Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 3401–3405, April 1995
Medical Sciences

Identification of two flavivirus-like genomes in the GB hepatitis agent

(hepatitis C virus/representational difference analysis)

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Communicated by Bernard Roizman, University of Chicago, Chicago, IL, January 5, 1995

ABSTRACT **A subtractive PCR methodology known as representational difference analysis was used to clone specific nucleotide sequences present in the infectious plasma from a tamarin infected with the GB hepatitis agent. Eleven unique clones were identified, seven of which were examined extensively. All seven clones appeared to be derived from sequences exogenous to the genomes of humans, tamarins, *Saccharomyces cerevisiae*, and *Escherichia coli*. In addition, sequences from these clones were not detected in plasma or liver tissue of tamarins prior to their inoculation with the GB agent. These sequences were detected by reverse transcription–PCR in acute-phase plasma of tamarins inoculated with the GB agent. Probes derived from two of the seven clones detected an RNA species of ≥8.3 kb in the liver of a GB-agent-infected tamarin by Northern blot hybridization. Sequence analysis indicated that five of the seven clones encode polypeptides that possess limited amino acid identity with the nonstructural proteins of hepatitis C virus. Extension of the sequences found in the seven clones revealed that plasma from an infected tamarin contained two RNA molecules >9 kb long. Limited sequence identity with various isolates of hepatitis C virus and the relative positions of putative RNA helicases and RNA-dependent RNA polymerases in the predicted protein products of these molecules suggested that the GB agent contains two unique flavivirus-like genomes.**

The first agent that induced hepatitis and that was passaged serially in primates originated from the serum of a 34-year-old surgeon (with the initials GB) obtained during the third day of jaundice (1). Passage of this "GB agent" in the *Sanguinus* sp. (tamarins) allowed extensive virological characterization (2–4). However, subsequent primate host range and cross-challenge experiments suggested that the GB agent was distinct from the currently known human hepatitis viruses (3–11). In addition, antibodies specific for hepatitis A, hepatitis B, hepatitis C, and hepatitis E viruses were not elicited by GB inoculation of tamarins as detected by current immunoassays (12). Thus, despite extensive characterization in tamarins, the relation of the GB agent to the previously identified viral agents of human hepatitis has not been elucidated. Clearly, the molecular characterization of the GB agent's genome would aid in deciphering the importance of this agent in cases of human viral hepatitis. To this end, we have characterized two RNA molecules found in the acute-phase plasma from a tamarin infected with the GB agent.†

MATERIALS AND METHODS

Virus. Material used in this study originated from a pool of known infectious tamarin GB sera (designated as H205 GB pass 11) that has been described (7, 13). Tamarin T-1053 was inoculated intravenously with 0.25 ml of a 1:50 dilution of H205 GB pass 11. On days 7 and 11 after inoculation, serum alanine transaminase levels were 67 and 172 units/liter, respectively (preinoculation baseline = 40.3 ± 6.6 units/liter), indicating acute hepatitis. T-1053 was euthanized on day 12 after inoculation, and plasma, liver, and kidneys were harvested. Titration experiments demonstrated ≥4×10^5 tamarin infectious doses/ml of T-1053 plasma was obtained at the time of sacrifice. Additional tamarin plasma used in this study are described in detail elsewhere (12).

