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Lack of Detection of Negative-Strand Hepatitis C Virus RNA in Peripheral Blood Mononuclear Cells and Other Extrahepatic Tissues by the Highly Strand-Specific rTth Reverse Transcriptase PCR

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To further explore the controversial potential for extrahepatic replication of hepatitis C virus (HCV), the highly strand-specific rTth method of reverse transcriptase PCR was used to examine sera, liver, peripheral blood mononuclear cells, and other extrahepatic tissues from HCV-infected chimpanzees and humans. Positive-strand HCV RNA was present in the liver at approximately 10-fold-higher levels than negative-strand HCV RNA. No negative-strand RNA was detected in peripheral blood mononuclear cells or other extrahepatic tissues despite the presence of abundant positive-strand RNA. These data demonstrate that within the limits of sensitivity of this highly strand-specific reverse transcriptase PCR method, no extrahepatic replication of HCV was detected.

The replication of hepatitis C virus (HCV) in extrahepatic tissues, especially peripheral blood mononuclear cells (PBMC), is highly controversial, and yet whether extrahepatic replication occurs is a critical question with regard to transmission and pathogenesis. One of the problems of determining the tropism of HCV for other tissues is the very low levels of virus in infected individuals. HCV can be routinely detected only by reverse transcriptase PCR (RT-PCR). The presence of HCV RNA is not sufficient to demonstrate replication of HCV in other tissues, since viral RNA may be present because of contamination with plasma and/or adherence of circulating virus. Methods for the detection of negative-strand HCV RNA are controversial. Several reports indicate that the standard method for detection of negative-strand HCV RNA lacks sufficient strand specificity to answer biological questions (5, 8, 9, 14). The lack of strand specificity is due to false priming of the incorrect strand during the cDNA step. With the high level of amplification provided by PCR, detection of the incorrect strand is not easily avoided.

We have recently described two methods to circumvent the problem of false priming and have used the methods to demonstrate the appearance of positive- and negative-strand HCV RNA following in vitro infection of primary chimpanzee hepatocytes (8). The rTth method of RT-PCR provided 10,000- to 100,000-fold differentials between the detection of the correct and incorrect strands in assays for HCV positive- and negative-strand synthetic RNA. To further explore the possibility of extrahepatic replication of HCV, we have examined serum, liver, PBMC, and other extrahepatic tissues from HCV-infected chimpanzees and humans by using rTth RT-PCR for negative- and positive-strand HCV RNA.

Strand-specific RT-PCR. In our earlier studies, tagged RT-PCR was used for detection of negative-strand RNA and rTth RT-PCR was used for detection of positive-strand RNA (8). In this study, the rTth method was used for the detection of both strands, because it is easier to perform and yields better strand specificity than tagged RT-PCR. With rTth RT-PCR, false priming of the incorrect strand is avoided by conducting cDNA synthesis at 70°C with the thermostable rTth RT. Strand specificity requires that no RT activity remain after cDNA synthesis when both primers are added for PCR amplification. In the rTth method, this was accomplished by chelating Mn^{2+} and adding Mg^{2+}, which results in DNA polymerase activity without significant RT activity.

To demonstrate the sensitivity and specificity of the assays, synthetic positive- and negative-strand HCV RNAs (nucleotides 1 to 582) were transcribed in vitro and purified extensively to remove DNA. RNA was diluted in normal cell RNA such that all samples contained 1 μg of cell RNA. RT-PCR was performed by a modification of the previously described rTth (8) procedure for both positive- and negative-strand RNA. RNA in 10 μl was layered with mineral oil and heated to 95°C for 1 min. The temperature was lowered to 70°C, and 10 μl of preheated cDNA reaction mixture was added. The reaction mixture consisted of 10 mM Tris (pH 8.3), 90 mM KCl, 1 mM $MnCl_2$, 200 μM each deoxynucleoside triphosphate, 50 ng of cDNA primer, and 5 U of rTth (Perkin-Elmer Cetus). The temperature was dropped to 60°C for 2 min for annealing and then raised to 70°C for 15 min for the cDNA reaction. The temperature was held at 70°C until 40 μl of a prewarmed buffer containing ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (20 mM Tris [pH 8.3], 200 mM KCl, 1.5 mM EGTA, 0.1% Tween 20, and 10% glycerol) was added to chelate the Mn^{2+} and inactivate the RT activity of rTth. Reaction tubes were held at 70°C while 40 μl of prewarmed PCR mixture (50 ng of forward PCR primer in 3.75 mM $MgCl_2$) was added. The PCR conditions consisted of an initial

