Specific Detection of Hepatitis C Virus Minus Strand RNA in Hematopoietic Cells

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Abstract

The presence of hepatitis C virus (HCV) negative strand RNA in extrahepatic compartments based on PCR detection assays has been suggested in many reports with a very heterologous detection rate (from 0 to 100%). In this study, we have analyzed the presence of HCV negative strand in hepatic (liver biopsies, $n = 20$) and extrahepatic (sera, $n = 32$; PBMC, $n = 26$ and fresh bone marrow cells, $n = 8$) compartments from infected patients with three different reverse transcriptase (RT)-PCR–based assays using primers located in the 5′ noncoding region, with or without a tag sequence, or in the nucleocapsid (CAP). Samples were selected to display different viral loads (10^5–3×10^7 genomic equivalent/ml or gram) and viral genotypes ($n = 5$). Using synthetic as well as biological templates, we could document extensive artifactual detection of negative strand RNA, due to self priming and mispriming events, when either 5′ noncoding region primer pair was used, whereas both artifacts were dramatically reduced (mispriming) or eliminated (self-priming) using CAP-based RT-PCR assay. Mispriming artifacts were directly correlated to the titer of positive strand RNA present in the sample. Using the CAP-PCR assay, the presence of HCV negative strand RNA was found in 75% of livers (16:20) and only 8% of PBMC, independent of the genotype involved, but could not be documented in sera (0:32) and fresh bone marrow cells (0:6). These findings suggest that caution regarding the type of RT-PCR assay used and the level of HCV positive strand RNA present in the biological sample analyzed has to be taken to avoid false identification of viral reservoirs. The findings suggest that hematopoietic peripheral cells can support HCV replication, although in a very limited number of carriers. (*J. Clin. Invest.* 1996. 97:845–851.) Key words: hepatitis C virus • replication • negative strand • amplification • peripheral blood mononuclear cells

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Received for publication 21 September 1995 and accepted in revised form 17 November 1995.

1. *Abbreviations used in this paper:* BMC, bone marrow cell; Eq, equivalent genomic molecules; HCV, hepatitis C virus; NCR, noncoding region; RT, reverse transcriptase.

J. Clin. Invest.

0021-9738/96/02/0845/07 $2.00
Volume 97, Number 3, February 1996, 845–851

Introduction

Hepatitis C virus (HCV),[1] a member of the Flaviviridae virus family (1), is the major agent responsible for parenterally transmitted non-A non-B hepatitis (2). The viral genome is represented by a single-stranded positive-sense RNA that is translated into a large polyprotein subsequently cleaved into the different viral proteins, structural and nonstructural (3). At least 6 different genotypes and 52 subtypes have been identified (4). Detection of the HCV genome in clinical specimens, which is particularly useful for evaluating antiviral therapies, is currently performed in most laboratories using PCR and primers from the highly conserved 5′ noncoding Region (5′NCR) (5, 6). As HCV presumably replicates via a negative strand RNA intermediate, active replication in infected cells is demonstrated by the detection of negative sense molecules. Different studies have recently reported the lack of specificity of the current techniques used for such detection, possibly due to false priming of the incorrect strand, self priming due to the complex 5′NCR secondary structure (7), or random priming by small cellular nucleic acids (8).

The presence of HCV replicative intermediates has not only been reported in liver, where viral replication is expected, but also in serum (9) and in PBMC (10–12) of many patients with chronic hepatitis C, with a detection range varying from 0 to 100% according to the different published reports. Although very heterologous and contradictory, these results suggest the existence of extrahepatic reservoirs for viral replication. Specific confirmation of the existence of such reservoirs and the precise analysis of the distribution and ratio of positive and negative strand RNA in different biological compartments would be particularly relevant for a precise evaluation of therapy and a better understanding of HCV cell tropism.

We have analyzed the presence of HCV negative strand in hepatic (liver biopsies, $n = 20$) and extrahepatic (sera, $n = 32$; PBMC, $n = 26$ and fresh BMC, $n = 8$) compartments using three different reverse transcriptase (RT)-PCR assays. Analyses were performed using synthetic or biological RNA templates and using three different primer combinations located in the 5′NCR, with or without a tag sequence, and in the nucleocapsid (CAP) of the viral genome. We assessed the specificity and the sensitivity of the three different primer pairs in the context of different viral genotypes and viral loads.