Representational Difference Analysis (RDA). RDA was performed as described (14) with minor modifications. Tester and driver amplicons were prepared from total nucleic acid isolated from tamarin T-1053 plasma obtained at the time of sacrifice and from preinoculation plasma (pooled from plasma taken 30 and 17 days before inoculation), respectively. Total nucleic acids extracted from 50 μl of filtered (0.1-μm pore size) plasma were converted to cDNA by random-primed reverse transcription (RT) with an RNA PCR kit (Perkin–Elmer). After heat denaturation of the RNA·cDNA hybrids, second-strand cDNA synthesis proceeded with the addition of 10× RP buffer [100 mM NaCl/420 mM Tris·HCl, pH 8.0/50 mM dithiothreitol/bovine serum albumin (100 μg/ml)], 2.5 μM random hexamers, and Sequenase Version 2.0 DNA polymerase at 0.13 unit/μl (United States Biochemical). The double-stranded DNA products of these reactions were digested with *Sau*3AI (New England Biolabs) and ligated to the R *Bgl* primer set as described by Lisitsyn *et al.* (14). Subsequent amplicon generation, hybridizations, and selective amplifications proceeded essentially as described (14). Products of the second and third rounds of selective amplification were cloned into pBluescript II (KS+) (Stratagene) for further analysis.

PCR and RT–PCR. PCR primers derived from the sequences of the RDA clones were qualified on plasmid templates. Genomic PCRs were performed on 300 ng of DNA from tamarin, human, *Saccharomyces cerevisiae*, and *Escherichia coli* utilizing the GeneAmp PCR kit (Perkin–Elmer). PCRs were performed with 0.5 μM primers for 35 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) followed by an extension at 72°C for 7 min. The PCR products were analyzed by agarose gel electrophoresis and Southern blot hybridization as described (15). Genomic DNAs from normal tamarin kidney and liver tissue were prepared as described (16). Genomic DNAs from human placenta, Rhesus monkey kidney, and *S. cerevisiae*

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Abbreviations: HCV, hepatitis C virus; RDA, representational difference analysis; RT, reverse transcription; RdRp, RNA-dependent RNA polymerase; GB-A, GB genome A; GB-B, GB genome B; GBV-A, GB virus A; GBV-B, GB virus B.

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U22303 and U22304).

were obtained from Clontech. *E. coli* DNA was obtained from Sigma.

Nucleic acids were extracted from tamarin plasma by using a total nucleic acid extraction kit (United States Biochemical). PCR and RT–PCR were performed using the GeneAmp PCR kit and the GeneAmp RNA PCR kit (Perkin–Elmer), respectively. RT reactions were primed by random hexamers. PCRs were performed with 1 μM primers for 35–40 cycles (94°C, 30 sec; 55°C, 30 sec; 72°C, 30 sec) followed by an extension cycle of 72°C for 3 min. The PCR products were analyzed as described above.

Northern Blot Analysis. Total cellular RNA was extracted from the liver of the GB-infected tamarin T-1053 and from the liver of an uninfected tamarin by using an RNA isolation kit (Stratagene). Total RNA (30 μg) was electrophoresed through a 1% agarose gel containing 0.6 M formaldehyde (33), transferred to Hybond-N nylon membrane (Amersham), and UV-crosslinked to the membrane (16). Membranes were prehybridized at 60°C for 2 h, hybridized at 60°C for 16 h, washed as described (17), and then exposed to Kodak X-Omat-AR film at −80°C. Plasmid-derived DNA fragments (50 ng) were radiolabeled by using a random-primer labeling kit (Stratagene) in the presence of [α-^{32}P]dATP. The specific activity of each probe was 10^9 cpm/μg.

Genome Extension. RT–PCR experiments utilizing different combinations of the clone-specific primers were used to extend and connect the RDA-derived sequences. Additional sequences were obtained as described by Sorensen *et al.* (18), by using RNA ligase-mediated 5′ RACE (using the 5′-AmpliFINDER RACE kit, Clontech), and genomic RNA circularization coupled with RT–PCR (19). Products from these reactions were cloned into pT7Blue T-vector (Novagen) for further analysis.

DNA Sequencing and Sequence Analysis. Clones obtained from RDA and extension experiments were sequenced by using the Sequenase Version 2.0 sequencing kit (United States Biochemical) as directed by the manufacturer. These sequences were analyzed by using programs in the GCG package Version 7.