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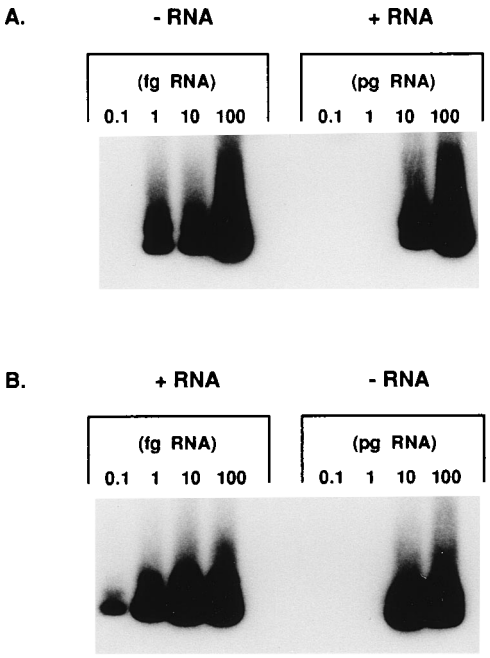

FIG. 1. Analysis of synthetic HCV RNA by strand-specific rTth RT-PCR. (A) Negative-strand-RNA assay of negative-strand (0.1 to 100 fg) and positive-strand (0.1 to 100 pg) RNA (−RNA and +RNA, respectively). (B) Positive-strand-RNA assay of positive-strand (0.1 to 100 fg) and negative-strand (0.1 to 100 pg) RNA.

cycle at 94°C for 3.5 min; 35 to 45 cycles at 94°C for 1.3 min, 60°C for 2 min, and 72°C for 3 min; and a final extension cycle at 72°C for 7 min. For positive-strand RNA, the cDNA reverse primer was 5′-TCGCGACCCAACACTACTC-3′ and the forward primer was 5′-GGGGGCGACACTCCACCA-3′. The same primers were used in reverse order for detection of negative-strand RNA. The amplified product spans nucleotides 15 to 274 of the 5′ noncoding region of HCV. One-fifth of the first-round product was analyzed by agarose gel electrophoresis and Southern hybridization with a ^{32}P-labeled probe internal to the PCR primers.

Analysis of highly purified synthetic HCV RNA with the rTth RT-PCR assays for positive- and negative-strand RNA revealed 10,000- to 100,000-fold differentials between detection of the correct and incorrect strands of RNA. In the experiment whose results are depicted in Fig. 1, the negative-strand assay detected negative-strand RNA at 1 fg and positive-strand RNA at 10 pg (Fig. 1A), while the positive-strand assay detected positive-strand RNA at 0.1 fg and negative-strand RNA at 10 pg (Fig. 1B). Either the detection of the incorrect strand is due to a lapse of strand specificity at levels 10,000-fold higher than required to detect the correct strand of RNA or it may reflect a trace contamination with the DNA of the transcriptional vector not removed by two rounds of DNase treatment and RNAzol extraction. This assay routinely yields a minimum of a 1,000-fold differential with synthetic RNA.

Analysis of chimpanzee and human sera for HCV positive- and negative-strand RNA. Sera from two acutely infected (×186 and ×187) and three chronically infected (×059, ×341, and ×174) chimpanzees were examined for HCV positive- and negative-strand RNA. The PCR titers for positive-strand RNA ranged from 10^2 to 10^5 PCR units per ml. The titers of RNA were extrapolated from the last dilution in which 10 μl was positive multiplied by 100 for PCR units per milliliter. No negative-strand HCV RNA was detected in the chimpanzee sera even with undiluted sera (Table 1). To determine whether negative-strand RNA might be present in sera having very high HCV RNA titers, two high-titer human sera were examined. One serum sample had titers of 10^6 and 10^2 PCR units per ml for positive- and negative-strand RNA, respectively. This serum sample was not routinely positive for negative-strand HCV RNA even with 10 μl of undiluted sera. The other serum sample had titers of 10^7 and 10^4 PCR units per ml for positive- and negative strand RNA, respectively; however, for this serum sample, the negative-strand signal was very weak at both of the dilutions tested (10^{-1} and 10^{-2} dilutions). Since the differentials in titer between the positive- and negative-strand-RNA assays for these sera were of the same order of magnitude as was observed with positive-strand synthetic RNA, it cannot be concluded that negative-strand RNA was definitively present in sera (see "Conclusions").

