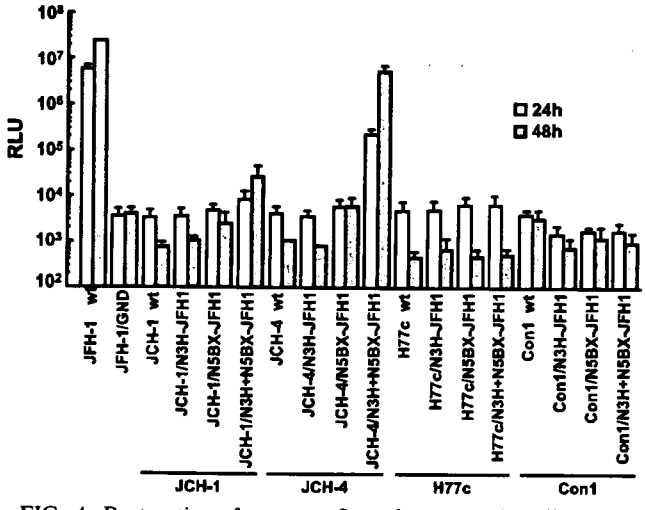

FIG. 4. Restoration of genotype 2a and genotype 1 replicon replication by the insertion of JFH-1 sequences. Two genotype 2a replicons, JCH-1 and JCH-4, a genotype 1a replicon, H77c, and a genotype 1b replicon, Con-1, were used in this assay. Three kinds of chimeric replicons, N3H-JFH-1, N5BX-JFH1, and N3H+N5BX-JFH-1, were prepared for all four HCV replicons. Wild-type (wt) or chimeric subgenomic RNAs were transfected into Huh7 cells and the luciferase activities of the transfected cells examined as described in the legend to Fig. 2B. The assays were performed three times independently, and data are presented as means and standard deviations for luciferase activity (RLU) at 24 h (white bars) and 48 h (gray bars) after transfection.

of wild-type JCH-1 at 48 h and recovered the JCH-4 replication to a level similar to that of wild-type JFH-1 at 48 h (Fig. 4, JCH-1/N3H+N5BX-JFH1 and JCH-4/N3H+N5BX-JFH1, respectively). On the other hand, insertion of the JFH-1 N5BX region or both the N3H and the N5BX regions did not restore H77c or Con1 replicon replication (Fig. 4, H77c/N5BX-JFH1, H77c/N3H+N5BX-JFH1, Con1/N5BX-JFH1, and Con1/N3H+N5BX-JFH1). HCV polyprotein processing is critically important for HCV RNA replication and virus production, and this processing may be affected by the chimeric RNA molecules between different isolates of genotype 2 as well as those between genotypes 1 and 2. However, our data indicated that HCV polyprotein processing did not differ among the chimeric constructs (data not shown). Thus, the JFH-1 N3H and N5BX regions can rescue the replication of genotype 2a replicons at different levels but not the replication of genotype 1 replicons.

The NS3 helicase and NS5B-3′X regions are both important for JFH-1 genomic RNA replication. Next, we applied the previously described results to genomic RNA replication. The structures of HCV, the template DNA for JFH-1, and the chimeric full-genomic RNAs are shown in Fig. 5A. Full-length HCV RNAs were synthesized as described above and their quality and integrity then confirmed by gel electrophoresis (data not shown). To analyze the transient RNA replication of these chimeric RNAs in Huh7 cells, the synthesized RNAs were transfected into Huh7 cells and total RNA was extracted from HCV RNA-transfected cells at various time points. Northern blot analysis was then performed. The equality of the transfection efficiencies was confirmed by the cotransfection of luciferase mRNA (data not shown). As shown in Fig. 5B, JFH-1 RNA decreased at 10 h after transfection but replicated efficiently at 24 to 48 h after transfection, as described previously (48). J6 chimeric RNA with the NS3 helicase and N5BX regions of JFH-1 (J6/N3H+N5BX-JFH1) replicated with similar kinetics but with lower efficiency. J6 chimeric RNA with JFH-1 N5BX (J6/N5BX-JFH1) showed no replication in this assay, like J6CF or JFH-1 GND, although this chimera replicated to a considerable extent in subgenomic-replicon assays. Taken together, these data indicate that the NS3 helicase-coding region and the NS5B-to-3′X region of JFH-1 are both essential for full-length genomic HCV RNA replication in Huh7 cells.

Core protein and infectious-chimeric-virus secretion from chimeric J6CF RNA-transfected cells. Finally, we tested whether chimeric RNA-transfected cells could secrete infectious virus particles. Figure 5C shows the core protein secretion into the culture medium from JFH-1, JFH-1/GND, J6CF, and chimeric-RNA-transfected cells. Core protein was efficiently secreted from cells transfected with JFH-1 RNA (Fig. 5C and Table 1) and those transfected with J6/N3H+N5BX-JFH1 RNA, but with efficiencies lower than that for JFH-1 (Fig. 5C and Table 1). J6/N5BX-JFH1, JFH-1/GND, and J6CF RNA-transfected cells, which showed no RNA replication by Northern blot analysis (Fig. 5B), did not secrete core proteins into the culture medium (Table 1). By the replicon assay, JFH-1/N5BX-J6 showed no replication in Huh7 cells (Fig. 2B, N5BX-J6), and full-length JFH-1/N5BX-J6 RNA-transfected cells did not secrete core protein into the culture medium (Table 1). On the other hand, JFH-1/N5B-J6 replicated to some extent in the replicon assay (Fig. 2B, N5B-J6), and full-length JFH-1/N5B-J6 RNA-transfected cells secreted a smaller amount of core protein than JFH-1 RNA-transfected cells (Fig. 5C and Table 1). Both JFH-1/N3H-J6 and JFH-1/3′UTR-J6 RNA-transfected cells secreted about half the amount of core protein that the JFH-1 RNA-transfected cells did (Fig. 5C and Table 1); however, the replication level of the JFH1/N3H-J6 replicon was markedly lower than those of the JFH-1 and JFH-1/3′UTR-J6 replicons (Fig. 2B, JFH-1 wt, N3H-J6, and 3′UTR-J6), and the replication level of full-length JFH-1/N3H-J6 RNA was also lower than those of the JFH-1 and JFH-1/3′UTR-J6 RNAs as determined by Northern blot analysis (data not shown). Transfection of the other two chimeric RNAs, JFH-1/N3H+N5B-J6 and JFH-1/N3H+N5BX-J6, did not induce core protein secretion (Table 1), and this is in agreement with the finding that neither chimeric replicon replicated (Fig. 2B, N3H+N5B-J6 and N3H+N5BX-J6).

Then, we tested the infectivity of the culture medium from the RNA-transfected cells by a focus formation assay. The infectivity of the culture medium from JFH-1 RNA-transfected cells was determined as $8.8 \times 10^3 \pm 5.7 \times 10^2$ FFU/ml (Table 1). The infectivity of the culture medium was also detected from cells transfected with J6/N3H+N5BX/JFH-1, JFH1/N3H-J6, JFH-1/N5B-J6, or JFH-1/3′UTR-J6 RNA but not with other chimeric RNAs (Table 1). This result thus indicates that efficient core protein secretion is at least indispensable for infectious-virus secretion. However, the levels of infectivity of culture medium did not correlate with core protein concentrations. In particular, JFH-1/N3H-J6 RNA-transfected cells secreted a rather higher level of core protein, but its infectious titer was low. The RNA replication capacity of JFH-1/N3H-J6 was lower than that of wild-type JFH-1 or JFH-1/3′UTR-J6