RESULTS

Isolation of GB-Agent Clones. RDA amplifies unique DNA sequences present in one complex source (the "tester") that are absent in a highly related source (the "driver") (14). It was hypothesized that the only qualitative nucleic acid difference between preinoculation plasma and infectious plasma from a GB-infected animal would be the presence of the GB-agent genome. Therefore, RDA of infectious plasma from a GB-inoculated tamarin (tester) and preinoculation plasma (driver) should amplify segments of the GB-agent genome.

Preinoculation and infectious plasma were obtained from T-1053, the GB-infected tamarin. Because the genome of the GB agent could be single- or double-stranded DNA or RNA, nucleic acids extracted from the T-1053 plasma were converted to double-stranded DNA by randomly primed RT and randomly primed second-strand synthesis. Double-stranded DNA derived from the infectious and preinoculation T-1053 plasma were subjected to RDA. Agarose gel analysis of the RDA products showed a marked reduction in the relative complexity of the tester amplicon (Fig. 1, lane 3) during the subsequent rounds of selective amplification (Fig. 1, lanes 4–6). The four bands with the highest molecular weights from the second round of selective amplification (Fig. 1, lane 5) and all products obtained after the third round of selective amplification were cloned into pBluescript. A total of 76 clones were isolated. Cross-hybridization experiments demonstrated that 11 unique sequences were represented, seven of which were analyzed extensively (Table 1).

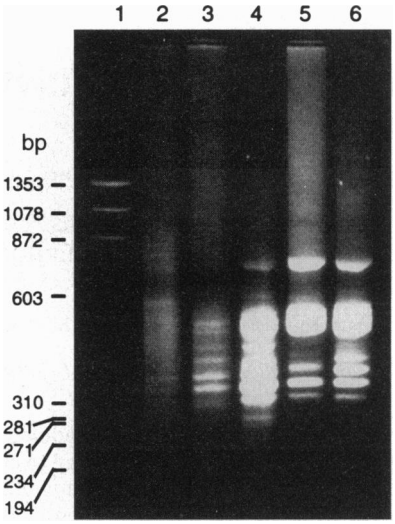

FIG. 1. RDA products from T-1053 plasma. DNA (≈0.5 μg per lane) from the driver amplicon (preinoculation plasma, lane 2) and tester amplicon (infectious plasma, lane 3) and products from the first, second, and third rounds of selective amplification (lanes 4–6, respectively) were electrophoretically separated on a 2% agarose gel and visualized by ethidium bromide staining. Lane 1 contains DNA markers with the sizes of the DNA fragments indicated.

Exogenicity of Clones. Genomic PCR analysis was utilized to determine whether the clones were derived from primate, human, *S. cerevisiae*, or *E. coli* sources. Clone 16 sequence was not detected in DNA from tamarin, rhesus monkey, human (Fig. 2*A*), *S. cerevisiae*, and *E. coli* (data not shown). Sensitivity of the clone 16-specific PCR was confirmed by the detection of <0.05 single copy gene equivalent of plasmid in normal tamarin liver and kidney DNA (Fig. 2*A*). The integrity of the human and primate DNAs was demonstrated by the PCR amplification of a 553-bp product from the human dopamine D_1 receptor gene (bases 1000–1552, ref. 20) and the primate

Table 1. Exogenous clones present in acute-phase T-1053 plasma and H205 GB passage 11

Clone	Acute-phase tamarin plasma, no. detected/ no. tested	T-1053 liver RNA size, kb	% HCV-1 identity
2	1/3	ND	ND
4	8/8	≥8.3	31.5 (NS4, 1805–1877)
10	1/1	ND	40.2 (NS5, 2637–2741)
16	3/8	ND	27.3 (NS2, 785–1011)
18	1/2	ND	ND
23	1/1	ND	29.3 (NS3, 1012–1133)
50	2/2	>8.3	33.0 (NS4, 1913–2021)

