HEPATOLOGY

HEPATOLOGY, VOL. 66, NO. 2, 2017

Hepatitis C Virus Double-Stranded RNA Is the Predominant Form in Human Liver and in Interferon-Treated Cells

Arielle Klepper,[1] Francis J. Eng,[1] Erin H. Doyle,[1] Ahmed El-Shamy,[1] Adeeb H. Rahman,[1] M. Isabel Fiel,[1] Gonzalo Carrasco Avino,[1] Moonju Lee,[1] Fei Ye,[1] Sasan Roayaie,[1] Meena B. Bansal,[1] Margaret R. MacDonald,[2] Thomas D. Schiano,[1] and Andrea D. Branch[1]

Hepatitis C virus (HCV) is unique among RNA viruses in its ability to establish chronic infection in the majority of exposed adults. HCV persists in the liver despite interferon (IFN)-stimulated gene (ISG) induction; robust induction actually predicts treatment failure and viral persistence. It is unclear which forms of HCV RNA are associated with ISG induction and IFN resistance during natural infections. To thoroughly delineate HCV RNA populations, we developed conditions that fully separate the strands of long double-stranded RNA (dsRNA) and allow the released RNAs to be quantified in reverse transcription/polymerase chain reaction assays. These methods revealed that dsRNA, a pathogen-associated molecular pattern (PAMP), comprised 52% (standard deviation, 28%) of the HCV RNA in the livers of patients with chronic infection. HCV dsRNA was proportionally higher in patients with the unfavorable *IL28B* TT (rs12979860) genotype. Higher ratios of HCV double-stranded to single-stranded RNA (ssRNA) correlated positively with ISG induction. In Huh-7.5 cells, IFN treatment increased the total amount of HCV dsRNA through a process that required *de novo* viral RNA synthesis and shifted the ratio of viral dsRNA/ssRNA in favor of dsRNA. This shift was blocked by ribavirin (RBV), an antiviral drug that reduces relapse in HCV patients. Northern blotting established that HCV dsRNA contained genome-length minus strands. *Conclusion:* HCV dsRNA is the predominant form in the HCV-infected liver and has features of both a PAMP and a genomic reservoir. Interferon treatment increased rather than decreased HCV dsRNA. This unexpected finding suggests that HCV produces dsRNA in response to IFN, potentially to antagonize antiviral defenses. (HEPATOLOGY 2017;66:357-370).

Despite the introduction of effective therapies, hepatitis C virus (HCV) remains a global challenge. The new therapies require adherence to lengthy and expensive regimens,[1] and treatment options remain limited for patients with decompensated cirrhosis. Human immunodeficiency virus coinfection or unabated high-risk behaviors can cause 5-year reinfection rates to reach 10%-15%.[2] To overcome these challenges, new strategies are needed.

Abbreviations: bp, base pairs; cDNA, complementary DNA; dsRNA, double-stranded RNA; HCV, hepatitis C virus; IFN, interferon; ISG, interferon-stimulated gene; MOI, multiplicity of infection; mRNA, messenger RNA; PAMP, pathogen-associated molecular pattern; PCR, polymerase chain reaction; qRT, quantitative reverse transcription; RBV, ribavirin; RF, replicative form; RI, replicative intermediate; RT, reverse transcription; SD, standard deviation; ssRNA, single-stranded RNA; UTR, untranslated region.

Received February 18, 2016; accepted August 27, 2016.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.28846/suppinfo.

This work was supported in part by National Institutes of Health grants AI081530, DA031095, DK090317, DK09031702, GM064118, CA152514, and DK00779211.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.28846

Potential conflict of interest: Sasan Roayaie owns stock in Gilead and Arqule.

Future efforts to control HCV may hinge on having a more complete understanding of interactions between HCV and antiviral defenses. Although HCV triggers an interferon (IFN) response,[3] induction of IFN-stimulated genes (ISGs) fails to contain the virus in most patients. Data suggest that IFN signaling may actually promote HCV persistence in some settings.[4-11] Higher ISG messenger RNA (mRNA) expression in the liver predisposes to failure of IFN-based therapy.[4-6] Patients with single nucleotide polymorphisms near the IFN-λ3 (*IL28B*) gene have higher ISG induction,[7] but they have impaired clearance of HCV[8,9] and higher rates of antiviral treatment failure.[10] These findings raise the possibility that HCV is among the growing list of viruses that exploit IFN signaling, rather than succumb to it, as discussed in the "IFN paradox."[11]

Viral double-stranded RNA (dsRNA) is a plausible viral product for involvement in both pro- and antiviral IFN responses because it is a pathogen-associated molecular pattern (PAMP) and also a potential genomic reservoir. Several *in vitro* studies demonstrate that HCV mitigates PAMP detection and downstream IFN signaling through cleavage and inactivation of dsRNA sensing (e.g., via cleavage of mitochondrial antiviral signaling protein[12] and TIR-domain-containing-adapter-inducing IFN β[13]). However, extensive ISG induction occurs in HCV-infected livers.[3] The highest levels are in HCV RNA-positive cells.[14] Wieland et al.[14] note that *in vivo*, "HCV neither prevents the induction of IFN nor signal transduction through the Jak-STAT pathway... another mechanism is more likely to explain the ability of HCV to persist in the face of a robust ISG mRNA response."

Potential mechanisms could involve HCV dsRNA. The replicative form (RF) of supergroup II viruses, the group that includes HCV, contains full-length plus (+) and minus (−) strands.[15] The poliovirus RF is capable of launching an infection, indicating that the full-length RNAs within the RF can function as genomic reservoirs.[16] Tomato bushy stunt virus uses dsRNA as a template for progeny plus strand synthesis.[17] Nascent Kunjin viral RNAs colocalize with dsRNA,[18] suggesting that this virus also uses dsRNA as a template. These findings establish dsRNA as a key intermediate in the life cycle of viruses closely related to HCV.

Although HCV dsRNA has not been characterized extensively, several investigations indicate that HCV produces dsRNA. Livers of HCV-positive patients and infected hepatoma cells react with the J2 antibody,[19] which binds to dsRNA 40 base pairs (bp) or longer. HCV-infected fetal hepatocytes harbor RNA that is resistant to RNase I, an RNase that degrades single-stranded RNA (ssRNA),[20] suggesting that these cells contain HCV dsRNA.

To investigate the role of HCV dsRNA in the viral life cycle, a major technical barrier must be overcome. Genome-length viral dsRNA is difficult to detect and quantify because of its extraordinary thermal stability. Standard reverse transcription (RT) protocols are used primarily to detect ssRNAs, such as the form of HCV RNA that is present in blood, and in cellular mRNAs. These assays typically employ a 65°C incubation step to melt-out secondary structures, which include the stem-loops in HCV ssRNA, before RT. However, 65°C is not hot enough to separate the strands of genome-length viral dsRNA, which has far greater thermal stability than dsDNA.[21-23] This is demonstrated by thermodynamic studies of reovirus dsRNA. Similar to HCV, reovirus RNA has a G+C content of approximately 50%. Notably, the melting temperature of reovirus dsRNA—the temperature at which 50% of the base pairs are disrupted, but the strands remain associated with each other—is 80°C-100°C.[24,25] Gomatos and Tamm[25] established that maximal strand-separation requires heating to temperatures above 102°C in very low ionic strength buffer. Given the high temperatures and low ionic strength needed for strand-separation of genome-length viral RNA duplexes, standard RT/PCR assays cannot be used to quantify HCV dsRNA because these conditions do not separate the (+) and (−) strands sufficiently to allow for quantitative

ARTICLE INFORMATION:

From the [1]Icahn School of Medicine at Mount Sinai, New York, NY; [2]The Rockefeller University, Laboratory of Virology and Infectious Disease, New York, NY.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Andrea Branch, Ph.D.
1425 Madison Avenue
Icahn 11-24
New York, NY 10029
E-mail: andrea.branch@mssm.edu
Tel: 212-659-8371

primer binding and reverse transcription (quantitative complementary DNA [cDNA] synthesis).

To establish the technical foundation for investigating HCV dsRNA, we established methods that fully separate the two strands of RNA duplexes and used them to measure HCV RNA populations in liver specimens and cultured cells. We discovered a population of thermally stable (i.e., long) viral dsRNA and examined its association with hepatic ISG induction and *IL28B* polymorphisms in HCV patients. Additionally, we established that in Huh-7.5 cells, treatment with IFN-α2b increased the amount of genome-length HCV dsRNA. The shift toward dsRNA could be blocked by inhibition of the viral RNA polymerase or by treatment with RBV. RBV is an antiviral drug that reduces relapse in patients treated with IFN-containing or IFN-free regimens.[26,27] The dsRNA contained full-length (−) strands. Collectively, our findings demonstrate that HCV responds to IFN by producing dsRNA that contains a complete and well-protected copy of the viral genetic information.