Methods

Biological samples. 32 patients with chronic hepatitis C, including 14 transplant patients, were included in the study. A total of 32 sera, 20 liver biopsies, 26 preparations of PBMC, and 8 fresh bone marrow cells (BMC) was analyzed. Sera were stored at −20°C and liver biopsies at −80°C before analysis. All patients were positive for HCV an-

tibodies as detected by the RIBA III assay (Abbott Laboratories, North Chicago, IL).

Purification of PBMC and BMC. Peripheral venous blood cells and bone marrow aspirates were collected in heparinized tubes. Mononuclear cells were obtained after separation on a discontinuous Ficoll-Hypaque gradient, washed five times in Hanks' buffer, and stored at −80°C in 10% DMSO.

Synthetic HCV RNA template. Synthetic HCV negative and positive strand RNAs were generated from a vector (pcDNA3; Invitrogen, San Diego, CA) containing the 5′NCR and capsid sequences (nt 1–1064 from a type 1a isolate). The plasmid DNA template was transcribed using T7 or SP6 RNA polymerase (Promega, Madison, WI) producing positive or negative strand RNA, respectively. BamHI and NotI restriction enzymes were chosen to linearize the plasmid DNA template. These enzymes generate protruding 5′ ends, less likely than protruding 3′ ends to result in the synthesis of RNA molecules that are initiated at the terminus of the template (13). Absence of residual plasmid DNA was controlled by DNase digestion (DNase I, 200 μg/ml, 30 min, 37°C), and incorporation in all PCR assays was performed of a control reaction excluding the primer used for cDNA synthesis and/or the RT step. Total cellular RNA extracted from normal livers (20 mg of tissue per assay) was added, when specified, to synthetic RNAs before cDNA synthesis.

Extraction of nucleic acids and cDNA synthesis. RNA was extracted from 10–30 mg of pulverized liver biopsies, 200 μl of serum, 2×10^6 PBMC, or 10^6 fresh BMC using two phenol acid guanidium thiocyanate extraction steps, followed by a chloroform extraction step and precipitation with ethanol (14). cDNAs were synthesized with specific primers described in Table I. RNA samples were preheated first for 10 min at 70°C with 0.2 μM of primer and 10 U rRNasin (Promega) and then incubated for 1 h at 42°C, with a reaction mixture containing RT-buffer 1X (Gibco Laboratories, Grand Island, NY), 10 mM DTT, 1 mM dNTP, 20 U rRNAsin (Promega), and 200 U MMLV RT (Gibco Laboratories). Samples were then heated to 100°C for 1 h. The different RT-PCR assays were performed simultaneously from the same RNA extraction product to avoid degradation of viral templates due to multiple freezing and thawing. Distilled water, normal sera, normal livers, and normal PBMC were used as negative controls in each extraction.

PCR amplification of cDNA. One-eighth of the generated cDNA was amplified as described previously (15) with the combination of primers previously listed in Table I. Briefly, reaction mixture consisted of 0.75× *Taq* buffer (Promega), 1.5 mM $MgCl_2$, 1 mM dNTP, 1 μM of each primer (Table I), and 1 U *Taq* DNA polymerase (Promega). The PCR conditions consisted of an initial cycle of 95°C for 5 min, 40 cycles of 95°C for 1 min (5′NCR) or for 1.5 min (tagged 5′NCR or CAP-based PCR), 64°C for 1 min (5′NCR) or 50°C for 2 min (tagged 5′NCR or CAP-based PCR), and 72°C for 2 min (5′NCR) or 3 min (tagged 5′NCR or CAP-based PCR), and a final extension cycle of 72°C for 10 min. Typically, one-fourth of the PCR product was analyzed by agarose gel and Southern hybridization with a ^{32}P-labeled probe internal to the PCR primers. All samples were analyzed at least in duplicate experiments. Hybridizations were done at 55°C in 6× SSC buffer (0.09 M sodium citrate, 0.9 M sodium chloride, pH = 7.0) containing 0.2% SDS, with the following probes: 5′-AGCCGAGTAGTGTTGGGTCGCG-3′ for the 5′NCR and 5′-ATGGGGTGGGCAGGATGGCTC-3′ for the capsid-derived PCR products.

bDNA assay. Positive strand HCV RNA was quantified by the bDNA assay (Chiron Corp., Emeryville, CA) using 50 μl of serum or RNA extracted from 10–50 mg of pulverized liver biopsies.