Clones were not detected in tamarin, human, *S. cerevisiae*, and *E. coli* DNAs by genomic PCR analysis able to detect ≤0.1 gene copy equivalent of plasmid added to tamarin DNA. In addition, these clones were detected in the infectious T-1053 plasma and H205 GB pass 11 by RT–PCR analysis. ND, not detected. For percent HCV-1 identity, amino acid translations of the clones were searched against the Swiss-Prot data base with the BLASTX program. Sequences showing limited identity with various strains of HCV were aligned to HCV-1 (Swiss-Prot accession no. P26664) by using the GAP program with a gap weight of 3.000 and a length weight of 0.100. Regions of HCV-1 nonstructural proteins (NS) showing similarity (gene product and residue numbers) are in parentheses.

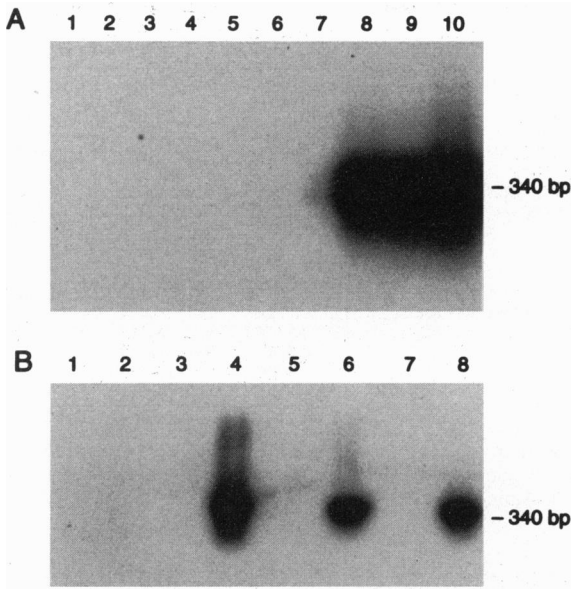

FIG. 2. PCR analysis of clone 16. (*A*) Absence of clone 16 sequence in genomic DNAs. Clone 16-specific genomic PCR/Southern blot hybridization does not detect clone 16 sequences in DNAs isolated from tamarin kidney and liver (lanes 1 and 2, respectively), rhesus monkey (lane 3), or human (lane 4). Clone 16 products generated from 1, 3, and 10 fg of clone 16 plasmid DNA are shown in lanes 6–8, respectively. Clone 16 PCR products generated from tamarin liver and kidney DNA containing 10 fg of clone 16 plasmid DNA are shown in lanes 9 and 10, respectively. Lane 5 is empty. (*B*) Presence of clone 16 sequence in GB-inoculated tamarins. Clone 16-specific RT–PCR/Southern blot hybridization of preinoculation (lanes 1, 3, 5, and 7) and acute-phase (lanes 2, 4, 6, and 8) plasma from tamarins T-1048 (lanes 1 and 2), T-1049 (lanes 3 and 4), T-1051 (lanes 5 and 6), and T-1055 (lanes 7 and 8) are shown.

homologues of this gene. In addition, clone 16 sequence was not detected in the genomes of tamarin, human, *S. cerevisiae*, and *E. coli*, as shown by Southern blot analysis designed to have single-gene-copy sensitivity (data not shown). Similarly, genomic PCR demonstrated that sequences from clones 2, 4, 10, 18, 23, and 50 are exogenous to the genomes of tamarin, human, *S. cerevisiae*, and *E. coli* (Table 1). These data are consistent with the RDA-derived clones being of viral origin.

Sequence Presence in Acute-Phase Plasma. RT–PCR was utilized to determine the presence of the seven RDA-derived sequences in tamarin plasma. Sequences from each of the seven clones were detected in the infectious T-1053 plasma and not the preinoculation T-1053 plasma (data not shown). In addition, these sequences could be detected in H205 GB pass 11, the inoculum used to infect tamarin T-1053. It should be noted that these sequences were detected only in samples that were subjected to RT, which suggests that the clones were derived originally from an RNA molecule, possibly an RNA virus.

