over 7.0 log copy/ml HBV DNA were diluted 10- or 100-fold with normal human serum and re-tested to obtain the end titer.

Serum concentrations of HBVcrAg were measured using the CLEIA method reported previously (10, 11). Briefly, 100 μL serum was mixed with 50 μL pretreatment solution containing 15% sodium dodecylsulfate and 2% Tween 60. After incubation at 70 °C for 30 min, 50 μL pretreated serum was added to a well coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61 and HB114) and filled with 100 μL assay buffer. The mixture was incubated for 2 h at room temperature and the wells were then washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies against denatured HBc and HBe antigens (HB91 and HB110) were added to the well, and the mixture was incubated for 1 h at room temperature. After washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBVcrAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBe antigen (amino acids, 10–183 of the precore/core gene product). The HBVcrAg concentration was expressed as units/ml (U/ml) and the immunoreactivity of recombinant pro-HBe antigen at 10 fg/ml was defined as 1 U/ml. In the present study, the cutoff value was tentatively set at 3.0 log U/ml. Sera containing over 7.0 log U/ml HBVcrAg were diluted 10- or 100-fold in normal human serum and re-tested to obtain the end titer.

Statistical analysis

The Mann–Whitney *U*-test and Wilcoxon signed-ranks test were utilized to analyze quantitative data, and Fisher's exact test was used for qualitative data. A log-rank test was used to compare the occurrence of lamivudine resistance. Statistical analyses were performed using the SPSS 5.0 statistical software package (SPSS, Inc., Chicago, IL). A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Table 1 shows a comparison of the clinical and virological backgrounds of the 27 patients who showed lamivudine resistance and the 54 patients who did not. Median age, gender distribution and median follow-up period did not differ between the two groups, and the positive rate of HBe antigen was similar. Both HBV DNA and HBVcrAg levels at the beginning of lamivudine administration were similar between the two groups; however, both HBV DNA and HBVcrAg levels at 6 months after the start of lamivudine administration were significantly lower in the lamivudine resistance negative group than in the positive group. ALT level was normal at the beginning in eight (15%) of the 54 patients without lamivudine resistance and in two (7%) of the 27 patients with it ($P > 0.2$).

Table 1. Comparison of the clinical and virological backgrounds of patients who showed lamivudine resistance and those who did not

Characteristics	Appearance of lamivudine resistance: Negative ($n = 54$)	Positive ($n = 27$)	P
Age (years)*	47.0 (24–79)	50.6 (34–67)	0.140†
Gender (male %)	74%	67%	>0.2‡
Follow-up period (months)*	16 (6–50)	21 (9–43)	>0.2†
HBV genotype (A/B/C)	2/2/50	0/1/26	>0.2‡
HBe antigen (positive %)	59%	70%	>0.2‡
ALT (IU/ml)*			
Initial	85 (22–713)	95 (20–1140)	>0.2†
At 6 months	27 (11–115)	30 (15–92)	>0.2†
HBV DNA (log copy/ml)*			
Initial	7.0 (3.5–9.1)	7.3 (4.2–9.2)	>0.2†
At 6 months	<2.6 (<2.6–4.8)	3.3 (<2.6–6.6)	<0.001†
HBVcrAg (log U/ml)*			
Initial	6.2 (<3.0–8.8)	7.3 (4.4–9.1)	0.073†
At 6 months	5.2 (<3.0–6.7)	5.8 (4.7–8.4)	<0.001†

HBe antigen, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; HBVcrAg, HBV core-related antigen. *Data are expressed as median (range). †Mann–Whitney *U* test. ‡χ^2-test.

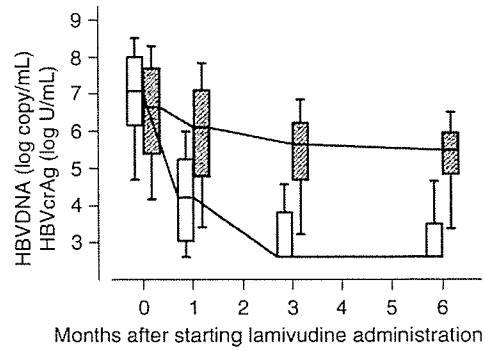

Fig. 1. Changes in the median levels of hepatitis B virus core-related antigen (HBVcrAg) and hepatitis B virus (HBV) DNA during lamivudine administration. The box plots show the 10th, 25th, 50th, 75th and 90th percentiles, with the open boxes indicating HBV DNA and shaded boxes indicating HBVcrAg. The median amount of decrease from the baseline in HBVcrAg levels was significantly smaller (Wilcoxon signed-ranks test) than that in HBV DNA level at 1 (2.80 log copy/ml vs. 0.27 log U/ml, $P < 0.001$), 3 (3.60 log copy/ml vs. 0.83 log U/ml, $P < 0.001$) and 6 months (3.90 log copy/ml vs. 1.15 log U/ml, $P < 0.001$) after the initiation of lamivudine administration.

Figure 1 shows changes in HBV DNA and HBVcrAg levels during lamivudine treatment in all patients. The level of HBV DNA decreased rapidly and became undetectable at 3 months after treatment was initiated. On the other hand, although HBVcrAg levels decreased continuously, the median amount of decrease from the base-line was significantly lower than that in HBV DNA levels at 1, 3 and 6 months after starting lamivudine administration (Wilcoxon signed-ranks test, $P<0.001$ at all analyzed points in time).

Changes in HBV DNA and HBVcrAg levels during lamivudine administration are compared in Fig. 2 between the 27 patients who showed lamivudine resistance and the 54 patients who did not. Serum HBV DNA levels were found to decrease rapidly and become undetectable within 6 months in 45 (83%) of the 54 patients without lamivudine resistance. On the other hand, only 11 (41%) of the 27 patients with lamivudine resistance showed a similar rapid decrease, and the HBV DNA levels of the remaining patients stayed above the detection limit during the follow-up period. HBVcrAg levels decreased but did not reach levels lower than 4.7 log U/ml (5000 U/ml) in the 27 patients with lamivudine resistance. In 19 (35%) of the 54 patients without lamivudine resistance, on the other hand, the levels decreased to levels below 4.7 log U/ml within 6 months after the start of lamivudine administration. The level of HBVcrAg increased rapidly as did the level of HBV DNA when lamivudine resistance occurred.

The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months after the initiation of treatment than in the remaining 25 patients (Fig. 3). The cumulative occurrence of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. There was no occurrence of lamivudine resistance during the follow-up period in the 19 patients whose HBVcrAg levels were less than 4.6 log U/ml at 6 months after the initiation of lamivudine therapy (Fig. 3). On the other hand, lamivudine resistance occurred in 50% of the remaining patients within 2 years.

Discussion

The HBVcrAg assay is a unique assay, which measures the amounts of e and core antigens