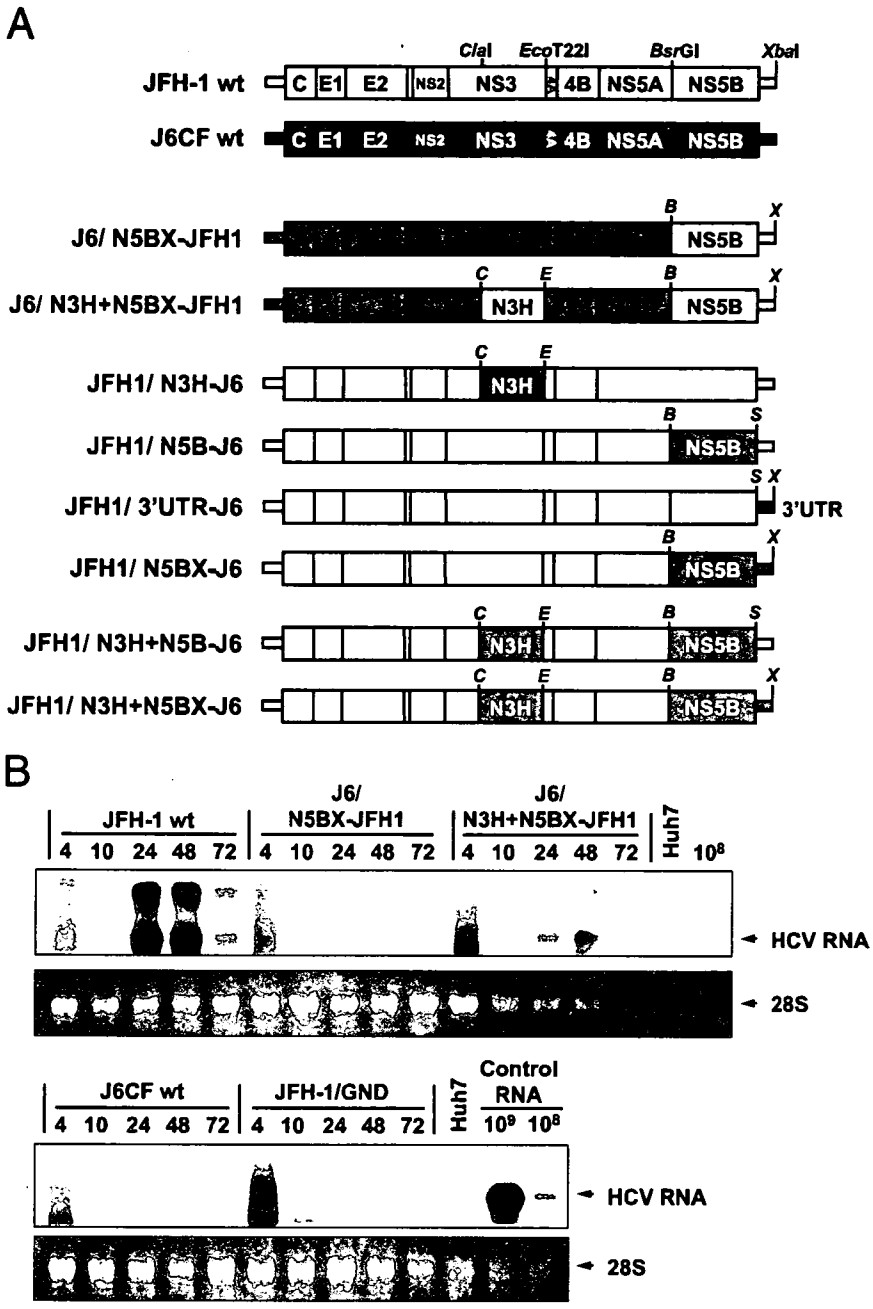

TABLE 1. Infectious titers of the media from chimeric HCV RNA-transfected cells

Construct[a]	Core protein level (fmol/ml)	Infectivity (FFU/ml)
JFH-1 (wild type)	50.7 ± 4.1	$8.8 \times 10^3 \pm 5.7 \times 10^2$
JFH-1/GND	0	0
J6CF (wild type)	0	0
J6/N5BX-JFH1	0	0
J6/N3H+N5BX-JFH1	7.7 ± 1.7	$9.1 \times 10^1 \pm 4.1 \times 10^1$
JFH-1/N3H-J6	26.3 ± 3.6	$1.7 \times 10^1 \pm 1.2 \times 10^1$
JFH-1/N5B-J6	0.1 ± 0.0	$6.7 \times 10^0 \pm 4.1 \times 10^0$
JFH-1/3'UTR-J6	23.6 ± 2.9	$2.6 \times 10^3 \pm 7.1 \times 10^2$
JFH-1/N5BX-J6	0	0
JFH-1/N3H+N5B-J6	0	0
JFH-1/N3H+N5BX-J6	0	0

[a] Culture media were collected from the RNA-transfected cells 72 h after transfection.

(Fig. 2B), and currently, there is no clear explanation for this discrepancy. This will be further examined in a future study.

Importantly, we found that the J6/N3H+N5BX-JFH1 chimera produced infectious virus. These results strongly indicate that the NS3 helicase and NS5B-to-3'X regions of JFH-1 are important for autonomous replication of the replication-incompetent J6CF strain and for secretion of infectious chimeric virus, although the virus secretion efficiency and the infection efficiency of the secreted virus were low.

DISCUSSION

In the present study, we identified the regions that are important for efficient JFH-1 replication in Huh7 cells by using chimeric constructs with other genotype 2a clones. Via transient replication assays of JFH-1 and J6CF chimeras, both the NS3 helicase-coding (N3H) region and the NS5B-to-3'X (N5BX) region of JFH-1 were found to be important for replication (Fig. 2 and 3). This was also confirmed by full-length genomic RNA replication, but the replication level of J6/N3H+N5BX-JFH1 was lower than that of wild-type JFH-1 (Fig. 5B). The N5BX region of JFH-1 was the minimum essential region for subgenomic-replicon replication (Fig. 3B, N5BX-JFH-1), but in full-length RNA replication, the NS3 helicase-coding region of JFH-1 was also necessary (Fig. 5B). This contradiction might be explained by differences in RNA length, because shorter RNAs such as subgenomic replicons are likely to replicate even with a less powerful replication engine. Alternatively, there could be some negative element for replication in the J6CF structural-protein-coding region or some positive element in the *neo* encephalomyocarditis virus internal ribosome entry site region of the subgenomic replicon. Furthermore, J6 chimeric RNA with the minimum essential regions of JFH-1 (J6/N3H+N5BX-JFH1) caused Huh7 cells to secrete infectious chimeric virus particles. However, the infection efficiency of J6/N3H+N5BX-JFH1 was lower than that of wild-type JFH-1. First, this may be due to the low RNA replication level. With JFH-1 NS3 helicase and N5BX, J6CF was able to replicate, but the replication efficiency was lower than that of JFH-1 (Fig. 5B). Because J6CF replication could occur only with JFH-1 NS3 helicase and N5BX, more *cis*-acting replication elements (CREs) of JFH-1 may be needed for more efficient replication of J6CF. Second, the levels of virus assembly may be low. This chimera had only the NS3 helicase, NS5B, and 3'UTR regions of JFH-1, possibly omitting some regions important for efficient virus particle secretion. Given that the NS2 region of JFH-1 is reportedly important in virus assembly and release (39), the NS2 region may be a possible candidate. JFH-1/N3H-J6 RNA-transfected cells secreted a substantial amount of core protein; however, its infectivity was much lower (Table 1). The JFH-1 N3H region may be important for the infectivity of the secreted virus and/or for virus particle secretion itself. This will be determined in a future study.

Significance of JFH-1 N5BX for replication. We demonstrated the importance of both the NS5B-coding region and the 3'UTR in JFH-1 replication in the present study. There are several reports regarding CREs within the NS5B-coding region and 3'UTR of Con1 (9, 28, 52). The importance of the interaction between CREs in NS5B and the 3'UTR for replication has also been reported for the Con1 strain (9). The nucleotide sequences involved in the kissing-loop interaction were conserved between JFH-1, J6CF, and Con-1. However, mutations in other regions may affect this interaction by disrupting the RNA secondary structures. On the other hand, given that the NS5B-coding region encodes an RNA-dependent RNA polymerase, the enzymatic activities of the polymerase may differ among the tested strains. The sequence similarities of the JFH-1 and J6CF NS5B regions are 92.2% for the nucleotide sequence and 95.1% for the amino acid sequence. Out of 591 amino acids, only 29 amino acids differ, and the GDD motif that is highly conserved among RdRps is conserved. There are many reports regarding the interaction between NS5B and other viral or cellular proteins, and some of the interactions have been reported to play a role in replication (6, 10, 12, 15, 17, 27, 41–43, 45, 46). Furthermore, the importance of the membrane localization of NS5B with respect to replication has also been reported (29, 35). Mutations in J6CF NS5B may affect these roles. It is thus important to examine the RdRp activities of JFH-1 and J6CF NS5B proteins in vitro.

FIG. 5. Analysis of transient replication of genomic chimeric HCV RNA. (A) Structures of full-length chimeric HCV RNAs. Each chimeric full-length construct was prepared by the insertion of the restricted fragments as indicated. The restriction enzyme recognition sites used for the plasmid constructions are indicated. *C*, ClaI; *E*, EcoT22I; *B*, BsrGI; *S*, StuI; *X*, XbaI; wt, wild type. (B) Northern blot analysis of total RNA prepared from cells transfected with transcribed genomic HCV RNA. Numbers of synthetic JFH-1 RNA (control RNA), RNA isolated from naïve cells (Huh7), and hours after transfection (4, 10, 24, 48, and 72) are indicated. Arrowheads indicate full-length HCV RNA (HCV RNA) and 28S rRNA (28S). A representative autoradiogram (6-h exposure) of three independent experiments is presented. (C) HCV core protein secretion from the RNA-transfected cells. Transcribed wild-type or chimeric full-length HCV RNAs (10 μg) were transfected into Huh7 cells. Culture medium was harvested at 4, 10, 24, 48, and 72 h after transfection. The amounts of core proteins in the harvested culture medium were measured using an HCV core enzyme-linked immunosorbent assay. The assays were performed five times independently, and data are presented as means and standard deviations.

On the other hand, the effect of the 3′UTR is very surprising, especially since the nucleotide sequences of this region are very similar between JFH-1 and J6CF. In this study, the 3′UTR includes four parts: 22 nucleotides at the 3′-end NS5B region (as a result of the cloning strategy), 39 nucleotides of variable region, the poly(U/UC) region, and a 98-nucleotide 3′X region. There are a single synonymous nucleotide mutation in the 3′-end NS5B region and three nucleotide mutations in the variable region. The poly(U/UC) regions are 99 and 132 nucleotides in JFH-1 and J6CF, respectively. There are no mutations in the 3′X region in either strain. It is thus quite interesting to pursue the mechanisms of these mutations in the 3′UTR that affect the HCV RNA replication levels. Further studies are important for precise elucidation of the efficient replication mechanisms of JFH-1.

Significance of the JFH-1 NS3 helicase region for replication. In the present study, we demonstrated the importance of the JFH-1 NS3 helicase region, especially in full-length genomic RNA replication. It has been reported that an active NS3 helicase is required for replication of subgenomic replicons (25). The NS3 helicase domain possesses helicase activity and ATPase activity, and it has been reported that the characters of these enzymes differ among the genotypes and the strains (26). NS3 has also been reported to interact with positive- and negative-strand RNA 3′UTRs (1). One possible model of the role of NS3 in RNA replication is that NS3 helicase unwinds RNA secondary structures and/or a double-stranded RNA intermediate before RNA synthesis by NS5B (37). The sequence similarity of the NS3 helicase regions of JFH-1 and J6CF is rather high, 89.5% for the nucleotide sequence and 93.8% for the amino acid sequence, and out of 487 amino acids, only 30 amino acids differ. These mutations may affect the enzymatic activities of NS3 helicase.

Furthermore, it has been reported that NS3 can stimulate NS5B RdRp activity (38). It has also been reported that the NS3 protease domain and NS5B stimulate NS3 helicase activity (53). Taken together, these findings show that not only the enzymatic activities themselves but also the combination or interaction of the NS3 and NS5B proteins could be important. However, it is still important to examine and compare the NS3 helicase enzymatic activities in vitro of JFH-1 and other HCV strains in a further study.