Genotypes. HCV isolates from sera were genotyped by the HCV LIPA assay (Innogenetics, Ghent, Belgium).

Results

Assessment of sensitivity and specificity of negative strand RT-PCR assays using synthetic RNA templates. We compared three combinations of primers for the ability to specifically prime and amplify HCV negative strand RNA. Two sets of primers are located in the 5′NCR region of the viral genome and have been described previously for HCV RT-PCR (7, 16), one of these contains a tag sequence unrelated to the HCV genome at the 5′ end. The third one is located in the nucleocapsid, i.e., outside of the highly structured 5′NCR (17). Synthetic viral RNA templates corresponding to the negative and positive strand RNAs were synthesized using plasmid DNA and used in serial dilution experiments (1:10) to determine the sensitivity limit of each primer combination. In some cases, total RNA from an uninfected liver was added before cDNA synthesis to mimic the cellular context found in biological samples.

Amplification of negative or positive strand templates was performed using conditions specific for the negative strand

Table I. Primers Used for RT-PCR Assays

Strand specificity	PCR name	Steps	Primers	Nucleotide position	Region	Polarity	References
Positive strand	5′NCR(+)	cDNA	NC4	285–311	5′-NCR	−	15
		Amplification	NC3	42–69	5′-NCR	+	
			NC4	285–311	5′-NCR	−	
Negative strand	5′NCR	cDNA	NC3	42–69	5′-NCR	+	15
		Amplification	NC3	42–69	5′-NCR	+	
			NC4	285–311	5′-NCR	−	
	Tagged	cDNA	TF*	81–96	5′-NCR	+	7
	5′NCR	Amplification	R	440–456	Capsid	−	
			T	Unrelated to HCV		+	
	CAP	cDNA	256(+)	480–499	Capsid	+	16
		Amplification	256(+)	480–499	Capsid	+	
			186	732–751	Capsid	−	

*TF: 5′-*TG*TCATGGTGGCGAATAA**GCCATGGCGTTAGTAT**-3′, adapted from Lanford et al. (7). HCV sequence is shown in bold, tag sequence is shown in regular character. The last two nucleotides (italic characters) at the 5′ end were extra compared with those from the Lanford sequence.

