

## ***In vitro* infection of adult normal human hepatocytes in primary culture by hepatitis C virus**

Chantal Fournier,<sup>1</sup> Camille Sureau,<sup>2</sup> Joliette Coste,<sup>3</sup> Jacques Ducos,<sup>4</sup> Georges Pageaux,<sup>5</sup> Dominique Larrey,<sup>1,5</sup> Jacques Domergue<sup>1,5</sup> and Patrick Maurel<sup>1</sup>

<sup>1</sup>INSERM U128, CNRS, 1919 Route de Mende, 34293 Montpellier cedex 5, France

<sup>2</sup>CNRS URA 1487, Montpellier, France

<sup>3</sup>Laboratoire de Biologie Moléculaire, ETS Languedoc Roussillon, Montpellier, France

<sup>4</sup>Unité Fonctionnelle des Hépatites Virales, Hôpital Lapeyronie, Montpellier, France

<sup>5</sup>Institut des Maladies de l'Appareil Digestif, Hôpital Saint Eloi, Montpellier, France

***In vitro* infection of adult normal human hepatocytes in primary culture has been performed for investigating the replication cycle of hepatitis C virus (HCV) in differentiated cells. Hepatocytes were prepared from liver tissue resected from donors who tested negative for HCV, and inoculation was performed 3 days after plating with 33 HCV serum samples of different virus load and genotype. The presence of intracellular HCV RNA, detected by a strand-specific *r7th* RT-PCR assay, was used as evidence of infection. A kinetics analysis of HCV replication revealed that intracellular negative-strand RNA appeared at day 1 post-infection with a maximum level at days 3 and 5, followed by a decrease until**

**day 14. At day 5, we estimated that the copy level of viral RNA was amplified at least 15-fold in infected cells. The level of intracellular HCV RNA in response to different serum samples was reproducible from one hepatocyte culture to another, suggesting that there is no inter-individual variability in the susceptibility of hepatocytes to HCV infection. These findings indicate that adult human hepatocytes in primary culture retain their susceptibility to *in vitro* HCV infection and support HCV RNA replication. This model should represent a valuable tool for the study of initial steps of the HCV replication cycle and for the evaluation of antiviral molecules.**

### **Introduction**

Hepatitis C virus (HCV) is a major causative agent of blood-transmitted hepatitis associated with the development of cirrhosis and hepatocellular carcinoma (Alter *et al.*, 1989; Choo *et al.*, 1989; Saito *et al.*, 1990). This virus is a new member of the *Flaviviridae* family (Miller & Purcell, 1990; Choo *et al.*, 1991; International Union of Microbiological Societies, 1995; Houghton, 1996). Its genome is a 9.4 kb single-stranded positive-sense RNA encoding a large polyprotein which is cleaved into structural and non-structural proteins (Houghton *et al.*, 1991; Grakoui *et al.*, 1993). The HCV replication cycle is poorly understood because its study has been hampered by the low titres of infectious virus present in serum or liver tissue and by the lack of a culture system capable of supporting efficient

virus replication. Despite recent progress made in this direction, efforts in developing a cell culture system susceptible to infection and permissive for replication of HCV remain a priority. *In vitro* culture systems for HCV infection have been described for peripheral blood mononuclear cells (Bouffard *et al.*, 1992; Zignego *et al.*, 1992; Müller *et al.*, 1993; Willems *et al.*, 1994; Cribier *et al.*, 1995), human B and T cell lines (Shimizu *et al.*, 1992, 1993; Bertolini *et al.*, 1993; Shimizu & Yoshikura, 1994; Kato *et al.*, 1995; Nakajima *et al.*, 1996) and human hepatocyte cell lines (Tagawa *et al.*, 1995; Kato *et al.*, 1996; Seipp *et al.*, 1997). Efficient long term *in vitro* replication has not been demonstrated and the results obtained with conventional RT-PCR assays for detection of replicating viral RNA must be interpreted with care (Willems *et al.*, 1993; Lanford *et al.*, 1994; McGuinness *et al.*, 1994; Lerat *et al.*, 1996; Laskus *et al.*, 1997). Although evidence of infection of non-hepatic cells has been reported (Lerat *et al.*, 1996), HCV is a hepatotropic virus with, presumably, a preference for infection of differentiated hepato-

**Author for correspondence:** Patrick Maurel.

Fax +33 4 67 52 36 81. e-mail maurel@crbm.cnrs-mop.fr

cytes (Takehara *et al.*, 1992; Nouri-Aria *et al.*, 1993; Gunji *et al.*, 1994; Lanford *et al.*, 1995; Laskus *et al.*, 1997). Primary cultures of chimpanzee and human foetal hepatocytes have been shown to be susceptible to HCV infection (Carloni *et al.*, 1993; Iacovacci *et al.*, 1993, 1997; Lanford *et al.*, 1994) and primary hepatocytes isolated from patients with chronic HCV infection were used for production of HCV particles *in vitro*. In the latter case, both positive- and negative-strand RNA was detected as well as the HCV core protein (Ito *et al.*, 1996).

In this report, we demonstrate the *in vitro* infection of adult human hepatocytes in primary culture using 33 separate serum samples from HCV-infected patients as inocula. To demonstrate infection, a strand-specific assay performed with the thermostable enzyme *rTth* (Lanford *et al.*, 1994) was used.

## Methods

**Primary cultures of human hepatocytes.** Hepatocytes were prepared either from liver lobectomy segments taken from adult patients for medical purposes unrelated to our research program or from a donor liver that had not been used for transplantation. The use of human hepatic specimens for the present study has been approved by the French National Ethics Committee. These patients tested negative for HCV (IMX, Abbott), hepatitis B virus (HBV) and human immunodeficiency virus (HIV) (VIDAS, Biomerieux) antibodies. Hepatocytes were isolated according to the previously published procedure (Pichard *et al.*, 1992). Viability, assessed by the trypan blue exclusion test, was between 70% and 90%. For each culture, hepatocytes were plated at confluence ( $14 \times 10^4$  cells/cm<sup>2</sup>) in 60 mm culture dishes precoated with collagen (Corning) in a total volume of 3 ml standard culture medium consisting of Williams' E and Ham's F-12 (Sigma) (1/1 in volume) supplemented as recommended (Isom & Georgoff, 1984). For the first 4 h, 5% foetal calf serum (Gibco) was present in the medium to favour cell attachment. The standard medium was then replaced with 3 ml of a serum-free medium as previously described (Lanford *et al.*, 1989; Ferrini *et al.*, 1997). Complete medium changes were then effected 24 h later and every 2–3 days thereafter. Cultures were incubated at 37 °C and 5% CO<sub>2</sub>.

