Detection of Hepatitis G Virus Replication Sites by Using Highly Strand-Specific Tth-Based Reverse Transcriptase PCR

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Received 3 November 1997/Accepted 17 December 1997

The replication sites of the recently discovered hepatitis G virus (HGV) remain unknown. Using highly strand-specific Tth-based reverse transcriptase PCR, we searched for the presence of viral RNA negative strand in multiple autopsy tissues from four patients with AIDS and in peripheral blood mononuclear cells from six other human immunodeficiency virus-positive patients. Negative-strand HGV RNA was detected in three of four bone marrow samples, in two of two spleen samples, and in one of four liver tissue samples. However, the specific cellular site of replication within the positive tissues was not determined. This study does not support HGV as a primary hepatotropic virus.

Recently, two independent groups of investigators described two isolates of the same novel flavivirus and named the virus hepatitis G virus (HGV) and hepatitis GB virus C (10, 13). Since the nomenclature of this new agent has not yet been decided, for the purpose of this article it will be referred to as HGV. HGV RNA sequences have been detected by reverse transcriptase (RT) PCR in 1 to 2% of volunteer blood donors and at significantly higher rates in persons with repeated parenteral exposure such as intravenous drug addicts (10, 14) or patients receiving multiple transfusions (4, 10, 16). Furthermore, HGV infection was found to be common in subjects with various forms of chronic liver disease, being particularly prevalent in subjects with chronic hepatitis C (2, 3, 10, 15). However, the association between hepatitis G and HGV infection is unclear since the vast majority of infected individuals do not show liver injury unless simultaneously infected with another hepatotropic virus (1, 11, 16). This raises the possibility that HGV is not a strictly hepatotropic virus but rather one which causes hepatitis only occasionally.

Although studies on the clinical effects of HGV infection are abundant, studies addressing the issue of viral replication sites are missing. In a previous article (7), we reported on the lack of evidence for HGV replication in the liver in a group of HGV-HCV-coinfected patients with cirrhosis, which implies that the liver is not the primary replication site for this virus. However, no other cell compartments have been studied so far.

The major obstacle to a study of HGV replication sites is the lack of availability of multiple tissue samples from infected individuals since such samples can be obtained only during autopsy. We reasoned that such an investigation could be conducted on postmortem tissues from intravenous drug addicts who died from AIDS, since HGV infection in this group is common and viral titers are expected to be elevated, facilitating positive identification of replication sites.

HGV genome organization was found to be similar to that of hepatitis C virus (HCV), with a single open reading frame and 5' and 3' untranslated regions (10, 13). In addition, analysis of the predicted amino acid sequences indicated the presence of structural and nonstructural proteins as well as a number of putative proteolytic cleavage sites in a relative position found in HCV (9). Taking into account these similarities, it can be assumed that HGV replicates through negative-strand RNA, the presence of which could be regarded as direct evidence of viral replication.

However, standard RT-PCR is not strand specific due to false priming of the incorrect strand or self-priming related to RNA secondary structures (5). An efficient way of avoiding these mispriming events is by conducting cDNA synthesis at high temperature with the thermostable enzyme Tth (5, 6, 8). In the current study, we employed this technique in the search for negative-strand HGV RNA in peripheral blood mononuclear cells (PBMCs) and multiple organs from HGV-positive patients with AIDS. The sensitivity and strand specificity of our assay were determined on synthetic RNA templates.

FIG. 1. Sensitivity and specificity of RT-PCR using the Tth assay. Synthetic positive and negative strands were generated by in vitro runoff transcription with T7 RNA polymerase from a vector (pGEM-3Z) containing the 3' and 5' untranslated regions (10, 13). In all cases, the number of target template copies was calculated from optical density readings. A positive-sense primer was present during cDNA synthesis, after which the enzyme was inactivated by chelation with Mn2+ and then negative-sense primer was added. Samples were amplified as described in the text. Twenty microliters (20%) of the reaction mixture was fractionated on agarose, transferred to a nylon membrane by Southern blotting, and subsequently hybridized to a 32P-labeled probe. When 1 or 6 µg of total cellular RNA extracted from normal human liver tissue was added, the sensitivity of the reactions was lowered by no more than 1 log, while the specificity of the assay was not affected.

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frozen at

tion, washed three times with phosphate-buffered saline (pH 7.4), and stored
by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifuga-
one had received any antiviral therapy prior to the study. PBMCs were isolated
virus type 1 (HIV-1)-positive drug addicts whose sera were found to be HGV
MMLV RT-based assay, while the presence of the negative strand was deter-

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TABLE 1. Detection of positive and negative strands of HGV RNA in serum samples and PBMCs in six HIV-infected patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>CD4 cells/mm³</th>
<th>HGV RNA titer in:</th>
<th>Serum (genomic eq/ml)</th>
<th>PBMCs (genomic eq/2 × 10⁶ to 3 × 10⁶ cells)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Positive strand</td>
<td>Negative strand</td>
<td>Positive strand</td>
</tr>
<tr>
<td>1</td>
<td>132</td>
<td>5 × 10⁶</td>
<td>Neg</td>
<td>10⁶</td>
</tr>
<tr>
<td>2</td>
<td>270</td>
<td>5 × 10⁶</td>
<td>Neg</td>
<td>10⁶</td>
</tr>
<tr>
<td>3</td>
<td>198</td>
<td>5 × 10⁶</td>
<td>Neg</td>
<td>10⁶</td>
</tr>
<tr>
<td>4</td>
<td>309</td>
<td>5 × 10⁶</td>
<td>Neg</td>
<td>10⁶</td>
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<tr>
<td>5</td>
<td>545</td>
<td>5 × 10³</td>
<td>Neg</td>
<td>10³</td>
</tr>
<tr>
<td>6</td>
<td>33</td>
<td>5 × 10³</td>
<td>Neg</td>
<td>10³</td>
</tr>
</tbody>
</table>