A panel of eight additional tamarins that had been experimentally infected with the GB agent all demonstrated biochemical hepatitis (serum alanine aminotransferase levels >3.75 SDs above the preinoculation mean; ref. 12). RT–PCR analysis was performed on preinoculation and acute-phase plasma from these animals. None of the sequences were detected in any of the preinoculation plasma tested, and each sequence was found in at least one of the GB-infected animals (Fig. 2*B*, Table 1, and data not shown). Clone 4 sequence was detected in the acute-phase plasma from all eight of the animals tested. However, clone 16 sequence was detected in only three of the eight animals tested (4 of 8 animals tested are shown in Fig. 2*B*). The absence of clone 16 sequence in acute-phase plasma from five of eight animals tested may be explained by the low sensitivity of the clone 16-specific RT–PCR and the apparent acute resolving nature of GB-agent infection assumed from biochemical analysis of infected tamarins (4). However, the detection limit of clone 4-specific and clone 16-specific PCRs is roughly equivalent (≈5000 copies of plasmid DNA by ethidium bromide staining), and plasma from all infected animals tested contained clone 4 sequences. Thus, the existence of clone 4-positive, clone 16-negative plasma may reflect the presence of RNA sequences of one virus (containing clone 4) and the absence of detectable RNA sequences from a second virus (containing clone 16).

Northern Blot Analysis. Northern blot analysis of GB-agent-infected and uninfected tamarin liver RNA was performed to determine the presence and size of any viral genome with which these clones may be associated. Probes for clones 4 and 50 hybridized with an RNA molecule of ≥8.3 kb present in the liver of T-1053, the infected tamarin (Fig. 3, lanes 4 and 6, respectively). Neither probe hybridized specifically with RNA extracted from the liver of an uninfected tamarin (Fig. 3, lanes 3 and 5). In contrast, no RNA species were detected by Northern blot hybridization of infected or uninfected tamarin liver RNA by using probes for clones 2, 10, 16, 18, or 23 (Table 1). Thus, consistent with the previous RT–PCR data, the clone 16 sequence is not detected in the presence of the clone 4 sequence by Northern blot analysis. Thus, the Northern blot and RT–PCR data suggest that clones 4 and 50 may be derived from one virus and clones 2, 10, 16, 18, and 23 may be derived from a second virus.

Sequence Analysis of GB Clones. Similarity searches of GenBank and Swiss-Prot databases demonstrated low but significant sequence similarity to hepatitis C virus (HCV) in five of the seven clones. Specifically, amino acid translations of clones 4, 10, 16, 23, and 50 are between 27.3 and 40.2% identical to portions of HCV-1 nonstructural proteins (Table 1). Of particular interest was the identity exhibited between a translation of clone 10 and the putative RNA-dependent RNA polymerase (RdRp) of HCV and other positive-strand RNA viruses (21). This includes the canonical Gly-Asp-Asp signature sequence (Fig. 4). In addition, only clone 10 had detectable identity with HCV at the nucleotide level. The best alignment was found to an HCV 3b genotype (108 of 176 identical bases by BLASTN search of GenBank). GAP alignment of clone 10 and HCV-1 demonstrated 52.1% identity. Analyses of clones 2 and 18 showed no significant nucleotide or amino acid identity to sequences in GenBank or Swiss-Prot databases.