**Inocula.** Thirty-three serum samples from HCV carriers were analysed. All patients were anti-HCV antibody-positive as detected by the EIA HCV 3.0 and Chiron RIBA HCV 3.0 SIA (Chiron Corporation) and in every serum sample HCV RNA was quantified and genotyped by a branched DNA (bDNA) assay (Quantiplex HCV RNA 2.0 assay; Chiron Diagnostics) and a line probe assay (Inno-LiPA HCV II, Innogenetics), respectively. In addition, HCV RNA was quantified in some serum samples using the Amplicor HCV Monitor test procedure (Roche Diagnostic Systems). None of the patients had received antiviral therapy prior to the study, and none was co-infected with HBV or HIV. All sera were stored at –80 °C until use.

**In vitro infections.** Infections were performed 3 days after plating by overnight incubation of cell monolayers with 100 µl inoculum in 3 ml culture medium. Following exposure, cells were washed three times with 3 ml Williams' E medium and incubated in 3 ml fresh serum-free medium. The medium was changed every 2–3 days until harvest. Cells were collected at various times during the culture period and stored at –80 °C.

**Analysis of viral RNA.** Positive- and negative-strand HCV RNA controls were generated from a pSP73 vector containing the full-length

HCV genome (nt 1–9401) (Lanford *et al.*, 1994). The plasmid was first digested by restriction enzymes *EcoRV* (New England Biolabs) and *KpnI* to obtain a DNA fragment (nt 1–582) containing the 5' non-translated region of the HCV genome. This fragment was inserted in between the *EcoRV* and *KpnI* restriction sites of plasmid pSP73 (Promega). The resulting plasmid DNA was linearized by *PvuII* and transcribed *in vitro* using SP6 RNA polymerase to produce synthetic positive-strand HCV RNA. Digestion with *EcoRV* and transcription by T7 RNA polymerase resulted in the synthesis of negative-strand HCV RNA. The DNA template was removed by extensive DNase digestion (DNase RQ1, Promega) and the absence of residual DNA was ascertained by performing the cDNA synthesis step of the *rTth* RT-PCR assay in the absence of specific primer. Dilutions of the synthetic RNA were made and 1 µg total cellular RNA extracted from normal livers was added to each reaction to mimic the conditions for analysis of cultured hepatocytes.

At the time of harvest, the medium was removed and the cultures were washed three times with PBS. RNA was purified either from 100 µl inoculum or from  $4 \times 10^6$  hepatocytes, using a guanidinium isothiocyanate acid–phenol extraction procedure (Chomczynski & Sacchi, 1987). The precipitated RNA was dissolved in 50 µl diethyl pyrocarbonate (DEPC)-treated water and quantified. One-fifth of the RNA extracted from the inoculum or 1 µg cellular RNA (1/70th to 1/150th of total extract) was analysed in our strand-specific *rTth* RT-PCR assay.

The strand-specific *rTth* RT-PCR assay was based on the thermostable reverse transcriptase *rTth* procedure (Lanford *et al.*, 1995). RNA dissolved in 10 µl DEPC-treated water was covered with mineral oil and heated at 95 °C for 1 min. The temperature was lowered to 70 °C and 10 µl preheated cDNA reaction mixture was added. The temperature was then dropped to 60 °C for 2 min for annealing and the cDNA reaction was performed for 15 min at 70 °C using the *rTth* DNA polymerase (Perkin-Elmer). The temperature was maintained at 70 °C while 40 µl prewarmed buffer containing EGTA as chelator of Mn<sup>2+</sup> was added to suppress the *rTth* RT activity. Reaction tubes were held at 70 °C while 40 µl prewarmed PCR mixture was added. The PCR conditions, performed on GeneAmp PCR System 9600 (Perkin-Elmer), consisted of an initial cycle at 94 °C for 1 min, 35 cycles at 94 °C for 20 s, 60 °C for 30 s, 72 °C for 30 s and a final extension step at 72 °C for 7 min. For positive-strand HCV RNA assay, the nucleotide sequence of the reverse primer P1 was: 5' TCGCGACCCAACACTACTC 3' (nt 274–256), and that of the forward primer P2 was: 5' GGGGGCGACACTCCACCA 3' (nt 15–32) (Lanford *et al.*, 1995). The same primers were used in reverse order for the detection of negative-strand RNA. In order to detect genotype 2 HCV RNA, primers P3 (5' TGG/ATGCACGGTCTACG-AGACCTC 3', nt 342–320) and P4 (5' CACTCCCCTGTGAGGAACT 3', nt 38–56) described by Laskus *et al.* (1997) were used. One-fifth of the PCR-amplified product was analysed by agarose gel electrophoresis followed by Southern hybridization with a <sup>32</sup>P-labelled HCV-specific probe (nt 138–167) internal to the PCR primers.

## Results

### Cell culture conditions

Four separate hepatocyte cultures were prepared from four different patients. The culture system used in this work was based on the one described previously for baboon and chimpanzee primary hepatocyte cultures (Lanford *et al.*, 1989; Jacob *et al.*, 1989). Slight modifications have been introduced, however, to take into account the specificity of human hepatocytes. We recently reported that, under these con-

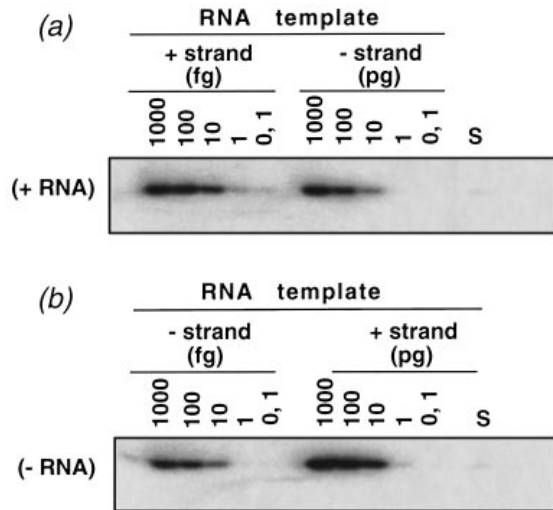


Fig. 1. Analysis of synthetic HCV RNA by the strand-specific *rTth* RT-PCR assay. (a) Positive-strand RNA assay (+RNA). Synthetic positive- (1000, 100, 10, 1 and 0.1 fg) and negative-strand (1000, 100, 10, 1 and 0.1 pg) HCV RNA was subjected to the *rTth* RT-PCR assay and the products were analysed by gel electrophoresis and Southern blot hybridization using a  $^{32}\text{P}$ -labelled probe internal to the amplification primers. (b) Negative-strand RNA assay (-RNA). Synthetic negative- (1000, 100, 10, 1 and 0.1 fg) or positive-strand (1000, 100, 10, 1 and 0.1 pg) HCV RNA was analysed by the same procedure. S, 20 pg HCV DNA fragment (nt 15–274) as Southern blot DNA control.

ditions, human hepatocytes retain several phenotypical characteristics of the adult human liver for at least 35 days (Ferrini *et al.*, 1997). During the present work no change in cell morphology was observed, as assessed by phase-contrast microscopy during the 14 days of culture following HCV infection.