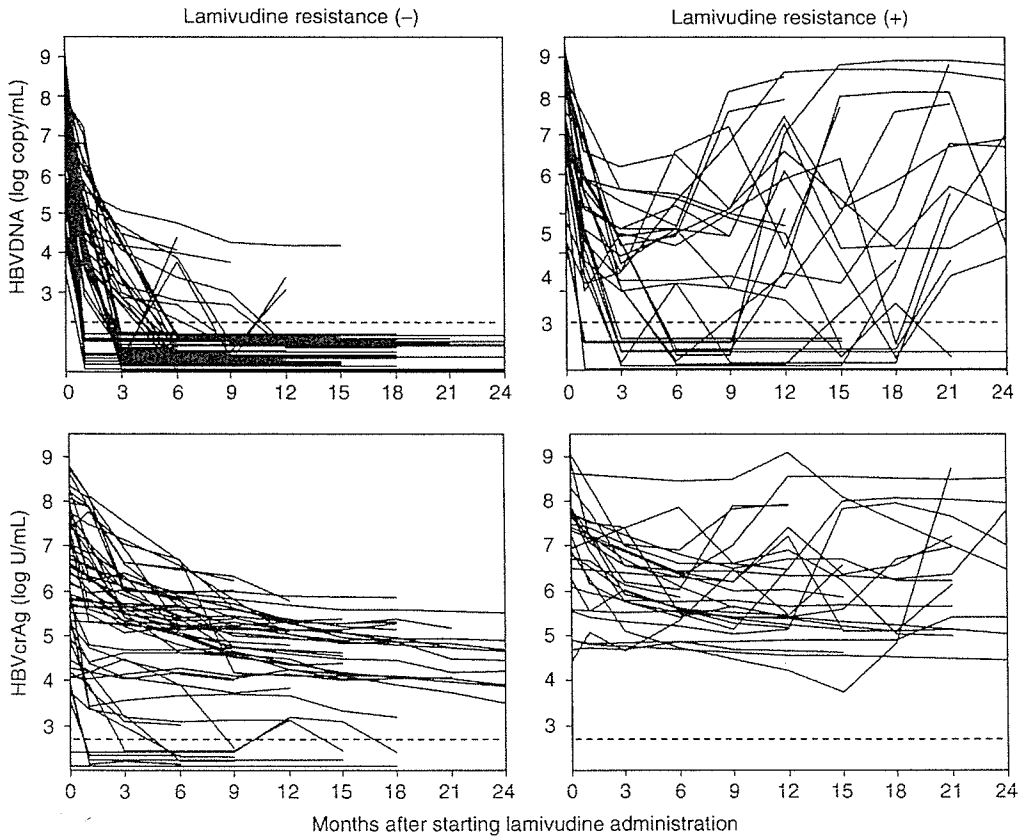

Fig. 2. Comparison of changes in serum hepatitis B virus (HBV) DNA and serum HBV core-related antigen (HBVcrAg) levels between patients who showed lamivudine resistance and those who did not. The broken lines indicate the detection limit of each assay.

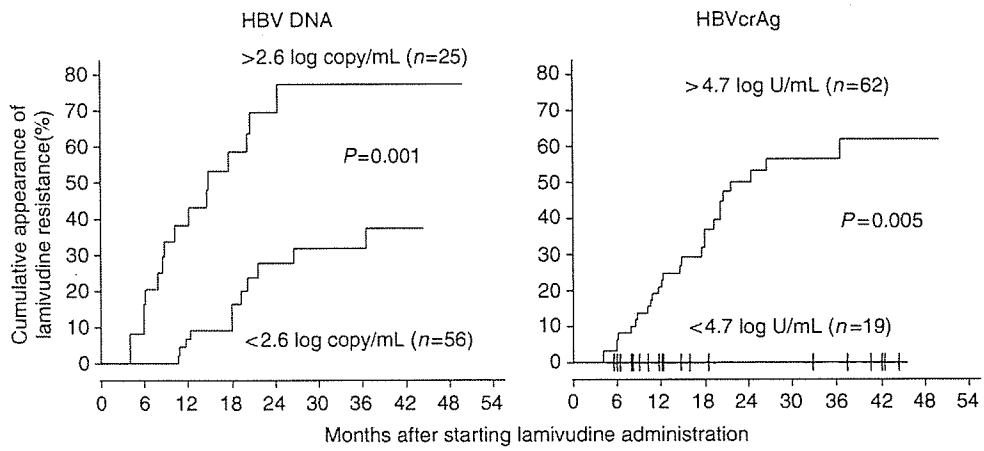

Fig. 3. Comparison of the cumulative occurrence of lamivudine resistance between patients who showed hepatitis B virus (HBV) DNA levels of less than the detection limit (2.6 log copy/ml) at 6 months after starting lamivudine administration and those who did not (left figure), and similarly between patients who showed HBV core-related antigen (HBVcrAg) levels of less than 4.7 log U/ml and those who did not (right figure).

coded by the core gene of the HBV genome with high sensitivity and a wide quantitative range. Serum HBVcrAg levels reflect the viral load in the natural course because these levels correlate linearly with those of HBV DNA (14, 15). On the other hand, the character of HBVcrAg is somewhat different from that of HBV DNA in patients undergoing anti-viral therapies such as lamivudine. That is, HBVcrAg levels decrease significantly more slowly than those of HBV DNA after the initiation of lamivudine administration.

HBV is an enveloped DNA virus containing a relaxed circular DNA genome, which is converted into a covalently closed circular DNA (cccDNA) episome in the nucleus of infected cells (18, 21–23). The cccDNA molecules serve as the transcriptional template for the production of viral RNAs that encode viral structural and non-structural proteins. Reverse transcription of the viral pregenomic RNA and second-strand DNA synthesis occur in the cytoplasm within viral capsids formed by the HBV core protein. Because lamivudine, a nucleoside analogue, inhibits reverse transcription of the pregenomic RNA, it directly suppresses the production of HBV virion. Thus, serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. On the other hand, the production of viral proteins is not suppressed by lamivudine because the production process does not include reverse transcription. Furthermore, it has been reported that the amount of cccDNA, which serves as a template for mRNA, decreases quite slowly after starting the administration of nucleoside analogues (24–26). Thus, it is reasonable that serum HBVcrAg levels decrease much more slowly than HBV DNA levels after the initiation of lamivudine therapy.

Significant markers that can predict the presence or absence of lamivudine resistance are clinically valuable because the emergence of this resistance and the subsequent recurrence of hepatitis are fundamental problems in lamivudine therapy. Serum markers that reflect the activity of HBV replication have been reported to be associated with the occurrence of lamivudine resistance (11, 12, 27, 28). However, neither the pretreatment existence of HBe antigen nor pretreatment levels of HBV DNA or HBVcrAg were found to be significant markers in the present study. These results may reflect a weak association between the pretreatment activity of HBV replication and the occurrence of lamivudine resistance (13, 29). Changes in HBV DNA and HBVcrAg levels after starting lamivudine administration clearly differed between patients with and without lamivudine resistance. Thus, HBV DNA and HBVcrAg levels at 6 months after starting lamivudine administration were analyzed to determine whether these levels might serve as predictive markers; both were found to be significantly lower in patients without lamivudine resistance at the tested point in time. Furthermore, patients who showed higher levels of HBV DNA and HBVcrAg at 6 months after the initiation of treatment were significantly more likely to develop lamivudine resistance than those who showed lower levels.

We believe that the measurement of HBV DNA levels is useful to identify patients who are at high risk for lamivudine resistance because as many as 70% of patients who were positive for HBV DNA at 6 months after starting lamivudine

administration developed lamivudine resistance within 2 years. However, a negative result of HBV DNA at 6 months does not necessarily guarantee the absence of lamivudine resistance because nearly 30% of such patients developed resistance within 2 years. On the other hand, HBVcrAg levels of less than 4.7 log U/ml at 6 months are a useful indicator of patients who are unlikely to develop lamivudine resistance, because no such patients developed resistance during the follow-up period in the present study. Lower serum HBVcrAg levels may reflect lower levels of cccDNA in hepatocytes because the mRNAs of HBVcrAg are transcribed from the cccDNA (18, 22, 23). This possibility may explain our finding that patients whose HBVcrAg levels decreased sufficiently were unlikely to develop lamivudine resistance, because cccDNA provides the templates for viral and pregenomic messenger RNA (18, 22, 23), which may be a source of lamivudine-resistant strains.

In conclusion, our results suggest that measurement not only of HBV DNA but also of HBVcrAg is useful for predicting the occurrence of lamivudine resistance. HBV DNA measurement is valuable for identifying patients who are at high risk of developing this resistance and HBcrAg measurement is valuable for identifying those who are at low risk.

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Cell-based models of sustained, interferon-sensitive hepatitis C virus genotype 1 replication

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Abstract

We have previously reported hepatitis C virus (HCV) replication using a novel binary expression system in which mammalian cells were transfected with a T7 polymerase-driven full-length genotype 1a HCV cDNA plasmid (pT7-flHCV-Rz) and infected with vaccinia-T7 polymerase. We hypothesized that the use of replication-defective adenoviral vectors expressing T7 (Ad-T7pol) or cell lines stably transfected with T7 (Huh-T7) would alleviate cell toxicity and allow for more sustained HCV replication.

CV-1, Huh7, and Huh-T7 cells were transfected with pT7-flHCV-Rz and treated with Ad-T7pol (CV-1 and Huh7 only). Protein and RNA were harvested from cells on days 1, 2, 3, 5, 7, and 9 post-infection. No cytotoxicity was observed at 9 days post-infection in any cell type. HCV positive- and negative-strand RNA expression were strongest during days 1–3 post-infection; however, HCV RNA remained detectable throughout the 9-day observation period. Furthermore, transfection with a replication-incompetent plasmid suggested that efficient HCV replication is dependent upon NS5B gene expression. Finally, after 1–2 days of IFN treatment, HCV positive-strand levels decreased significantly compared to HCV-infected but untreated samples ($p < 0.05$).

In conclusion, these refined binary systems offer more durable and authentic models for identification of host cellular processes critical to HCV replication and will permit longer-term analysis of virus–host interactions critical to HCV pathogenesis and the treatment of genotype 1 infections.