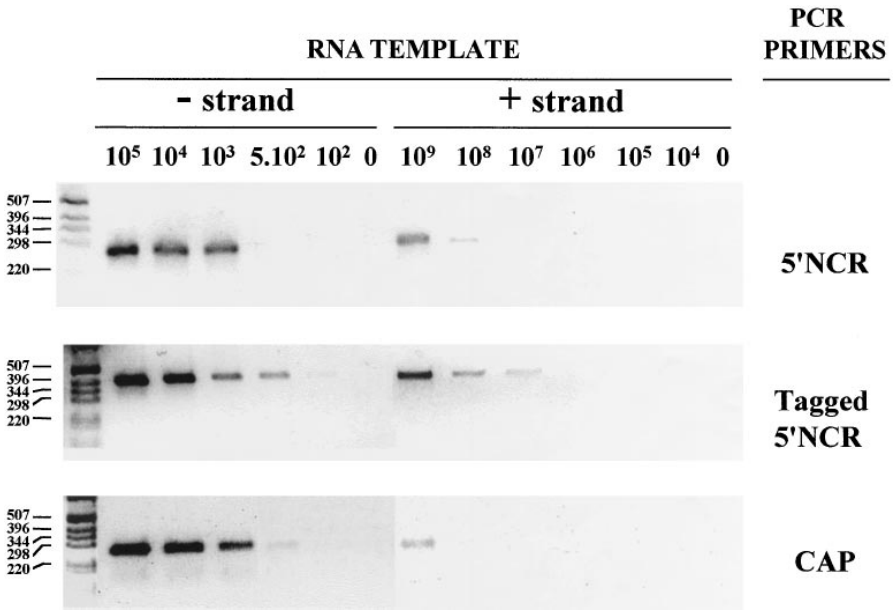

Figure 1. RT-PCR sensitivity and specificity using different combinations of primers. Viral negative (− *strand*) or positive (+ *strand*) strand RNA templates were synthesized from a vector (pcDNA3) containing the 5′NCR and capsid HCV sequence and serially diluted in DEPC-treated water. The number of viral copies (represented on top) was determined from the OD measurement (260 nm) and confirmed by electrophoresis in agarose gel. RT-PCR was performed using 5′NCR, tagged 5′NCR, or capsid (*CAP*)-derived primers. PCR products were fractionated on a 2.5% agarose gel and stained by ethidium bromide. Molecular weight markers are shown on the left (base pair).

template to determine the sensitivity of the assay and its strand specificity (18). With the nontagged 5′NCR-derived primers (Fig. 1, *5′NCR*), we detected 10^3 equivalent genomic molecules (Eq) of HCV negative strand RNA. Using these primers, detection of a signal was observed in the sole presence of $\geq 10^8$ Eq of positive strand RNA molecules. The use of a tagged primer (Fig. 1, *Tagged 5′NCR*) increased the sensitivity of the assay to the detection of 10^2 Eq, but the nonspecificity was also increased since a signal could be observed in the presence of 10^7 Eq of positive strand template. Using capsid-derived primers (Fig. 1, *CAP*), the detection limit was 5×10^2 Eq. In this case, specificity was improved such that the presence of at least 10^9 Eq of positive strand template was required to produce a signal. Addition of cellular RNA in the reactions described above slightly lowered the sensitivity of the detection (less than one log) with all three primer pairs used (data not shown). In all of the above experiments, the absence of residual plasmid DNA was confirmed by the absence of specific signal obtained after PCR amplification of cDNA products synthesized when priming oligonucleotides were omitted from the cDNA synthesis reaction (data not shown). Thus, the sole presence of positive strand RNA templates, although at different concentrations depending on the primer combination used, can result in the detection of signals leading to the false identification of negative strand templates. None of the primer combinations evaluated here allowed the complete elimination of mispriming events.

Self-priming events possibly due to the existence of hairpin structures in the 5′NCR (7) were analyzed in experiments in which primers were systematically excluded from the cDNA synthesis reactions before PCR amplification. Self-priming of RNA templates was never observed in experiments using the synthetic RNAs, even in the presence of added cellular RNAs (data not shown). In that case, sensitivity was uniformly reduced by approximately one-half of a log.

Comparative detection of HCV negative strand RNA in liver and sera using different RT-PCR assays. To confirm the previously made observations using synthetic RNA templates and to assess the presence of replicative intermediates in extrahepatic reservoirs, we evaluated the different RT-PCR assays using liver tissues and sera from HCV carriers. Positive and negative strand HCV RNA was analyzed in 32 sera and 20 liver biopsies of infected patients. Patients were chosen to represent infection by a variety of viral isolates (five genotypes were represented: genotype 1a, $n = 3$; 1b, $n = 37$; 2a, $n = 3$; 3a, $n = 2$; 4a, $n = 1$) as well as a wide range of viral loads (from < 4 to 10,400 Eq/ml).