Materials and Methods

HUMAN SUBJECTS

Liver tissue was obtained from patients following liver resection or liver transplantation at The Mount Sinai Hospital (no tissue was obtained from executed prisoners or other institutionalized persons). Informed consent was obtained from liver transplant recipients before transplantation. Patients were either infected with HCV or were without viral infections: control 1 had hepatocellular carcinoma and steatohepatitis, control 2 had hepatocellular carcinoma and alcoholic cirrhosis, control 3 had hepatocellular carcinoma with no known underlying liver disease, and control 4 had cholangiocarcinoma. In addition, we also obtained deidentified surgical discard tissue anonymously from HCV-positive patients undergoing liver transplantation or resection (n = 14); these specimens could not be evaluated for genomics or other protected health information. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the appropriate institutional review committee.

SAMPLE PREPARATION AND RNA PURIFICATION

RNA from liver and cell culture specimens were extracted in Trizol (Life Technologies) and purified using RNeasy mini kits (Qiagen); details are provided in the Supporting Information. To ensure high-quality RNA, only total liver RNA with RNA integrity number scores greater than 7 were analyzed.

THERMAL STRAND-SEPARATION AND qRT/PCR

To strand-separate dsRNA prior to quantitative reverse transcription and polymerase chain reaction (qRT/PCR), samples were resuspended in 10 mM Tris/1 mM EDTA (TE), pH 7.4, drawn into pulled glass capillary tubes, sealed, heated to 106°C in an oil bath, and then snap-cooled in ice water to prevent reannealing. For further details, including cycling conditions, primer sequences, and reaction conditions, see the Supporting Information.

CELL CULTURE, INFECTION, INTERFERON TREATMENT, NORTHERN BLOTTING, AND FLOW CYTOMETRY

Huh-7.5 cells were maintained and virus stocks were prepared as described previously.[28] Cultures were infected for 12 hours at 37°C at a multiplicity of infection (MOI) of 0.01 or 1.0. IFNα-2b (Merck) and RBV (Sigma-Aldrich) were resuspended in water and stored at 4°C. Northern hybridization was performed as described previously.[29] Huh-7.5 cells were stained with J2 anti-dsRNA antibody (English & Scientific Consulting, Budapest, Hungary) and anti-NS5A antibody 9E10 and frozen in 70% ethanol at −20°C. For further details, including Northern blot probes and conditions, see the Supporting Information.

Results

MOST HCV RNA IN EXTRACTS OF HUMAN LIVER IS IN STABLE dsRNA DUPLEXES

To enhance understanding of HCV-host interactions, we characterized HCV RNA in extracts of liver using techniques that fully separate the two strands of RNA duplexes before qRT/PCR. To achieve strand separation, RNA samples were heated to 106°C in 1 × TE and snap-cooled in ice water to prevent reannealing. RNAs were then reverse-transcribed under standard conditions. The resulting cDNAs represent

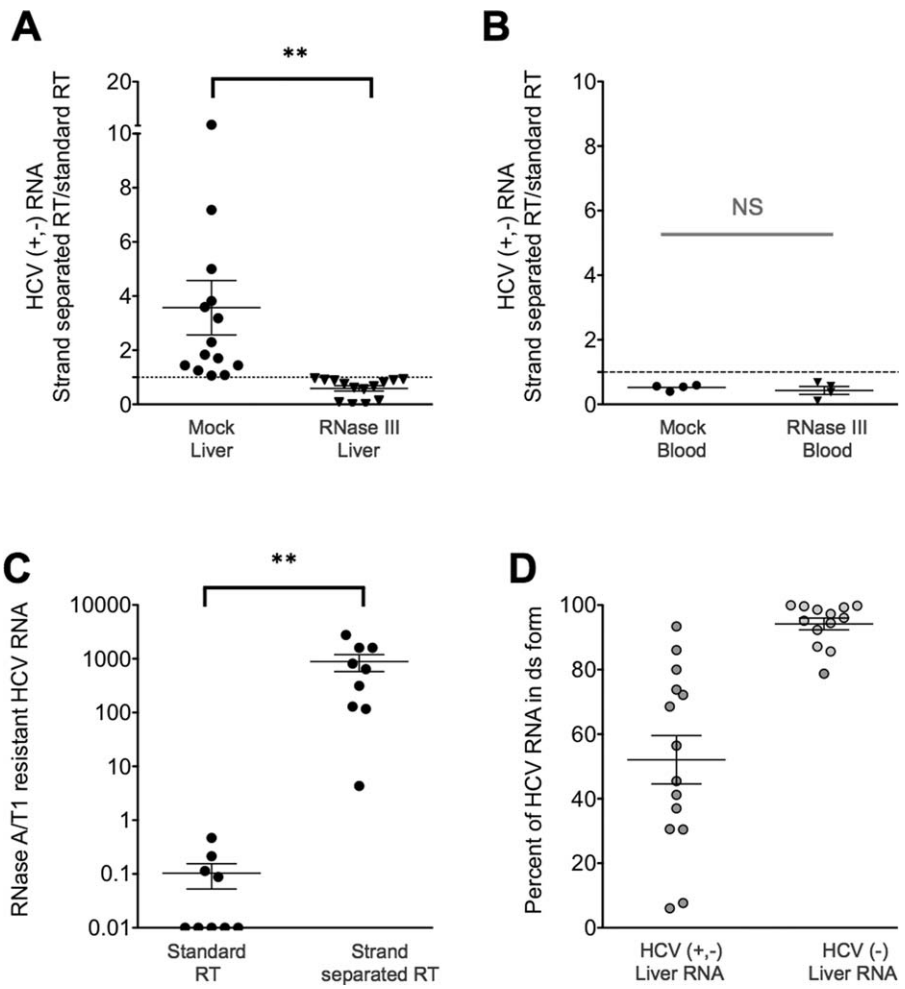
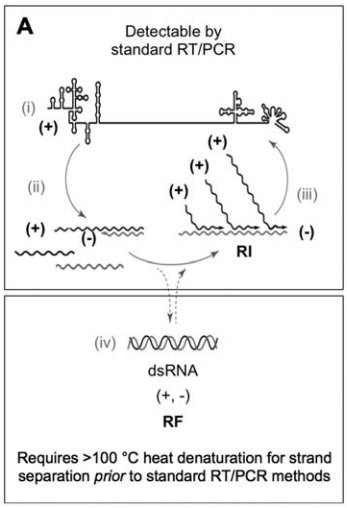


FIG. 1. Analysis of human liver and serum specimens. Quantitation of HCV RNA from (A) HCV patient livers (n = 14) or (B) HCV-infected patient serum (n = 4); RNA was quantified using an assay targeting the 5′ UTR with detection of (+) and (−) strands of the virus. Samples were treated with glycerol (mock, black circles) or RNase III (black triangles). RNA was either quantified directly by qRT/PCR (standard RT) or preheated to 106°C before qRT/PCR (strand-separated RT) to liberate dsRNA from duplex structures. Data were normalized to the signal from the same sample without preheating (standard RT) and are expressed as a ratio of strand-separated RT/standard RT (*y* axis). $^{**}P < 0.01$ (paired *t* test). NS, not significant. (C) Absolute quantitation of RNaseA/T1-resistant HCV RNA from patient livers (n = 9) using primers targeting the 5′ UTR of the (+) strand and the complimentary region of the (−) strand; samples were quantified using unheated, standard RT methods (left) or strand-separated RT (right) and were preheated to 106°C before RT (right) $^{*}P < 0.05$ (paired *t* test). (D) Percentage of HCV RNA present in double-stranded form (*y* axis, n = 14) in HCV-infected patient livers; percent dsRNA was calculated by taking the difference in the signal between samples quantified using strand-separated RT (preheated to 106°C before qRT/PCR) and standard RT (no preheating), and dividing by the total, strand separated signal; qRT/PCR was performed using an assay targeting both strands [(+), (−)] of HCV (left) or only the (−) strand (right).

the total population of HCV RNA in liver. This includes both the HCV RNA released from dsRNA as a result of heating to 106°C as well as the population of HCV RNA that was already in single-stranded form (and thus accessible to RT) before heating to 106°C. For comparison, parallel samples were subjected to the standard RT reaction without having been heated to 106°C. These standard RT conditions were used to produce cDNA, which represents the population of free HCV RNA that was in single-stranded form before heating to 106°C.