Extension of GB-Agent Sequences. Compelling evidence for the viral origin of clones 4 and 50 is provided by (*i*) the inability

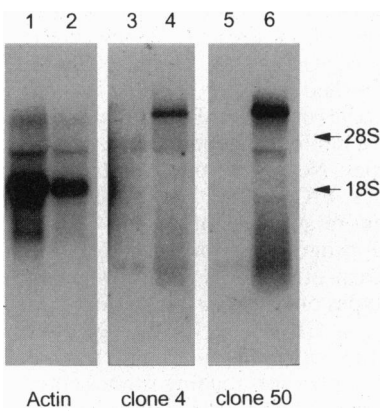

FIG. 3. Northern blot hybridization of tamarin liver RNA. Lanes 1, 3, and 5 contain liver RNA from an uninfected tamarin, and lanes 2, 4, and 6 contain liver RNA from T-1053, the H205 GB pass 11-inoculated tamarin. Lanes 1 and 2 were hybridized with a human β-actin cDNA probe, lanes 3 and 4 were hybridized with the clone 4 probe, and lanes 5 and 6 were hybridized with the clone 50 probe. Exposure times were as follows. Lanes: 1 and 2, 5 h; 3–6, 56 h. The positions of the 28S and 18S rRNAs are indicated.

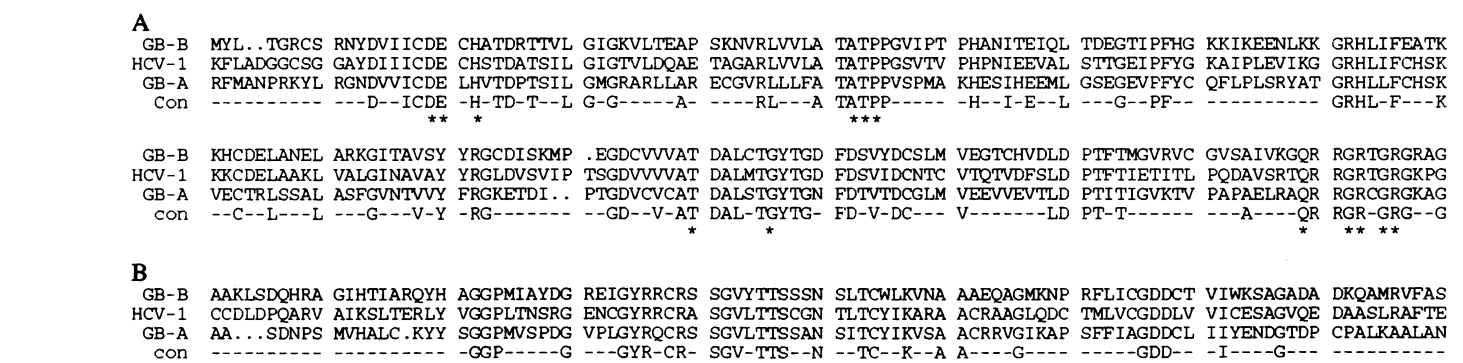

FIG. 4. Amino acid sequence comparisons. Alignments were performed with the GCG package program PILEUP. (*A*) Alignment of the putative NS3 RNA helicase domain of HCV-1 (residues 1298–1497), GB genome A (GB-A) (residues 1242–1439), and GB genome B (GB-B) (residues 1212–1408). Amino acids conserved between the RNA helicases of flaviviruses, pestiviruses, and poty-related viruses (21) are denoted by *. (*B*) Alignment of the putative RdRp domains of HCV-1 (residues 2662–2761), GB-A (residues 2634–2729), and GB-B (residues 2513–2612). Amino acids conserved in the RdRps of positive-strand RNA viruses (21) are denoted by #. Amino acid residues encoded by clone 10 correspond to GB-A residues 2609–2718.