Replication in vitro and in vivo. We previously reported that JFH-1 RNA could replicate efficiently in Huh7 cells. Cell-cultured JFH-1 virus was also found to be infectious in chimpanzees; however, the virus was cleared immediately after transient viremia (48). In contrast, J6CF does not replicate in Huh7 cells, but it is infectious in chimpanzees (49). J6/JFH-1 chimeric RNA replicated efficiently in Huh7 cells (39) and Huh7-derived cell lines (30), and cell-cultured chimeric J6/JFH-1 virus was infectious in chimpanzees and in chimeric uPA-SCID mice (31). Replication efficiency in vitro may not necessarily correlate with that in vivo. The H77, Con-1, and HCV-N strains were infectious in chimpanzees (3, 5, 23, 50). However, the H77 and Con-1 strains need adaptive mutations for efficient replication in cultured cells (4, 24) and HCV-N replicates relatively efficiently in cultured cells (16). On the other hand, H77-S containing five adaptive mutations can produce infectious virus particles (51), but the Con-1 and HCV-N strains do not produce virus particles (16, 40). It is still unclear what viral or host factors are important for efficient replication and infectious-virus production in vitro and in vivo. However, understanding HCV replication mechanisms by using cell culture models is still important for elucidation of the HCV life cycle.

Significance of the regions responsible for JFH-1 replication. Using two HCV strains, JFH-1 and J6CF, which are very closely related but have different characteristics, we were able to determine which regions are important for replication in cultured cells. Replication of two other genotype 2a strains, JCH-1 and JCH-4, was also recovered by replacement of the N3H and N5BX regions of JFH-1 at the lower levels compared to replication of the J6 replicon (Fig. 3B and 4). This may be because J6CF is an infectious clone in chimpanzees, but the JCH-1 and JCH-4 strains are clinical isolates from chronic-hepatitis patients (21) and may include critical mutations in other important regions. Furthermore, replication of genotype 1 HCV replicons was not restored by the same procedure as that for genotype 2a replicons (Fig. 4). Functional complementation in the nonstructural region and 3′UTR may be difficult beyond the genotypes.

Obtaining virus particles is an important step in antiviral research. Although infection efficiency is improved in permissive cell lines, most HCV strains still cannot replicate or produce virus particles in cultured cells. Therefore, chimeric virus particles with the JFH-1 replication engine may be suitable substitutes. Furthermore, analyses using chimeric viruses that have structural proteins and other regions from various strains may give us new information regarding strain-specific effects on HCV life cycles. Consequently, applying the findings of the present study to replication-incompetent strains may be useful not only for analyses of virus strain specificity and precise analyses of the HCV life cycle but also for antiviral studies.

In conclusion, we analyzed the mechanism underlying efficient JFH-1 replication by using intragenotypic chimeras of JFH-1 and J6CF and clearly showed the importance of the JFH-1 NS3 helicase region and the NS5B-to-3′X region for efficient replication of HCV genotype 2a strains.

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Rapid Genome Sequencing of RNA Viruses

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We developed a system for rapid determination of viral RNA sequences whereby genomic sequence is obtained from cultured virus isolates without subcloning into plasmid vectors. This method affords new opportunities to address the challenges of unknown or untypeable emerging viruses.

Over the past few years, global migration has led to emerging infectious diseases that pose substantial risks to public health. To prevent potential outbreaks, early detection of infectious pathogens is necessary. In particular, the recent outbreak of severe acute respiratory syndrome (SARS) provided important lessons on how unknown viruses should be detected rapidly. Thus, a standardized and qualified system is required for rapid nucleic acid sequence determination for newly emerging viruses.

Recently, we developed a new method for detecting RNA viruses. This method, based on cDNA representational difference analysis (cDNA RDA), uses 96 hexanucleotides that are not suitable for priming ribosomal RNAs but that normally prime most of the genome of an RNA virus as primers for reverse transcription in cDNA RDA (*1*). However, the RDA method with a cloning step requires at least 1 week for the determination of the nucleic acid sequence.

The Method

Our new system for rapid determination of viral RNA sequence (RDV) uses whole-genome amplification and direct sequencing techniques (Figure 1). The RDV method comprises 6 procedures: 1) effective destruction of cellular RNA and DNA for semipurification of viral particles, 2) effective elimination of DNA fragments by using a prefiltration column system and elution of small amounts of RNA, 3) effective synthesis of first- and second-strand cDNAs, 4) construction and amplification of a cDNA library, 5) construction of a second cDNA library, and 6) direct sequencing using optimized primers. The RDV method enables a broad range of partial nucleotide sequences within the entire viral RNA genome to be obtained within 2 days without cloning into plasmids.

To eliminate contaminating cellular RNA and DNA from the samples, 0.001 μg of RNase A (Qiagen, Hilden, Germany) and 1 μL (2 U) of Turbo DNA-free DNase I (Ambion, Austin, TX, USA) with 1× Turbo DNA-free buffer were incubated at 37°C for 30 min under conditions that prevented destruction of viral RNA in the viral particles. The RNA in the viral particles was then extracted within 30 min by using a total RNA isolation mini kit (Agilent Technologies Inc., Palo Alto, CA, USA). We confirmed that DNA was effectively eliminated by this RNA extraction kit.

In accordance with the Invitrogen manual, cDNA was synthesized, by using random hexamers (Takara Bio Inc., Kyoto, Japan) and Superscript III (Invitrogen, Carlsbad, CA, USA) lacking RNase H activity, at 50°C for 1 h. Then 60 U of RNase H (Takara Bio Inc.) added before synthesis of second-strand cDNA at 50°C for 1 h. In accordance with the manual, a whole genome amplification system (WGA; Sigma-Aldrich, Saint Louis, MO, USA), which was developed for amplification of genomic DNA, was used to amplify viral double-stranded cDNA. This process was

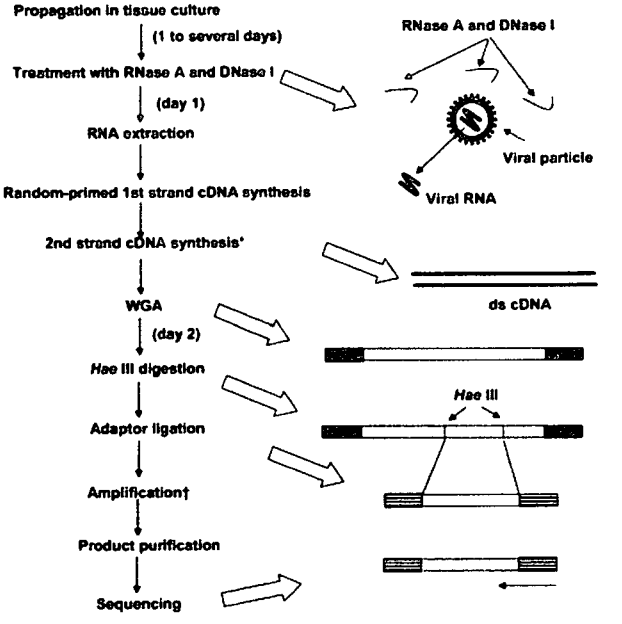

Figure 1. Overall scheme of the rapid determination of viral RNA sequence method. *By adding RNase H; WGA, whole genome amplification; †With specially designed primer sets as shown in Figure 2.

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performed within 90 min. Instead of the Taq polymerase recommended in the kit, we used 1.25 U of AmpliTaq Gold LD (Applied Biosystems, Foster City, CA, USA) to obtain a high yield of the PCR products. Primers were provided in the WGA kit, but no information regarding their sequences was obtained. The reaction mixture was heated at 95°C for 9 min (for activation of AmpliTaq Gold), followed by 70 cycles of amplification using Mastercycler (Eppendorf AG, Hamburg, Germany). Each PCR cycle consisted of annealing at 68°C for 1 min, primer extension at 72°C for 5 min, and denaturation at 94°C for 1 min.

The 1st cDNA library was digested with 40 U of *Hae*III (Takara Bio Inc.) at 37°C for 30 min. DNA was purified by using the MonoFas DNA isolation system (GL Science, Tokyo, Japan), and a blunt *EcoR*I-*Not*I-*Bam*HI adaptor (10 pmol; Takara Bio Inc.) was ligated at 16°C for 30 min by using DNA Ligation Kit, Mighty Mix (Takara Bio Inc.). The second cDNA library was amplified by PCR with specially designed primer sets in which 6 nucleotides composed of CC (*Hae*III-digested sequence) and 4 variable nucleotides were added to the 3′ end of the adaptor sequence (Figure 2). For example, 1 primer set was as follows: forward primer, H1–1: 5′-AATTCGGCGGCCGCG GATCC*CC*GGGG-3′; reverse primer H9–3: 5′-AATT CGGCGGCCGCGGATCC*CC*AGGA-3′ (the adaptor sequence is underlined, and the *Hae*III-digested sequence is shown in italics) (Figure 2).

We always used >12 primer sets and 0.83 μmol of each primer per cDNA library. PCR was performed with AmpliTaq Gold Master Mix (Applied Biosystems). The reaction mixture was heated at 95°C for 12 min, followed by 70 cycles of amplification. Each PCR cycle consisted of annealing and primer extension at 72°C for 30 s and denaturation at 94°C for 30 s. A single band was consistently obtained in ≈50% of the reactions. DNA was purified from the PCR by using MonoFas. Occasionally, we purified DNA fragments from the gels when >2 bands were detected. Direct sequencing was performed with the forward primer, reverse primer, or both.

When the number of viral particles in the sample was high, we omitted the RNase A and DNase I treatments and used the RNeasy Mini Kit (Qiagen) for RNA extraction. We occasionally used a whole transcriptome amplification kit (Rubicon Genomics Inc, Ann Arbor, MI, USA) instead of the WGA kit because both kits yielded similar amplification results.

