Toward a Surrogate Model for Hepatitis C Virus: An Infectious Molecular Clone of the GB Virus-B Hepatitis Agent

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GB virus-B (GBV-B) is a member of the Flaviviridae family of viruses. This RNA virus infects tamarins, but its natural host is not known. GBV-B has special interest because it is the virus that is most closely related to hepatitis C virus (HCV), an important human pathogen. In the present study, we identified a previously unrecognized sequence at the 3' end of the GBV-B genome. This new 3' terminal sequence can form several predicted stem-loop structures as is typical for other members of the Flaviviridae family. We constructed molecular clones and showed that the new 3' UTR sequence was critical for infectivity. After intrahepatic transfection of two tamarins with RNA transcripts of the full-length GBV-B clone, we detected high viral titers from Week 1 postinoculation with peak titers of ~10^9 genome equivalents/ml. The viremic pattern of GBV-B infection in the transfected animals was the same as in animals inoculated intravenously with the virus pool used as the cloning source. The sequence of the recombinant virus was recovered from one of the tamarins and shown to be identical to that of the infectious clone. The development of severe hepatitis in both tamarins infected with the recombinant GBV-B virus provides formal proof that GBV-B is a true hepatitis virus.

INTRODUCTION

Transmission studies of potential human hepatitis agents were reported in 1967 (Deinhardt et al., 1967). Four tamarins inoculated with acute-phase serum from a surgeon with acute hepatitis (Patient GB) developed hepatitis, as did most tamarins inoculated in serial passage studies. Subsequent studies indicated that the etiological agent responsible for the development of hepatitis in these animals was not any of the known human hepatitis viruses (Purcell, 1993). In 1995, two related RNA viruses named GB virus-A (GBV-A) and GB virus-B (GBV-B) were identified in acute phase sera of a tamarin that developed hepatitis after inoculation with serum of the 11th tamarin passage of the putative GB agent (Simons et al., 1995b). GBV-B infection of tamarins resulted in acute resolving hepatitis (Schlauder et al., 1995). The natural host of GBV-B is still unknown because it has not been detected in uninoculated animals or in humans. In contrast, GBV-A is an indigenous tamarin virus rather than a component of the original GB inoculum (Bukh and Apgar, 1997; Erker et al., 1998). Experimental infection of tamarins with GBV-A did not cause hepatitis (Schlauder et al., 1995). Subsequently, a human agent, GBV-C or hepatitis G virus, closely related to GBV-A, was identified (Simons et al., 1995a; Linnen et al., 1996). It is still not clear whether this virus actually causes hepatitis, but it most likely does not (Alter et al., 1997; Bukh et al., 1998b). Thus among the GB viruses only GBV-B may be a true hepatitis virus.

Based on analysis of their genomic sequences, GBV-A, GBV-B, and GBV-C were classified as members of the Flaviviridae family (flaviviruses, pestiviruses, and hepacivirus; Rice, 1996). GBV-B is of particular interest because among the known viruses, it is the virus most closely related to hepatitis C virus (HCV) (Muerhoff et al., 1995; Erker et al., 1998; Robertson et al., 1998), which is an important human pathogen (Houghton, 1996). The GBV-B virus contains a positive-sense, single-stranded RNA genome of 9143 nts (Muerhoff et al., 1995; Simons et al., 1995b). A single long open reading frame (ORF) is bracketed by 5' and 3' untranslated regions (UTR). Based on known motifs, structural proteins were predicted to be encoded in the 5' portion of the ORF and nonstructural (NS) proteins in the 3' portion of the ORF (Muerhoff et al., 1995). The hydropathy plots of the polyproteins of GBV-B and HCV are very similar even though the overall homology of the predicted polyproteins between GBV-B and HCV is only ~25–30% (Muerhoff et al., 1995). The putative envelope proteins (E1 and E2) of GBV-B and HCV share common structural features.