* The presence and tilters of the positive strand were determined by the MMLV RT-based assay, while the presence of the negative strand was deter-

MATERIALS AND METHODS

Biological samples. PBMCs were collected from six human immunodeficiency
virus type 1 (HIV-1)-positive drug addicts whose sera were found to be HGV
RNA positive. All were HCV positive and hepatitis B surface antigen negative; none had received any antiviral therapy prior to the study. PBMCs were isolated
by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifuga-

TABLE 2. Detection of positive (+) and negative (−) strands of HGV RNA in serum samples and various tissues from four patients with AIDS

<table>
<thead>
<tr>
<th>Patient</th>
<th>Serum (genomic eq/ml)</th>
<th>Liver</th>
<th>Spleen</th>
<th>Bone marrow</th>
<th>Lymph node</th>
<th>Pancreas</th>
<th>Thyroid</th>
<th>Adrenal gland</th>
<th>Kidney</th>
<th>Lung</th>
<th>Muscle</th>
<th>Skin</th>
<th>Spinal cord</th>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>5 × 10⁴ N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>5 × 10⁴ N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>3</td>
<td>5 × 10⁵ N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>5 × 10⁶ N</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>

* The presence and tilters of the positive strand were determined by the MMLV RT-based assay, while the presence and tilters of the negative strand were determined by the Tth-based assay. N, negative; P, positive in 6 μg of total RNA; ND, not done.
By using the Tth-based strand-specific assay, the presence of negative-strand HGV RNA was documented in some bone marrow, spleen, and liver tissue samples at titers which were 1 to 2 logs lower than the titers of the positive-strand HGV RNA (Table 2). In patient 1, negative-strand HGV RNA was found in bone marrow only; in patient 2, it was found in bone marrow and spleen; in patient 3, it was present in bone marrow, spleen, and liver; while in the remaining patient, all studied samples were persistently negative for negative-strand HGV RNA (Table 2; Fig. 2). These results were confirmed in two independent experiments using two separate extraction procedures.

In contrast, positive-strand HCV RNA was detected in liver tissue from all four patients at titers ranging from $10^3$ to $10^7$ genomic eq of RNA/μg, and negative-strand HCV RNA strand, as determined by Tth assay, was present in all liver samples at titers that were 1 to 2 logs lower than those of the positive strand. Titers of positive-strand HCV RNA in serum samples ranged from $5 \times 10^3$ to $5 \times 10^5$ genomic eq/ml (Table 3).

The present study is the first to positively identify HGV replication sites in humans. By examining autopsy material from AIDS patients, we found the presence of viral negative-strand RNA, a putative viral replicative form, in bone marrow, spleen, and liver tissue. Moreover, negative-strand HGV RNA titers were 1 to 2 logs lower than titers of the positive strand, which is the same proportion as that found for another flavivirus, HCV, at its respective replication site. However, negative-strand HGV RNA detection was not consistent from patient to patient—in one case, no negative-strand RNA was found in any of the organs tested. Since the tissue samples were collected at the time of autopsy, some RNA might have been degraded and low-level replication could have been missed. Nevertheless, detection of high titers of positive- and negative-strand HCV RNA in all four liver samples suggests the presence of relatively well preserved template.

Negative-strand HGV RNA was detected in only one of four studied liver samples, although the overall HGV titers in liver tissue were significantly higher than those of previously studied HIV-negative patients (7). In striking contrast, significant titers of negative-strand HCV RNA were detected in liver tissue from all four patients. These findings support our previous conclusion that HGV is not a strictly hepatotropic virus and that even in the presence of severe HIV-related immunosuppression, its replication in the liver is low or absent.

However, we cannot exclude the possibility of a very low level of HGV replication in the liver in the remaining three subjects. In cells supporting HCV replication, negative RNA strands are generally detected at a level that is 1 to 2 logs lower than the levels of positive strands (6, 7). Since the same seems likely to be true for HGV, it would mean that replication is below the sensitivity level of our Tth-based strand-specific assay. Obviously, the same applies to other tissues and PBMCs, where low-level replication or replication confined to a small subset of cells would remain undetected.

It is currently unclear which particular cells are infected at the identified replication sites. Replication in bone marrow precursor cells could manifest itself clinically, but so far no hematological disturbances have been associated with HGV infection. Alternate candidates for supporting bone marrow viral replication are stromal endothelial cells, fibroblasts, and macrophages. Interestingly, macrophages are richly represented at the sites where HGV replication was detected and are known to be permissive for a wide range of viruses, including some other flaviviruses (12). Alternatively, HGV could infect various cells at different locations.

In summary, by using a strand-specific Tth-based assay on a variety of autopsy samples from AIDS patients, we identified the presence of negative-strand HGV RNA in bone marrow, spleen, and liver tissue. However, the cell lineage supporting viral replication at these sites remains to be determined.

### REFERENCES