In preliminary studies that used referential RNA viruses, we attempted to determine the nucleic acid sequences of SARS coronavirus, mouse hepatitis virus, West Nile virus, Japanese encephalitis virus, and dengue virus type 2 in culture supernatants (10–100 μL) by using the RDV method. The percentages of positive fragments (number of fragments containing viral nucleic acid/total number of sequenced fragments) in the reactions for detection of these 5 viruses were 60% (3/5), 45% (5/11), 100% (12/12), 50% (5/10), and 40% (4/10), respectively. As a clinical application, a throat swab specimen from a patient with fever and upper respiratory infection was characterized. Although the specimen exhibited enterovirus-like cytopathic effect by inoculation into HEF and GMK cells when cell culture system for virus isolation was used (*2*), extracted RNA from the supernatant of the cells showed no amplification by reverse transcription–PCR (RT-PCR) when 1 of the conventional primer sets for human enteroviruses was used (*3*,*4*). In the cell culture supernatant analysis by the RDV method, the specimen exhibited amplification of the partial nucleotide sequences of coxsackie A14 virus (nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under accession nos. AB275848–AB275853). Thus, the RDV method could detect unidentified cytopathic-effect agents such as enterovirus that could not be detected by RT-PCR when the conventional primer set for enteroviruses was used.

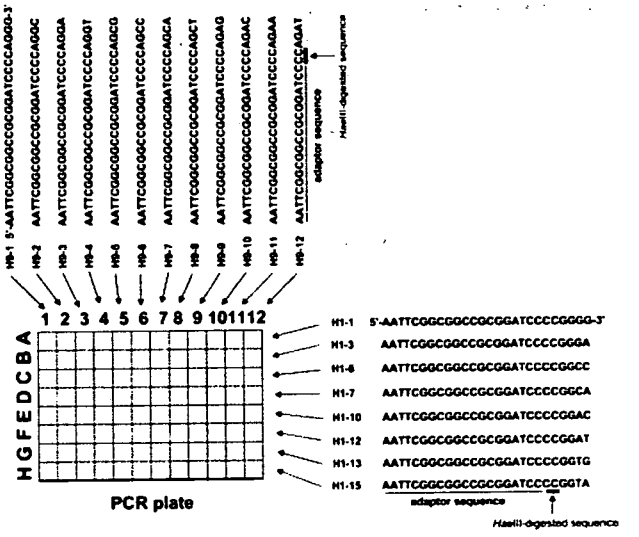

Figure 2. Primers used in rapid determination of viral RNA sequence method.

Conclusions

The RDV method is a rapid method for the direct determination of viral RNA sequences without using the cDNA cloning procedure. The limitations of the RDV method are the requirement for cell culture isolate and the large number of steps. However, RDV would be useful for species-independent detection of RNA viruses including unknown or untypeable emerging RNA viruses. Furthermore, with minor modifications, this method would also be applicable to the detection of DNA viruses and bacteria.

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Original article

GBV-B as a pleiotropic virus: distribution of GBV-B in extrahepatic tissues *in vivo*

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Abstract

GB virus B (GBV-B) infection of New World monkeys is considered to be a useful surrogate model for hepatitis C virus (HCV) infection. GBV-B replicates in the liver and induces acute resolving hepatitis but little is known whether the other organs could be permissive for the virus. We investigated the viral tropism of GBV-B in tamarins in the acute stage of viral infection and found that the viral genomic RNA could be detected in a variety of tissues. Notably, a GBV-B-infected tamarin with marked acute viremia scarcely showed a sign of hepatitis, due to preferential infection in lymphoid tissues such as lymph nodes and spleen. These results indicate that GBV-B as well as HCV is a pleiotropic virus *in vivo*.

Keywords: GB virus B; Hepatitis C virus; Tamarin; Surrogate model

1. Introduction

Over 100 million people worldwide are carriers of hepatitis C virus (HCV) and the viral infection is a significant cause of human morbidity and mortality; chronic HCV infection in many cases will lead to liver cirrhosis and hepatocellular carcinoma. Furthermore, HCV infection manifests a variety of extrahepatic, at least in part due to the extrahepatic tropisms of HCV, particularly lymphotropism diseases (for review see [1]).

Other than humans, only chimpanzees that are endangered as species can be productively infected by HCV. Together with ethical issues regarding animal experiments, it has becomes increasingly difficult to access chimpanzees for experimental studies. Tamarins (*Saguinus* species), one of the new world monkeys, develop acute, self-limited hepatitis upon infection with the GB virus B (GBV-B), which is most closely related to HCV [2–4]. Although the acute nature of GBV-B infection in tamarins has been distinguished this hepatitis from HCV infection in humans, recent studies demonstrated that tamarins could be persistently infected by GBV-B and developed chronic hepatitis [5,6]. Therefore, the GBV-B infection of tamarins is proposed as a good surrogate model for hepatitis C. While GBV-B appeared to infect liver, comprehensive documentation of the *in vivo* tropism of GBV-B has not been

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reported yet. A previous report that GBV-B RNA was observed in peripheral blood mononuclear cells (PBMCs) from a GBV-B-infected marmoset [7] suggests that GBV-B may be lymphotropic as well as HCV. Considering the close similarity between HCV and GBV-B, we examined the viral distribution and tropism in tamarins in the acute phase of the viral infection.

2. Materials and methods

2.1. Animals

Adult white-lipped and Red-handed tamarins (*Saguinus labiatus* and *Saguinus midas*, respectively) were housed in individual cages at the Tsukuba Primate Research Center. All animal studies were conducted in accordance with the protocols of experimental procedures that were approved by the Animal Welfare and Animal Care Committees of the National Institute of Biomedical Innovation and National Institute of Infectious Diseases. The details of tamarins used in this study were summarized in Table 1.

2.2. GBV-B infection in tamarins

GBV-B RNA was transcribed *in vitro* with T7 RNA polymerase (Promega, Madison, WI) from 10 μg of *Xho*I-digested pGBB [2] that harbors infectious cDNA for GBV-B (kind gift of Dr. J. Bukh, National Institutes of Health, USA). The integrity of the RNA was checked by electrophoresis through an agarose gel stained with ethidium bromide. Each transcription mixture (400 μg of GBV-B RNA) was diluted with 400 μl of ice-cold water and then immediately frozen on dry ice and stored at −80 °C. Transcription mixtures were injected into each tamarin intrahepatically. For transmission of GBV-B, animals were infected intrahepatically with 100 μl of GBV-B infectious plasma containing 8×10^8 genome equivalents (GE) of the viral RNA. Blood samples were periodically collected from the monkeys from femoral vein under anesthetization and were tested for plasma ALT level.

2.3. Quantification of GBV-B genomic RNA

GBV-B-infected tamarins were euthanized and perfused with saline thoroughly before the collection of specimens including plasma, PBMCs and a variety of tissues (esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver, pancreas, submandibular gland, trachea, lung, bone marrow, thymus, spleen, submandibular lymph nodes, axillary lymph nodes, intestinal lymph nodes, mesenteric lymph nodes, inguinal lymph nodes, tonsil, heart, kidney, adrenal gland, bladder, brain, spinal cord, testis, uterus and ovary). GBV-B RNA from these specimens was quantified by a real-time, 5′ exonuclease PCR (TaqMan) assay using a primer-probe combination that recognized a portion of the GBV-B capsid gene. The primers 558F [5′AACGAGCAAAGCGCAAAGTC] and 626R [5′CATCATGGATACCAGCAATTTTGT] and probe 579P [5′6FAM-AGCGCGATGCTCGGCCTCGTATAMRA] [8] were obtained from PE Biosystems. The primers were used at 15 pmol/50 μl reaction, and the probe was used at 10 pmol/50 μl reaction. Synthesized GBV-B RNA was used as a reference standard of GBV-positive plasma. PBMCs were isolated from whole blood by density-gradient centrifugation. Approximately 10 mg of tissues were removed under sterile conditions and immediately homogenized in 1 ml of TRIzol (Invitrogen, Carlsbad, CA) to extract RNA. We set our lowest detection cutoff at 10^2 GE per ml. All the specimens were evaluated in duplicates and the averages were shown.

Table 1
Summary of the results of GBV-B RNA levels in the tissues of the virus-infected tamarins

		Tm3	Tm4	Tm5	Tm6
Animals		*S. labiatus*	*S. midas*	*S. labiatus*	*S. midas*
Sex		Female	Female	Male	Female
GBV-B inoculum		Plasma	Plasma	RNA	RNA
Weeks at necropsy		4	4	3	ND[a]
ALT		321	522	38	554
Viral loads in:					
Blood	Plasma	3.8×10^8	5.9×10^8	1.3×10^{10}	2.8×10^9
	PBMC	270	1630	35650	ND
Spleen		(−)[b]	540	5980	ND
Lymph nodes	Inguinal	(−)	(−)	3090	ND
	Intestinal	(−)	(−)	640	ND
Liver		70080	33480	16080	ND
Kidney		(−)	(−)	380	ND
Testis				600	ND
Ovary		1290	150		ND
Bone marrow		120	(−)	750	ND

Viral loads in each tissues were presented as GE/mg except for plasma (GE/ml) and PBMC (GE/10^6 cells). Data for Tm6 were obtained at week 4.
[a] ND: not done.
[b] (−): undetectable.

2.4. Detection of anti-GBV-B core and NS3 antibodies by ELISA

The TrpE-core (aa 1 to 132) fusion protein and TrpE-NS3 (aa 1135 to 1378) fusion protein, representing a portion of NS3 identified as being immunogenic in infected animals [9], was expressed in *Escherichia coli* [10] to serve as an antigen to generate polyclonal rabbit antisera. Tamarin sera were tested for the presence of antibodies to GBV-B core and NS3 by ELISA as described previously [8].

2.5. Cloning of entire GBV-B genome from plasma, liver and PBMCs of infected tamarins

GBV-B RNA was isolated from plasma, liver and PBMCs as described above. GBV-B cDNA was synthesized using SuperScript reverse transcriptase II (Invitrogen) with GB-5145R primer (5′-GCG AGT GCG GCT GTC CCA GAA GTA TTG ACT-3′) or GB-9051R primer (5′-AAT TTG GGG GTT CAG CTG ATG GCT AAT CCA-3′). After RNase H (Invitrogen) treatment at 42 °C, a cDNA mixture was subjected to PCR with LA-taq DNA polymerase (TaKaRa), GB-5145R primer and GB-35S primer (5′-ACC ACA AAC ACT CCA GTT TGT TAC ACT CCG CTA GG-3′) or GB-9051R primer and GB-3999S primer (5′-CGT ACG GCG TGA ATC CAA ATT GCT ATT TTA-3′) for 30 cycles of denaturation at 94 °C for 20 s and extension at 68 °C for 5 min. PCR products were purified from the gel using a QIA-quick gel kit (Qiagen), and then cloned into pGEM-T Easy vector (Promega). Four clones of each fragment were determined using a CEQ-2000XL analysis system with a DTCS quick start kit and GBV-B specific primers according to the manufacturer's instructions. Sequence data were analyzed on Macintosh computers with the Sequencer (Gene Code Corp.) and MacVector (Accelrys) software packages.