#### Sensitivity and specificity of *rTth* RT-PCR assays

HCV is a positive-stranded RNA virus and its replication is thought to occur via the synthesis of a negative (complementary)-strand RNA intermediate which, in turn, is used as a template for the synthesis of the positive strand. Since the positive strand is the only form of viral RNA present in the inoculum, detection of the negative strand in infected cells is indicative of active viral RNA replication and thus demonstrates infection. Using the *rTth* RT-PCR assay, false priming of the incorrect strand is much reduced by conducting cDNA synthesis at 70 °C with the thermostable *rTth* enzyme. To assess the sensitivity and specificity of the method, synthetic positive and negative strands of HCV RNA encompassing the 5' non-coding region (nt 1–582) were transcribed *in vitro* and purified extensively to remove the DNA template before they were used as standards in our assays. The results presented in Fig. 1 (a) (positive-strand RNA assay) and Fig. 1 (b) (negative-strand RNA assay) indicate that, under these conditions, we were able to detect 1 fg of the correct-strand RNA ( $3 \times 10^3$  molecules), while at least 1–10 pg of the incorrect-strand RNA was required to produce a signal (3–4 log of specificity). The

addition of 1 µg cellular RNA to mimic cellular extract conditions reduced the sensitivity by no more than 1 log and did not affect the specificity of the assay. Although the assay is not quantitative, the use of a single-round PCR followed by Southern hybridization resulted in marked differences in the signal obtained with 1000, 100, 10 and 1 fg RNA, respectively.

#### Analysis of positive- and negative-strand HCV RNA in human serum samples

A total of 33 serum samples to be used as the inocula for *in vitro* infection was obtained from 32 patients. Two serum samples, 23 and 33, were collected from the same patient 8 months apart. For every patient, the serum sample was characterized for HCV genotype and viraemia using conventional methods. The genotypes were 1a or 1b in 73% of cases (24/33), 2, 2a or 2a/2c in 15% (5/33), and 3 in 10% (3/33). The viraemia was highly variable between an undetectable level and > 120 Meq/ml ( $10^6$  genome equivalents/ml). These serum samples were also analysed with our *rTth* RT-PCR strand-specific assay using primers P1 and P2. The results are presented in Fig. 2 (a, b). After *rTth* RT-PCR reaction and hybridization with a  $^{32}\text{P}$ -labelled HCV-specific probe, the blots were scanned in a PhosphorImager for quantification. For samples of genotypes 1 and 3, no signal was detected when the titre was estimated at < 1.7 Meq/ml by bDNA assay. Overall, there was a very significant correlation (Fisher's R to Z correlation coefficient 0.771,  $P < 0.0001$ ) between the *rTth* RT-PCR positive-strand RNA signals (Fig. 2a, b) and the bDNA viraemia. No signal was detected for serum samples with HCV genotype 2 (see samples 4, 6, 18, 31 and 32 in Fig. 2a, b). This may result from the subtype-specific polymorphism of the 5' non-coding region as reported recently (Smith *et al.*, 1995). New primers (P3 and P4) were therefore used in order to detect genotype 2 HCV RNA (Laskus *et al.*, 1997) and the results are reported in Fig. 2(c). The *rTth* RT-PCR assay using this new set of primers was estimated as described in Fig. 1 for primers P1 and P2. Similar results were obtained in terms of sensitivity and specificity (data not shown). Using these primers, the positive strand was then detected in samples 4, 6, 18 and 31, but serum 32, which exhibited a bDNA titre < 1.0 Meq/ml, remained negative. As expected from recent findings (Lanford *et al.*, 1995; Laskus *et al.*, 1997), the negative strand was detected in none of these serum samples, in spite of a very high level of positive-strand RNA in some of them (samples 12, 13, 19, 20, 21, 29, 30 and 31).

#### Kinetics of viral RNA replication in HCV-infected human hepatocytes

For *in vitro* infections, hepatocyte cultures were inoculated with 100 µl HCV-positive serum added directly to the culture medium as described in Methods. In a preliminary study (culture FT120), serum samples from patients 1–25 were