Fig. 2 illustrates an example of detection obtained after analysis of a serum and a liver sample performed using the different primer pairs described previously. As shown in Fig. 2, the use of nontagged 5′NCR-derived primers led to a specific signal even when the primer had been omitted from the cDNA synthesis reaction in both serum (lane *5*) and liver samples

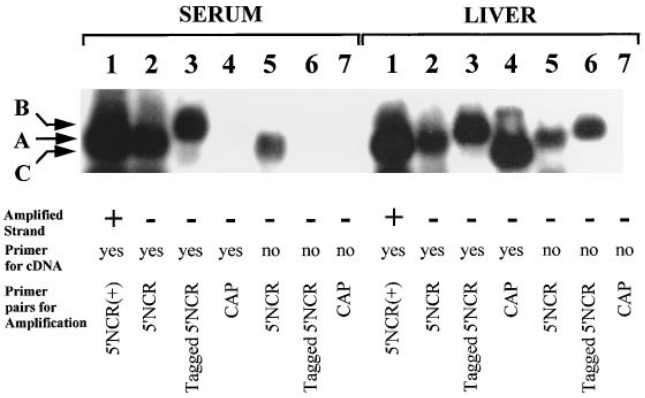

Figure 2. HCV negative strand RNA detected in sera and liver biopsies using different combinations of primers. cDNA synthesis reactions for both the negative and positive strand RNA were run in the presence and/or absence of primers followed by amplification with the corresponding primer pairs. PCR products were fractionated on a 2.5% agarose gel and analyzed by Southern blot. Amplified products from the 5′NCR (289 bp), tagged 5′NCR (393 bp), and CAP (271 bp) RT-PCR reactions from a representative experiment are shown (*arrows* in *A*, *B*, and *C*, respectively).

(lane *5*) reflecting self-priming events. Amplification using a tagged primer resulted in the disappearance of a positive signal from the serum (lane *6*) but not the liver sample (lane *6*) when the primer had been omitted from the cDNA synthesis reaction. In absence of primers for the cDNA synthesis reaction, similar results as those observed with the tagged 5′NCR primers were observed with capsid-derived primers. No amplification was observed from the serum sample (lane *7*). In contrast to data obtained with the tagged primers, no signal was obtained from the liver sample with the capsid primers (lane *7*) when the primer had been omitted from the cDNA synthesis reaction (although positive signals could be observed using other liver samples, data not shown). Thus, in contrast to observations obtained from experiments using synthetic viral templates, self-priming can occur when templates are directly analyzed from biological material. Such priming can apparently be eliminated when analyzing sera either with tagged or capsid-derived primers, but not when liver samples are analyzed. In this latter cellular material, a specific folding of the genomic RNA, possibly due to interactions of the viral genome with cellular RNAs and/or proteins, may occur which contributes to self-priming of template molecules.

The overall results obtained comparing cDNA synthesis performed in the absence or presence of primers followed by amplification of the negative strand RNA with the three different primer pairs in the 20 livers and 32 sera included in the study are shown in Fig. 3. The presence of positive strand RNA was also evaluated and confirmed in all samples. A specific signal for negative strand RNA was observed in 88% of sera and 95% of biopsies when nontagged 5′NCR-derived primers were used. With these primers, a signal was also observed in 31% of sera and 75% of biopsies in the absence of primer for cDNA synthesis reflecting self-priming of the template molecules. When tagged primers were used, in the presence of primer for cDNA synthesis, negative strand RNA could be found in 47 and 75% of sera and biopsies, respectively. With these primers, a specific signal for the negative strand RNA in the absence of primer for cDNA synthesis was never observed in sera but was observed in 20% of biopsies. Thus, addition of a tag sequence could totally eliminate self-priming artifacts in serum samples and greatly reduce them when analyzing liver samples. When capsid-derived primers

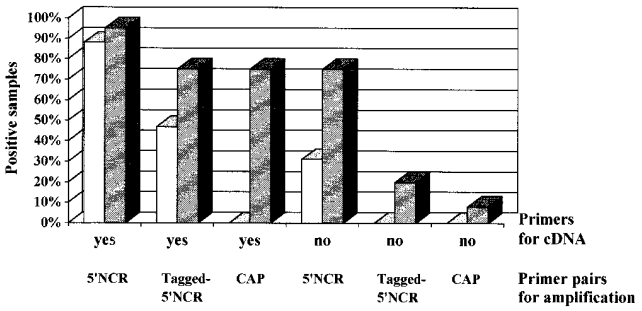

Figure 3. Comparative PCR detection of HCV negative strand RNA in sera and liver biopsies using tagged or nontagged primers from the 5′NCR, or from the nucleocapsid. RT-PCR using different sets of primers was performed as described in Fig. 2. Presence of primers for cDNA synthesis (*yes*, primers included; *no*, primers excluded) and primer pairs subsequently used in the PCR amplification are shown on the axis. The percentages of samples showing a specific signal are shown. Symbols represent either serum (*light bars*) or liver (*dark bars*) samples.