Initially, extracts of human liver from HCV patients were analyzed using qRT/PCR targeting both the HCV plus (+) and minus (−) strand. Using an assay



B

Detection of HCV RNA forms: free (+) and (-), HCV RI, and HCV RF

	(+,-) Standard RT	(+,-) Strand separated RT	(-) Standard RT	(-) Strand separated RT	RNase III Digests dsRNA	RNase A/ T1 Digests ssRNA	Northern	J2 Antibody
Free (+) *Fig. 2a, i,ii*	Detected	Detected	Not Detected	Not Detected	Not Digested	ss Digested	Detected	Not Detected
Free (-) *Fig. 2a, ii*	Detected	Detected	(-) Detected	(-) Detected	Not Digested	ss Digested	Detected	Not Detected
RI (+,-) *Fig. 2a, iii*	(+) and (-) form ss regions detected	(+) and (-) form ss and ds detected	(-) form ss regions detected	(-) form ss and ds detected	ds regions digested	ss regions digested	Detected	Detected
RF (+,-) *Fig. 2a, iv*	Not Detected	(+) and (-) Detected	Not Detected	(-) Detected	ds Digested	Not Digested	Detected	Detected

FIG. 2. Model of viral RNAs in the HCV life cycle. (A) The top of the diagram shows viral RNAs that are predicted to be detectable in standard qRT/PCR assays: (i) HCV ssRNA, the genomic (+) RNA has many stem-loops, but no perfect duplexes more than 12 bp long; (ii) short double-stranded regions form when (−) strands are copied from (+) RNA; (iii) short double-stranded regions form when (+) strands are copied from (−) strands in the RI. The bottom portion (iv) shows genome-length dsRNA, the RF, which requires >100°C heat for dsRNA strand separation before subsequent quantitation by means of qRT/PCR. (B) Table delineating the ability of various assays to detect different forms of HCV RNA: free (+) strand, free (−) strand, replicative intermediate (RI), and replicative form (RF).

that detects the 5′ untranslated region (UTR) of the HCV plus (+) strand and the complementary region of the minus (−) strand (Fig. 1A, Supporting Table S1), samples heated to 106°C had a 3.6-fold higher signal than samples that did not undergo strand separation (Fig. 1A, circles, Supporting Table S1). Similar results were obtained using primers targeting the 3′ UTR of the (+) strand and the complementary region of the (−) strand (Supporting Figs. S1 and S2). Figure 2B summarizes the ability of various assays to detect free and duplexed forms of HCV RNA.

To determine the percentage of HCV RNA in human liver that was present in double-stranded form prior to heating to 106°C, the quantity of free HCV RNA (measured in the standard RT reaction) was subtracted from the total HCV RNA (measured in the strand-separated RT reaction), and this value was divided by the total RNA and multiplied by 100 (Fig. 1D, Supporting Table S1). The average was 52% (standard deviation [SD], 28%). These results indicate that dsRNA is the predominant form of HCV RNA in extracts of human liver. The percentage of total HCV (+) and (−) RNA present in double-stranded form varied between patients across a wide range (6%-93%; Fig. 1D, Supporting Table S1).

Further investigations are needed to determine what percentage of HCV dsRNA is present in replicative intermediates (RIs) and what percentage is present in RFs. The RI, which is composed of a single minus (−) strand and multiple progeny plus (+) strands, is included in most depictions of the HCV replication cycle and is denoted as structure (iii) in Fig. 2A. The high thermal stability of the dsRNA we detected in infected liver suggests that HCV may also produce an RF, denoted as structure (iv) in Fig 2A.

Experiments were performed to confirm that the molecular species responsible for the heating boost (seen using strand-separated RT conditions) was, indeed, dsRNA. RNase III cleaves dsRNA and provides a reagent that can be used to verify the double-stranded nature of an RNA molecule. Treatment of RNA from liver with RNase III eliminated the heating boost (Fig. 1A, triangles). HCV RNA from blood did not show a heating boost and was insensitive to RNase III treatment (Fig. 1 B), as expected. Treatment of HCV RNA from liver with RNAseA/T1, which cleaves ssRNA, confirmed the double-stranded nature of the molecular species giving rise to the heating boost (Fig. 1C).

An established strand-specific qRT/PCR assay[30] was used to quantify HCV (−) strands in extracts of human liver and to determine the percentage of (−) strand RNA in double-stranded form. This assay has

several features that improve strand specificity, including an RT reaction temperature of 70°C, use of tagged RT primers, and post-RT RNase H treatment.[31] The assay was validated using (+) and (−) ssRNA *in vitro* transcripts (Supporting Fig. S3) and was then applied to liver RNA extracts (Fig. 1D). The percentage of (−) strands present in double-stranded form averaged 94% (SD, 6.5%), with a range of 78% to nearly 100%. The high percentage of (−) strand RNA that is sequestered in double-stranded form is in accordance with published data showing that intrahepatic HCV (−) strand RNA is relatively inaccessible to treatment with short hairpin RNAs[32] and is cleared more slowly than (+) strand RNA during IFN treatment.[33]

Before characterizing HCV dsRNA further, the qRT/PCR methods were validated by way of a three-part series of control studies (Supporting Fig. S4): (1) Heating a 500 base-pair duplex at 106°C for 30-60 seconds produced a maximum heating boost (Supporting Fig. S4A, top), but caused only minimal thermolysis of HCV ssRNA (Supporting Fig. S4A, bottom). (2) Heating at 106°C increased the signal from dsRNA, but had minimal impact on the signal from ssRNA (whose sequence is identical to one strand of the dsRNA molecule) or from HCV (+) ssRNA (Supporting Fig. S4B,C). (3) Heating at 95°C or 99°C had minimal effect on the signal from dsRNA (Supporting Fig. S4D), which is as expected because these temperatures are too low to achieve sufficient strand separation.[24,25] These results were confirmed further by way of a gel-based assay (Supporting Fig. S4E). Heating a 500-bp dsRNA molecule at 106°C for 45 seconds before electrophoresis caused the dsRNA band to be replaced by a slower band that comigrated with a 500-bp ssRNA. Heating at 65°C had no impact on the mobility of the dsRNA.

HCV dsRNA IS ASSOCIATED WITH HIGHER ISG INDUCTION AND THE UNFAVORABLE *IL28B* GENOTYPE

The HCV-infected liver is in a state of chronic innate immune activation as indicated by up-regulation of ISGs such as *IFIT1* and *ISG15*.[14,34] Paradoxically, higher levels of ISG induction predispose HCV patients to nonresponse to IFN treatment.[4-6] The presence of HCV dsRNA in patient livers raised the question of whether this viral PAMP was associated with the magnitude of ISG induction. To investigate this, we compared expression of *IFIT1* and *ISG15* in the livers of HCV-infected patients to that of control patients (Supporting Fig. S5) and correlated these findings with the ratio of HCV dsRNA/ssRNA. Our results demonstrate that expression of *IFIT1* and *ISG15* mRNA correlated closely with the ratio of HCV dsRNA/ssRNA (Fig. 3A,C). This correlation is of biological interest because it suggests a possible connection between HCV dsRNA and higher intrahepatic ISG induction and because it implies that HCV dsRNA was present *in vivo*. If the HCV dsRNA in extracts of human liver had been created during the extraction process, it is unlikely that the proportion of this species would be related to the expression of cellular ISGs. Consistent with published data, the titer of intrahepatic HCV ssRNA did not correlate with *IFIT1* or *ISG15* expression (Fig. 3B,D).[35]

ISGs such as *IFIT1* are up-regulated in patients with certain *IL28B* alleles.[7] Patients with the rs12979860 TT genotype are more likely to progress to chronic HCV infection[8,9] and to fail IFN-based therapies.[10] Among the HCV patients in this study for whom genotypic data were available, those with the rs12979860 TT genotype had a higher ratio of dsRNA/ssRNA than those with CC or CT genotypes (Fig. 3E).