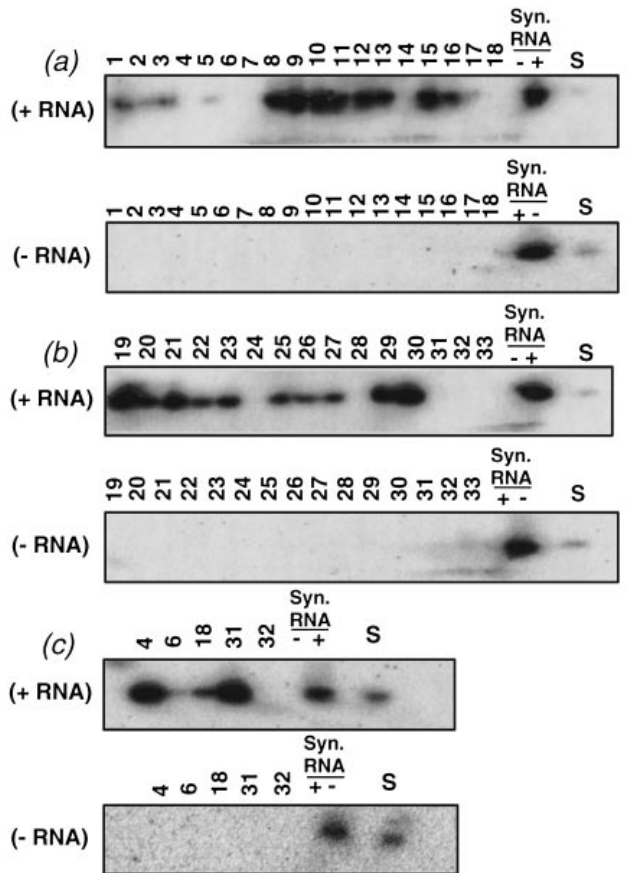


Fig. 2. Detection of HCV RNA in human serum samples using a strand-specific *rTth* RT-PCR assay. Genotype data and HCV RNA titre (Meq/ml) of each inoculum are as follows: 1 (1b, 2·82), 2 (1b, 0·31), 3 (1b, 3·5), 4 (2a, 3·27), 5 (1a, 2·05), 6 (2a, 1·59), 7 (1a, 1·03), 8 (1b, 22·69), 9 (1b, 4·33), 10 (3, 4·57), 11 (1b, 6·67), 12 (1b, 64·65), 13 (1a, 32·40), 14 (1b, 1·75), 15 (1b, 11·76), 16 (1b, 5·45), 17 (1b, 41·89), 18 (2a/2c, 3·98), 19 (1a, 49·47), 20 (1b, > 120), 21 (3a, > 120), 22 (1b, 11·42), 23 (1b, 104·5), 24 (3, 0·27), 25 (1b, 5·4), 26 (1b, 29·14), 27 (1b, > 120), 28 (undefined, 0), 29 (1b, 41·07), 30 (1a, 112), 31 (2, 50·14), 32 (2a/2c, 0·87), 33 (1b, 0·59). One-fifth of the RNA extracted from 100 µl serum 1–18 (a) and 19–33 (b) was analysed by the strand-specific *rTth* RT-PCR assay using primers P1 and P2. RT-PCR products were analysed by gel electrophoresis and Southern blot hybridization using a <sup>32</sup>P-labelled probe internal to the amplification primers. Strand-specific assays were standardized by amplification of 100 fg of the incorrect strand for specificity control and 10 fg of the correct strand for sensitivity control of synthetic HCV RNA (Syn RNA) as negative and positive markers, respectively, and 1/5 of each reaction was loaded on the gel. All reactions were performed in the presence of 1 µg cellular RNA to approximate the conditions used to detect HCV RNA in infected hepatocytes. Specific detection of genotype 2 HCV RNA in serum samples 4, 6, 18, 31 and 32 was performed using primers P3 and P4 (c). S, 20 pg HCV DNA fragment (nt 15–274) as Southern blot DNA control. + RNA, Positive-strand RNA assay; –RNA, negative-strand RNA assay; +, positive-strand synthetic HCV RNA; –, negative-strand synthetic HCV RNA.

tested. Intracellular negative-strand HCV RNA was detected at 13 days post-inoculation with serum samples 8, 12, 13, 17, 19, 20, 23 (data not shown). A kinetics study was then conducted using a new culture (FT122) and inocula 23 and 19, which produced, respectively, a strong and a low intracellular

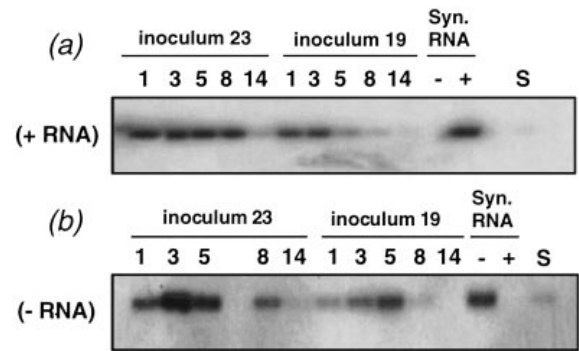


Fig. 3. Kinetic analysis of positive- and negative-strand HCV RNA in infected hepatocytes. Cells (FT122) were infected 3 days after plating with 100 µl human sera 23 and 19 (see Fig. 2), which produced, respectively, a strong and a weak negative-strand RNA signal 13 days post-infection in infected cells with a previous culture (FT120). Total cellular RNA was harvested at the indicated times after inoculation and analysed for HCV positive- (a) and negative-strand (b) RNA using *rTth* RT-PCR. Positive-strand (+) and negative-strand (–) synthetic HCV RNAs were included as markers for strand specificity and sensitivity, as described in the legend to Fig. 2. S, 20 pg HCV DNA fragment (nt 15–274) as Southern blot DNA control. + RNA, Positive-strand RNA assay; –RNA, negative-strand RNA assay.

negative-strand RNA signal at day 13 post-infection in culture FT120. The results are reported in Fig. 3(a) (positive-strand RNA assay) and Fig. 3(b) (negative-strand RNA assay). Positive-strand RNA was detected at day 1 and was maintained for 8 and 3 days in cells infected with serum samples 23 and 19, respectively. It then gradually decreased to become undetectable after day 14. In contrast, the negative strand exhibited transient expression, being detectable as early as day 1, reaching a maximum at day 3 and 5 in cells infected with sera 23 and 19, respectively, and becoming undetectable at day 14, in this culture.

In addition, in order to evaluate the efficiency of infection and the level of intracellular HCV replication, we measured the number of viral RNA molecules in inoculum 23 and in cells harvested at day 5 post-infection using our positive-strand *rTth* RT-PCR assay. By comparison with a known amount of synthetic HCV RNA, we estimated that 100 µl serum 23 contained approximately  $1·5 \times 10^5$  copies of HCV RNA. This result was confirmed with the Amplicor HCV monitor test procedure ( $1·6 \times 10^6$  copies/ml). Using the same method, we evaluated the number of intracellular HCV RNA copies at day 5 post-infection at  $2–4 \times 10^6$ . Compared with the amount of RNA in the inoculum ( $1·5 \times 10^5$  copies) this represents a 15–25-fold amplification.