TABLE 1. Positive- and negative-strand HCV RNA in sera

Serum sample[a]	PCR titer (units/ml of sera) with[b]:	
	+RNA	−RNA
Chx059	10^5	$<10^2$
Chx174	10^3	$<10^2$
Chx341	10^4	$<10^2$
Chx186	10^2	$<10^2$
Chx187	10^4	$<10^2$
HS1	10^6	10^2
HS2	10^7	10^4

[a] RNA was purified from 10-fold dilutions of chimpanzee (Ch) and human (HS) sera prepared in fetal bovine serum. HS1 was a gift from Paul Holland and Ken Quramoto and has been previously described (8). HS2 was a gift from Robert Purcell and is the original H strain inoculum of HCV (2).

[b] rTth RT-PCR was performed for both positive-strand (+RNA) and negative-strand (−RNA) HCV RNA by using RNA prepared from 10 μl of each serial dilution. The PCR products were detected by Southern hybridization. Titers were extrapolated from the last dilution in which 10 μl was positive for PCR multiplied by 100 for a PCR titer per milliliter of sera.

Detection of HCV positive- and negative-strand RNA in the livers of HCV-infected chimpanzees. To determine the level of positive- and negative-strand HCV RNA in the liver, biopsy samples were obtained from the five chimpanzees mentioned above. Dilutions of the liver RNA were assayed for positive- and negative-strand HCV RNA. For each liver sample, positive-strand HCV RNA was detected at a 10-fold-higher level than negative-strand HCV RNA (Fig. 2). Higher levels of positive-strand RNA than negative-strand RNA have been observed in cells infected with other positive-strand RNA viruses.

Lack of detection of HCV negative-strand RNA in PBMC derived from HCV-infected chimpanzees and humans. To examine PBMC for the presence of negative-strand HCV RNA, PBMC were purified from the plasma of the five chimpanzees mentioned above. Serial dilutions of PBMC RNA were tested for the presence of positive- and negative-strand RNA. Positive-strand RNA could be detected in the dilution of PBMC RNA containing 10 ng of RNA for ×187 and 1 μg of RNA for the remaining four animals (Fig. 3). No negative-strand RNA was detected in any of the dilutions of PBMC RNA. Since a 10-fold-lower level of HCV negative-strand RNA than positive-strand RNA was detected in the liver RNA of the same animals, detection of negative-strand RNA may have been expected only in the PBMC RNA of ×187, in which case the

