table 1), these results suggest that some other sites supporting HGV replication exist in the infected host.

However, we cannot definitely conclude from our study that HGV is not a hepatotropic virus, as there remains a possibility that the ostensible lack of replication in liver is an extreme manifestation of interference caused by replicating HCV. Indeed, it was reported that HCV may exert a suppressive effect on HBV and HDV replication in vivo, although a total cessation of the latter is unusual (9). Nevertheless, in the sole patient without HCV coinfection, HGV RNA negative strand was not detected either. Obviously, further studies of a larger number of patients infected with HGV alone are needed to address this issue. In addition, patients with mild forms of hepatitis should be studied, as viral replication in liver tissue could be more efficient in less advanced liver disease.

In summary, we did not find any evidence of HGV replication in the livers of chronic hepatitis C patients coinfected with HGV. This implies that the liver is not the primary replication site for this newly discovered virus, at least in the population studied. Absence of replication in liver tissue explains the reported lack of influence of HGV coinfection on the course of chronic hepatitis C.

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