1 The sequence reported in this paper has been deposited in the GenBank database (accession no. AF179612).
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 Furthermore significant homology was observed among the NS3 serine protease, the NS3 RNA helicase, the NS5 RNA-dependent RNA polymerase regions, and the consensus sequences at the predicted cleavage sites in the NS regions, respectively, of GBV-B and HCV (Muerhoff et al., 1995). The function and substrate specificity of the GBV-B and HCV NS3 serine proteases are also similar (Scarselli et al., 1997). The genomic structure and organization of GBV-B and HCV share additional features of interest. First, colinear regions with significant sequence homology were identified in the 5′ UTRs (Muerhoff et al., 1995). The predicted internal ribosome entry site (IRES) structure of GBV-B is similar to that of HCV (Lemon and Honda, 1997). Second, both viruses begin the 3′ terminal sequence of HCV forms a stable stem-loop structure (Tanaka et al., 1995, Kolykhalov et al., 1996), this was not the case for the published 3′ terminal sequence of GBV-B.

Despite the recent development of infectious clones of HCV (Kolykhalov et al., 1997; Yanagi et al., 1997, 1998), molecular studies are severely limited by the lack of an efficient cell culture system for this virus and by the absence of a small animal model. We are exploring the possibility of using experimental infections of tamarins with GBV-B as a surrogate model for the study of HCV. In the present study, a detailed sequence analysis of the complete genome of GBV-B led to the identification of additional 3′ terminal sequence. A molecular clone of GBV-B that contained this new sequence was infectious in vivo.

RESULTS

Course of GBV-B in experimentally infected tamarins

To generate virus pools of the GB agent, we inoculated tamarins intravenously with pooled sera of the 11th tamarin passage of this agent. Acute phase serum from one Saguinus mystax tamarin that developed hepatitis was pooled (GB 8/93) and inoculated into additional S. mystax tamarins to generate a second pool of acute phase serum (GB 2/94). Each serum pool contained ~10^7 genome equivalents (GE)/ml of GBV-B. A 10% liver homogenate (CT 11/91) was prepared from one S. oedipus tamarin that developed hepatitis following inoculation with the 12th passage of the GB agent. The titer of GBV-B in the liver homogenate was ~10^7 GE/ml. The GB 2/94 serum and CT 11/91 liver samples were used as GBV-B-cloning sources in the present study.

Inoculation of eight S. mystax tamarins with 10-fold serial dilutions of the GB 2/94 pool demonstrated that its infectivity titer for GBV-B was 10^8 tamarin 50% infectious doses (TID_{50}) (Fig. 1). The five GBV-B-infected tamarins all developed acute resolving hepatitis characterized by early appearance of viremia (Weeks 1 or 2 postinoculation), peak viral titers of 10^7–10^8 GE/ml and clearance of viremia after 9–16 weeks (Fig. 1). A single S. mystax tamarin inoculated with the liver homogenate also developed acute resolving hepatitis with peak GBV-B titers of 10^7 GE/ml and clearance of viremia after 11 weeks. The infectivity titer of GBV-B in the GB 8/93 pool was 10^{6.5} TID_{50}. The four infected S. mystax inoculated with dilutions of this pool had acute resolving hepatitis with clearance of the GBV-B virus after 11–26 weeks. Thus the natural history of GBV-B in S. mystax tamarins was characterized by acute hepatitis, early appearance of viremia, high peak viral titers, and viral clearance. There was a good correlation between infectivity titer and GBV-B genome titer; one TID_{50} was equivalent to one GE in the GB 2/94 pool and to 32 GE in the GB 8/93 pool.

Sequence analysis of the GBV-B cloning source: discovery of a novel 3′ terminal sequence

The consensus sequence of the complete 5′ UTR of GBV-B (nts 1–445) was deduced from 13 clones containing nts 1–283 and 3 clones containing nts 31–445. In addition, the entire 5′ UTR sequence was determined by direct sequencing of the amplicons. The sequence of the various clones was highly conserved and the consensus 5′ UTR sequence of GBV-B from this pool was identical to that published previously for GBV-B (Simons et al., 1995b). It is noteworthy that 13 of 15 clones analyzed from the 5′ RACE procedure contained the published GBV-B 5′ terminus (A residue) and that the same 5′ terminus was obtained whether the 5′ RACE was performed with dC or dA tailing.