Keywords: Hepatitis C virus; HCV; Replication; Genotype 1; Adenovirus vector; Huh-T7

1. Introduction

Hepatitis C virus (HCV) is a leading cause of chronic liver disease, including hepatitis, cirrhosis, and hepatocellular carcinoma (Alter et al., 1999). The combination of interferon (IFN) and ribavirin (RBV) is the standard treatment for chronic HCV infection; however, their effectiveness remains limited (McHutchison and Poynard, 1999). The lack of a full-length HCV tissue culture model has limited not only the ability to screen novel antiviral agents but also the ability to precisely characterize the antiviral effect of IFN, particularly against genotype 1 infections.

We recently reported successful cell-based HCV replication using a novel binary expression system in which mammalian cells were transfected with a T7 polymerase-driven full-length genotype 1a HCV cDNA plasmid and infected with a recombinant vaccinia vector encoding T7 polymerase (Chung et al., 2001). However, HCV replication driven by vaccinia-based vectors is restricted to short-term studies due to the cytotoxic

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effects of vaccinia. Moreover, vaccinia encodes two proteins, E3L (Chang et al., 1992; Watson et al., 1991) and K3L (Carroll et al., 1993; Gale et al., 1996), that act as potent inhibitors of the IFN-induced double-stranded RNA-activated protein kinase (PKR). Due to these limitations, we sought to further refine our HCV replication model using alternative, less disruptive modes of T7 polymerase delivery.

We hypothesized that the use of replication-defective adenoviral vectors expressing T7 or cell lines stably expressing T7 would alleviate cell toxicity and allow for more sustained HCV replication.

Recombinant replication-defective adenoviral vectors have comparable infectivity to vaccinia vectors. These adenoviral vectors cannot replicate inside infected cells, because they lack the E1A and E1B proteins necessary for viral vector replication. Moreover, these vectors lack the E3 gene that inhibits immune responses by interacting with cytoplasmic MHC class-I molecules (Wold and Gooding, 1989). Thus, adenoviral vectors maintain infectivity and protein delivery with minimal cytotoxicity. By transfecting the HCV cDNA construct into Huh7 cell lines stably expressing T7 polymerase (Huh-T7) (Schultz et al., 1996), the need for viral delivery systems was removed altogether. Using these alternative delivery methods, we have established a refined HCV replication model that produces more sustained viral RNA replication, leads to less perturbation of host genes, and represents a more authentic system for studying virus–host interactions relevant to HCV pathogenesis. These refined models were also utilized to characterize the antiviral kinetics of IFN on HCV replication.

2. Materials and methods

2.1. Cell lines

CV-1 cells (American Type Culture Collection, Manassas, VA) and Huh7 (Dr. Robert Lanford, Southwest Foundation for Biomedical Research) and Huh-T7 (Dr. Stanley Lemon, University of Texas) (Schultz et al., 1996) were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum.

2.2. Plasmids and transfection-infection

The binary replication system has been described previously and is capable of successful positive-strand and negative-strand HCV RNA synthesis, efficient HCV protein production, and quasispecies generation (Chung et al., 2001; Contreras et al., 2002). Briefly, a plasmid containing the infectious full-length genotype 1 cDNA sequence corresponding to the H77 prototype strain (Yanagi et al., 1997) was adapted at its 5′ and 3′ termini with the T7 promoter and a hepatitis delta virus ribozyme sequence, respectively, to yield pT7-flHCV-Rz (hereafter referred to as H77). As a negative control, a mutant plasmid in which the GDD active site of the NS5B RNA-dependent RNA polymerase (RdRp) was mutated to AAG (hereafter referred to as $H77_{GDD \rightarrow AAG}$) was generated by site directed mutagenesis (Quick Change; Stratagene; La Jolla, CA). This substitution is associated with replication-incompetence in replicon models (Blight et al., 2000). H77 and $H77_{GDD \rightarrow AAG}$ were used to transfect CV-1, Huh7, or Huh-T7 cells at 70% confluency on 6-well plates with Lipofectamine (Invitrogen, Carlsbad, CA). Plasmids were transfected at concentrations of 1 μg/well for CV1 cells and 3 μg/well for Huh7 and Huh-T7 cells. Transfection efficiency was assessed by co-transfection with 0.1 μg/well of phRL-TK (Int^-) (Promega, Madison, WI) and luciferase activity quantified using the Dual-Luciferase reporter assay system (Promega). For CV-1 and Huh7 cells, T7 polymerase was delivered using a recombinant vaccinia virus vector (vTF7-3) (Fuerst et al., 1986) or a recombinant adenovirus vector (Ad-T7pol) 24 h after H77 transfection. In control experiments, a replication-defective adenovirus vector lacking the T7 polymerase gene (Ad-Psi5) was used. Adenoviral vectors were provided by the Harvard Gene Therapy Initiative's Viral Vector core (Boston, MA).

2.3. X-gal staining of pOS8-transfected cells

To compare the transfection and infection efficiency of the vaccinia and adenovirus vectors, the pOS8 plasmid, which contains a T7 promoter flanking the β-galactosidase gene, was co-transfected into cells. After 48 h, cultured cells were washed with PBS, fixed with 0.25% glutaraldehyde for 1 h at 4 °C, and stained with 0.1% 5-bromo-5-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as described previously (Hiasa et al., 1998; Miyake et al., 1996).

2.4. Interferon experiments

Interferon alpha 2b was obtained from Schering Plough (Kenilworth, NJ). For CV-1 and Huh7 cells, 100–1000 IU/mL of IFN was added 5 h after infection with adenovirus vector. For Huh-T7 cells, 100–1000 IU/mL of IFN was added 5 h after transfection with H77. Medium with or without IFN was changed at day 1 post-infection and every 2 days thereafter.

2.5. Cellular RNA extraction and qualitative strand-specific rTth RT-PCR

Cells were washed three times with phosphate-buffered saline. RNA was extracted using TRIzol (Invitrogen; Carlsbad, CA), and treated two times for 4 h with DNase I using the DNA-free kit (Ambion; Austin, TX) following the manufacturer's protocol. RNA was quantified by UV spectrum analysis, and adjusted to 0.3 μg/μL. HCV RNA was detected utilizing a previously described qualitative strand-specific r*Tth* reverse transcription PCR (RT-PCR) assay (Castet et al., 2002; Lanford et al., 1995). For detection of negative-strand HCV RNA, 1 μg of RNA in 10 μL of dietyl pyrocarbonate-treated water was layered with mineral oil and heated at 95 °C for 1 min, and lowered to 70 °C. A 20 μL mixture containing 10 pM of HCV-II sense primer (5′-CAC TCC CCT GTG AGG AAC T-3′, nucleotides [nt] 38–56 of the 5′UTR) (Laskus et al., 1997), 1× RT buffer (Applied Biosystems; Foster City, CA), 1 mM $MnCl_2$, 200 μM (each) deoxynucleoside triphosphate, and 5 U of r*Tth* enzyme

(Applied Biosystems) was then added. The temperature was dropped to 60 °C for 2 min for annealing and then raised to 70 °C for 20 min for the cDNA reaction. To inactivate the RT activity of r*Tth*, chelating buffer (Applied Biosystems) was added. Forty microliters of the prewarmed PCR mixture containing 10 pM of HCV-I antisense primer (5′-TGG ATG CAC GGT CTA CGA GAC CTC-3′, nt 342–320 of the 5′UTR) (Laskus et al., 1997) and 3.75 mM $MgCl_2$ was added. Twenty-five cycles of PCR (94 °C 15 s, 58 °C 30 s, 72 °C 30 s) were performed.

For GAPDH measurements, RT was carried out using an oligo $d(T)_{16}$ primer under standard conditions (Hiasa et al., 2003). The cDNA product was subjected to 25 cycles of PCR (95 °C 1 min, 60 °C 2 min, 73 °C 2 min), using 50 pM of the GAPDH sense and antisense primers (forward primer 5′-GAA GGT GAA GGT CGG AGT-3′, reverse primer 5′-GAA GAT GGT GAT GGG ATT TC-3′), 0.1 mM of each dNTP, 2.5 mM $MgCl_2$, and 0.5 U *Taq* polymerase. Reaction products were separated on 1.5 % agarose gels.

To ensure efficient removal of plasmid DNA after DNase I treatment, a qualitative PCR was performed. The plasmid DNA was completely digested as no PCR products were observed using this approach.