were used, in the presence of primers for cDNA synthesis, negative strand RNA could be found in 0% of sera and 75% of biopsies. In absence of primers for cDNA synthesis, no signal was detected in sera and only in 8% of biopsies. This was not due to a decrease in the sensitivity of the RT-PCR reaction which is, as shown in Fig. 1, comparable to the sensitivity of the RT-PCR using tagged primers. It therefore appears possible, using such primers, to further reduce self-priming events when analyzing liver-derived samples.

Influence of viral load on detection of HCV negative strand RNA in livers and sera. In view of the results obtained in vitro using synthetic RNA templates, we analyzed the correlation between titers of positive strand RNA (i.e., viral load) and the frequency of detection of the negative strand RNA using the three different primer pairs. Table II shows the results obtained. When nontagged 5′NCR primers were used for amplification, a signal was detected independently of the sample titer, except in very low titer containing sera, i.e., $< 10 \times 10^5$ Eq/ml (3:7). With the 5′NCR tagged primer, we observed a correlation between positive strand RNA titers and the detection of negative strand RNA, in sera as well as in biopsies. A signal

Table II. Correlation between Positive Strand HCV RNA Titers in Sera or Liver Biopsies and Frequency of Detection of HCV Negative Strand RNA Using Different Primer Sets*

			Primer sets		
Range ($\times 10^5$ Eq/ml)	Genomic Eq/reaction	*n*	5′NCR	Tagged 5′NCR	Capsid
Sera					
0–10	$0–2 \times 10^5$	7	3 (43%)	0 (0%)	0 (0%)
11–100	$2 \times 10^5–2 \times 10^6$	8	8 (100%)	2 (25%)	0 (0%)
101–300	$2–6 \times 10^6$	6	6 (100%)	5 (83%)	0 (0%)
> 300	$> 6 \times 10^6$	6	6 (100%)	6 (100%)	0 (0%)
Liver biopsies					
0–1	$0–2 \times 10^6$	3	3 (100%)	1 (33%)	1 (33%)
1–2	$2–4 \times 10^6$	3	3 (100%)	2 (67%)	2 (67%)
> 2	$> 4 \times 10^6$	5	5 (100%)	5 (100%)	5 (100%)

*All biopsies were taken from transplant patients.

was detected in all sera (6:6) and in all biopsies (5:5) with titers $> 300 \times 10^5$ Eq/ml and 2×10^5 Eq/mg, respectively. Negative strand RNA was only detected in 83% of sera (5:6) containing $100–300 \times 10^5$ Eq/ml, 25% of sera (2:8) containing $11–100 \times 10^5$ Eq/ml and 0% (0:7) of sera containing $< 10 \times 10^5$ Eq/ml. In liver samples, detection was positive in only 33% of livers (1:3) containing $< 10^5$ Eq/mg, 67% of livers (2:3) containing between 10^5 and 2×10^5 Eq/mg and 33% (1:3) of livers containing $< 10^5$ Eq/mg were positive. With capsid-derived primers, no specific signal was observed in any of the sera tested whereas 72% (8:11) of liver samples, identical to those detected with tagged 5′NCR primers, were found positive. The correlation between detection and viral load was in these cases identical to that described with the tagged 5′NCR primers. Thus, the use of capsid-derived primers to amplify negative strand RNA from sera and livers resulted in a more specific detection than that obtained with tagged 5′NCR primers. The differences were particularly dramatic in the analysis of serum samples (0:27 vs. 13:27 of positive signal frequency).

These data combined with data shown in Fig. 1 suggest that, in sera, presence of negative strand RNA is probably due to (*a*) self-priming when detection is performed with nontagged 5′NCR derived primers, and (*b*) mispriming of positive strand RNA when detection is performed with tagged 5′NCR primers. Using the highly specific CAP-based RT-PCR assay, negative strand RNA was never detected in any of the 32 sera, thus excluding this biological compartment from being a site for replication.