IFN TREATMENT INCREASES HCV dsRNA

The human liver data suggested an association between IFN signaling and an increase in the ratio of HCV dsRNA/ssRNA. Cell culture experiments were performed to directly investigate the impact of IFN on HCV RNA populations. In the first set of studies, Huh-7.5 cells were infected with HCV at an MOI of 1.0 under standard conditions for viral infection.[28] At 24 hours after infection, cultures were either left untreated (control) or treated with 1000 IU/mL IFN for 48 hours, as reported previously.[36]

The impact of IFN on HCV dsRNA was first investigated using the assay quantifying (−) strands. IFN treatment decreased the titer of single-stranded minus (−) strands detected using standard RT methods only slightly (Fig. 4A), but decreased the titer of total ss HCV RNA [(+) and (−) strands] by about one log (Supporting Fig. S6B). To calculate the titer of HCV (−) strand RNA in double-stranded form, the signal from the standard RT assay [which represents free (−) strands, Fig. 2B] was subtracted from the signal from the strand-separated RT assay [which

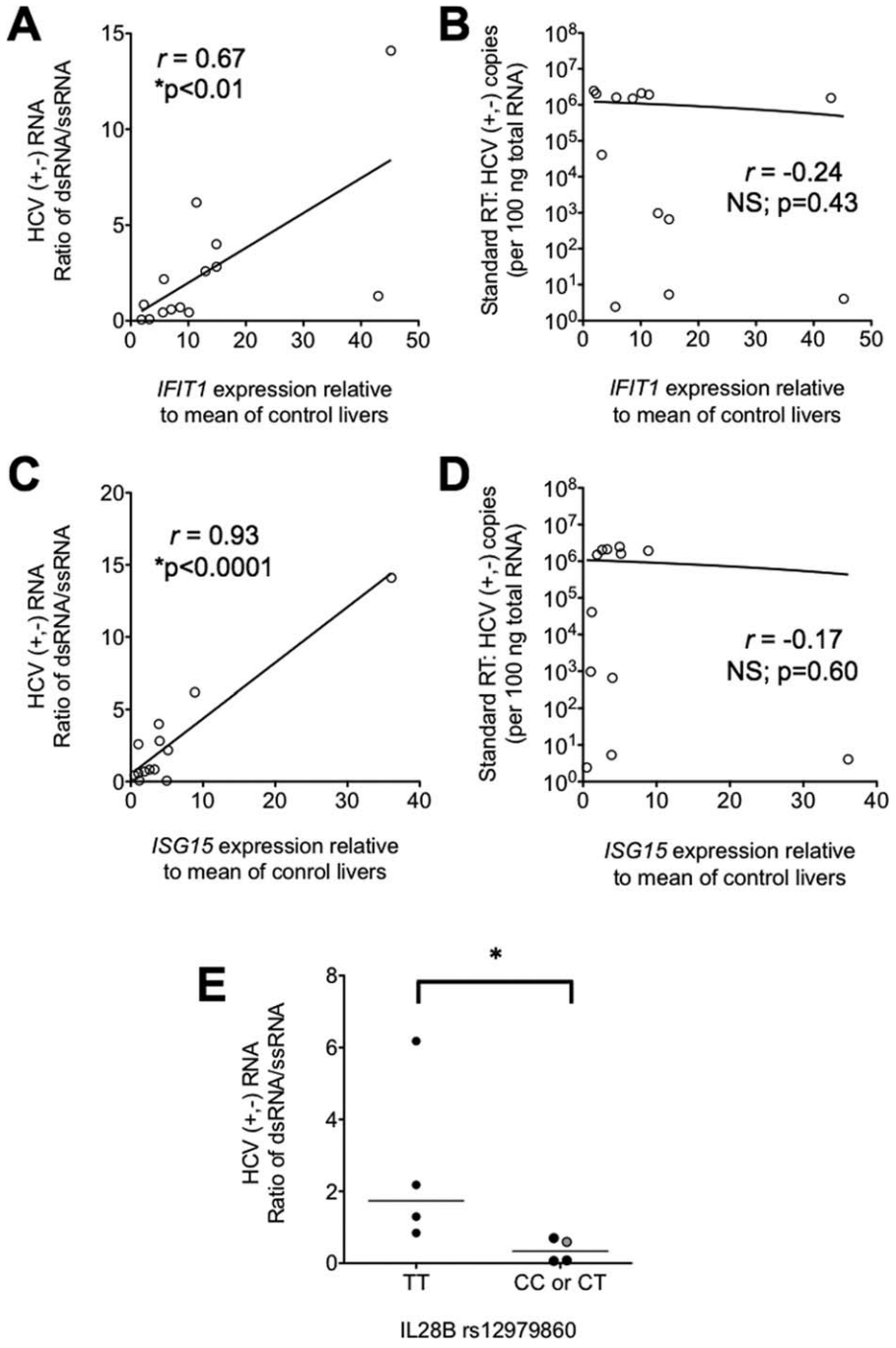


FIG. 3. Association between HCV dsRNA, ISG induction, and *IL28B* genotype. (A) The relationship between *IFIT1* mRNA expression and the titer of HCV dsRNA (calculated as the strand-separated RT signal normalized to standard RT) was analyzed by means of linear regression; the ratio of HCV dsRNA/ssRNA significantly correlates with dsRNA titers (n = 14). (B) *IFIT1* expression did not significantly correlate with HCV ssRNA copy number (measured by standard RT). NS, not significant. (C) The relationship between *ISG15* mRNA expression and the titer of HCV dsRNA (calculated as the strand-separated RT signal normalized to standard RT) was analyzed by means of linear regression; the ratio of HCV dsRNA/ssRNA significantly correlates with dsRNA titers (n = 13). (D) *ISG15* expression did not significantly correlate with HCV ssRNA copy number (measured by standard RT; n = 13). NS, not significant. (E) Patients who provided consent for genotype analysis underwent *IL28B* rs12979860 SNP typing; the ratio of HCV dsRNA/ssRNA was significantly higher in patients with the unfavorable *IL28B* rs12979860 TT minor genotype (horizontal bar represents the median). *$P < 0.05$ (Mann-Whitney U test).

represents all (−) RNA in the sample]. The amounts of dsRNA in IFN-treated and control cultures were compared with each other using *t* tests (Fig. 4A,B); IFN treatment increased HCV dsRNA almost 10-fold, from 3.8×10^5 per 100 ng of extracted RNA to 26.0×10^5 per 100 ng ($P < 0.05$). In the absence of IFN treatment, only 31% (SD = 11%, Fig. 4A) of (−) strands were in dsRNA form, which is lower than that observed in liver (Fig. 1D), likely because IFN signaling is blunted *in vitro*.[12,13] IFN increased the percentage of (−) strands in dsRNA to 81% (SD, 9%; Fig. 4A).

To discern the role of viral RNA synthesis in the IFN-induced increase in HCV dsRNA, cells were

A

HCV (-) strand RNA copies per 100 ng total RNA		
	Untreated	IFNα
Strand separated RT (Total HCV RNA)	$1.2x10^6$	$3.2x10^6$
Standard RT mean (Free HCV ssRNA)	$8.2x10^5$	$5.9x10^5$
Difference (Total – Free = dsRNA amount)	$3.8x10^5$	$2.6x10^6$
Percent dsRNA (Total – Free/Total x 100% ± SD)	31% ± 11	81% ± 9