2.6. RNase protection assay

Antigenomic HCV RNA was detected as described previously (Chung et al., 2001). Briefly, utilizing the sense-oriented [α-^{32}P] UTP-labeled probe (corresponding to 98 nucleotides of the 3′ terminal HCV genome), antigenomic RNA was generated by in vitro transcription using T7 polymerase from the vector pHCV-3′T (Chung and Kaplan, 1999). Transcripts were generated using the RPA III kit according to the manufacturer's instructions (Ambion).

2.7. Real-time quantification of HCV positive- and negative-strand RNA

Positive- and negative-strand HCV RNAs were quantified by real-time PCR using LightCycler technology (Roche Diagnostics, Mannheim, Germany) and SYBR green I dye as described previously (Blackard et al., 2005). One microgram of RNA was used for cDNA synthesis in a mixture containing 5 U of r*Tth* and 10 pM of the appropriate RT primer (HCV-I for positive-strand HCV RNA or HCV-II for negative-strand HCV RNA). cDNA was purified with the High Pure PCR product purification kit (Roche Diagnostics).

Positive- and negative-strand HCV PCR amplifications were performed with 2 μL of purified cDNA in a reaction mixture containing 1 μL of LightCycler Fast Start DNA Master SYBR Green I, 4 mM of $MgCl_2$, and 5 pM of antisense primer KY78 (5′-CTC GCA AGC ACC CTA TCA GGC AGT-3′, nt 311–288 of the 5′UTR) and 5 pM of sense KY80 (5′-GCA GAA AGC GTC TAG CCA TGG CGT-3′, nt 68–91 of the 5′UTR). The PCR consisted of an initial denaturation step of 10 min at 95 °C, followed by 40 cycles of the following thermal conditions: 15 s at 95 °C, 5 s at 70 °C, and 15 s at 72 °C. All samples were analyzed in triplicate. The sensitivity of the PCR for HCV was previously determined to be approximately 230 copies HCV/μL.

For quantification of GAPDH mRNA, RT was performed with the same amount of RNA used for HCV positive- and negative-strand analysis, using the oligo $d(T)_{16}$ primer under standard conditions. For real-time PCR amplification of GAPDH, a commercial GAPDH primer set (Roche Search LC, Mannheim, Germany) was used under the recommended conditions. For real-time PCR amplification of LacZ, sense (5′-GCC TGC GAT GTC GGT TTC CGC GAG G-3′) and antisense primers (5′-GCC AGC GCG GAT CAT CGG TCA GAC G-3′) were utilized under the following conditions: 10 s at 95 °C, 10 s at 68 °C, 16 s at 72 °C (Dobson et al., 1990). The sensitivity of detection was approximately 210 copies/μL.

DNA was quantified measuring SYBR green I dye incorporation into PCR products at 530 nm following manufacturer's instructions. An HCV standard curve was generated using a PCR product corresponding to nucleotides 38–342 of the 5′UTR. At the end of each run, a DNA melting curve was performed to control for sample homogeneity and quality. In a subset of samples, electroporation and sequencing were performed to confirm the correct identity of the amplified PCR product. Data were expressed as the copy number of HCV positive-strand (or negative-strand) RNA per molecule of GAPDH. This analysis was done in quadruplicate for each sample and presented as the mean and standard deviation. Each value was analyzed statistically using the SPSS 10.0 software (SPSS, Chicago, IL). Differences in mean values were compared using the Mann–Whitney *U*-test.

2.8. Western blotting analysis

Cells were washed twice with PBS, and lysed with 100 μL of Nonidet P-40 buffer (0.5% Nonidet P-40, 10 mM Tris, pH 7.4, 150 mM NaCl, 1% SDS). Protein lysate concentrations were measured using the DC protein assay Kit (Bio-Rad, Hercules, CA). Forty microliters of protein lysate were utilized. Separated products were then blotted onto Immobilon-P membranes, and each membrane was incubated with the relevant antibody. The ECL Kit (Amersham Pharmacia, Buckinghamshire, UK) was used for detection. Monoclonal antibody to HCV core protein (515s) (Kashiwakuma et al., 1996) was provided by Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan; monoclonal antibody to β-galactosidase was purchased from Promega. Appropriate species-specific conjugated secondary antibodies were obtained commercially (Amersham Pharmacia).

2.9. ELISA for HCV core antigen

Cell culture lysates were adjusted to 0.2 mg/mL. HCV core antigen concentrations were quantified using the HCV core protein ELISA kit (Ortho-Clinical Diagnostics, Raritan, NJ) following the manufacturer's instructions (Bouvier-Alias et al., 2002). Core ELISA data were expressed as fmol of HCV core antigen per μg of total protein. The lower level of detection for this assay was less than 1.5 pg/mL.

3. Results

3.1. Replication-defective adenoviral vectors successfully replicate HCV RNA without cytotoxicity

To compare the transfection and infection efficiency of the vaccinia and adenovirus vectors, the pOS8 plasmid, which contains a T7 promoter flanking the β-galactosidase gene, was transfected into cells that were then infected with either vaccinia-T7 (vTF7-3) or Ad-T7pol at a multiplicity of infection (MOI) of 10. The parental replication-incompetent vector Ad-Psi5 was used as an adenoviral vector control. At an MOI of 10, each viral vector efficiently expressed β-galactosidase in approximately 50% of cells 24 h after infection (Fig. 1A). Using trypan-blue staining, cell injury was observed in cells transfected with vaccinia-T7 but not in cells transfected with the Ad-T7pol or Ad-Psi5 vectors (data not shown).

Ribonuclease protection assay for negative-strand HCV RNA (Fig. 1B) and Western blotting for HCV core protein (Fig. 1C) were performed 24 h after infection. Expression of negative-strand HCV RNA was lower after Ad-T7pol infection compared to vTF7-3 infection, yet was clearly detectable. Similarly, HCV