2.6. Synthesis of positive and negative standard RNAs for RT–PCR controls

Recombinant positive and negative strand RNAs were generated from pGBB containing 3′ sequences of GBV-B. Positions 8569–9359 were amplified and inserted into pGEM-T easy vector. Clones were selected for sense and antisense orientation of the insert corresponding to positive and negative strands, respectively. Ten micrograms of the selected plasmids were linearized using *PstI* and positive- and negative-strand RNAs were synthesized by transcription from the upstream T7 RNA polymerase promoter by Ambion MEGAscript T7 kit (Ambion, Austin, TX).

2.7. Detection of strand-specific viral RNA by tagging PCR system

One microgram of total RNA obtained from tissues or cells was subjected to RT–PCR. cDNAs were synthesized using Superscript III first strand synthesis system (Invitrogen). In order to overcome the detection of falsely primed cDNA products and make the PCR system strand-specific, additional nucleotides (TCATGGTGGCGAATAA) were added to the 5′ end of the reverse transcription primer (5′-TCATGGTGGC GAATAATTGGATTAGCCATCAGCTGAACC-3′), forming a "tag" (underlined) [11,12]. This "tag" sequence was neither complementary nor homologous to any part of the GBV-B genome. PCR amplification of a tagged cDNA was performed using only the tag portion of the cDNA primer (5′-TCATGG TGGCGAATAA-3′) as one of the primers and a GBV-B specific oligonucleotide for the opposing primer (5′-CTTGGTAC TACGCTCTGCACA-3′, positions 9339–9359). For the first round of PCR using 2 μl of cDNA in a final volume of 25 μl, the reactions were performed using a TaKaRa PCR kit (TaKaRa) with following conditions; a 20 s and 94 °C denaturation step followed by 20 s and 55 °C annealing and 2 min and 72 °C extension steps. After 30 cycles of first round amplification, 2 μl of reaction samples were subjected to 30 cycles of nested PCR using 5′-TTTTAGGGCAGCGGCAACAG-3′ (positions 9105–9124) and 5′-CACACAGCCAGGACTCCTCA-3′ (positions 9260–9279) as primers.

2.8. Histopathology

Five tamarin livers were used in this study. Of these, three livers were from GBV-B-infected tamarins (Table 1), and two were from uninfected tamarins. Liver samples obtained by necropsy were fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 4 μm thick-sections. Deparaffinized sections were stained with hematoxylin and eosin (H&E) for histopathological analyses. To investigate apoptotic cells in the livers, we also examined both DNA fragmentation and immunohistochemistry for an active form of caspase-3. To diminish autofluorescence mainly caused by lipofuscin, sections were pre-stained with 1% Sudan black B. DNA fragmentation was evaluated by a TUNEL assay with an ApopTag Direct *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA) according to the manufacturer's instructions. Briefly, the specimens were digested with a solution of proteinase K (20 μg/ml) in PBS for 5 min and then incubated with terminal deoxynucleotidyl transferase (TdT) and fluorescein-labeled nucleotides (ApopTag Direct) in a humid atmosphere at 37 °C for 1 h. Specimens were viewed with a BX-FLA fluorescence microscope (Olympus, Tokyo, Japan). To control for nonspecific incorporation of nucleotides and nonspecific binding of TdT, cells were treated with proteinase K as usual, but staining was performed in the absence of active TdT. This served as a negative control. In parallel, immunohistochemistry for an active form of caspase-3 was examined by using an FITC-conjugated monoclonal antibody against the active caspase-3 (C92-605; BD Pharmingen, San Jose, CA) in order to confirm the degree of apoptotic cells detected by TUNEL staining. Sections were deparaffinized followed by autoclaving for 5 min at 121 °C, and then incubated free floating in the primary antibody solution overnight at 4 °C. Following brief washes, sections were then incubated with DAPI (1:800; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. These sections were examined with a Digital Eclipse C1 confocal microscope (Nikon, Japan).

3. Results

3.1. GBV-B infection in tamarins

Firstly, two tamarins were intrahepatically inoculated with RNA transcripts from GBV-B infectious molecular clone pGBB (Fig. 1). Both monkeys showed viremia at 2 weeks post inoculation; peak viral titers in plasma reached up to 10^9 GE/ml and both monkeys developed hepatitis with dramatically elevated plasma ALT levels. The viremia was maintained up to 8 weeks, followed by rapid decline in parallel with the resolution of the ALT abnormalities. Within 6–8 weeks of the inoculation, the development of antibodies reactive with the viral core and NS3 proteins was observed (Fig. 1). Multiple plasma samples collected at later time points contained no detectable viral RNA and showed no ALT abnormalities; however, antibodies against GBV-B core and NS3 proteins were maintained at relatively high levels at least until 28 weeks after inoculation (Fig. 1). These results confirmed that inoculation of GBV-B viral RNA caused acute hepatitis in parallel with typical viremia in tamarins.

Next, in order to examine the tissue tropism of GBV-B *in vivo*, four tamarins were inoculated intrahepatically with week 2 plasma of tamarin Tm1 containing 8×10^8 GE of GBV-B (Tm3 and Tm4) or synthetic GBV-B RNA as described above (Tm5 and Tm6). These tamarins developed a typical acute infection that were marked by high levels of viremia, indicating that inoculation of either viral RNA or plasma of the infected tamarin resulted in comparable outcome (Fig. 2). It is noteworthy that in Tm5 the plasma ALT level was scarcely elevated in contrast with other three tamarins during the acute period of GBV-B infection, although this tamarin developed highest viremia (1.3×10^{10} GE/ml).

3.2. Histopathological analyses of GBV-B infection

Histopathological analyses in Tm3 and Tm4 livers showed inflammatory responses including inflammatory cell invasions around central and/or portal veins and hemorrhages, hepatocytic degenerations, and disruptions of sinusoids (Fig. 3A,B,E,F). Although there were only minimal pathological changes, hepatocytic degenerations and dilation of sinusoids were also found in the Tm5 liver (Fig. 3C and G) in contrast to uninfected tamarins (Fig. 3D and H, data not shown). To further evaluate the levels of apoptotic hepatocytes in these monkeys, we employed two different methods, detecting fragmented DNA (TUNEL assay) and an active form of caspase-3 as previously described [13]. It was found that substantial numbers of fragmented DNA-positive cells were observed in the Tm3 and Tm4 livers while much less in the Tm5 liver (Fig. 3I–K). Consistent results were obtained when the active form of caspase-3 was stained (Fig. 3M–O). On the other hand, we found neither DNA fragmentation nor caspase-3 activation in uninfected tamarin livers (Fig. 3L and P, data not shown). The minimal levels of pathological changes in the Tm5 liver were well correlated with a lower level of plasma ALT in Tm5 (Fig. 2, Table 1).

3.3. Tissue distribution of GBV-B

The results described above suggested the possibility that the substantial levels of viral replication occurred in other tissues rather than in the liver of Tm5. To ascertain the possibility, we euthanized three tamarins (Tm3, Tm4 and Tm5) and the viral levels in a variety of tissues were compared. Table 1 summarizes the data obtained in this experiment. It is reasonable to consider that GBV-B replicated in the liver accounts for majority of the viral load *in vivo*. However,

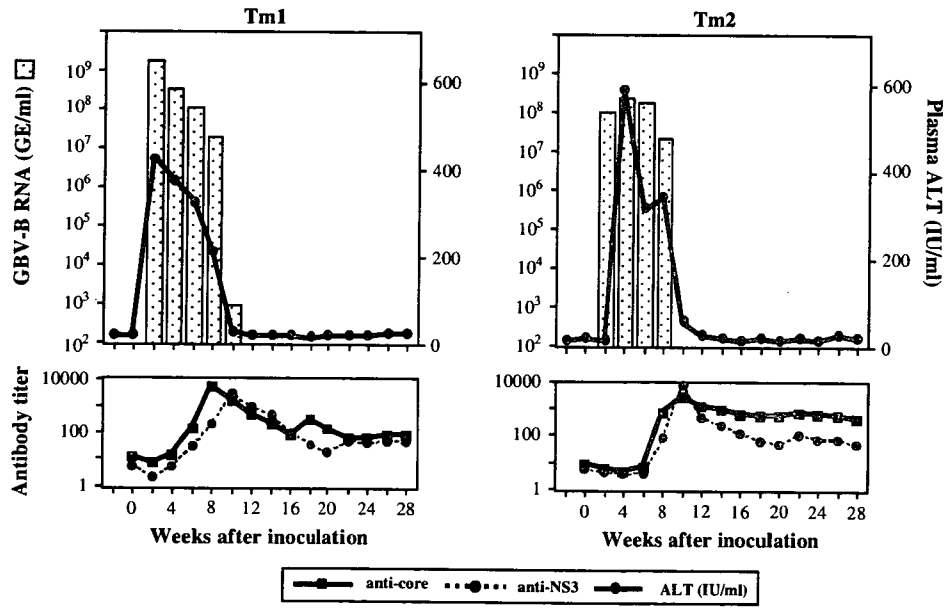

Fig. 1. Course of GBV-B infection in tamarins Tm1 and Tm2. Synthesized infectious RNA transcript of GBV-B from a pGBB molecular clone was inoculated into each tamarin intrahepatically. Plasma samples were collected from each tamarin at 2-week intervals post inoculation. The viral RNA copies, ALT levels, and titers of anti-viral antibodies (anti-core and anti-NS3) in the plasma samples until 28 weeks after inoculation were shown.

substantial levels of GBV-B RNA were detected not only in the liver but also in a variety of extrahepatic tissues such as hematolymphoid and genital tissues, suggesting that GBV-B may infect and replicate in these organs. Notably, the viral RNA levels of Tm5 were much greater in the lymphoid tissues but lower in the liver as compared with those of other two tamarins, indicating that the highest plasma viral load in Tm5 derived from extrahepatic tissues, mainly hematolymphoid tissues. We could not detect GBV-B RNA from other tissues tested (data not shown). From these results, we concluded that the preferential distribution of GBV-B in the extrahepatic tissues rather than in the liver of Tm5 may attribute to the highest plasma viral load in spite of the mild disorder and the lower viral load in the liver.