Evaluation of replication sites in hematopoietic cells. Using the capsid-optimized RT-PCR assay, we looked for evidence of HCV negative strand RNA in 26 PBMC (including those from three transplant patients) and 8 fresh BMC of chronically infected patients (Table III). Detection of positive strand RNA was done simultaneously in all samples. Negative strand RNA was also analyzed in all corresponding sera.

Positive strand RNA was detected in 15:23 (65%), 3:3 (100%), and 3:8 (38%) of PBMC (chronic carriers), PBMC (transplant), or fresh BMC, respectively. Titers of positive strand RNA in PBMC of both chronic and transplant patients varied from 10^5 to 10^6 Eq/10^6 cells. The presence of serum negative strand RNA was never documented in any of the samples. Negative strand RNA in peripheral blood mononuclear cells could only be documented in 1:23 (4%) of chronic carriers but never in fresh bone marrow cells (0:6). One out of three (33%) transplant patients also displayed the presence of replicative intermediates in PBMC. In both cases where negative strand RNA was detected, serial dilution experiments suggested the presence of $10^3–10^5$ copies/100 μl (data not shown). Thus, the ratio between positive strand and negative strand RNA in PBMCs for the two positive cases identified in our study is approximately 1:10–1:100. This ratio is comparable to the ratio found in infected liver tissues. This observation further suggests active viral replication in PBM cells.

Table III. Detection of HCV Genomic RNA (Positive and Negative Strand) in Different Putative Viral Reservoirs Using Strand-specific RT-PCR

	PBMC‡		
Strand detected*	Chronic	Transplant	Fresh BMC‡
Positive strand§	15/23 (65%)	3/3 (100%)	3/8 (38%)
Negative strand§	1/23 (4%)	1/3 (33%)	0/6 (0%)

*Negative strand was detected using capsid-derived primers. §Number of samples showing a specific RT-PCR signal versus total number of samples tested. ‡Samples were thoroughly washed before testing. No signals were ever detected in the last wash. Fresh BMC were BMC before culture.

Discussion

Many traps can lead to the detection of positive signals in HCV negative strand RT-PCR assay. Some of these traps have been described by Willems et al. (19) and McGuinness et al. (18) as: (*a*) insufficient inactivation of the RT enzyme after cDNA synthesis; (*b*) possible RT activity of the *Taq* DNA polymerase, (*c*) existence of a thermostable hairpin structure in the 5′NCR that leads to self-priming of the HCV RNA template; or (*d*) mispriming of the incorrect strand. Whereas many studies have reported the detection of positive strand RNA in serum of HCV carriers (9, 10, 20–23) only a few have actually attempted to demonstrate the presence of negative strand RNA using tightly controlled experiments (9, 23). All reported studies have been performed using nontagged primers from the 5′NCR in RT-PCR assays. Controls for self-priming events, such as reactions excluding primers for cDNA synthesis, were usually described, but analysis of mispriming events related to the presence of positive strand RNA templates was never documented. The presence of HCV negative strand RNA in sera or PBMC of HCV chronic carriers has been found in as much as 40–60% of the populations studied (9, 24, 25). In a recent study Shindo et al. (24) proposed that separate HCV structures may exist that contain positive and negative strand RNA. More specifically, they proposed that negative strand molecules could be found in association with a membrane structure containing one of the viral envelopes and would thus not represent replicative forms of the virus.

We present here a systematic analysis of two major events likely to generate a false diagnosis of the presence of HCV replicative intermediates in biological samples, mispriming due to the positive strand RNA and self-priming of the negative strand template. We show, as originally predicted and demonstrated by Chaves et al. (26) for the detection of minus strand hepatitis A virus RNA and Lanford et al. (7) for the detection of HCV negative strand RNA from tissue culture samples, that the use of tagged (or tailed) primers enhances the sensitivity and reduces self-priming events for the detection of negative strand RNA molecules. We demonstrate in this study that mispriming or artifactual priming due to the positive strand RNA was not eliminated with the use of tagged primers. This was shown in experiments using synthetic viral RNA templates and strongly suggested by the correlation found between titers of positive strand RNA templates present in a biological sample and the detection rate of negative strand RNA in the same sample.