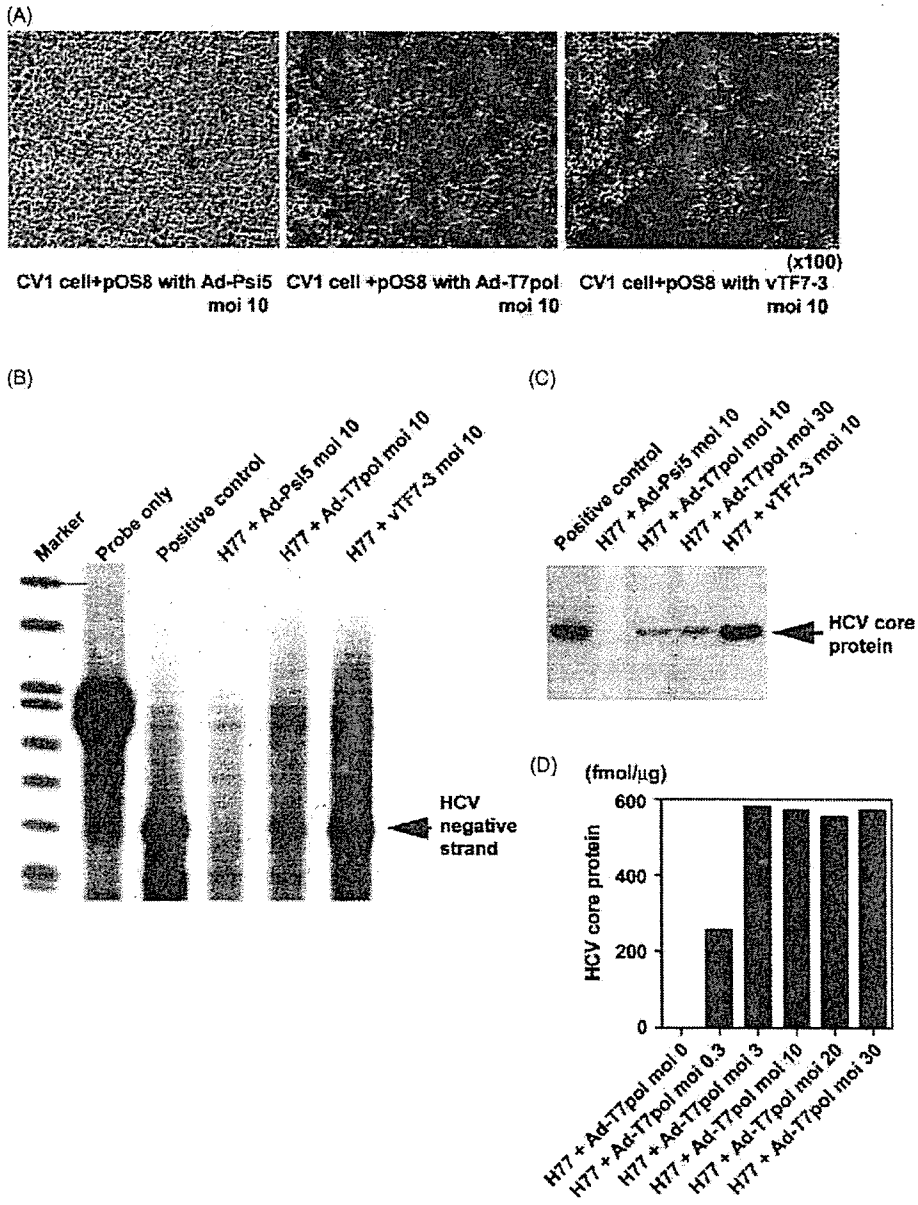

Fig. 1. Comparison of the transfection and infection efficiency of HCV replication system using vaccinia-T7 and adeno-T7 vectors: (A) after transfection with the pOS8 plasmid, cells were infected with virus vectors using control adenovirus (Ad-Psi5), recombinant adeno-T7 polymerase (Ad-T7pol), or vaccinia-T7 polymerase (vTF7-3) at an MOI of 10. (B) RPA for negative-strand HCV RNA was performed with H77 plasmid as a positive control. (C) Western blotting for HCV core protein was performed on CV-1 cell lysates 24 h after infection. (D) Quantitative HCV core ELISA results indicated that an MOI of 10 was optimal for adenoviral-driven HCV protein production.

core protein production was less robust after Ad-T7pol infection; nonetheless, it was clearly detectable. Negative-strand HCV RNA and HCV core protein were not detected when the control Ad-Psi5 vector was used. Quantitative HCV core ELISA results suggested that an MOI of 10 was optimal for adenoviral-driven HCV protein production (Fig. 1D); therefore, an MOI of 10 was selected for all subsequent experiments.

In contrast to increased HCV RNA synthesis and protein production in transfected/infected cells, LacZ mRNA levels decreased rapidly after day 1 and were not detectable after day 7 (data not shown).

3.2. Adenoviral-T7-driven HCV replication is dependent on an intact HCV polymerase gene

The $H77_{GDD-AAG}$ mutant (Fig. 2A) was used to assess whether the HCV RNA polymerase gene (NS5B) was necessary for viral replication. By qualitative RT-PCR of the 5′UTR, HCV negative-strand synthesis was detectable only in the presence of both H77 and Ad-T7pol (Fig. 2B) in CV-1 cells. The absence of detectable negative-strand HCV RNA upon transfection of the mutant plasmid ($H77_{GDD-AAG}$) indicates that replication was dependent on an intact polymerase sequence. H77 + Ad-T7pol expressed significantly higher core protein levels compared to $H77_{GDD\rightarrow AAG}$ + Ad-T7pol (Fig. 2C), further suggesting an intact polymerase sequence is necessary for robust HCV protein production. Ribonuclease protection assay demonstrated the presence of HCV negative-strand in CV-1, Huh7, and Huh-T7 cell lines on day 2 (Fig. 2D). However, negative-strand HCV RNA was not detected when the $H77_{GDD-AAG}$ mutant was transfected, indicating that an intact NS5B sequence was necessary for negative-strand HCV RNA synthesis.

3.3. Kinetic analysis of HCV RNA synthesis and core protein production

The data described above suggest that the Ad-T7pol replication system results in efficient HCV RNA and protein

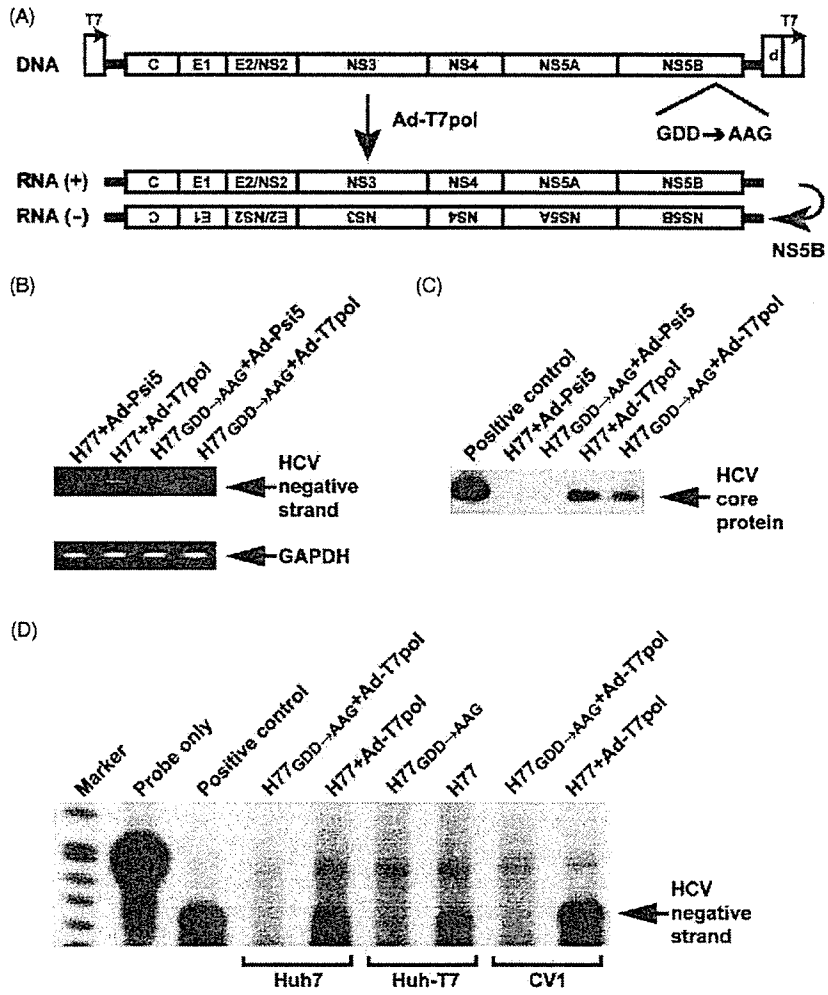

Fig. 2. (A) A control plasmid was prepared by mutating the active site motif from GDD to AAG in the NS5B RNA-dependent RNA polymerase sequence ($H77_{GDD\rightarrow AAG}$). (B) A qualitative strand-specific RT-PCR for negative-strand HCV RNA was performed as previously described (Lanford et al., 1995). (C) Western blotting analysis demonstrated that transfection/infection with H77 + Ad-T7-pol also resulted in HCV core protein production. (D) Ribonuclease protection assay demonstrated detectable negative-strand HCV RNA in CV-1, Huh7, and Huh-T7 cell lines on day 2.

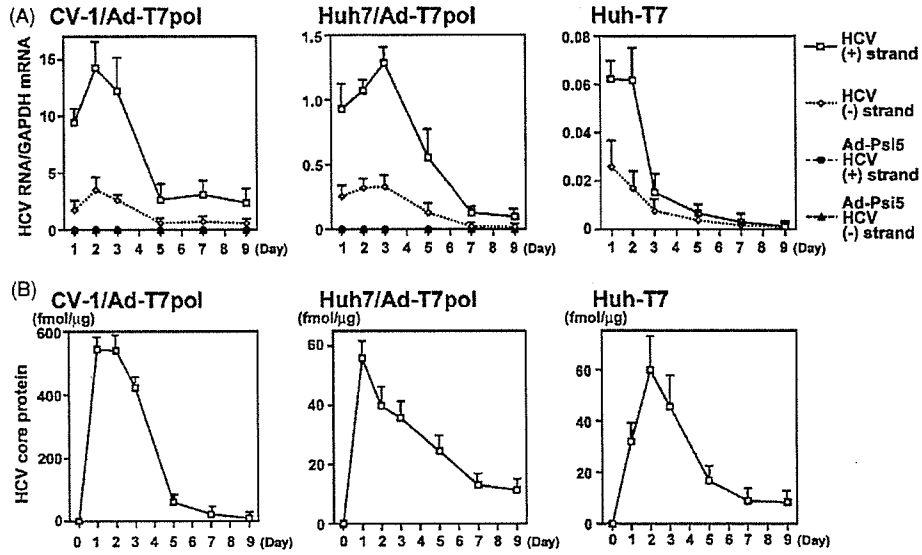

Fig. 3. (A) Real-time PCR of HCV positive- and negative-strand RNA was performed as described. Data are expressed as the strand-specific HCV copy number per molecule of GAPDH. Error bars indicate the mean ± standard error (S.E.) of four replicates. (B) A quantitative core ELISA measured HCV protein production in CV-1, Huh7, and Huh-T7 cell lines. Data are expressed as fmol (mean ± S.E.) of HCV core per μg of total protein for three replicates.