The consensus sequence of the ORF (nts 446–9037) was determined by direct sequencing of PCR products obtained by using long RT–PCR (Yanagi et al., 1997). In addition, we analyzed three clones containing nts 446–7135 (one of these clones had a deletion of nts 3041–3643), two clones containing nts 2019–3373, 5 clones containing nts 7151–8261, and 7 clones containing nts 7521–9037. The sequence of GBV-B in this pool was very homogeneous. Evidence of microheterogeneity was found at only 70 (0.8%) nucleotide and 36 (1.3%) amino acid positions, scattered throughout the ORF. The proportion of amino acid positions with heterogeneity ranged from 0.5–2.6% in different putative gene regions (lowest in NS5B; highest in E2). The GBV-B ORF sequence differed from the published sequence of GBV-B (Simons et al., 1995b) at 34 (0.4%) and 12 (0.4%) nucleotide and deduced amino acid positions, respectively (Table 1).

The consensus sequence we determined for the 3′ UTR of GBV-B is shown in Fig. 2. The sequences among the various 3′ UTR clones analyzed were highly conserved with only three mutations found downstream of the poly(U) tract. We initially identified additional 3′ UTR sequence by performing RT–PCR across 5′-to-3′-end-
ligated viral RNA, extracted from serum. In all of four clones with GBV-B sequences, the 5′ UTR was truncated. However, whereas one clone had the exact 3′ terminus previously published by Simons et al. (1995b), the three other clones had 150 additional terminal nucleotides. Compared with the sequence previously published, all four clones had a single nucleotide insertion (C residue) at position 9134. We next performed 5′ RACE (using dC-tailing only) on the 5′ end of the negative-strand RNA extracted from the liver homogenate. We analyzed 11 clones and found that all had additional sequences at the 3′ terminus. Compared with the published GBV-B sequence two clones had 259 additional nucleotides, 8 clones had 237 additional nucleotides, and 1 clone had 233 additional nucleotides. Also, all of these clones had the insertion at position 9134. To demonstrate that the terminal 22 nucleotides found only in the two longest clones existed in circulating viruses, we performed RT-nested PCR on 10-fold serially diluted RNA extracted from the serum pool GB 2/94 using an RT and external antisense primer deduced from this sequence. We detected GBV-B RNA at a dilution of $10^{-7}$ (a $10^{-8}$ dilution was detected with the 5′ UTR primers) and the sequence of the amplicon was identical to the sequence recovered from the liver homogenate. Thus the 3′ UTR of GBV-B consists of a short sequence of 30 nts followed by an 11–24 nucleotide-long poly(U) tract (single C residues were observed in GBV-B from the liver homogenate) and

**FIG. 1.** Course of GBV-B infection in tamarins (S. mystax) inoculated with a dilution series of the pool GB 2/94. All animals were inoculated intravenously at Week 0 with 1 ml of the indicated dilution. Results of qualitative RT-nested PCR for GBV-B in serum are shown at the top (filled circles, positive; empty circles, negative). Serum levels of isocitrate dehydrogenase [ICD (U/ml); shaded area] and the estimated log$_{10}$ GBV-B GE titer/ml (vertical columns) were plotted against time. Two tamarins that were inoculated with $10^{-9}$ dilutions of the pool and were not infected are not shown. None of the eight tamarins became infected with GBV-A, which was present in the GB 2/94 pool. S. mystax 750, 760, and 782 were infected with GBV-ASM, a GBV-A variant indigenous to S. mystax, before inoculation (Bukh et al., 1997).
a 3’ terminal sequence of at least 309 nts. A “Blast” search indicated that the new GBV-B 3’ UTR sequence did not have significant homology to any of the sequences deposited in the GenBank database. A prediction of the secondary structure of the 3’ UTR sequence is shown in Fig. 2. Inclusion of the GBV-B 5’ UTR sequence in the analysis did not disrupt the putative stem-loop structures at the 3’ end. Most notable is a highly stable stem-loop structure, consisting of 47 nucleotides, at the very 3’ end.