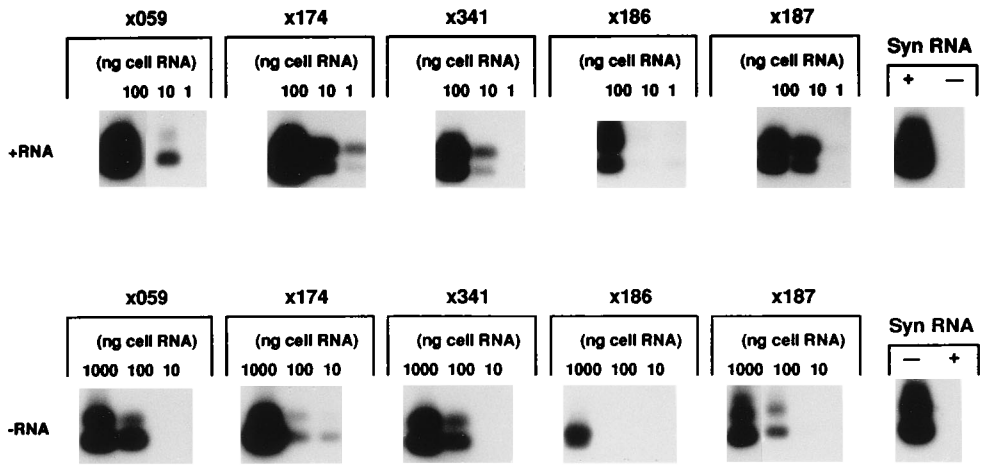

FIG. 2. Detection of positive- and negative-strand HCV RNA in chimpanzee liver RNA. Total cell RNA was purified from liver biopsy samples from three chronically HCV-infected chimpanzees (×059, ×174, and ×341) and two chimpanzees during acute infection (×186 and ×187). Tenfold dilutions of liver RNA were prepared in normal cell RNA to yield samples with 1,000 to 1 ng of liver RNA and 1 μg of normal cell RNA per 10 μl. All assays included several negative controls consisting of normal cellular RNA. In addition, assays were controlled by amplification of 1 or 10 fg of the correct strand (sensitivity control) and 100 fg of the incorrect strand (specificity control) of synthetic HCV RNA (Syn RNA). Serum, plasma, and liver needle biopsy samples were taken by standard methods under a protocol approved by the institutional animal research committee.

level of positive-strand RNA was sufficiently high to expect detection of negative-strand RNA.

To extend these observations to HCV-infected humans, PBMC RNA was purified from 10 HCV-infected individuals. In general, higher levels of positive-strand RNA were detected in human PBMC than in chimpanzee PBMC. Positive-strand HCV RNA was detected at titers of 10^3 PCR units per μg of PBMC RNA for two of the PBMC samples, 10^2 PCR units per μg of PBMC RNA for three of the PBMC samples, and 10^1 PCR units per μg of PBMC RNA for two of the PBMC samples. No positive-strand RNA was detected in the RNA from three of the PBMC preparations. No negative-strand RNA was detected in any of the PBMC RNA preparations (Table 2 and Fig. 4). If positive-strand RNA were present at 10-fold-higher levels than negative-strand RNA, as in the liver, negative-strand RNA should have been detectable in five of the PBMC samples. If present, negative-strand RNA must have been at a level at least 1,000-fold lower than that of positive-strand RNA for the two PBMC samples with the highest titers of positive-strand RNA.

Analysis of extrahepatic tissues for the presence of HCV positive- and negative-strand RNA. To extend the observations to other extrahepatic tissues, multiple tissues were obtained from a chimpanzee that was euthanized for humanitarian reasons because of kidney failure. The kidney failure may have been due to long-term HCV infection (6, 7, 12). Tissues were obtained precisely at the time of death and frozen in liquid nitrogen. Serial dilutions of liver RNA were examined, while all other tissues were analyzed at 1 μg of RNA per PCR. An intense reaction for positive-strand RNA was obtained with 10 ng of liver RNA. For all other tissues, positive-strand RNA was detected from 1 μg of RNA with various degrees of intensity (Fig. 5). The reactions for the spleen, muscle, and lymph nodes were the most intense. The pancreas and kidney were weakly positive with a short exposure of the autoradiogram (4 h [Fig.

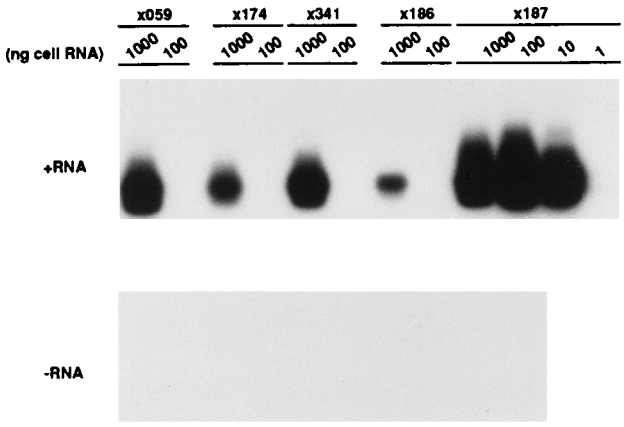

FIG. 3. Lack of detection of negative-strand HCV RNA in PBMC from chimpanzees infected with HCV. PBMC were prepared from the same chimpanzees described in the legend to Fig. 2, and dilutions of the PBMC RNA were subjected to strand-specific RT-PCR for HCV positive-strand (+RNA) and negative-strand (−RNA) RNA. PBMC were isolated on Histopaque 1077, and the PBMC layer was washed three times with serum-free RPMI 1640.