expression without inducing cell toxicity. Thus, the time course of strand-specific HCV RNA synthesis (Fig. 3A) and HCV protein production (Fig. 3B) was examined further in CV-1 and Huh7 cells (transfected with H77 and infected with Ad-T7pol), as well as Huh-T7 cells (transfected with H77). Both positive- and negative-strand HCV RNA were detectable for the entire 9-day experiment in each cell line. Positive-strand HCV RNA levels increased significantly after infection and continued to be expressed at high levels for 3 days and diminished thereafter. Negative-strand HCV RNA synthesis paralleled that of positive-strand throughout the time course; however, the quantity of negative-strand HCV RNA was approximately 10% of positive-strand levels. This is consistent with positive-/negative-strand ratios reported from infected human liver samples (Komurian-Pradel et al., 2004; Laskus et al., 1998). As expected, HCV RNA was not detected in CV-1 or Huh7 cells infected with the Ad-psi5 control vector.

Using an identical experimental approach, HCV core protein production was expressed strongly during days 1–3 in all cell lines examined, and diminished with similar kinetics as HCV RNA (Fig. 3B). Similar to HCV RNA, HCV core protein was detectable for the entire 9-day experiment in each cell line.

To circumvent potential perturbations in the cellular environment due to transfection/infection with viral vectors, experiments in a Huh7 cell line stably transfected with T7 polymerase (Huh-T7) were performed. After transfection of H77 into these cells, positive- and negative-strand HCV RNA were detected (Fig. 3A), as well as HCV core protein (Fig. 3B), throughout the entire 9-day experiment. Interestingly, HCV RNA levels were lower in Huh-T7 cells than in CV-1 and Huh7 cells, although core levels were not appreciably different between Huh7 and Huh-T7 cells.

3.4. IFN efficiently inhibits HCV expression

Utilizing these refined models of HCV replication, the inhibitory effects of IFN on HCV expression were examined. To determine the potential effects of IFN on cellular gene translation, the plasmid OS8 was transfected, and LacZ mRNA levels were measured in the presence of several doses of IFN. LacZ mRNA expression was slightly reduced; however, no significant toxicity in cells exposed to IFN was observed using trypan-blue staining (data not shown).

In CV-1 cells (Fig. 4A), HCV positive-strand RNA levels were significantly decreased in the presence of 1000 IU/mL IFN at day 2 (14.21 ± 3.95 versus 8.55 ± 0.61, $p < 0.05$). A trend toward reduced HCV RNA was also observed on day 3 (12.23 ± 5.43 versus 4.13 ± 0.74, $p < 0.10$). In Huh7 cells, a significant decrease of HCV positive-strand was observed on days 2 (1.07 ± 0.07 versus 0.63 ± 0.14, $p < 0.05$) and 3 (1.29 ± 0.13 versus 0.44 ± 0.15, $p < 0.05$). In Huh-T7 cells, a significant decrease was also observed on day 2 (0.06 ± 0.03 versus 0.03 ± 0.007, $p < 0.05$). For HCV negative-strand RNA (Fig. 4B), only day 3 IFN-treated CV-1 cells had significantly decreased levels compared to untreated cells (2.60 ± 0.41 versus 0.97 ± 0.31, $p < 0.05$).

HCV core protein expression was approximately 10-fold higher in CV-1 cells compared to either Huh7 or Huh-T7 cells (Fig. 4C). IFN treatment of CV-1 cells did not appear to have a large effect on HCV core protein levels; however, HCV core levels were decreased in IFN-treated CV-1 cells compared to untreated cells at days 3 (423.51 ± 25.73 fmol/μg versus 190.92 ± 35.25 fmol/μg, $p < 0.05$) and 5 (60.24 ± 12.89 fmol/μg versus 34.15 ± 0.76 fmol/μg, $p < 0.05$). For Huh7 and Huh-T7 cells, HCV core expression was significantly reduced when treated with IFN compared to untreated cells at all time points ($p < 0.05$).

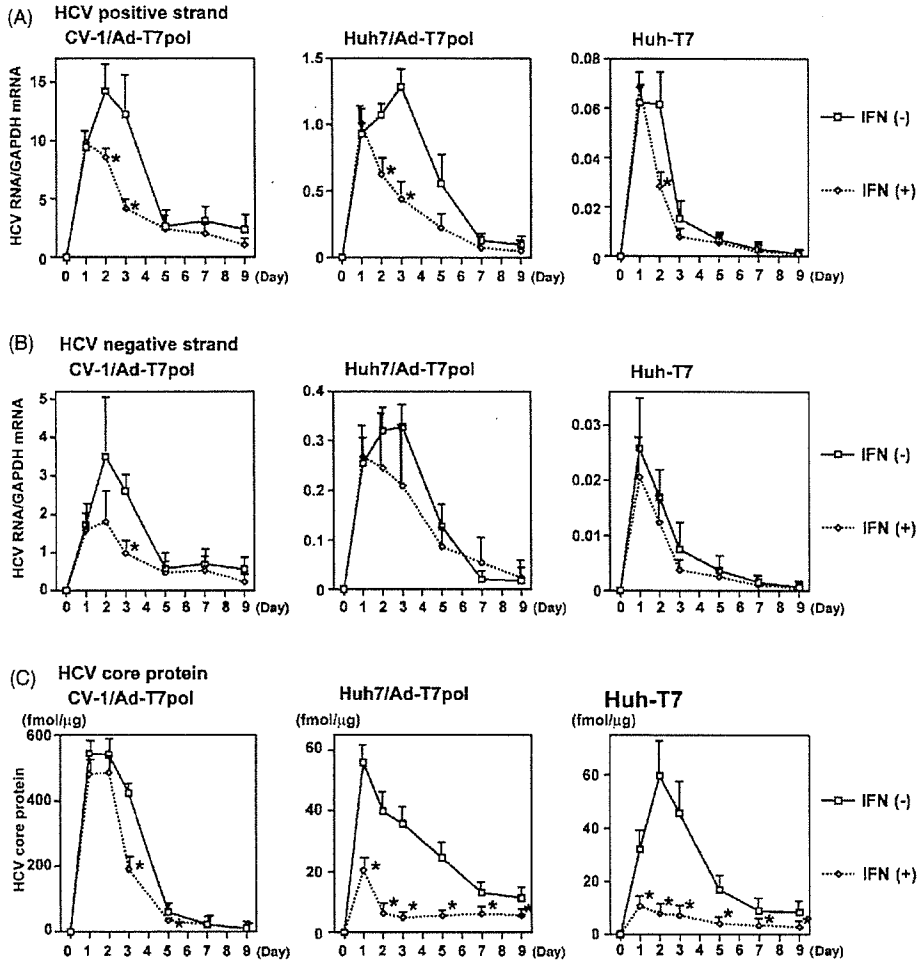

Fig. 4. (A) Real-time PCR of positive-strand HCV RNA was performed in the presence of 1000 IU/mL IFN. Error bar indicates mean ± S.E. for four replicates ($^{*}p < 0.05$; $^{**}p < 0.10$). (B) Real-time PCR of negative-strand HCV RNA was also performed in the presence of 1000 IU/mL IFN. (C) Quantitative core ELISA also demonstrated decreased HCV protein production in IFN-treated cells.

4. Discussion

Because of the cytotoxic nature of vaccinia virus, long-term evaluation of HCV RNA synthesis and protein production, as well as characterization of the inhibitory effects of antiviral agents, such as IFN and RBV, was not possible using our previous replication model. By using adenovirus-derived T7 vectors, vaccinia-induced cytotoxicity was removed, allowing sustained detection of HCV replication and protein production for 9 days in multiple cell types. The refined binary HCV replication system efficiently synthesized HCV negative-strand RNA, an important indicator of ongoing, active viral replication, in an NS5B-dependent manner, as no negative-strand HCV RNA was detected upon transfection of an NS5B mutant. Using Huh7 cell lines stably expressing T7 polymerase (Huh-T7), dependence of the replication models on any viral vectors was removed. Quantities of HCV RNA synthesis and protein production in Huh-T7 cells were not as robust as in CV-1 or Huh7 cells transfected with Ad-T7. However, sustained HCV replication in Huh-T7 cells, with no obvious signs of cytotoxicity, suggests that this viral vector-independent replication model will be useful for future studies of virus–host interactions and the development of antiviral agents with activity against HCV genotype 1.