to detect these sequences in genomic DNA, (*ii*) the presence of these sequences in all acute-phase plasma tested, (*iii*) the hybridization of these sequences with ≥8.3-kb RNA species found in infected liver, and (*iv*) the limited HCV sequence identity found in translations of these clones. Similar evidence exists to support the viral origin of clones 2, 10, 16, 18, and 23 despite the apparent absence of these sequences in some acute-phase tamarin plasma and in T-1053 liver RNA, and the absence of HCV sequence identity in translations of clones 2 and 18. Therefore, to obtain additional sequences from the putative viral genome(s) that are contiguous with the previously characterized sequences, several PCR-based experiments were performed. Specifically, combinations of clone-specific oligonucleotide primers were used in RT–PCR experiments in attempts to amplify sequences that may be present between the clones. In addition, PCR techniques for obtaining sequences found upstream and downstream of known sequences (18) and sequences at the 5′ and 3′ ends of RNA molecules (19, 22) were utilized. These experiments led to the cloning of sequences between the previously characterized clones in addition to sequences on the 5′ and 3′ sides of their termini. Sequence analysis demonstrated the presence of two distinct RNA molecules found in the acute-phase T-1053 plasma that we have termed GB-A and GB-B. GB-A consists of at least 9493 nt and contains the sequences found in clones 2, 10, 16, 18, and 23. GB-B consists of at least 9143 nt and contains clones 4 and 50. A detailed analysis of the GB-A and GB-B genomes will be presented elsewhere (A.S.M., T.P.L., J.N.S., T.J.P.-M., G.J.D., J.C.E., M.L.C., G.G.S., S.M.D. & I.K.M., unpublished data).

BLASTN searches of the GenBank database reveal that GB-A and GB-B have limited nucleotide sequence identity to various HCV genotypes. No significant identity is detected to other viral sequences in GenBank. Nucleotide sequence comparisons of the entire genomes of GB-A, GB-B, and HCV-1 by using the GAP program demonstrate that they are all <44% identical to each other (data not shown) and, thus, are not distinct genotypes of HCV (23). In addition, the low sequence identity between GB-A and GB-B suggests that GB-A and GB-B are not genotypes of the same virus. GB-A and GB-B contain single large open reading frames coding for 2972 and 2864 amino acids, respectively. BLASTX searches of the Swiss-Prot database with the translation products of GB-A and GB-B showed limited identity among GB-A, GB-B, and various HCV genotypes (data not shown). Comparison of regions from the putative RNA helicases of GB-A, GB-B, and HCV-1 demonstrates that in these regions, GB-A and GB-B are 47 and 55% identical to HCV-1, respectively, and 43.5% identical to each other (Fig. 4*A*). Similarly, regions encoding the putative RdRp of GB-A and GB-B are 36 and 41% identical to HCV-1, respectively, and 43% identical to each other (Fig. 4*B*). These similarities and the relative genomic position of the putative RNA helicase and RdRp suggest that GB-A and GB-B are the genomic sequences of two distinct flavi-like viruses.

DISCUSSION

RDA was used to amplify and clone unique sequences present in infectious tamarin plasma containing the GB agent. Sequences from these clones were not detected in genomic DNAs of human, tamarin, *S. cerevisiae*, or *E. coli*, in preinoculation plasma, or in infectious tamarin plasma that have not been subjected to RT prior to PCR amplification. The RDA-isolated sequences were detected by RT–PCR in the infectious tamarin plasma from which they were isolated and in the GB-agent inoculum, H205 GB pass 11. In addition, amino acid translations of five clones contained low but significant sequence similarity to nonstructural proteins of HCV. Extension of the seven clones demonstrated the existence of two flavivirus-like genomes in the GB-agent inoculum, GB-A and GB-B.

Flaviviruses are enveloped viruses that contain a single positive-sense genomic RNA molecule of ≈10 kb. The existence of two flavivirus-like genomes suggests that H205 GB pass 11 contains two distinct viruses. The presence of two viruses is consistent with the detection of clone 4 and 50 sequences, but not clone 2, 10, 16, 18, and 23 sequences, in liver RNA from the GB-infected animal T-1053 by Northern blot analysis, and the existence of clone-4-positive clone-16-negative acute-phase plasma. The existence of two viruses is further supported by studies demonstrating that the two RNA species present in the GB-agent inoculum can be filtered, diluted, and passaged separately in tamarins (12). Thus, GB-A and GB-B are derived from the genomes of two viruses: GB virus A (GBV-A) and GB virus B (GBV-B).