In addition, the unique viral distribution implied that the GBV-B disseminated in Tm5 might acquire novel tissue tropism as a result of genomic mutation. To ascertain the possibility, we amplified the entire viral genomes by RT–PCR from the liver, PBMCs and plasma collected from Tm5 at euthanasia and compared with the original nucleotide sequence. The sequences determined were completely identical to the original sequence of GBV-B (data not shown), indicating that the sequence heterogeneity of GBV-B was not responsible for the different tropism observed in Tm5 and thus GBV-B intrinsically exhibits pleiotropism in a host-dependent manner.

3.4. Detection of strand-specific viral RNA in the tamarin tissues

To confirm that the virus was actually replicated in the tissues other than the liver, we sought to deferentially determine negative-strand viral RNA which is shown to be a viral replication intermediate in case of HCV. We thus newly developed an assay system for detecting replication intermediate of GBV-B.

To determine the sensitivity of this method, synthetic positive- and negative-strand GBV-B transcripts (ranging from 10^8 to 10^0 copies of GBV-B) in 100-fold serial dilutions were subjected to RT–PCR. As shown in Fig. 4A, at least 100 copies of GBV-B negative-strand RNA could be detected by this method. When the primer for cDNA synthesis was omitted, no PCR products were obtained (Fig. 4A, negative control), indicating that the PCR signals were derived specifically from the GBV-B negative-strand RNA. In the presence of 10^8 copies of positive-strand HCV RNA, false positive PCR signals appeared (Fig. 4A). We then analyzed the samples from liver, spleen, pancreas, stomach and PBMCs from Tm5 using the GBV-B strand-specific PCR assay and found that the negative-strand viral RNAs were detected in the liver, spleen and PBMC samples (Fig. 4B). No negative-strand or replicating forms of the virus were detected from RNA extracted from pancreas, stomach and HeLa cells.

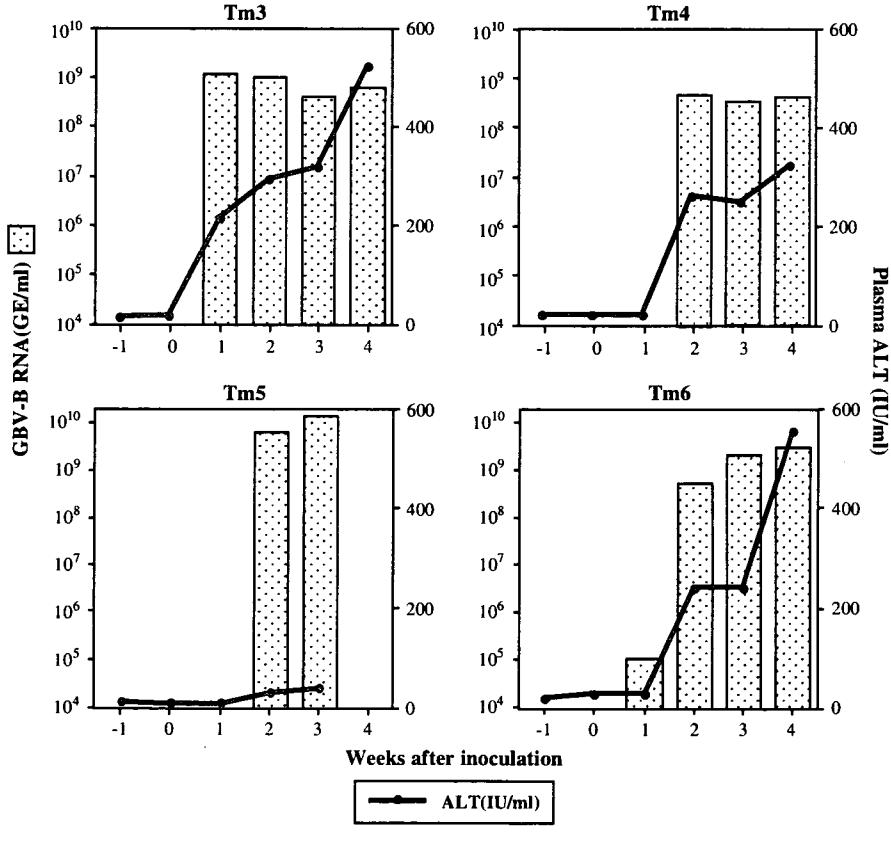

Fig. 2. Acute course of GBV-B infection in tamarins (Tm3 and Tm4) by *in vivo* passage of plasma (7.9×10^8 GE/head) obtained from the GBV-B RNA-inoculated Tm1 in comparison with GBV-B RNA transcript-inoculated tamarins (Tm5 and Tm6). The viral RNA copies and ALT levels in the plasma samples collected from each tamarin were indicated.

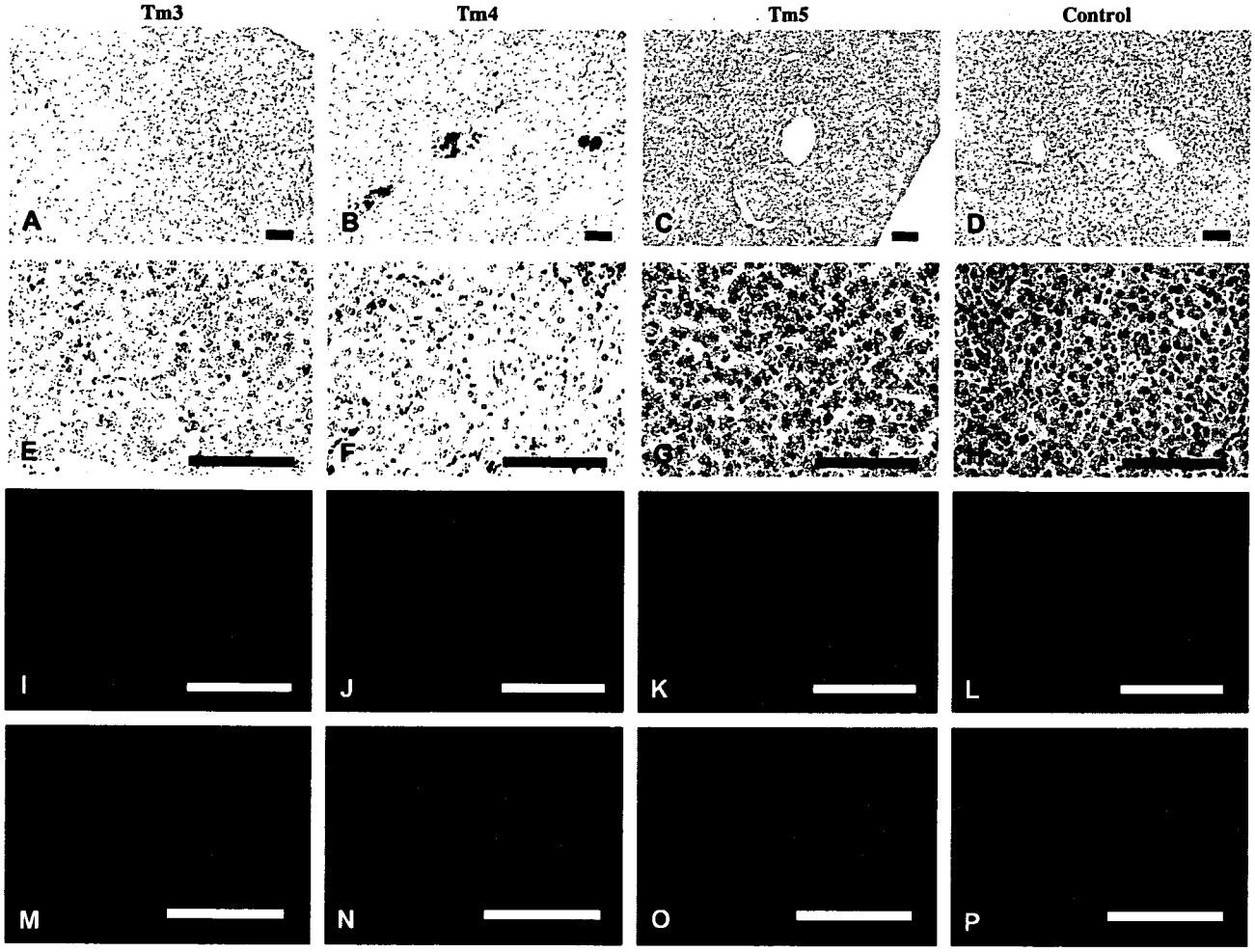

Fig. 3. Photomicrographs of liver sections from Tm3 (A, E, I, M), Tm4 (B, F, J, N), Tm5 (C, G, K, O), and an uninfected tamarin (D, H, L, P). A–H show sections with H&E staining, while I–L and M–P indicate sections with a TUNEL assay and immunohistochemistry for an active form of caspase-3, respectively. Sections immunostained for an active form of caspase-3 (green fluorescent) were counterstained with DAPI (blue fluorescent). Scale bars: 100 μm.

4. Discussion

GBV-B is most closely related to HCV and induces acute resolving hepatitis in tamarins. It is therefore reasonable that GBV-B has been considered to be a hepatotropic virus; in this study, however, we show for the first time that GBV-B is a pleiotropic virus and can disseminate to not only liver but also a variety of extrahepatic tissues such as hematolymphoid and genital tissues. Of note, there is ample evidence that persistent HCV infection manifests a variety of extrahepatic diseases, at least in part due to the extrahepatic tropisms of HCV (for review see [1]). This also suggests that extrahepatic tissues may serve as alternative reservoirs for HCV, while further analyses should still be required to understand the viral dynamics *in vivo*. Considering the similar pleiotropism of HCV and GBV-B, our results support and extend the usefulness of New World primates infected with GBV-B as a surrogate model for the study of pathogenesis and tropism of HCV infection.