These binary systems offer several advantages over currently available HCV replication systems. First, these replication models do not require continuous antibiotic selection as do current replicon systems (Blight et al., 2000; Frese et al., 2001; Guo et al., 2001; Lohmann et al., 1999). Second, the requirement of highly adaptive viral mutations for efficient replicon activities that are not necessarily viable in vivo (Bukh et al., 2002) may limit the interpretability of certain findings obtained from replicon systems. Because the refined replication models do not require continuous selection and do not possess highly 'adaptive' viral mutations, they are more authentic for characterization of antiviral agents, virus–host interactions, and viral fitness. Third, these replication systems can be used to study HCV replication in a variety of hepatocyte- and non-hepatocyte-derived cell types; in contrast, replicon systems only replicate efficiently in Huh7 cells. Most importantly, these replication systems use a full-length infectious genotype 1a cDNA construct that yields an

Table 1
Several similarities and differences between the vaccinia and adenovirus systems exist

	Vaccinia	Ad-T7
T7 polymerase delivery	Vaccinia virus	Adenovirus
Cytotoxicity	Yes	No
HCV replication	(+) and (−) strand synthesis: high levels	(+) and (−) strand synthesis; low but effective levels
Duration of replication	24 h	9 days
HCV protein production	Yes	Yes
Quasispecies generation	Yes	Yes
IFN inhibits replication	Yes	Yes

authentic dual-function template in vivo that is both translated and transcribed. Moreover, transfected cells in our replication systems are able to express all HCV structure and non-structural proteins (Lin et al., 2005). Thus, they are more likely to carry out authentic HCV RNA replication than replicon systems based on sub-genomic constructs.

Several significant differences exist between the vaccinia and adeno-T7 replication systems (Table 1). Both are capable of positive- and negative-strand HCV RNA synthesis, protein production, and quasispecies generation without the need for cell culture adaptive mutations (Chung et al., 2001; Contreras et al., 2002; Blackard and Hiasa et al., unpublished data). HCV replication is inhibited significantly by IFN in both systems. However, the vaccinia-based system replicates at much higher levels than the adeno-T7-based system; yet, HCV RNA synthesis occurs for at least 9 days in the former due to the lack of vector-induced cytotoxicity. Nonetheless, the decrease of HCV RNA synthesis and protein production after 3 days suggests an inhibitory effect exerted by key host cells proteins, such as protein kinase R (PKR), since adenoviruses do not inhibit PKR function as does vaccinia virus (Chang et al., 1992; Watson et al., 1991). Further examination of host antiviral pathways that limit robust long-term viral replication in culture is necessary.

We used these refined replication systems to explore the kinetics of HCV RNA synthesis and protein production in the presence of IFN. When 1000 IU/mL of IFN was added to the culture medium of HCV-expressing cells, there was no difference in HCV positive- or negative-strand quantity compared to untreated cells at day 1. Despite this lack of short-term antiviral activity, HCV RNA was significantly decreased in IFN-treated cells at days 2 and 3, suggesting that the full effects of IFN may require at least 24 h.

This cell-based HCV replication system has already been used to examine the interaction between HCV protein expression and host type I IFN signaling components in the Jak-STAT kinase pathway (Lin et al., 2005). Recently, in vitro systems that support infectious HCV production have been reported. However, these systems are based on HCV genotype 2a (Wakita et al., 2005; Zhong et al., 2005) and do not support replication in cells other than the highly permissive Huh-7 cell line and its derivatives. Importantly, the replication systems described here are based on genotype 1a isolate and replicate in several hepatocyte- and non-hepatocyte-derived cell lines. Thus, these refined replication models provide the opportunity to explore HCV molecular biology and the interactions between antiviral agents and specific HCV and/or host proteins that are relevant to genotype 1 infection.

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MAJOR ARTICLE

Detection of Hepatitis C Virus (HCV) in Serum and Peripheral-Blood Mononuclear Cells from HCV-Monoinfected and HIV/HCV–Coinfected Persons

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It has been speculated that hepatitis C virus (HCV) replicates in peripheral-blood mononuclear cells (PBMCs), which, therefore, may be a site for interaction with human immunodeficiency virus (HIV). We used strand-specific real-time polymerase chain reaction to detect HCV RNA in 28 HCV-monoinfected and 20 HIV/HCV–coinfected women. At the first visit, positive-strand HCV RNA was detected in serum samples from 89% of the women, whereas positive-strand HCV RNA was detected in PBMC samples from 32% and 55% of the HCV-monoinfected and HIV/HCV–coinfected women, respectively. After initiation of antiretroviral therapy, the HIV/HCV–coinfected women were significantly more likely to have detectable positive- and negative-strand HCV RNA in the PBMC compartment than were the HCV-monoinfected women. HIV and HCV RNA levels were not correlated. Serum HCV RNA levels were correlated over time; HCV RNA levels in the serum and PBMC compartments were not. These data suggest differential regulation of HCV RNA in the serum and PBMC compartments and may partially explain the limited HCV antiviral response rates observed in coinfected persons.

Hepatitis C virus (HCV) is a positive-strand RNA virus that infects >170 million people worldwide. Because of the inability to infect small animals with HCV and the lack of efficient cell-culture models, much of the current understanding of the HCV life cycle has been inferred from studies that use samples from infected humans. Although hepatocytes are the major site of infection, there is a broad clinical spectrum of disease and extrahepatic complications, including cryoglobulinemia, non-Hodgkin lymphoma, and porphyria cutanea tarda [1].

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Some studies have reported evidence for extrahepatic replication of HCV in peripheral-blood mononuclear cells (PBMCs); however, these studies have typically involved a small number of patients and have often yielded contradictory results [2–7]. Other studies have reported evidence for HCV replication in granulocytes, monocytes/macrophages, dendritic cells, and B lymphocytes, as well as in extrahepatic tissues [8–16]. Because certain amplification methods lack strand specificity, which may influence the reliable detection of replication intermediates (i.e., negative-strand HCV RNA), it has been challenging to definitively demonstrate extrahepatic HCV replication. Recently, modification of the real-time polymerase chain reaction (rtPCR) assay to include the *Tth* enzyme, which has high strand specificity and independent reverse-transcriptase and DNA-dependent polymerase activity, has been used to detect negative-strand HCV RNA in the liver and/or PBMC compartment [7, 9, 17–19].

In the United States, 150,000–300,000 people are coinfected with HCV and HIV [20]. Multiple studies have demonstrated the adverse effects of HIV coinfec-

tion on liver fibrosis, HCV RNA levels, HCV disease progression [21], and treatment response rates [22–24]. The mechanisms by which these 2 viruses interact remain unclear, because no direct virus-virus interactions have been demonstrated to date. However, recent in vitro data suggest that HCV and HIV proteins cooperatively induce hepatic apoptotic pathways [25, 26] and secretion of proinflammatory cytokines [27] without requiring cell infection and viral replication. Thus, it is reasonable to speculate that similar signaling cascades in PBMCs may also permit indirect interactions between HCV and HIV.

We have previously investigated serum HCV diversity in HIV/HCV–coinfected persons initiating antiretroviral therapy (ART) for HIV infection [28]. We found significant evolution of the hypervariable region 1, but not the adjacent envelope 1 region, after ART initiation. However, few studies have addressed the effects of ART on HCV in HIV/HCV–coinfected persons in compartments other than serum. The demonstration of extrahepatic HCV replication in the PBMC compartment would have important implications for transmission of the virus and efficient treatment of HCV infection. Nonetheless, previous studies have not assessed this phenomenon in HIV/HCV–coinfected persons in a longitudinal manner, nor have they addressed it in coinfected persons initiating ART [15, 29, 30]. Therefore, we sought to investigate whether HCV replication could be detected in the serum and PBMC compartments of persons coinfected with HIV and HCV and to assess the effect of ART on extrahepatic HCV replication.

PARTICIPANTS, MATERIALS, AND METHODS

Study population. From April 1993 to February 1995, the HIV Epidemiology Research (HER) Study, a prospective natural-history study of HIV infection, enrolled 871 HIV-infected women and 439 demographically matched HIV-uninfected women [31]. The women participated in clinic visits at 6-month intervals through 1999. By study design, one-half of the women reported injection drug use (IDU), and the other half reported only sexual risk behavior.