TABLE 2. Lack of detection of negative-strand HCV RNA in human PBMC

PBMC sample[a]	PCR titer (units/μg of RNA) with[b]:	
	+RNA	−RNA
1	10^3	−
2	10^2	−
3	−	−
4	−	−
5	10^2	−
6	10^3	−
7	10^2	−
8	10^1	−
9	10^1	−
10	−	−

[a] Total cell RNA was prepared from purified PBMC, and 10-fold dilutions of 1 μg of cell RNA were prepared in normal cell RNA.

[b] rTth RT-PCR was performed for positive-strand (+RNA) and negative-strand (−RNA) HCV RNA. The PCR titer represents the highest positive dilution. The largest amount of RNA tested was 1 μg; samples negative at this level are designated by a minus sign.

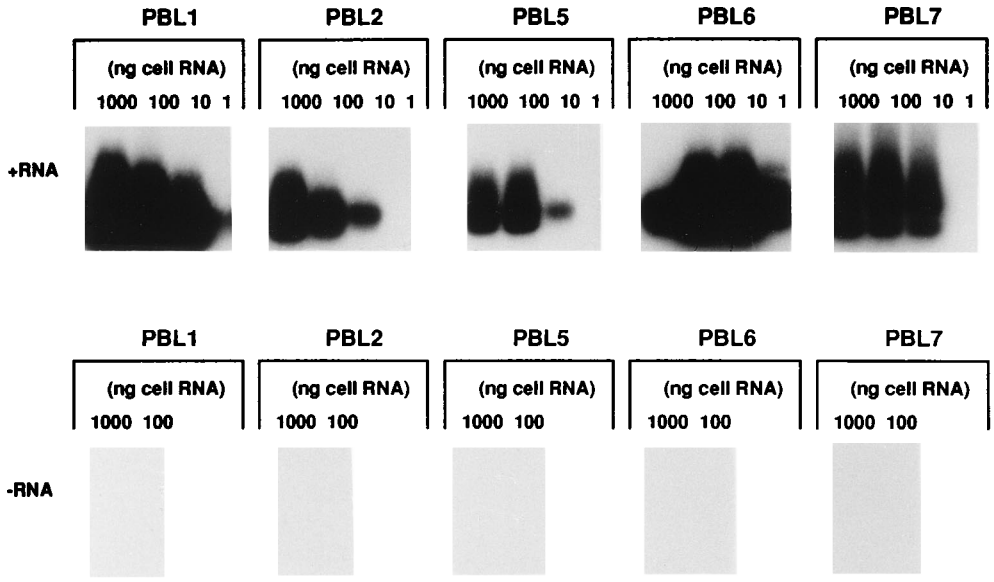

FIG. 4. Lack of detection of negative-strand HCV RNA in PBMC from humans infected with HCV. PBMC RNA was purified, diluted in normal cell RNA, and subjected to strand-specific RT-PCR for positive-strand (+RNA) and negative-strand (−RNA) HCV RNA. PBMC were obtained from 10 patients with chronic HCV infection. The diagnosis of chronic infection was based on standard clinical parameters and serological assays. All patients were anti-HCV antibody positive as determined by the second-generation (c200/c22-3) HCV ELISA test system (Ortho Diagnostics, Inc., Raritan, N.J.) as previously described (1). PBMC were separated on Ficoll-Hypaque density gradients, washed three times in Hank's balanced salt solution, and resuspended with RPMI 1640 as previously described (1). Peripheral blood lymphocyte (PBL) samples correspond to the PBMC samples in Table 2.

5A]), while detection of the reaction for PBMC and bone marrow required a longer exposure (data not shown). Negative-strand RNA was not detected in any tissue other than liver, which was positive with 100 ng of RNA (Fig. 5B). These data are consistent with the virus being present in other tissues because of contamination with serum.