Tamarins infected with GBV-B generally develop semi-acute viremia, of which peak levels regularly ranged from 10^7 to 10^9 GE/ml on the basis of previous reports [2,5,6, 14,15]. From this point of view, the peal viremia (1.3×10^{10} GE/ml) in Tm5 euthanized at the acute phase of the viral infection appeared to be much greater than other cases. It seems likely that in Tm5 the lymphoid tissues but not liver were responsible for the highly efficient viral production, because (i)

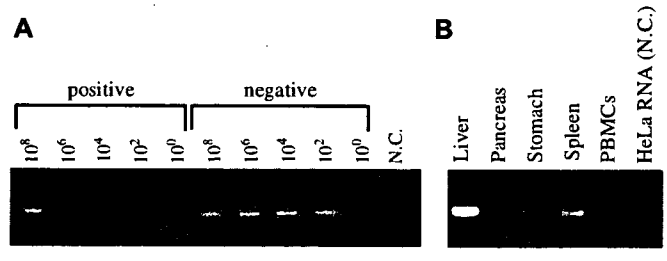

Fig. 4. (A) Titration of synthetic GBV-B RNA transcripts. Synthetic RNA transcripts corresponding to the positive- and negative-strands of part of the GBV-B were serially diluted and each transcript was subjected to amplification using strand-specific RT–PCR to determine the specificity and sensitivity of the assays. (B) Detection of negative-strand GBV-B RNA from various tissues. One microgram of total RNA obtained from tissues or cells was subjected to RT–PCR.

viral titer in the liver was lowest among three monkeys, which was consistent with minimal plasma ALT level and liver damage; (ii) yet, Tm5 exhibited highest viremia levels, (iii) the viral RNA levels in PBMCs, spleen and inguinal and intestinal lymph nodes of Tm5 were much greater than others, and (iv) we could detect negative-strand GBV-B RNA from not only liver but also spleen and PBMCs. Supposing that the entire virus in Tm3 plasma (3.8×10^8 GE/ml) was produced in the liver of which RNA titer was highest among tamarins, most of the virus in Tm5 plasma (1.3×10^{10} GE/ml) should be derived from extrahepatic tissues. Taken together, our data demonstrate preferential dissemination of GBV-B in extrahepatic tissues. In order to further define the cell type(s) in which GBV-B replicates efficiently, *in situ* histological analysis should be needed as indicated in the case of HCV [16].

It was possible that differential lymphotropism among GBV-B-infected tamarins could be due to adaptive mutation in the viral genome. From this point of view, we cloned the viral RNA obtained from plasma and liver; however, we did not find any sequence heterogeneity in the viral genome (data not shown). Furthermore, challenge of Tm5 plasma to naïve tamarins developed typical semi-acute hepatitis with regular viremia and did not reproduce the preferential lymphotropism (data not shown). These results indicate that GBV-B intrinsically has pleiotropism in a host-dependent manner. It is possible that multiple surface molecules in the host cells, which act as alternative receptors, would determine the pleiotropism of GBV-B. It remains to be investigated whether host molecules which are used as receptors for HCV [17] would also be used by GBV-B.

Histopathological studies showed the inflammatory responses in Tm3 and Tm4 livers; especially, the Tm4 liver developed strong degenerative changes, which was consistent with high ALT levels (Fig. 2G,H). Furthermore, the livers of Tm3 and Tm4 showed substantial proportions of apoptotic cells as revealed by greater signals of DNA fragmentation and caspase-3 activation, both of which were popular markers of apoptosis, than those in Tm5 (Fig. 3). It needs to be clarified whether such cytopathic effects could be directly induced by GBV-B infection into hepatocytes or whether effector cytotoxic T lymphocytes would be responsible for the cytopathicity.

Acknowledgments

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Hepatitis C viral life cycle ☆

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Abstract

Hepatitis C virus (HCV) has been recognized as a major cause of chronic liver diseases worldwide. Molecular studies of the virus became possible with the successful cloning of its genome in 1989. Although much work remains to be done regarding early and late stages of the HCV life cycle, significant progress has been made with respect to the molecular biology of HCV, especially the viral protein processing and the genome replication. This review summarizes our current understanding of genomic organization of HCV, features of the viral protein characteristics, and the viral life cycle.

Keywords: Hepatitis C virus; Translation; Polyprotein processing; RNA replication; Viral assembly

Contents

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1. Introduction

Since its discovery in 1989, representing a turning-point in the search for infectious agents associated with post-transfusion non-A, non-B hepatitis, hepatitis C virus (HCV) has been recognized as a major cause of chronic liver disease and affects approximately 200 million people worldwide at the present time [1–3]. Persistent infection with HCV is associated with the development of chronic hepatitis, hepatic steatosis, cirrhosis, and hepatocellular carcinoma [3–8]. In general, people with chronic hepatitis C are relatively asymptomatic and have few, if any, clinical manifestations prior to the development of cirrhosis.

HCV is a small, enveloped RNA virus belonging to the *Hepacivirus* genus of the *Flaviviridae* family, which also includes several classical flaviviruses, including dengue virus and yellow fever virus, as well as pestiviruses, such as bovine viral diarrhea virus and the unassigned GB viruses [9,10]. This review summarizes our current understanding of genomic organization of HCV, as well as features of the viral protein characteristics, and the viral life cycle.

2. Genomic organization

The HCV genome consists of a single-stranded positive-sense RNA of approximately 9.6 kb, which contains an open reading frame (ORF) encoding a polyprotein precursor of approximately 3000 residues flanked by untranslated regions (UTRs) at both ends [11]. The precursor is cleaved into at least 10 different proteins: the structural proteins Core, E1, E2 and p7, as well as the non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B (Fig. 1).

An important feature of the HCV genome is its high degree of genetic variability [12,13]. Mutation rates, however, vary in different regions. The E1 and E2 regions are the most variable, while the 5′UTR and terminal segment of the 3′UTR have the highest degree of sequence conservation among various isolates. The 5′ UTR, which is ~341 nucleotide (nt) in length, contains an internal ribosomal entry site (IRES), which is essential for cap-independent translation of viral RNA, from which four highly structured domains (domains I-IV) are produced (Fig. 1) [14–19]. These are largely conserved among HCV and related viruses [15,16]. As with other RNA viruses with IRES-mediated expression, the HCV 5′NTR is thought to contain determinants for translation, as well as cis-acting elements for RNA replication. It has been shown that (i) the sequence upstream of the IRES is essential for viral RNA replication, (ii) sequences within the IRES are required for high-level HCV replication, and (iii) the stem–loop domain II of the IRES is crucial for replication [20]. A recent study has revealed that the 5′UTR is capable of binding to a liver-specific microRNA, miR-122, resulting in enhanced HCV RNA replication [21]. (Fig. 2).

The 3′UTR varies between 200 and 235 nt in length, including a short variable region, a poly(U/UC) tract with an average length of 80 nt, and a virtually invariant 98-nt X-tail region [22–24] The X region forms three stable stem–loop structures that are highly conserved among all genotypes and, as a result, the HCV genome likely ends with a double-strand stem structure. It appears that the 3′X region, as well as the 52 nt upstream of the poly(U/C) tract, are crucial for RNA replication, while the remainder of the 3′UTR plays a role in enhancement of replication [25,26].

To date, hepaciviruses are divided into six principal genotypes of HCV that differ in their nucleotide sequences by 31–34%, and in their amino acid sequences by ~30%. HCV, like many other RNA viruses, circulates in infected individuals as a population of diverse but closely related variants referred to as quasispecies [12]. HCV heterogeneity is primarily due to a high error rate of the RNA-dependent RNA polymerase encoded by the NS5B gene. The existence of different quasispecies of the HCV genome appears to contribute to viral persistence. It has been shown that patients with chronic hepatitis C have greater genetic complexity in terms of the population of quasispecies they possess than patients with spontaneous clearance [13]. During the course of chronic infection, random genetic drift steadily induces the development of quasispecies primarily due to changes in the

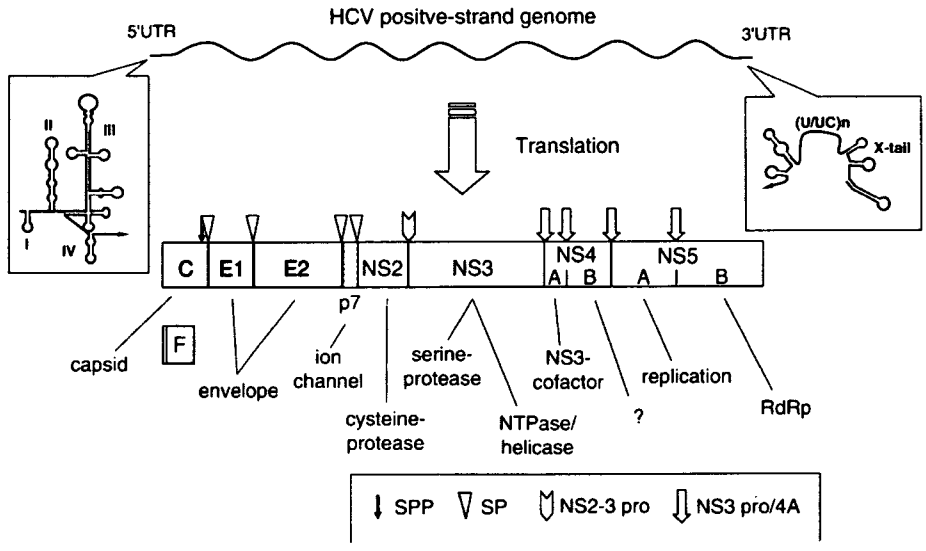

Fig. 1.