#### Detection of positive- and negative-strand HCV RNA in hepatocytes infected with a panel of 33 serum samples

Human hepatocytes were plated and inoculated with 33 separate serum samples under the conditions described in

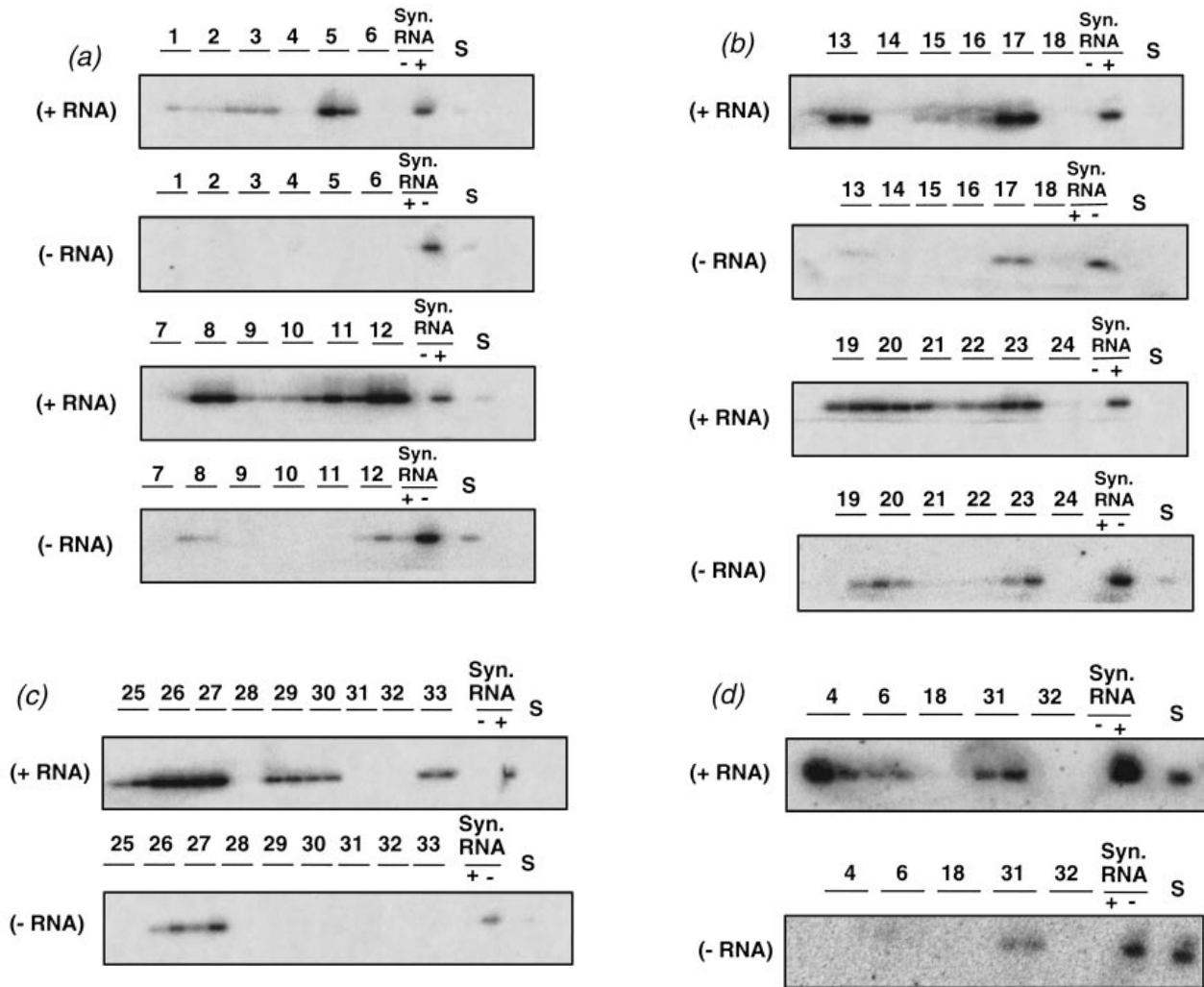


Fig. 4. *In vitro* infectivity analysis of 33 characterized serum samples. Infections (FT128) were performed in duplicate, 3 days after plating, with 100  $\mu$ l serum 1–12 (a), 13–24 (b) and 25–33 (c). Five days after infection with 33 separate serum samples, hepatocytes were harvested, RNA was purified and 1  $\mu$ g cellular RNA was analysed by *rTth* RT–PCR using primers P1 and P2. Strand-specific assays were standardized by amplification of 100 fg of the incorrect strand (specificity) and 10 fg of the correct strand (sensitivity) of synthetic HCV RNA (Syn RNA) as negative and positive markers, respectively. Similar analysis was carried out with primers P3 and P4 in order to detect the genotype 2 HCV RNA in cells infected with serum samples 4, 6, 18, 31 and 32 (d). S, 20 pg HCV DNA fragment (nt 15–274) as Southern blot DNA control. + RNA, Positive-strand RNA assay; – RNA, negative-strand RNA assay; +, positive-strand synthetic HCV RNA; –, negative-strand synthetic HCV RNA.

Methods. All experiments were carried out in duplicate. At day 5 post-infection, when maximum expression of the negative strand occurs, as revealed by the previous experiments (Fig. 3), cells were harvested and the total cellular RNA was extracted and analysed with the strand-specific *rTth* RT–PCR assays for the presence of positive- and negative-strand HCV RNA. The results obtained with culture FT128 are presented in Fig. 4, but similar results were obtained with cultures FT122 and FT126 (data not shown).

Positive-strand HCV RNA was detected by *rTth* RT–PCR using primers P1 and P2 in cells infected with all serum samples except samples 4, 6, 7, 14, 18, 24, 28, 31 and 32. These either contained a very low amount of HCV RNA or belonged to

genotype 2 (Fig. 2). In general, there was good agreement between serum and intracellular positive-strand HCV RNA signals (Figs 2a, b and 4a–c). For example, inocula which exhibited a bDNA HCV RNA titre of between 20 and 120 Meq/ml (samples 8, 12, 13, 17, 19, 20, 21, 23, 26, 27, 29 and 30) gave rise to a strong intracellular positive-strand HCV RNA signal, whereas inocula with titres less than 20 Meq/ml (1, 2, 3, 7, 9, 10, 15 and 16) led to lower signals in infected cells. In contrast, strong signals for intracellular positive-strand RNA were obtained after infection with sera of low or moderate HCV RNA titre, as observed with samples 5, 25 and 33. When infection experiments with genotype 2 inocula 4, 6, 18, 31 and 32 were analysed using primers P3 and P4 in the *rTth* RT–PCR

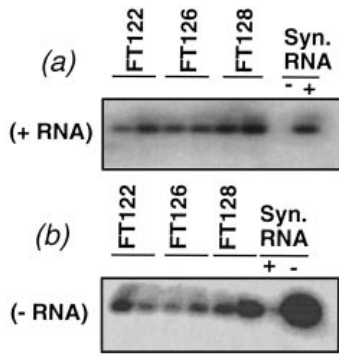


Fig. 5. Lack of variability in the susceptibility of hepatocyte cultures to HCV. Analysis of positive-strand (a) and negative-strand (b) HCV RNA was performed from cellular RNA of hepatocyte cultures FT122, FT126 and FT128 harvested 5 days after infection with serum sample 23. One hundred fg of the incorrect strand and 10 fg of the correct strand of synthetic (Syn) RNA was included in the assays as standard. +RNA, Positive-strand RNA assay; -RNA, negative-strand RNA assay; +, positive-strand synthetic HCV RNA; -, negative-strand synthetic HCV RNA.

assay (Laskus *et al.*, 1997), intracellular positive-strand RNA was detected after inoculation with sera 4, 6 and 31 only (Fig. 4d).