The presence of two flavi-like viruses in the GB-agent inoculum may explain the controversy regarding the human origin of the GB agent. Parks and Melnick (24) argued that the GB agent is a tamarin virus based on their finding of cases of spontaneous hepatitis in tamarin colonies that could be passaged, and the similarity of these isolates with the GB agent as demonstrated by cross-challenge experiments. However, evidence for a human virus in the GB agent is provided by the similarity of GB and WW-55. WW-55, a human plasma sample obtained from blood donated 1 day prior to the development of hepatitis with jaundice, induced hepatitis in 5 of 10 human volunteers (25). WW-55 also causes hepatitis in tamarins (1) and prior infection with WW-55 renders tamarins resistant to

GB-agent hepatitis (5). Thus, the two viruses identified in the GB-agent inoculum may have two sources: one virus originating from GB, the human, and the other virus originating from tamarins during the passage of the GB agent in tamarins.

Which virus caused hepatitis in GB? The fact that clones 2, 10, 16, 18, and 23 (from GBV-A) are not detected in the liver of T-1053 by Northern blot hybridization (Table 1) despite equivalent amounts of GBV-A (clone 16) and GBV-B (clone 4) in T-1053 plasma as determined by limiting-dilution PCR analysis (data not shown) may indicate that GBV-A does not replicate in the liver of tamarins. In contrast, GBV-B (clone 4) appears in all acute-phase tamarin plasma tested (Table 1) and GBV-B (clones 4 and 50) is detected in T-1053 liver RNA by Northern blot hybridization. Thus, these data suggest that GBV-B causes hepatitis in tamarins. However, whether GBV-B causes hepatitis in humans remains an open question. Studies of human samples related to the GB agent in cross-challenge experiments (e.g., WW-55, Berlin agent, and WAL; refs. 5 and 34) in addition to the original serum from GB may resolve this issue.

Previous methodology utilized for the molecular cloning of hepatitis viruses from infectious primate source material relied on differential hybridization and immunoscreening of 4×10^4 and 10^6 λ phage clones, respectively (26, 27). Besides being labor intensive, these methodologies required the existence of relatively large amounts of highly purified viral nucleic acids and/or well-qualified immunoscreening sera. In contrast, RDA performed with 50 μl of preinoculation and infectious plasma yielded 11 unique clones, 7 of which were demonstrated to be of viral origin. In addition, sequence analysis of the four original clones isolated by RDA but not analyzed extensively indicated that one was derived from GB-A and two were derived from GB-B. A BLASTN search of GenBank with the sequence from the one clone not found in GB-A or GB-B indicated 99% identity to a segment of the *E. coli lepA* gene (GenBank accession no. K00426), suggesting bacterial origin. Thus, RDA produced 10 of 11 clones of viral origin by using 50 μl of preinoculation and infectious T-1053 plasma. Clearly, RDA presents an extremely efficient way of identifying viral sequences.

The known viral agents of hepatitis (hepatitis A virus, hepatitis B virus, HCV, hepatitis D virus, hepatitis E virus, cytomegalovirus, and Epstein–Barr virus) do not account for all of the cases of hepatitis of purported viral etiology. Specifically, the screening of donated blood for serologic markers of HCV has not prevented all cases of non-A, non-B post-transfusion hepatitis, suggesting the existence of a "non-A, non-B, non-C" agent (28). In addition, recent publications have suggested that additional viral agents may be responsible for water-borne hepatitis (29), hepatitis-associated aplastic anemia (30), fulminant hepatic failure (31), and community-acquired acute and/or chronic hepatitis (32). Diagnostic reagents developed from the genomic sequences of GBV-A and GBV-B may determine whether either of these viruses are the causative agents in hepatitis cases of unknown etiology.

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