We report that the use of primers located outside of the highly structured 5′NCR such as in the nucleocapsid eliminates self-priming events (at least to the degree of sensitivity reached by the techniques used) and greatly reduces or eliminates mispriming ones when either sera or liver samples are analyzed. At least six major genotypes of HCV have been de-

scribed (27) with genomes that differ mostly in coding domains such as the envelope proteins. Accumulating clinical evidence suggests that some of these genotypes may be more virulent, have higher circulating titers or can be more readily transmitted as reflected by differential sensitivity of various genotypes to IFN treatment, or the particularly high prevalence of some isolates in endemic areas (28–30). The 5′NCR has been the target of choice for amplification of HCV genomic strands because it is the most highly conserved genomic domain. Nonetheless, recent data from the literature (21) have made it possible to adapt capsid-derived primers (16) for PCR amplification of negative (or positive) strand RNA to tightly match the various HCV genotype sequences so far described, thus allowing the unambiguous amplification of distinct HCV genotypes (Inchauspé, G., unpublished data). With capsid-derived primers copied from conserved domains, we failed to detect negative strand RNA in sera, whatever the positive strand RNA titer and the viral genotype. This observation strongly suggests that serum detection of negative strands observed by us with tagged primers or by others using 5′NCR nontagged primers was probably due to mispriming of the incorrect strand. In our study, such signals were never observed in samples from noninfected patients, indicating that viral positive strand RNAs are implicated in the artifactual detection observed. Residual nonspecific signals observed in livers with capsid or tagged 5′NCR-derived primers are probably due to random priming of small cellular or viral nucleic acids as previously suggested (8). Those residual nonspecific signals may still bias the detection levels of specific signals and therefore may provide an overestimation of actual viral replicating viruses. Within the limits of the detection assays, our data exclude the existence of replicative intermediates in serum of infected patients.

The results obtained using a capsid-optimized RT-PCR assay suggest that HCV negative strand RNA is never detected in serum of HCV chronic carriers, whereas the same patient's PBMC can be the site of active replication. Our data suggest that such replication is a particularly rare event as only a minority of HCV carriers appear to harbor replicative viruses in hematopoietic cells (4%). We are in the process of confirming this observation in a larger scale study. Infected PBMC appear to be a more privileged site for replication in the case of transplant patients (1:3 or 33%). Higher viral titers may be found in these patients due to their state of immunodepression which may favor overall enhanced replication. This observation remains to be confirmed by the analysis of additional cases. It has been suggested that the disappearance of positive strand viral RNA from serum or plasma during IFN therapy does not always correlate with the disappearance of replicative forms in PBMC (31–33). In these cases, failure to sustain a long-term response to treatment as documented by recurrence of ALT elevation and rebound of viremia may be linked to the nonelimination of HCV from hematopoietic cells. Although intriguing, because of the nonspecificity of the assays used in these studies, the relevance of HCV replicative intermediate detection in such reservoirs before and during therapy will have to be reevaluated using more specific detection tools such as CAP-based PCR assays.

In conclusion, studies aimed at demonstrating the existence of HCV negative strand RNA, either for the purpose of identifying extrahepatic viral reservoirs, monitoring successful therapeutic eradication of the virus, characterizing active in vitro or in vivo replication, or for studying implication of the virus in the development of hepatocellular carcinomas (25), should be carefully controlled for the various priming events that can dramatically affect the significance of results. Whereas HCV is clearly a hepatoprotic virus, our data provide evidence for the existence of reservoirs from hematopoietic origin.

Acknowledgments

We thank M. Brinton for useful discussion and advice and F. Zoulim for reviewing the manuscript. The authors are thankful to the Chiron Corporation and Dr. J.P. Bonn for providing us with the bDNA assay.

This work was partly financed by l'Association pour la Recherche sur le Cancer; H. Lerat is supported by a grant from INSERM, and M. Major is a recipient of a fellowship from Fondation Merieux.

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