Intracellular negative-strand HCV RNA signals were detected in hepatocytes infected with only 9 serum samples (8, 12, 13, 17, 19, 20, 23, 26 and 27) in the *rTth* RT-PCR assay using primers P1 and P2 (Fig. 4a-c). These inocula all exhibited a high HCV titre (> 22 Meq/ml) and belong to genotype 1a or 1b (Fig. 2). Nevertheless, using primers P3 and P4 in the *rTth* RT-PCR assay, we could detect an intracellular negative-strand RNA signal in hepatocytes infected with serum 31 also of HCV RNA titre > 22 Meq/ml (Figs 2 and 4d). However, it should be noted that sera 21, 29 and 30 of high HCV RNA titre did not lead to the detection of intracellular negative-strand HCV RNA after inoculation (Figs 2 and 4c). It appears therefore that a high titre of HCV RNA in the inoculum is necessary but not sufficient for *in vitro* infection of human hepatocytes in primary culture.

In order to demonstrate the reproducibility of infection *in vitro* with a given inoculum, three different cultures, FT122, FT126 and FT128, were infected with serum sample 23, and analysed using the same procedure. The results are presented in Fig. 5(a) (positive-strand RNA assay) and Fig. 5(b) (negative-strand RNA assay). Positive- and negative-strand HCV RNA was detected at day 5 after infection with serum 23 in all three cultures. It clearly appeared that all these cultures were susceptible to infection in addition to culture FT120. Similar observations were made with all other infectious sera.

## Discussion

Here we demonstrate that primary cultures of adult human hepatocytes prepared from uninfected patients can sustain HCV infection *in vitro* and support at least in part the

replication of HCV RNA. The use of a strand-specific *rTth* RT-PCR assay clearly revealed the presence of both positive- and negative-strand HCV RNAs as evidence of infection in cells inoculated with 10 out of 33 different sera. In addition, infectivity assays performed on separate cultures using the same inocula led to consistent results, demonstrating the reliability of these cultures in their susceptibility to infection *in vitro*.

Previous reports have described different systems for the propagation of HCV *in vitro* including: (i) primary cultures of hepatocytes from experimentally infected chimpanzees (Jacob *et al.*, 1990) or from chronically infected patients (Ito *et al.*, 1996); (ii) normal primary culture of hepatocytes from chimpanzee (Lanford *et al.*, 1994) or human foetus (Carloni *et al.*, 1993; Iacovacci *et al.*, 1993, 1997); and (iii) human hepatocyte or non-hepatocyte cells lines (Bouffard *et al.*, 1992; Shimizu *et al.*, 1992, 1993; Zignego *et al.*, 1992; Bertolini *et al.*, 1993; Müller *et al.*, 1993; Shimizu & Yoshikura, 1994; Willems *et al.*, 1994; Cribier *et al.*, 1995; Kato *et al.*, 1995; Tagawa *et al.*, 1995; Nakajima *et al.*, 1996; Seipp *et al.*, 1997). Although these systems are quite valuable in allowing *in vitro* investigations of some aspects of the HCV replication cycle, our system presents specific advantages. Primary human adult hepatocytes remain differentiated and metabolically active for at least 35 days when cultured as described (Ferrini *et al.*, 1997). They represent therefore the closest *in vitro* model to natural infection in man. In particular, we have reported that under these conditions the hepatocyte enzyme systems (cytochromes P450) involved in drug metabolism and pro-drug activation are maintained and functional. This aspect is important if this system is to be used for the screening of antiviral molecules, some of which may require bioactivation to become pharmacologically active. Susceptibility of hepatocytes to infection *in vitro* offers the possibility of investigating the early steps of the virus cycle such as the interaction between virus and receptor on the hepatocyte.

In our study, the kinetics of positive- and negative-strand HCV RNA expression after infection of human adult hepatocytes appear to be different from those observed in chimpanzee and human foetal hepatocytes. Lanford *et al.* (1994) observed that both positive- and negative-strand RNA was maintained at constant levels for at least 25 days post-infection, whereas Iacovacci *et al.* (1997) observed an increase in expression of the positive strand over a period of 30 days post-infection. Our data show that in primary adult human hepatocytes the level of positive-strand RNA decreased gradually after a few days post-infection but remained detectable for at least 8 days, while the negative strand first appeared at day 1, reached a maximum level at 3-5 days post-infection and became undetectable at day 14. Although the pattern of expression for both RNA strands is qualitatively the same from one culture to another, their levels at day 14 vary slightly. The absence or a low level of intracellular positive- and negative-strand HCV RNA as observed in some assays in this study cannot be ascribed to a

deterioration of cells upon ageing because further experimental analysis performed on the same cultures, including liver-specific protein production and expression of cytochromes P450, provided clear evidence in favour of the maintenance of the differentiated cellular phenotype. The difference between our cultures and the chimpanzee or human foetal hepatocytes could be inherent in the proliferative abilities of the latter or in the culture conditions including isolation conditions and medium formulation. It is possible that the process of virus infection and the mechanism of HCV RNA replication are dependent on the proliferative status of the cells. Cell-cycle markers are currently analysed in our laboratory and preliminary results indicate that the cells are in the early G<sub>1</sub> phase after 1–2 weeks in culture and exhibit neither DNA synthesis (phase S) nor mitosis (data not shown). Although the proliferative status of chimpanzee and human foetal hepatocytes has not been reported in corresponding culture systems, it is expected to be different from that of adult human hepatocytes. Contrary to a study reported recently using human foetal cells (Iacovacci *et al.*, 1997), we had no direct evidence for *de novo* synthesis and release of virus particles in the culture medium of infected adult hepatocytes. This was assayed by performing *rTth* RT-PCR on 100 µl different culture medium harvested at day 1, 3, 5, 8, 11 and 14 post-infection.