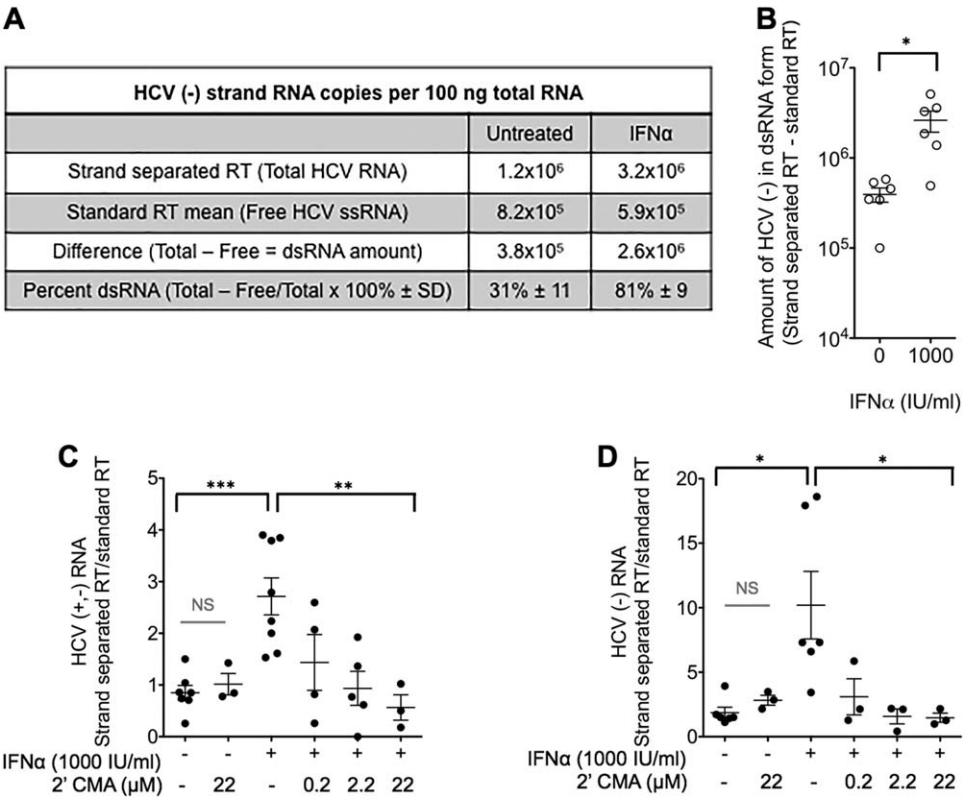


FIG. 4. Impact of IFN and HCV polymerase inhibition on HCV dsRNA in Huh-7.5 cells. Comparison of HCV dsRNA levels in HCV-infected Huh-7.5 cells. (A) Calculation of the total amount of HCV dsRNA observed using the HCV (−) assay. Total dsRNA was calculated by taking the total signal, quantified using the strand-separated RT method (heated to 106°C prior to RT/PCR), and subtracting the free RNA signal, quantified using standard RT (no preheating). This is expressed as the difference, which is also the amount of HCV in dsRNA form, (B) Impact of IFN on the amount of HCV (−) RNA in dsRNA form analyzed using a strand-specific assay with primers complementary to the 3′ UTR of the (−) strand. IFN treatment led to a significant increase in the amount of HCV dsRNA when compared with untreated cultures (n = 6). $^*P < 0.05$ (*t* test). (C) Quantitation of HCV RNA analyzed by means of a qRT/PCR assay detecting both (+) and (−) strands of HCV. IFN treatment led to a significant increase in the proportion of HCV dsRNA when compared with untreated cultures (n = 3-7). $^{***}P < 0.005$ (*t* test). This increase in HCV dsRNA can be blocked by the addition of 2′ CMA in a dose-dependent manner. $^{**}P < 0.01$ (*t* test). NS, not significant. (D) Impact of IFN on the amount of HCV (−) RNA in dsRNA form analyzed using a strand-specific assay with primers complementary to the 3′ UTR of the (−) strand; IFN treatment led to a significant increase in the proportion of HCV dsRNA when compared with untreated cultures. $^*P < 0.05$ (*t* test). This increase in HCV dsRNA can be blocked by the addition of 2′ CMA in a dose-dependent manner (n = 3-6). $^*P < 0.05$ (*t* test). NS, not significant.

either maintained as controls, treated with IFN alone, or in combination with an HCV polymerase inhibitor, 2′-C-methy-adenosine. IFN shifted the HCV RNA population in favor of dsRNA, as determined using either an assay that detects both (+) and (−) strands (Fig. 4C) or the (−) strand-specific assay (Fig. 4D). The addition of 2′-C-methy-adenosine reduced this shift significantly (Fig. 4C,D). Sofosbuvir, a polymerase inhibitor used clinically, had similar effects (Supporting Fig. S6B,C). These experiments demonstrated that IFN increased the amount of HCV dsRNA present in Huh-7.5 cells and established that the shift toward HCV dsRNA depended on *de novo* synthesis of viral RNA.

FULL-LENGTH MINUS (−) STRANDS IN IFN-TREATED CELLS

To assess the generalizability of the observation that IFN treatment increases the ratio of double-stranded/single-stranded HCV RNA, and to determine the size of the (−) strands protected in HCV dsRNA, Huh-7.5

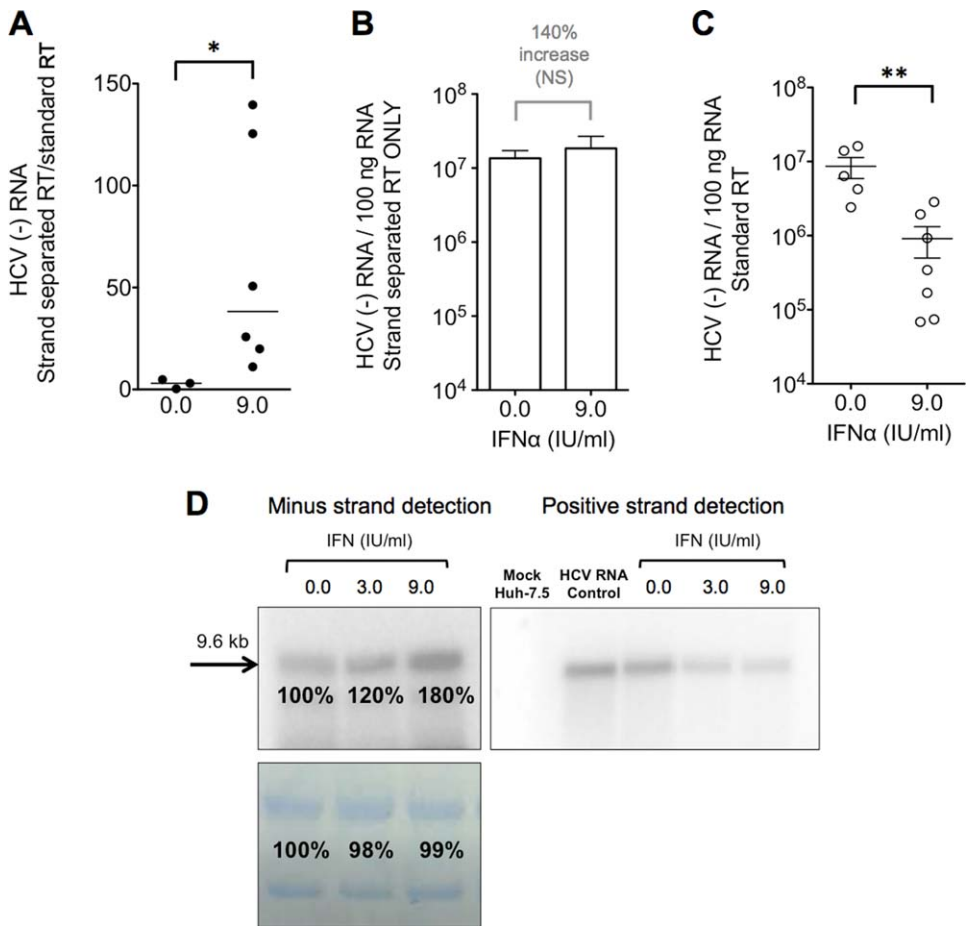


FIG. 5. Impact of IFN on HCV (−) strand RNA in Huh-7.5 cells. (A) The relative amount of HCV (−) strand RNA present in double-stranded form was compared between cells with and without IFN treatment. IFN led to a significant increase in the proportion of HCV dsRNA (horizontal bar represents the median; n = 5-7). $^{*}P < 0.05$ (*t* test). (B) The total copy number of HCV (−) strands were quantified by heating at 106°C before RT/PCR (strand-separated RT), with and without IFN treatment (n = 5-7). NS, not significant. (C) The free HCV (−) strand RNA copy number was analyzed in cells with or without 9.0 IU/mL IFN treatment using a strand-specific assay (n = 5-7). $^{**}P < 0.01$ (*t* test). (D) Northern blot analysis of HCV RNA from mock and HCV-infected cells treated with IFN (0.0, 3.0, or 9.0 IU/mL) analyzed using probes complementary to the 5′ UTR-E1 region of the (−) strand (left) or NS4B region of the (+) strand (right). The position of the 9.6 kb full-length genome is indicated by *in vitro*-transcribed genomic RNA (control). Methylene blue staining (bottom) performed before hybridization confirmed the integrity, even loading, and transfer of 18S and 28S rRNA.

cells were infected and treated under a second set of culture conditions. Cells were infected at an MOI of 0.01 for 12 hours, washed, maintained for 7 days to allow the infection to spread, and then treated with 9 IU/mL of IFN for 2 days. In keeping with the results in Fig. 4A,B, IFN treatment significantly increased the proportion of HCV (−) strands in dsRNA (Fig. 5A, Supporting Table S2) and had no significant effect on the amount of total (−) strands (Fig. 5B). In the absence of IFN treatment, the low MOI model had a higher mean percentage of (−) strands in duplex form than the high MOI model (Fig. 4A versus Fig. 5A, Supporting Table S2), perhaps because the longer duration of infection increased ISG induction, as reported previously.[37] IFN treatment reduced free (−) strands (Fig. 5C) in the low MOI model and increased the percentage of (−) strand RNA in duplex form to 95% (SD, 4%) (Supporting Table S2), similar to patient livers (Fig. 1D). Thus, in the low MOI model, virtually all the (−) RNA in IFN-treated cells was present as dsRNA.

The population of IFN-resistant viral RNA included full-length (−) strands, as shown by Northern blot

analysis using a probe for the UTR-E1 region of the (−) strand (Fig. 5D, left). These blots establish that full-length HCV (−) strands survive IFN treatment. It is an inescapable conclusion that some of these full-length (−) strands were present in dsRNA prior to the denaturation step of the Northern procedure because >95% of the (−) strands were present in double-stranded form in the IFN-treated cells (Fig. 5A, Supporting Table S2).