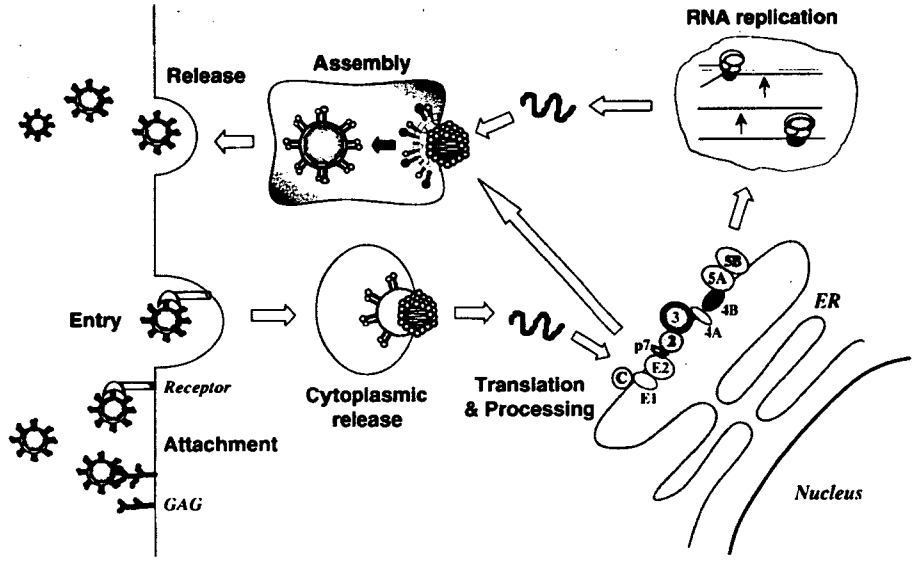

Fig. 2.

hypervariable region 1, involving the 27 N-terminal 27 residues of the E2 envelope protein [27–29].

3. Features of the viral proteins

3.1. Core protein

The HCV core protein, which is derived from the N-terminus of the polyprotein, most likely forms the viral nucleocapsid given similarities between its position and that of sequences encoding viral nucleocapsids in other flavivirus genomes. The amino acid sequence of the core protein is highly conserved among different HCV strains, compared with other HCV proteins. HCV core protein has been extensively used in a number of serologic assays since anti-core antibodies are highly prevalent among HCV-infected individuals. Although several core proteins of varying molecular weights have been identified [30–33], the core protein is released as a 191-residue precursor of 23 kDa and further processing yields the predominant form of 21 kDa. The N-terminal domain of the core protein is highly basic, while its C-terminus is hydrophobic. Several groups have reported a complex intracellular localization of the core protein [30,33–42]. The core protein is primarily detected in the cytoplasm, in association with the endoplasmic reticulum (ER), lipid droplets, and mitochondria. In some studies, a fraction of the core protein has also been found in the nucleus.

The ubiquitin–proteasome pathway, a major route by which selective protein degradation occurs in eukaryotic cells, is involved in post-translational modification of the core protein [32,43–45]. An initial report indicated that processing at the carboxyl-terminal hydrophobic domain of the core protein produced efficient polyubiquitylation and proteasomal degradation [32]. Recently, ubiquitin ligase E6AP has been identified as an HCV core-binding protein that enhances ubiquitylation and degradation of mature, as well as carboxyl-terminus truncated-core protein, and it has been suggested that E6AP-dependent degradation of the core protein is common to a variety of HCV isolates and plays a critical role in the HCV life cycle [45].

The core protein is likely multifunctional and essential for viral replication, maturation, and pathogenesis. It is involved not only in formation of the HCV virion, but also has a number of regulatory functions, including modulation of signaling pathways, cellular and viral gene expression, cell transformation, apoptosis, and lipid metabolism [reviewed in 46].

3.2. E1 and E2 envelope proteins

The E1 and E2 proteins are essential components of the virion envelope and are necessary for viral entry. These glycosylated proteins extend from aa 192–383 (E1) and from aa 384–746 (E2) of the polyprotein, and have molecular weights of 33–35 and 70–72 kDa, respectively [47]. Along the precursor polyprotein, it has been suggested that the C-terminal transmembrane domains of E1 and E2 form hairpin structures that pass through the membrane twice, thereby allowing processing by a signal peptide in the ER lumen [48]. Upon signal peptidase cleavage, the C-termini are thought to translocate into the cytoplasm in order to generate the type I membrane topology of mature E1 and E2. Mature E1 and E2 remain noncovalently associated, interacting in part through their C-terminal transmembrane domains, which also mediates retention of the E1–E2 complex in the ER. It has recently been demonstrated that, in addition to this conventional type I membrane topology, E1 protein also adopts a polytopic topology, in which the protein twice spans the ER membrane with an intervening cytoplasmic loop spanning aa 288–360 [49].

3.3. p7 protein

The p7 protein is a small (63 aa) hydrophobic polypeptide that adopts a double membrane-spanning topology. This protein is essential for the production of infectious virions in vivo [50] and may belong to a small protein family of viroporins, which are known to enhance membrane permeability. It has been revealed

that p7 protein forms an ion channel in artificial lipid bilayers, suggesting it may function as a viroporin [51,52].

3.4. NS2 protein

The NS2 protein is a transmembrane protein of 21–23 kDa, with 96 highly hydrophobic N-terminal residues, forming either three or four transmembrane helices that insert into the ER membrane. The C-terminal part of NS2 presumably resides in the cytoplasm enabling zinc-stimulated NS2/3 autoprotease activity together with the N-terminal domain of NS3. Efficient cleavage at the NS2/3 site requires the 130 C-terminal residues and first 180 aa of the NS3. Site-directed mutagenesis has revealed that His-952, Glu-972, and Cys-993 may comprise the active site for proteolytic activity [53,54]. Deletion of NS2 from the nonstructural polyprotein has not been observed to abolish HCV RNA replication in cell cultures, indicating that NS2 is not essential for viral RNA replication [55,56]. However, the NS2 protein is essential for completion of the viral replication cycle in vitro and in vivo [57,58]. A recent report regarding the crystal structure of the C-terminus of NS2 suggests that the cytoplasmic domain of NS2 forms a dimeric cysteine protease with two composite active sites, in which His-952 and Glu-972 comprise the active site of one monomer, and Cys-993 contributes to the active site of the other [59].

3.5. NS3–4A complex

NS3–4A is a complex bifunctional molecule essential for viral polyprotein processing and RNA replication. NS3 is a fairly hydrophobic protein of 69 kDa with a serine protease encoded by its N-terminal one-third region that non-covalently binds the NS4A cofactor, which is a 54-aa polypeptide [reviewed in 60]. The catalytic triad is formed by residues His-1083, Asp-1107 and Ser-1165 of NS3. The central portion of NS4A is important for efficient processing of the nonstructural proteins by NS3. It has been suggested that the N-terminus of NS4A might form a transmembrane helix that anchors the NS3–4A complex to the cellular membrane [61]. Crystal structural analyses of the NS3/4A complex have demonstrated structural similarities between the NS3 serine protease and trypsin, with two large domains primarily composed of six-stranded beta barrels separated by a cleft containing the active site and substrate binding pocket [62–64]. Of note, NS4A forms an integral part of this structure and interacts with the extreme N-terminal residues of NS3 to form two additional anti-parallel beta-strands. The NS3–4A complex has a shallow substrate-binding pocket, thus requiring extended interaction sites with the substrate.

The final 442 aa of the C-terminal of NS3 comprise the helicase-NTPase domain, which is a member of the superfamilily-2 DexH/D-box helicase, which unwind RNA-RNA substrates in a 3′-to -5′ direction [reviewed in 65]. This is supported by crystal structure analysis indicating the presence of NTPase domains and RNA binding within the protein [66]. During RNA replication, the NS3 helicase is believed to translocate along the nucleic acid substrate by changing its protein conformation, utilizing the energy of NTP hydrolysis [67–69]. Its helicase activity is positively modulated by the NS3 protease domain and NS4A [70].

3.6. NS4B protein

NS4B is an integral membrane protein of 27 kDa, which is predicted to contain at least four transmembrane domains and an N-terminal amphipathic helix that is responsible for membrane association [71]. NS4B has the ability to induce the formation of a specialized membrane compartment, a sort of membranous web where viral RNA replication may take place [72,73].

3.7. NS5A protein

NS5A is a membrane-anchored phosphoprotein that is observed in basally phosphorylated (56 kDa) and hyperphosphorylated (58 kDa) forms. Based on the results of a comparative sequence analysis following limited proteolysis of purified protein, NS5A is predicted to contain three domains: domain 1 (aa 1–213), domain 2 (aa 250–342) and domain 3 (aa 356–447) [74]. A recent structural study has demonstrated that domain 1 immediately follows the membrane-anchoring alpha-helix and forms a dimeric structure with an unconventional zinc-coordinating motif [75]. Thus, it may interact with viral and cellular proteins, as well as membranes and RNA.

While its function has not fully been elucidated, NS5A is believed to be important in viral replication. A large number of cell culture-adaptive mutations mapped to the NS5A have been shown to enhance RNA replication [76–78]. These adaptive mutations often affect hyperphosphorylation of NS5A, suggesting that the phosphorylation status of NS5A might influence replication efficiency. NS5A has been reported to interact with other HCV nonstructural proteins [79–81]. In addition, several cellular proteins interact with NS5A, resulting in assembly of the viral replication complex and/or regulation of RNA replication, as described bellow.

3.8. NS5B protein

NS5B is a 68-kDa protein with a conserved sequence motif characteristic of viral RNA-dependent RNA polymerase (RdRp), including a hallmark GDD motif that produces catalytic activity. NS5B is a tail-anchored protein and its C-terminal 21-aa region forms an alpha-helical transmembrane domain, which is dispensable for polymerase activity in vitro but is responsible for post-translational targeting to the cytoplasmic side of the ER [82,83]. Analysis of the crystal structure of NS5B has revealed that the HCV RdRp resembles a right hand and contains fingers, palm, and thumb subdomains, similar to other template-dependent polymerases [84–86]. Unlike the more open structures of other template-dependent DNA polymerases, such as the Klenow Fragment and the human immunodeficiency virus 1 reverse transcriptase, the HCV RdRp has a fully encircled active site through extensive interactions between the fingers and thumb subdomains, resulting in a protein that predominantly exists in a "closed" conformation. HCV RdRp also has an unusual hairpin loop that protrudes into the active site and helps position the 3′-