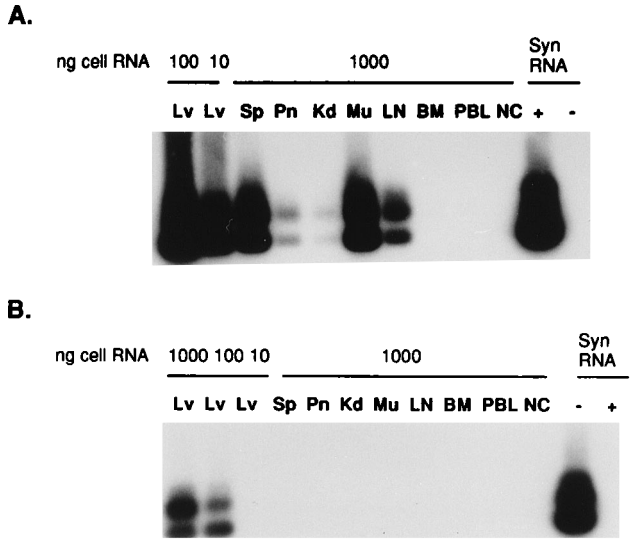

FIG. 5. Lack of detection of negative-strand HCV RNA in extrahepatic tissues from a chronically HCV-infected chimpanzee. (A) Positive-strand-RNA assay. (B) Negative-strand-RNA assay. Tissues were obtained from a chimpanzee immediately at the time of death and were frozen in liquid nitrogen. Tenfold dilutions of RNA from each tissue were prepared in normal cell RNA as described in Fig. 2. Liver RNA (Lv) was examined at 100 and 10 ng for positive-strand HCV RNA (A) and at 1,000 to 10 ng for negative-strand HCV RNA (B). RNA samples from other tissues were examined at 1,000 ng, including spleen (Sp), pancreas (Pn), kidney (Kd), muscle (Mu), lymph node (LN), bone marrow (BM), and PBL. Positive-strand (+) and negative-strand (−) synthetic HCV RNAs (Syn RNA) were included as controls for strand specificity and sensitivity, as described in the legend to Fig. 2.

Conclusions. Whether extrahepatic replication occurs is an important question with regard to the transmission and pathogenesis of HCV infections. Several investigations have reported the detection of HCV negative-strand RNA in PBMC (3, 4, 10, 11, 13, 15), with detection of high levels of negative-strand RNA in plasma in approximately 50% of the studies. In most instances, the differentials, if determined, between negative- and positive-strand RNA in PBMC and sera were 10- to 100-fold. In this study, PBMC from 10 humans chronically infected with HCV were examined. For the PBMC from five of these patients, the level of positive-strand RNA was quite high yet no negative-strand RNA could be detected. The data suggest that if negative-strand RNA were present in these PBMC, it was present at 100- to 1,000-fold-lower levels than positive-strand RNA.

The potential for extrahepatic replication was also examined in a series of tissues from a chimpanzee chronically infected with HCV. Low levels of positive-strand RNA were detected in all tissues examined, but no negative-strand RNA was detected. The detection of positive-strand RNA in all tissues in the absence of detectable negative-strand RNA suggests that the positive-strand RNA may be due to contamination with circulating virus.

Although these data do not exclude the potential for replication of HCV in extrahepatic tissue, they do suggest that several criteria should be met in order to determine whether replication occurs. First, titration assays should be performed with synthetic RNA in the presence of a high level of heterologous RNA to determine the degree of strand specificity and the level of sensitivity for the RT-PCR assay being employed. Second, the cellular RNA samples should be titrated to determine the endpoints of detection for positive- and negative-strand RNA and thus the ratio of negative- to positive-strand RNA. Negative-strand RNA must be detected at a level higher

than can be accounted for by false priming of the incorrect strand before replication can be definitively documented. In this respect, our examination of sera from HCV-infected humans is a good example of borderline results. Low levels of negative-strand RNA were detected in two human serum samples that had exceptionally high titers of positive-strand RNA. Since the level of negative-strand RNA was 1,000- to 10,000-fold lower than the level of positive-strand RNA, and the differential in strand specificity with synthetic RNA was in the same order of magnitude, it cannot be concluded that negative-strand RNA was detected in plasma. The detection of negative-strand RNA in plasma has been attributed to the release of replicative complexes from damaged hepatocytes. However, this argument also suggests that any negative-strand RNA detected in PBMC could be accounted for by adherence of circulating negative-strand RNA. Thus, in this case, to document replication of HCV in PBMC would require detection of a higher ratio of negative- to positive-strand RNA in PBMC than is detected in plasma from the same samples.

Certainly, resolution of the dilemma created by the need for RT-PCR to detect HCV, and of the problem of using RT-PCR for strand-specific detection of RNA, will require additional studies to resolve the problem of extrahepatic HCV replication.

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