### TABLE 1

<table>
<thead>
<tr>
<th>Genomic regiona</th>
<th>Position</th>
<th>Nucleotide</th>
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<td>nt [aa]</td>
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<td>T</td>
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<td>T</td>
<td>C (t)</td>
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<td></td>
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<td>ND</td>
<td>259 nts</td>
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Note. ND: Not determined. Nucleotides and amino acids shown in parenthesis were found as a minor species in the cloning source (GBV-B, 2/94).

a Nucleotide positions corresponding to pGBB. Putative gene borders defined as suggested by homology with HCV (Muerhoff et al., 1995; Scarselli et al., 1997).

b Positions that differ between the cloning source (GBV-B 2/94) and the infectious clone of GBV-B (pGBB). The change introduced into pGBB at position 7138 introduced an artificial SalI site.
A molecular clone of GBV-B is infectious in vivo

We first tested the infectivity of RNA transcripts from the consensus clone pGBB5–1, which encompassed only the GBV-B sequence previously published (Simons et al., 1995b). Within the GBV-B sequence there were no deduced amino acid differences and only two nucleotide differences (at nucleotide positions 3475 and 7138) from the consensus sequence of GBV-B as determined in the present study. In addition, in the 3' UTR this clone had a deletion at nucleotide position 9134 and was missing the 3' terminal 259 nts. Before transcription, the pGBB5–1 clone was linearized at the BamHI site with digestion at the exact GBV-B 3' terminus. The RNA transcripts from pGBB5–1 were injected into the liver of two tamarins (S. mystax 797 and 815). However, we did not detect GBV-B RNA in weekly serum samples collected during 17 weeks of follow-up. Furthermore both tamarins had normal liver enzyme values throughout follow-up. The susceptibility of the two tamarins to GBV-B was subsequently demonstrated by experimental infection using a GBV-B virus pool. Thus this consensus clone was not infectious in vivo.

We next tested the infectivity of RNA transcripts from a consensus cDNA clone of GBV-B (pGBB) which included the nucleotide insertion at position 9134 and the additional 259 nts at the 3' terminus. Except for the new 3' UTR sequence inserted, this clone was identical to pGBB5–1. Thus this cDNA clone of GBV-B contained a 5' UTR of 445 nts, an ORF of 8592 nts encoding 2864 amino acids and a 3' UTR of 362 nts (short sequence of 30 nts followed by a 23-nucleotide-long poly(U) tract and the 3' terminal sequence of 309 nts). pGBB was linearized at the XhoI site, which added up to five extra residues at the 3' end of the transcribed GBV-B RNA. When RNA transcripts from the pGBB clone were injected into the liver of two tamarins (S. mystax 816 and 817) both became infected with GBV-B, with viremia at Week 1 post-inoculation and peak viral titers of 10^8 GE/ml (Fig. 3). The consensus sequence of PCR products of the complete ORF, amplified from serum obtained during Week 2 post-inoculation from one tamarin (S. mystax 817), was identical to the sequence of pGBB, including at the two positions that differed from the consensus sequence of the cloning source and from the published sequence of GBV-B (Table 1). We demonstrated, using RT–PCR as described above, that the very 3' terminal GBV-B sequence of pGBB existed in the circulating viruses in this tamarin. Within 3 weeks of the transfection, both tamarins developed hepatitis with dramatically elevated serum liver enzyme levels and necroinflammatory changes in liver biopsy samples (Fig. 3). The GBV-B infection was resolved in both animals (Fig. 3).

**DISCUSSION**

The agent GBV-B, a virus of unknown origin, infects tamarins. In the present study, we confirmed the sequence of the GBV-B 5' terminus previously published by Simons et al. (1995b) and identified additional nucleotides at the 3' terminus of GBV-B. The 3' terminus previously published by Simons et al. (1995b) was lacking...
defined secondary structures typical for flaviviruses, pestiviruses, and HCV (Kolykhalov et al., 1996). However, the 3' UTR with the additional nucleotides could potentially form several putative stem-loop structures; the 3' terminal 47 nucleotides form a highly stable stem-loop structure. The additional sequence was critical for infectivity of an infectious molecular consensus clone of GBV-B (pGBB); an identical molecular clone lacking the new 3' UTR sequence was not infectious.