THE IFN-MEDIATED SHIFT TOWARD dsRNA IS DOSE-DEPENDENT, INCREASES OVER TIME, AND CAN BE BLOCKED BY RBV, AN ANTIVIRAL DRUG THAT PREVENTS RELAPSE IN PATIENTS

In additional experiments, Huh-7.5 cells were infected at an MOI of 0.01 for 12 hours, washed, and maintained for 7 days and then treated with a range of IFN concentrations (Supporting Fig. S7) or for variable periods of time (Fig. 6A, Supporting Table S3). Treatment with 10 IU/mL of IFN for 1, 2, or 3 days reduced the amount of free HCV [(+) and (−)] RNA over time (Fig 6A, left), while simultaneously shifting the population toward dsRNA (Fig. 6A, right, Supporting Table S3). Treatment with 0.0, 3.0, or 9.0 IU of IFN reduced the amount of free HCV [(+) and (−)] RNA in a dose-dependent manner (Fig. 6B, left) while shifting the population toward dsRNA (Fig. 6B, right, circles, Supporting Table S4, Supporting Fig. S8), and increasing the percent in double-stranded form from 43% (SD, 12%) in untreated cultures to 62% (SD, 20%) in cultures treated with 3 IU/mL of IFN, to 80% (SD, 17%) in cultures treated with 9 IU/mL of IFN. Treatment with RNase III confirmed that the shift was due to dsRNA (Fig. 6B, right, triangles, Supporting Table S4). To complement the RNase III treatment, samples from IFN-treated and control cells were digested with a mixture of single-stranded specific ribonucleases (pancreatic RNase A and RNaseT1) to cleave free HCV ssRNA, while preserving HCV dsRNA. After digestion, RNAs were repurified and then analyzed by way of qRT/PCR. Samples from control and IFN-treated cells contained similar, very low amounts of free HCV ssRNA (Fig. 6C). However, samples from IFN-treated cells had higher amounts of RNase A/RNase T1-resistant dsRNA than control cells (Fig. 6D). The signal originating from the dsRNA was delineated by subtracting the signal from the standard RT assay (in which the qRT/PCR signal represents residual ssRNA) from the signal from the strand-separated RT assay (in which the qRT/PCR signal represents both residual ssRNA and dsRNA). Of interest, dual treatment with IFN and RBV, a drug with a unique ability to prevent relapse in patients, caused a dose-dependent decrease in the proportion of HCV RNA in double-stranded form (Fig. 6E, Supporting Table S5).

IFN-TREATMENT INCREASED THE PERCENTAGE OF CELLS CONTAINING dsRNA BUT NOT DETECTABLE VIRAL PROTEIN

Flow cytometry was used to confirm that HCV dsRNA existed prior to RNA extraction and to investigate the impact of IFN on HCV RNA using an independent approach. Experiments were performed using 9E10, an antibody that detects the HCV NS5A protein, and J2, an antibody that detects RNA duplexes of 40 bp or longer.[19] The J2 antibody is expected to react with both HCV dsRNA in RFs, which are comprised of full-length duplexes, and RIs, provided that the duplex regions in the RIs contain at least 40 bp (see Fig. 2B). Huh-7.5 cells were infected at an MOI of 0.01, maintained for 7 days to allow infection to spread throughout the culture, and then either left untreated or treated with 0, 3, or 9 IU/mL of IFN for 2 days. Uninfected Huh-7.5 cells were also assayed in parallel as a control. The gating strategy is shown in Supporting Fig. S9. The dsRNA antibody allows duplexes to be detected *in situ* without nucleic acid extraction but does not distinguish short and long duplexes.

Uninfected Huh-7.5 cells did not react with either antibody (Fig. 7A, top panel). Most cells in highly infected cultures reacted with both antibodies (Fig. 7A, bottom panel); however, approximately 17.5% reacted with the dsRNA antibody alone (Fig. 7A, bottom panel, upper left quadrant). IFN treatment caused a dose-dependent increase in the percentage of dsRNA single-positive cells (up to 33%) and increased the mean fluorescence intensity of the J2 signal (Fig. 7B,C). The flow cytometry data suggest that IFN shifted the cell population from one in which nearly all cells were actively producing viral proteins (suggested by NS5A-positivity) to a population in which many cells contained HCV in a quiescent state where viral dsRNA was present, but viral protein production was