With a limited number of serum samples tested, we found a reasonable correlation at the level of positive-strand HCV RNA between serum and cells post-infection (compare Figs 2 and 4) for all genotypes 1, 2 and 3. A notable exception is the case of serum 33 which, in spite of exhibiting a very low HCV RNA level, led to a high synthesis of intracellular positive-strand RNA after infection (see Fig. 4). This serum sample had been collected from the same patient as serum 23, but 8 months later.

In this study, we also observed that HCV RNA titres greater than 20 Meq/ml are necessary but not sufficient for *in vitro* infection. For example, sera 21, 29 and 30, while exhibiting a high HCV RNA titre, are apparently non-infectious. These preliminary results, obtained with a highly sensitive and specific method, demonstrate that differentiated adult human hepatocytes in primary culture retain their susceptibility to HCV infection and their permissivity for HCV RNA replication, but do not support the production of progeny virions. This work provides a new and reliable *in vitro* model for investigating the initial steps of the replication cycle of HCV and for the evaluation of antiviral molecules.

We are grateful to C. Segarra and J. Viladomat (ETS Languedoc Roussillon) for helpful technical advice. We wish to express our thanks to Drs M. F. Saint Marc and S. Dantin (Produits Roche) and to J. B. Ferrini (INSERM U128) for critical comments and discussions.

This study is supported by Produits Roche (Neuilly sur Seine, France) and by grants from la Fondation pour la Recherche Médicale Française, le Conseil Régional Languedoc-Roussillon, and l'Association pour la Recherche sur le Cancer.

## References

- Alter, H. J., Purcell, R. H., Shih, J. W., Melpolder, J. C., Houghton, M., Choo, Q. L. & Kuo, G. (1989). Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. *New England Journal of Medicine* **32**, 1494–1500.
- Bertolini, L., Iacovacci, S., Ponzetto, A., Gorini, G., Battaglia, M. & Carloni, G. (1993). The human bone-marrow-derived B-cell line CE, susceptible to hepatitis C virus infection. *Research in Virology* **144**, 281–285.
- Bouffard, P., Hayashi, P. H., Acevedo, R., Levy, N. & Zeldis, J. B. (1992). Hepatitis C virus is detected in a monocyte/macrophage subpopulation of peripheral blood mononuclear cells of infected patients. *Journal of Infectious Diseases* **166**, 1276–1280.
- Carloni, G., Iacovacci, S., Sargiacomo, M., Ravagnan, G., Ponzetto, A., Peschle, C. & Battaglia, M. (1993). Susceptibility of human liver cell cultures to hepatitis C virus infection. *Archives of Virology* **8**, 31–39.
- Chomczynski, P. N. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162**, 156–159.
- Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W. & Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**, 359–362.
- Choo, Q. L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Median-Selby, A., Barr, P. J., Weiner, A. J., Bradley, D. W., Kuo, G. & Houghton, M. (1991). Genetic organization and diversity of the hepatitis C virus. *Proceedings of the National Academy of Sciences, USA* **88**, 2451–2455.
- Cribier, B., Schmitt, C., Bingen, A., Kirn, A. & Keller, F. (1995). *In vitro* infection of peripheral blood mononuclear cells by hepatitis C virus. *Journal of General Virology* **76**, 2485–2491.
- Ferrini, J. B., Pichard, L., Domergue, J. & Maurel, P. (1997). Long-term primary cultures of adult human hepatocytes. *Chemico-Biological Interactions* **107**, 31–45.
- Grakoui, A., Wychowski, C., Lin, C., Feinstone, S. M. & Rice, C. M. (1993). Expression and identification of hepatitis C virus polyprotein cleavage products. *Journal of Virology* **67**, 1385–1395.
- Gunji, T., Kato, N., Hijikata, M., Hayashi, K., Saitoh, S. & Shimotohno, K. (1994). Specific detection of positive and negative stranded hepatitis C viral RNA using chemical RNA modification. *Archives of Virology* **134**, 293–302.
- Houghton, M. H. (1996). Hepatitis C viruses. In *Fields Virology*, 3rd edn, pp. 1035–1058. Edited by B. N. Fields, D. N. Knipe & P. M. Howley. Philadelphia: Lippincott Raven.
- Houghton, M., Weiner, A., Han, J., Kuo, G. & Choo, Q. L. (1991). Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. *Hepatology* **14**, 381–388.
- Iacovacci, S., Sargiacomo, M., Parolini, I., Ponzetto, A., Peschle, C. & Carloni, G. (1993). Replication and multiplication of hepatitis C virus genome in human foetal liver cells. *Research in Virology* **144**, 275–279.
- Iacovacci, S., Manzin, A., Barca, S., Sargiacomo, M., Serafino, A., Valli, M. B., Macioce, G., Hassan, H. J., Ponzetto, A., Clementi, M., Peschle, C. & Carloni, G. (1997). Molecular characterization and dynamics of hepatitis C virus replication in human fetal hepatocytes infected *in vitro*. *Hepatology* **26**, 1328–1337.
- International Union of Microbiological Societies (1995). In *Virus Taxonomy*, 7th edn, pp. 424–427. Edited by F. A. Murphy, D. H. L. Bishop, S. A. Ghabrial, A. W. Jarvis, G. P. Martelli, M. A. Mayo & M. D. Summers. New York: Springer Verlag.
- Isom, H. & Georgoff, I. (1984). Quantitative assay for albumin-