Because of the lack of cell cultures known to support the replication of GBV-B, we tested infectivity of RNA transcripts from the molecular clones in vivo, as previously established for RNA transcripts of infectious clones of hepatitis A virus in tamarins (Emerson et al., 1992) and HCV in chimpanzees (Kolykhalov et al., 1997; Yanagi et al., 1997). After transfection of tamarins with the shorter clone (pGBB5–1), we did not detect viremia during 17 weeks of follow-up, indicating that the clone was not infectious. In contrast, transfection with an equivalent amount of RNA transcripts of the longer clone (pGBB) produced high viral titers beginning by Week 1 postinoculation. The sequence of the recombinant virus was recovered from one of the tamarins and shown to be identical to that of the infectious clone.

A clear association between experimental infection with GBV-B and the development of acute hepatitis in tamarins was previously demonstrated (Schlauder et al., 1995; Bukh et al., 1997). However, as illustrated by the simultaneous transmission of GBV-A from the GB-agent pools (Schlauder et al., 1995; Bukh et al., 1997), other unrecognized viruses could have been passaged as well and theoretically could have caused the hepatitis. The development of hepatitis in both tamarins transfected with the recombinant GBV-B transcripts provides formal proof of GBV-B as the disease-causing agent. The pattern of GBV-B infection in animals inoculated intravenously with the virus pool used as the cloning source was the same as that of transfected animals and was characterized by early appearance of viremia, peak viral titers of ~10^8 GE/ml, and viral clearance.

The infection of tamarins with GBV-B could potentially serve as a surrogate animal model for infection of humans with HCV; this important human pathogen, which causes liver disease worldwide, can be propagated only in chimpanzees. GBV-B is a logical first choice because it is the virus most closely related to HCV and it infects tamarins, which are more readily available than chimpanzees. There are, however, important differences in the natural history of HCV and GBV-B in their respective animal hosts. Although both HCV and GBV-B cause acute hepatitis, acute hepatitis after infection of chimpanzees (and humans) is most often not seen until >8 weeks postinoculation. Conversely, GBV-B causes acute hepatitis in experimentally infected tamarins as early as 1 week postinoculation. However, the most important difference is that HCV infection often becomes chronic, whereas GBV-B infection is apparently resolved in all cases.

From a virological point of view, there are also important similarities and differences between HCV and GBV-B. HCV is so genetically heterogeneous that it is divided into at least six major genotypes with multiple subtypes and displays a quasispecies distribution in an infected host (reviewed in Bukh et al., 1995). Furthermore HCV has a hypervariable region in the E2 protein believed to be of importance for viral persistence. In contrast, only one viral strain of GBV-B has been identified. In the present study, we found that the virus populations of GBV-B in an acute phase virus pool generated from three infected tamarins were quite homogeneous. Although we did not find evidence of hypervariable regions, we did observe several differences between the GBV-B sequence of this virus pool compared with the published sequence (Simons et al., 1995b).
Perhaps the most important application of the GBV-B model is the possibility of indirectly or directly studying the molecular biology of HCV. This could be done by studying motifs that are conserved between these viruses (Muerhoff et al., 1995; Scarselli et al., 1997); mutagenesis in the GBV-B clone would be followed by the determination of infectivity for tamarins. Alternatively, the construction of GBV-B chimeras containing HCV elements would permit analysis of HCV elements in vivo. For instance, chimeric viruses might potentially serve as a model for testing HCV protease, helicase, or polymerase inhibitors as well as therapeutic agents that target the UTR regions or their interaction with host or viral factors. The 5’ UTR of both viruses contain IRES-like structures (Lemon and Honda, 1997). We found that uncapped GBV-B RNA was infectious, suggesting that cap-independent translation initiation via the IRES is used for gene expression in vivo. It was recently found that the IRES of poliovirus or bovine viral diarrhea virus could be replaced with that of HCV (Lu and Wimmer, 1996; Frolov et al., 1998; Zhao et al., 1999). Thus it might very well be possible to make similar chimeras between GBV-B and HCV and study IRES function in vivo.