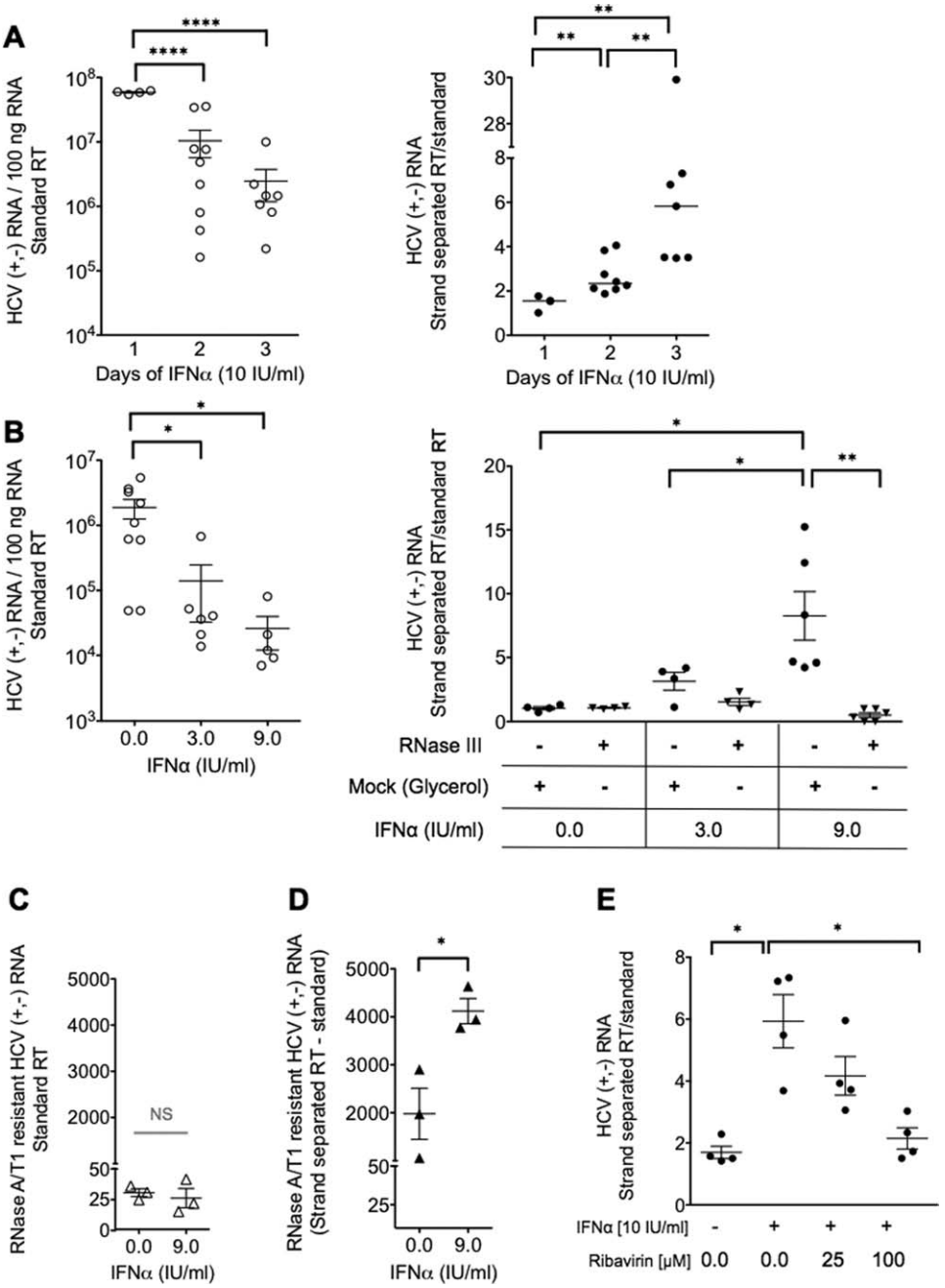


FIG. 6. Time course and dose response analysis of IFN-induced synthesis of HCV dsRNA in Huh-7.5 cells after 1 week of HCV infection. (A) HCV RNA in cultures treated with 10.0 IU/mL of IFN for 1, 2, or 3 days quantified using an assay targeting the 5′ UTR of the (+) strand and its (−) strand counterpart. Left: IFN treatment led to a significant decrease in free HCV RNA detected over time using standard RT methods (n = 4-8). $^{****}P < 0.001$ (*t* test). Right: The ratio of strand-separated RT/standard RT was determined (*y* axis), and a significant increase in the relative amount of HCV dsRNA over time was observed (n = 3-8). $^{**}P < 0.01$ (*t* test). (B) The effect on HCV RNA titer on 48 hours of treatment with 0.0, 3.0, or 9.0 IU/mL IFN was quantified using an assay targeting the 5′ UTR of the (+) strand and its (−) strand counterpart. Left: Samples were analyzed using standard RT methods; an IFN dose-dependent decrease in free HCV ssRNA was observed (n = 5-9). $^{*}P < 0.05$ (*t* test). Right: Samples were treated with glycerol (black circles) or RNase III (black triangles), and an IFN-induced, dose-dependent increase in HCV dsRNA was observed (n = 4-6). $^{*}P < 0.05$ (*t* test). This HCV dsRNA detection was reduced significantly in samples digested with RNase III before qRT/PCR when compared with glycerol-treated control (n = 4-6). $^{**}P < 0.01$ (*t* test). (C) HCV dsRNA was quantified in cells treated with 48 hours of 0.0 or 9.0 IU/mL of IFN; all samples were treated with RNase A/T1 to digest ssRNA and were analyzed directly using standard RT to determine the titer of free HCV ssRNA (n = 3). NS, not significant. (D) Strand-separated RNA was used to analyze remaining HCV RNA resistant to treatment with RNaseA/T1. The HCV dsRNA copy number is presented; this number was calculated as the difference in the signal between samples quantified using strand-separated RT and standard RT. The HCV dsRNA content of cells treated with 0.0 IU/mL of IFN was compared with that of cells treated with 9.0 IU/mL of IFN (n = 3). $^{*}P < 0.05$ (*t* test). (E) The impact of IFN/RBV treatment on HCV dsRNA; cultures were untreated or treated with 10 IU/mL IFN, or 10 IU/mL IFN plus 25 or 100 μM RBV for 2 days (n = 4). $^{*}P < 0.05$ (*t* test).

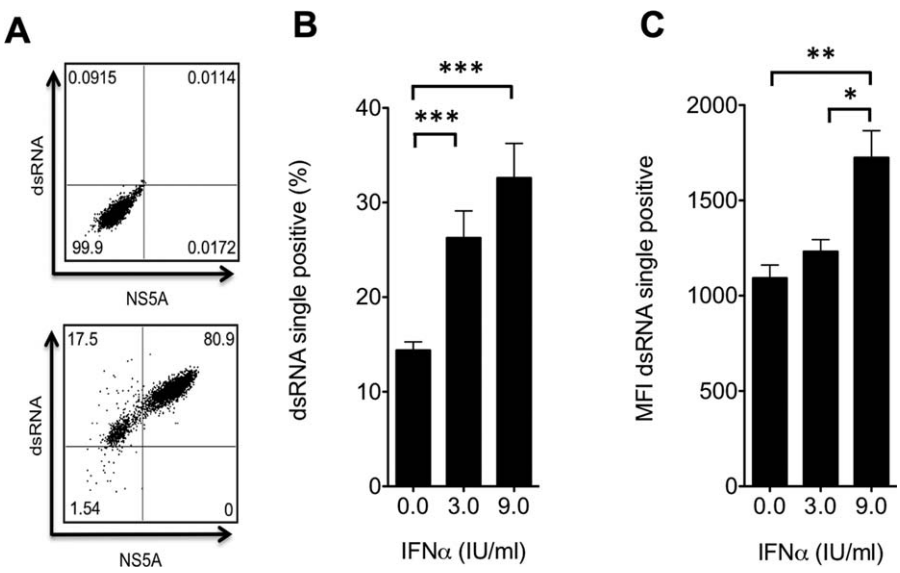


FIG. 7. Flow cytometric analysis of dsRNA *in situ*. (A) Uninfected control cultures (top, n = 5) compared with HCV-infected cultures treated with IFN (9.0 IU/mL; bottom, n = 3). Controls and infected cultures were stained in parallel with anti-NS5A (*x* axis) and anti-dsRNA (*y* axis) antibodies. (B, C) Impact of IFN treatment on the percentage of HCV dsRNA single-positive cells (B) and the mean fluorescence intensity (MFI) of dsRNA single-positive cells (C). $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.005$.

below the level of detection. In experiments using the high MOI model, treatment with the HCV polymerase inhibitor 2′-C-methy-adenosine eliminated the population of dsRNA single-positive cells (Supporting Fig. S10).

Discussion

HCV dsRNA has been difficult to characterize due to the technical limitations of conventional qRT/PCR assays. To overcome these limitations, we added a strand-separation step to standard methods. We show that heating to 106°C prior to reverse transcription increased the total amount of HCV RNA detectable in human liver specimens by 3.6-fold. Of great interest, we demonstrated a predominance of HCV dsRNA in human liver and established that the ratios of HCV dsRNA/ssRNA were higher in patients with the *IL28B* TT (rs12979860) genotype and correlated with *IFIT1* expression, an indicator of IFN signaling and predictor of IFN-based treatment failure.

Cell culture studies directly demonstrated that IFN causes a shift in the ratio HCV dsRNA/ssRNA and led to a nearly 10-fold increase in the amount of HCV dsRNA in one model of infection. This IFN effect is dose-dependent and requires *de novo* viral RNA synthesis. The IFN-resistant viral RNA population includes full-length (−) strands, which provide a complete copy of the viral genomic information. To our knowledge, this stimulation of viral dsRNA [including some dsRNA that harbored full-length (−) strands] is a newly described effect of IFN.

Of potential clinical significance, the percentage of HCV RNA in duplex form did not increase in cells treated with the combination of IFN and RBV, identifying dsRNA formation as a potential target of RBV. RBV reduces relapse when used in both IFN-containing[26] and IFN-free[27] regimens. If further investigations establish that RBV blocks dsRNA formation, elucidation of the mechanism could lead to new (less toxic) broad-spectrum antiviral drugs that inhibit production of viral RNA duplexes.

We propose that HCV dsRNA and the full-length (−) strands contained within it merits consideration as a genomic reservoir contributing to viral persistence. The ability of HCV to establish chronic infection in the majority of exposed, immunocompetent individuals is well established,[38] but the mechanisms of persistence are not completely understood. In several settings, viral RNA is temporarily undetectable before chronic infection becomes established. Patients with newly acquired HCV often cycle between periods when viral RNA can and cannot be detected in blood.[39] During times of maximal viral suppression in chimpanzees, HCV RNA was not detectable using standard assays in either blood or liver,[40] although it later reappeared. In the clinic, HCV RNA levels can be undetectable in patients receiving antiviral therapy but rebound after the end of treatment, leading to relapse.[41] Cycles of intermittent HCV RNA positivity and negativity even occur in HCV cell culture systems.[42] If HCV dsRNA can serve

as a template for viral RNA synthesis, the inability of conventional qRT/PCR assays to detect this form of viral RNA could account for the ability of HCV to reappear from an unseen reservoir. The use of viral dsRNA as a genomic reservoir has many precedents. Reoviruses go so far as to package dsRNA in their infectious particles.[43]

Now that methods have been developed to identify HCV dsRNA, it will be of interest to determine whether HCV dsRNA contains full-length (+) strands, as well as full-length (−) strands (i.e., to determine whether the HCV replication cycle includes an RF or only RIs). It will also be important to learn how additional direct-acting antiviral drugs impact HCV dsRNA synthesis. Agents that effectively block viral dsRNA formation might have utility beyond HCV. During hepatitis A virus infection, the liver harbors viral RNA in the liver for weeks in absence of detectable levels of hepatitis A virus RNA in blood,[44] suggesting that this virus might establish an intrahepatic reservoir. Hepatitis E virus and respiratory syncytial virus, both sensitive to RBV treatment,[45,46] are top candidates for additional human viruses that may use dsRNA to resist host clearance mechanisms. HCV is unique among known human RNA viruses in its ability to persist indefinitely in a high percentage of immunocompetent individuals, but viruses that establish transient infections may benefit from strategies that delay host clearance mechanisms, even temporarily.

In conclusion, this study established that IFN stimulates the production of HCV dsRNA. This is a newly discovered activity of IFN. The study also demonstrated that HCV dsRNA is the predominant form in human liver and showed that RBV blocks the IFN-mediated shift in the ratio of single-stranded to double-stranded HCV RNA.

Acknowledgments: We thank Charles Rice and Costica Aloman for experimental advice, guidance, and critical reading of the manuscript.