- producing liver cells after simian-virus 40 transformation of rat hepatocytes maintained in chemically defined medium. *Proceedings of the National Academy of Sciences, USA* **81**, 6378–6382.
- Ito, T., Mukaigawa, J., Zuo, J., Hirabayashi, Y., Mitamura, K. & Yasui, K. (1996).** Cultivation of hepatitis C virus in primary hepatocyte culture from patients with chronic hepatitis C results in release of high titre infectious virus. *Journal of General Virology* **77**, 1043–1054.
- Jacob, J. R., Eichberg, J. W. & Lanford, R. E. (1989).** In vitro replication and expression of hepatitis B virus from chronically infected primary chimpanzee hepatocytes. *Hepatology* **10**, 921–927.
- Jacob, J. R., Burk, K. H., Eichberg, J. W., Dreesman, G. R. & Lanford, R. E. (1990).** Expression of infectious viral particles by primary chimpanzee hepatocytes isolated during the acute phase of non-A non-B hepatitis. *Journal of Infectious Diseases* **161**, 1121–1127.
- Kato, N., Nakazawa, T., Mizutani, T. & Shimotohno, K. (1995).** Susceptibility of human T-lymphotropic virus type I infected cell line MT-2 to hepatitis C virus infection. *Biochemical Biophysical Research Communications* **206**, 863–869.
- Kato, N., Ikeda, M., Mizutani, T., Sugiyama, K., Noguchi, M., Hirohashi, S. & Shimotohno, K. (1996).** Replication of hepatitis C virus in cultured non-neoplastic human hepatocytes. *Japanese Journal of Cancer Research* **87**, 787–792.
- Lanford, R. E., Carey, K. D., Estlack, L. E., Smith, G. C. & Hay, R. V. (1989).** Analysis of plasma protein and lipoprotein synthesis in long-term primary cultures of baboon hepatocytes maintained in serum-free medium. *In Vitro Cellular and Developmental Biology* **25**, 174–182.
- Lanford, R. E., Sureau, C., Jacob, J. R., White, R. & Fuerst, T. R. (1994).** Demonstration of in vitro infection of chimpanzee hepatocytes with hepatitis C virus using strand-specific RT/PCR. *Virology* **202**, 606–614.
- Lanford, R. E., Chavez, D., Chisari, F. V. & Sureau, C. (1995).** 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. *Journal of Virology* **69**, 8079–8083.
- Laskus, T., Radkowski, M., Wang, L.-F., Cianciara, J., Vargas, H. & Rakela, J. (1997).** Hepatitis C virus negative strand RNA is not detected in peripheral blood mononuclear cells and viral sequences are identical to those in serum: a case against extrahepatic replication. *Journal of General Virology* **78**, 2747–2750.
- Lerat, H., Berby, F., Trabaud, M. A., Vidalin, O., Major, M., Trepo, C. & Inchauspe, G. (1996).** Specific detection of hepatitis C virus minus strand RNA in hematopoietic cells. *Journal of Clinical Investigations* **97**, 845–851.
- McGuinness, P. H., Bishop, G. W., McLaughan, R., Trowbridge, R. & Gowans, E. J. (1994).** False detection of negative-strand hepatitis C virus RNA. *Lancet* **343**, 551–552.
- Miller, R. H. & Purcell, R. H. (1990).** Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. *Proceedings of the National Academy of Sciences, USA* **87**, 2057–2061.
- Müller, H. M., Pfaff, E., Goeser, T., Kallinowski, B., Solbach, C. & Theilmann, L. (1993).** Peripheral blood leukocytes serve as a possible extrahepatic site for hepatitis C virus replication. *Journal of General Virology* **74**, 669–676.
- Nakajima, N., Hijikata, M., Yoshikura, H. & Shimizu, Y. K. (1996).** Characterization of long-term cultures of hepatitis C virus. *Journal of Virology* **70**, 3325–3329.
- Nouri-Aria, K. T., Sallie, R., Sangar, D., Alexander, G. J. M., Smith, H., Byrne, J., Portmann, B., Eddleston, A. L. W. F. & Williams, R. (1993).** Detection of genomic and intermediate replicative strands of hepatitis C virus in liver tissue by in situ hybridization. *Journal of Clinical Investigations* **91**, 2226–2234.
- Pichard, L., Fabre, I., Daujat, M., Domergue, J., Joyeux, H. & Maurel, P. (1992).** Effect of corticosteroids on the expression of cytochromes P450 and on cyclosporin A oxidase activity in primary cultures of human hepatocytes. *Molecular Pharmacology* **41**, 1047–1055.
- Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Ohta, Y., Choo, Q. I., Houghton, M. & Kuo, G. (1990).** Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proceedings of the National Academy of Sciences, USA* **87**, 6547–6549.
- Seipp, S., Mueller, H. M., Pfaff, E., Stremmel, W., Theilmann, L. & Goeser, T. (1997).** Establishment of persistent hepatitis C virus infection and replication in vitro. *Journal of General Virology* **78**, 2467–2476.
- Shimizu, Y. K. & Yoshikura, H. (1994).** Multicycle infection of hepatitis C virus in cell culture and inhibition by alpha and beta interferons. *Journal of Virology* **68**, 8406–8408.
- Shimizu, Y. K., Iwamoto, A., Hijikata, M., Purcell, R. H. & Yoshikura, H. (1992).** Evidence for in vitro replication of hepatitis C virus genome in a human T-cell line. *Proceedings of the National Academy of Sciences, USA* **89**, 5477–5481.
- Shimizu, Y. K., Purcell, R. H. & Yoshikura, H. (1993).** Correlation between the infectivity of hepatitis C virus in vivo and its infectivity in vitro. *Proceedings of the National Academy of Sciences, USA* **90**, 6037–6041.
- Smith, D. B., Mellor, J., Jarvis, L. M., Davidson, F., Kolberg, J., Urdea, M., Yap, P.-L., Simmonds, P. & The International HCV Collaborative Study Group (1995).** Variation of the hepatitis C virus 5' non-coding region: implications for the secondary structure, virus detection and typing. *Journal of General Virology* **76**, 1749–1761.
- Tagawa, N., Kato, N., Yokosura, I., Ishikawa, T., Ohto, M. & Omata, M. (1995).** Infection of human hepatocyte cell lines with hepatitis C virus in vitro. *Journal of Gastroenterology and Hepatology* **10**, 523–527.
- Takehara, T., Hayashi, N., Mita, E., Hagiwara, H., Ueda, K., Katayama, K., Kasahara, A., Husamoto, H. & Kamada, T. (1992).** Detection of the minus strand of hepatitis C virus RNA by reverse transcription and polymerase chain reaction: implications for hepatitis C virus replication in infected tissue. *Hepatology* **15**, 387–390.
- Willems, M., Moshage, H. & Yap, S. H. (1993).** PCR and detection of negative HCV RNA strands. *Hepatology* **17**, 526.
- Willems, M., Peerlinck, K., Moshage, H., Deleu, I., Van den Eynde, C., Vermynen, J. & Yap, S. H. (1994).** Hepatitis C virus-RNAs in plasma and in peripheral blood mononuclear cells of hemophiliacs with chronic hepatitis C: evidence for viral replication in peripheral blood mononuclear cells. *Journal of Medical Virology* **42**, 272–278.
- Zignego, A. L., Macchia, D., Monti, M., Thiers, V., Mazetti, M., Foschi, M., Maggi, E., Romagnani, S., Gentilini, P. & Brechot, C. (1992).** Infection of peripheral mononuclear blood cells by hepatitis C virus [see comments]. *Journal of Hepatology* **15**, 382–386.

---

Received 8 April 1998; Accepted 30 June 1998