The composition of the 3’ UTR of GBV-B is most similar to that of HCV. A short sequence after the ORF termination codon is followed by a poly(U) tract and additional sequence at the 3’ terminus. Except for the poly(U) tract, however, the 3’ UTR sequence of GBV-B shows no significant homology with the 3’ UTR of HCV or any other sequences in the GenBank database. The poly(U) tract in GBV-B is much shorter than that of HCV. The sequence after the poly(U) tract is three times longer in GBV-B (309 nts) than in HCV (98 nts). Importantly, however, the 3’ UTR of both of these viruses can form a similar stem-loop structure at the very 3’ end, consisting of 47 nts in GBV-B and 46 nts in HCV (Fig. 2). We recently demonstrated that this 3’ terminal stem-loop structure is critical for infectivity of HCV in vivo (Yanagi et al., 1999). The availability of infectious clones of both GBV-B and HCV should permit more detailed analysis of these 3’ UTR sequences, which are believed to be important for initiation of RNA replication.

In summary, we have determined the complete sequence of GBV-B, a member of the Flaviviridae family of viruses that infects tamarins, and have constructed a genetically stable infectious molecular clone. Infection of tamarins with recombinant GBV-B virus results in the development of acute hepatitis. Because GBV-B is the virus most closely related to HCV, the availability of this new GBV-B infectious clone could represent an important tool for the study of the molecular biology of HCV and for the development of therapeutics for this important human pathogen.

MATERIALS AND METHODS

Source of GBV-B

Two pools of tamarin serum containing GBV-A and GBV-B [VR-806 (American Type Culture Collection) and H205], supplied by the late Prof. F. Deinhardt, were used for experimental transmission of the GB agents.

Amplification, cloning, and sequence analysis of GBV-B

Viral RNA was extracted from 10 to 100 μl of serum or liver homogenate with the TRIzol system (GIBCO/BRL). The RNA pellet was resuspended in 10 mM dithiothreitol (DTT) containing 5% (vol/vol) of RNAsin (20–40 u/μl) (Promega). Primers used in cDNA synthesis and PCR amplification were based on the genomic sequence of GBV-B (Simons et al., 1995b). Long RT–PCR was performed by using Superscript II reverse transcriptase (GIBCO/BRL) and the Advantage cDNA polymerase mix (Clontech) as described previously (Tellier et al., 1996). Four subgenomic regions of GBV-B covering the entire published sequence (Simons et al., 1995b) were amplified from serum and the PCR products were purified and cloned into pGEM-9Zf(−) (Promega) or pCR2.1 vector (Invitrogen) using standard procedures.

The 5’ terminus of GBV-B was amplified from serum by using the 5’ RACE with dC or dA tailing (GIBCO/BRL) and GBV-B-specific antisense primers. To determine the 3’ terminal sequence of GBV-B, two different approaches were used. In one approach GBV-B RNA extracted from serum was circularized with T4 RNA ligase (Promega) and the 5’-to-3’-end-ligated viral RNA was amplified in RT-PCR using specific GBV-B primers. In the second approach, the 5’ end of the negative strand GBV-B RNA extracted from the liver homogenate was amplified by using the 5’ RACE with dC tailing and GBV-B-specific sense primers. The PCR products were cloned directly into pCR2.1-TOPO by using the TOPO TA Cloning Kit (Invitrogen).

The consensus sequence of GBV-B was determined by direct sequencing of PCR products (nts 1–9078 and 9130–9359) and by sequence analysis of clones (nts 1–7135 and 7151–9399). Nucleotide positions correspond to those of the infectious clone (pGBB) reported in this paper. Analyses of genomic sequences were performed with GeneWorks (Oxford Molecular Group) (Bukh et al., 1995). The predicted secondary structures of the GBV-B and HCV 3’ UTR sequences were determined by the program “mFold” (Genetics Computer Group).