REFERENCES

1) Bichoupan K, Martel-Laferriere V, Sachs D, Ng M, Schonfeld EA, Pappas A, et al. Costs of telaprevir-based triple therapy for hepatitis C: $189,000 per sustained virological response. HEPATOLOGY 2014;60:1187-1195.
2) Simmons B, Saleem J, Hill A, Riley RD, Cooke GS. Risk of late relapse or reinfection with hepatitis C virus after achieving a sustained virological response: a systematic review and meta-analysis. Clin Infect Dis 2016; doi: 10.1093/cid/civ948.
3) Thomas E, Gonzalez VD, Li Q, Modi AA, Chen W, Noureddin M, et al. HCV infection induces a unique hepatic innate immune response associated with robust production of type III interferons. Gastroenterology 2012;142:978-988.
4) Chen L, Borozan I, Feld J, Sun J, Tannis LL, Coltescu C, et al. Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection. Gastroenterology 2005;128:1437-1444.
5) Feld JJ, Nanda S, Huang Y, Chen W, Cam M, Pusek SN, et al. Hepatic gene expression during treatment with peginterferon and ribavirin: identifying molecular pathways for treatment response. HEPATOLOGY 2007;46:1548-1563.
6) Sarasin-Filipowicz M, Oakeley EJ, Duong FH, Christen V, Terracciano L, Filipowicz W, et al. Interferon signaling and treatment outcome in chronic hepatitis C. Proc Natl Acad Sci U S A 2008;105:7034-7039.
7) Honda M, Sakai A, Yamashita T, Nakamoto Y, Mizukoshi E, Sakai Y, et al. Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. Gastroenterology 2010;139:499-509.
8) Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 2009;461:798-801.
9) Tillmann HL, Thompson AJ, Patel K, Wiese M, Tenckhoff H, Nischalke HD, et al. A polymorphism near IL28B is associated with spontaneous clearance of acute hepatitis C virus and jaundice. Gastroenterology 2010;139:1586-1592.
10) Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399-401.
11) Odorizzi PM, Wherry EJ. Immunology. An interferon paradox. Science 2013;340:155-156.
12) Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, et al. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature 2005;437:1167-1172.
13) Li K, Foy E, Ferreon JC, Nakamura M, Ferreon AC, Ikeda M, et al. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. Proc Natl Acad Sci U S A 2005;102:2992-2997.
14) Wieland S, Makowska Z, Campana B, Calabrese D, Dill MT, Chung J, et al. Simultaneous detection of hepatitis C virus and interferon stimulated gene expression in infected human liver. HEPATOLOGY 2014;59:2121-2130.
15) Koonin EV. The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. J Gen Virol 1991;72:2197-2206.
16) Detjen BM, Lucas J, Wimmer E. Poliovirus single-stranded RNA and double-stranded RNA: differential infectivity in enucleate cells. J Virol 1978;27:582-586.
17) Kovalev N, Pogany J, Nagy PD. Template role of double-stranded RNA in tombusvirus replication. J Virol 2014;88:5638-5651.
18) Westaway EG, Khromykh AA, Mackenzie JM. Nascent flavivirus RNA colocalized in situ with double-stranded RNA in stable replication complexes. Virology 1999;258:108-117.
19) Liang Y, Shilagard T, Xiao SY, Snyder N, Lau D, Cicalese L, et al. Visualizing hepatitis C virus infections in human liver by two-photon microscopy. Gastroenterology 2009;137:1448-1458.
20) Iacovacci S, Manzin A, Barca S, Sargiacomo M, Serafino A, Valli MB, et al. Molecular characterization and dynamics of

hepatitis C virus replication in human fetal hepatocytes infected in vitro. HEPATOLOGY 1997;26:1328-1337.
21) Kropinski AM. Bacteriophage DNA: correlation of buoyant density, melting temperature, and the chemically determined base composition. J Virol 1974;13:753-756.
22) Ueno S, Tachibana H, Husimi Y, Wada A. "Thermal stability" maps for several double-stranded DNA fragments of known sequence. J Biochem 1978;84:917-924.
23) Wienken CJ, Baaske P, Duhr S, Braun D. Thermophoretic melting curves quantify the conformation and stability of RNA and DNA. Nucleic Acids Res 2011;39:e52.
24) Steger G, Muller H, Riesner D. Helix-coil transitions in double-stranded viral RNA. Fine resolution melting and ionic strength dependence. Biochim Biophys Acta 1980;606:274-284.
25) Gomatos PJ, Tamm I. The secondary structure of reovirus RNA. Proc Natl Acad Sci U S A 1963;49:707-714.
26) Davis GL, Esteban-Mur R, Rustgi V, Hoefs J, Gordon SC, Trepo C, et al. Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. N Engl J Med 1998;339:1493-1499.
27) Curry MP, O'Leary JG, Bzowej N, Muir AJ, Korenblat KM, Fenkel JM, et al. Sofosbuvir and velpatasvir for HCV in patients with decompensated cirrhosis. N Engl J Med 2015;373:2618-2628.
28) Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623-626.
29) Lohmann V. HCV replicons: overview and basic protocols. Methods Mol Biol 2009;510:145-163.
30) Komurian-Pradel F, Perret M, Deiman B, Sodoyer M, Lotteau V, Paranhos-Baccala G, et al. Strand specific quantitative real-time PCR to study replication of hepatitis C virus genome. J Virol Methods 2004;116:103-106.
31) Lin L, Libbrecht L, Verbeeck J, Verslype C, Roskams T, van Pelt J, et al. Quantitation of replication of the HCV genome in human livers with end-stage cirrhosis by strand-specific real-time RT-PCR assays: methods and clinical relevance. J Med Virol 2009;81:1569-1575.
32) Lisowski L, Elazar M, Chu K, Glenn JS, Kay MA. The anti-genomic (negative) strand of hepatitis C virus is not targetable by shRNA. Nucleic Acids Res 2013;41:3688-3698.
33) Guo JT, Bichko VV, Seeger C. Effect of alpha interferon on the hepatitis C virus replicon. J Virol 2001;75:8516-8523.
34) Marukian S, Andrus L, Sheahan TP, Jones CT, Charles ED, Ploss A, et al. Hepatitis C virus induces interferon-lambda and interferon-stimulated genes in primary liver cultures. HEPATOLOGY 2011;54:1913-1923.
35) Wieland S, Makowska Z, Campana B, Calabrese D, Dill MT, Chung J, et al. Simultaneous detection of hepatitis C virus and interferon stimulated gene expression in infected human liver. HEPATOLOGY 2014;59:2121-2130.
36) Metz P, Dazert E, Ruggieri A, Mazur J, Kaderali L, Kaul A, Zeuge U, et al. Identification of type I and type II interferon-induced effectors controlling hepatitis C virus replication. HEPATOLOGY 2012;56:2082-2093.
37) Walters KA, Syder AJ, Lederer SL, Diamond DL, Paeper B, Rice CM, et al. Genomic analysis reveals a potential role for cell cycle perturbation in HCV-mediated apoptosis of cultured hepatocytes. PLoS Pathog 2009;5:e1000269.
38) Micallef JM, Kaldor JM, Dore GJ. Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies. J Viral Hepat 2006;13:34-41.
39) Thimme R, Oldach D, Chang KM, Steiger C, Ray SC, Chisari FV. Determinants of viral clearance and persistence during acute hepatitis C virus infection. J Exp Med 2001;194:1395-1406.
40) Shimizu YK, Igarashi H, Kiyohara T, Shapiro M, Wong DC, Purcell RH, et al. Infection of a chimpanzee with hepatitis C virus grown in cell culture. J Gen Virol 1998;79:1383-1386.
41) Bauhofer O, Ruggieri A, Schmid B, Schirmacher P, Bartenschlager R. Persistence of HCV in quiescent hepatic cells under conditions of an interferon-induced antiviral response. Gastroenterology 2012;143:429-438.
42) Mizutani T, Kato N, Ikeda M, Sugiyama K, Shimotohno K. Long-term human T-cell culture system supporting hepatitis C virus replication. Biochem Biophys Res Commun 1996;227:822-826.
43) Silverstein SC, Christman JK, Acs G. The reovirus replicative cycle. Annu Rev Biochem 1976;45:375-408.
44) Lanford RE, Feng Z, Chavez D, Guerra B, Brasky KM, Zhou Y, et al. Acute hepatitis A virus infection is associated with a limited type I interferon response and persistence of intrahepatic viral RNA. Proc Natl Acad Sci U S A 2011;108:11223-11228.
45) Kamar N, Izopet J, Tripon S, Bismuth M, Hillaire S, Dumortier J, et al. Ribavirin for chronic hepatitis E virus infection in transplant recipients. N Engl J Med 2014;370:1111-1120.
46) Turner TL, Kopp BT, Paul G, Landgrave LC, Hayes D Jr, Thompson R. Respiratory syncytial virus: current and emerging treatment options. Clinicoecon Outcomes Res 2014;6:217-225.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.28846/suppinfo.