Testing for GBV-B was performed by an RT-nested PCR assay with primers from the 5’ UTR (external primer pair: 5’-CCT AGA AGG CGG TGG GGG ATT TCC-3’ and 5’-AGG TCT GCG TCC TTG GTA GTG ACC-3’; internal primer pair: 5’-GGA TTT CCC CTG CCC GTC TG-3’ and 5’-CCC CGG TCT TCC CTA CAG TG-3’). The RT was
performed with avian myeloblastosis virus reverse transcriptase (Promega), and the external anti-sense primer and nested PCR was performed with AmpliTaq DNA polymerase or AmpliTaq Gold DNA polymerase (Perkin–Elmer) as described in detail previously (Bukh et al., 1998a,b). Specificity was confirmed by sequence analysis of selected DNA products. Each set of experiments included a positive control sample [a 10⁻⁶ dilution of the GB 8/93 serum pool, estimated titer 100 genome equivalents (GE)] and appropriate negative control samples. The GE titer of GBV-B in positive samples was determined by RT-nested PCR on 10-fold serial dilutions of the extracted RNA (Bukh et al., 1998b). One GE was defined as the number of GBV-B genomes present in the highest dilution positive in RT-nested PCR. Testing for GBV-A and GBV-A variants was performed by RT-nested PCR assays as described previously (Bukh and Apgar, 1997).

Construction of consensus cDNA clones of GBV-B

We first constructed a consensus clone of GBV-B, clone pGBB5–1, which had the 3' terminus of GBV-B as published by Simons et al. (1995b). The core sequence of the T7 promoter, a 5' guanosine residue and the sequence of GBV-B (9139 nts) were cloned into pGEM-9Z(–) vector using NotI and SacI sites. A BamHI site was included at the GBV-B 3' terminus. Digested fragments containing the consensus sequence were purified from subclones and ligated using convenient sites. A second consensus clone of GBV-B, clone pGBB, was constructed by inserting the new 3' UTR sequence, amplified by PCR from one of the clones obtained by the RACE procedure, into pGBB5–1 by using XmaI (at position 9114) and BamHI sites. A XhoI site was inserted following the GBV-B 3' terminus. DH5α competent cells (GIBCO BRL) were transformed and selected on LB agar plates containing 100 μg/ml ampicillin (SIGMA) and amplified in LB liquid cultures at 30°C for 18–20 h (Yanagi et al., 1997). Each cDNA clone was retransformed to select a single clone, and large-scale preparation of plasmid DNA was performed with a QIAGEN plasmid Maxi kit as described previously (Yanagi et al., 1997). Each clone was genetically stable because the digestion pattern was as expected after retransformation and the complete sequence was the expected one.

Intrahepatic transfection of tamarins (S. mystax) with transcribed GBV-B RNA

In 100 μl reactions, RNA was transcribed in vitro with T7 RNA polymerase (Promega) from 10 μg of linearized template plasmid. The plasmid pGBB5–1 was linearized with BamHI (Promega), and the plasmid pGBB was linearized with XhoI (Promega). The integrity of the RNA was checked by electrophoresis through agarose gel stained with ethidium bromide. Each transcription mixture was diluted with 400 μl of ice-cold phosphate-buffered saline without calcium or magnesium (SIGMA) and then immediately frozen on dry ice and stored at −80°C. Within 24 h, two transcription mixtures were injected into each tamarin by percutaneous intrahepatic injection guided by ultrasound (Yanagi et al., 1998, 1999). If the tamarin did not become infected, the same transfection was repeated once. All transfected animals were negative for GBV-A and GBV-A variants (Bukh and Apgar, 1997).

Transmission studies in tamarins

We primarily used tamarins of the species Saguinus mystax. A single tamarin of the species S. oedipus was also used. Animals were maintained under conditions that met or exceeded all requirements for their use in an approved facility. Serum samples were collected weekly from the tamarins and monitored for liver enzyme levels [alanine aminotransferase (ALT), γ-glutamyltranspeptidase (GGT), and isocitrate dehydrogenase (ICD)] by standard methods. In addition, liver biopsy samples were collected weekly from the two transfected animals, and fixed liver tissue was examined for necroinflammatory changes.

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