As described elsewhere, HCV serostatus was determined by either Abbott HCV EIA (version 2.0) or Ortho HCV ELISA (version 3.0) [32]. Overall, the seroprevalence of HCV was 56.5%, with rates of 48.0% and 60.8% in HIV-uninfected and HIV-infected women, respectively. Of the women who acknowledged prior IDU, 88.3% were HCV seropositive; of these women, 76.9% had detectable HCV RNA [33]. Because the HER Study cohort was formed before the widespread use of combination therapy, only 30% were receiving ART at the beginning of the study. By 1999, 31.3% were still not receiving any ART [34].

HER Study participants were included in the present study if they (1) were HCV seropositive, regardless of their HIV status; (2) had serum and PBMC samples available from at least 2 consecutive study visits conducted at the Providence, RI, site; and (3) were not receiving ART at the beginning of the study (for the HIV-infected women). For the HIV-infected women, study visits corresponded to the visit immediately before ART initiation (denoted "visit A") and the visit immediately after ART initiation (denoted "visit B"). The median intervals between visits were 5.8 months and 6.6 months for the HCV-monoinfected and HIV/HCV–coinfected women, respectively. The drug regimens initiated by the HIV-infected women were as follows: ≥2 nucleoside reverse-transcriptase inhibitors (NRTIs) ($n = 4$); ≥1 NRTI plus ≥1 protease inhibitor (PI) ($n = 11$); ≥2 NRTIs plus 1 nonnucleoside reverse-transcriptase inhibitor (NNRTI) ($n = 4$); and 2 NRTIs plus 1 NNRTI plus 2 PIs ($n = 1$). One HIV/HCV–coinfected woman was missing serum samples at both visits, and 3 HIV/HCV–coinfected women were missing PBMC samples at visit B.

Cellular RNA extraction and strand-specific* Tth *rtPCR. RNA was extracted from serum samples by use of the QIAamp Viral RNA Kit (Qiagen). For PBMC samples, the number of cells available was limited. Because the number of cells varied per sample (range, 1.4–7.6 × 10^6 cells/mL), we normalized all quantitative HCV RNA data on the PBMC compartment to the copy number of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Five hundred microliters of a PBMC suspension was washed with diethopyrocarbonate (DEPC)–treated dH_2O, and cellular RNA was extracted by use of TRIzol (Invitrogen). The resultant RNA was resuspended in 40 μL of DEPC-treated dH_2O and treated 2 times with DNase I (Ambion). Positive- and negative-strand HCV cDNAs were quantified by a validated strand-specific rtPCR assay using SYBR green dye I, as described elsewhere [17, 19]. Extracted RNA was heated at 95°C for 1 min and then incubated at 70°C. A mixture containing 10 pmol/μL HCV-1 antisense primer (5′-TGGATGCACGGTCTACGAGACCTC-3′; nt 342–320, according to the numbering of H77 [35]; GenBank accession number AF009606) for HCV positive-strand synthesis or HCV-2 sense primer (5′-CACTCCCCTGTGAGGAACT-3′; nt 38–56) for HCV negative-strand synthesis, 1× reverse-transcriptase buffer, 1 mmol/L $MnCl_2$, 200 mmol/L each deoxynucleoside triphosphate, and 5 U of *Tth* enzyme (Applied Biosystems) was added. The cDNA reaction consisted of an annealing step for 2 min at 60°C, followed by an extension step for 20 min at 70°C. To inactivate the reverse-transcriptase activity of the *Tth* enzyme, chelating buffer was added after cDNA synthesis. cDNA was purified by use of the High Pure PCR Template Preparation Kit (Roche Diagnostics).

Positive- and negative-strand HCV PCR amplification was performed with 2 μL of purified cDNA in a mixture containing LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics), 4 mmol/L $MgCl_2$, and 5 pmol/L each antisense primer KY78 (5′-CTCGCAAGCACCCTATCAGGCAGT-3′; nt 311–288) and sense primer KY80 (5′-GCAGAAAGCGTCTAGCCA-

Table 1. Characteristics of the study cohort.

Characteristic, parameter	HCV monoinfected	HIV/HCV coinfected	*P*
Risk factor			NS[a]
Injection drug use	24 (86)	18 (90)	
Heterosexual contact	4 (14)	2 (10)	
Age at enrollment, mean ± SD, years	34.4 ± 6.0	35.2 ± 3.9	NS[b]
Race			.008[a]
Black	4 (14)	11 (55)	
White	21 (75)	8 (40)	
Hispanic	3 (11)	1 (5)	
HCV genotype			NS[a]
1	7 (25)	11 (55)	
2	3 (11)	0 (0)	
3	3 (11)	1 (5)	
4	2 (7)	3 (15)	
Unknown	13 (47)	5 (25)	
Visit A			
CD4 cell count, mean ± SD, cells/μL	1139 ± 345	285 ± 144	<.0001[c]
Plasma HIV RNA level, median (IQR), $\log_{10}$ copies/mL	...	4.1 (2.9–4.6)	...
Receipt of ART	...	0 (0)	...
Visit B			
CD4 cell count, mean ± SD, cells/μL	1101 ± 264	376 ± 191	<.0001[c]
Plasma HIV RNA level, median (IQR), $\log_{10}$ copies/mL	...	2.1 (<1.7–3.4)	...
Receipt of ART	...	18 (90)	...

NOTE. Data are no. (%) of women, unless otherwise noted. ART, antiretroviral therapy; HCV, hepatitis C virus; IQR, interquartile range; NS, not significant ($P > .05$).

[a] Fisher's exact test.

[b] Student's *t* test for normal data.

[c] Wilcoxon rank sum test.

TGGCGT-3′; nt 68–91). The PCR consisted of an initial denaturation step for 10 min at 95°C, then 40 cycles under the following conditions: 15 s at 95°C, 5 s at 70°C, and 15 s at 72°C. For generation of GAPDH mRNA, cDNA synthesis was performed with an oligo d(T) primer under standard conditions. For PCR amplification, we used a commercial GAPDH primer set (Roche Search LC), with the conditions recommended by the manufacturer.

For each PBMC sample, we determined the positive- and negative-strand HCV RNA copy numbers and normalized them to the GAPDH copy number, to provide standardized values (i.e., positive-strand HCV RNA copies and negative-strand HCV RNA copies per molecule of GAPDH). Serum HCV quantities were expressed as HCV RNA copies per microliter (extracted from 140 μL of serum). Previous studies have reported very low rates of negative-strand HCV RNA detection in serum [11, 14, 15]; thus, we did not systematically measure negative-strand HCV RNA in this compartment. To avoid potential cross-contamination, samples for each time point and each compartment from an individual were handled separately. Additionally, all rtPCR amplifications included a negative control that contained no template.

Statistical analyses. Demographic and clinical data were compared by Fisher's exact test for categorical variables and either Student's *t* test or the Wilcoxon rank sum test for continuous variables. The Wilcoxon rank sum test was used to compare HCV RNA levels between the HCV-monoinfected and HIV/HCV–coinfected women; values for undetectable levels were set at 0 for serum samples ($\log_{10}$ transformed) and at 0.01 for PBMC samples (untransformed). Spearman's correlation test was used to investigate the linear relationships between CD4 cell count, plasma HIV RNA level, and serum and PBMC HCV RNA levels. All *P* values reported are 2-sided; $P < .05$ was considered to be statistically significant. No adjustments were made for multiple comparisons. All analyses were performed by use of SAS software (version 9; SAS Institute).

RESULTS

Study cohort characteristics. Twenty-eight HCV-monoinfected and 20 HIV/HCV–coinfected women from the HER Study cohort were selected for the present study. These 2 groups of women did not differ with respect to the reporting of IDU as the main risk factor for HCV acquisition, age at enrollment, or HCV genotype; however, HIV/HCV–coinfected women were more likely to be black (table 1). None of the women reported receiving HCV treatment during the visits included in the present study. Mean CD4 cell counts were lower in the HIV/HCV–

Table 2. Strand-specific hepatitis C virus (HCV) RNA detection rates.

Visit, clinical variable	Total	HCV monoinfected	HIV/HCV coinfected	*P*
Visit A				
Serum positive-strand HCV RNA	89 (42/47)	82 (23/28)	100 (19/19)	NS
PBMC positive-strand HCV RNA	42 (20/48)	32 (9/28)	55 (11/20)	NS
PBMC negative-strand HCV RNA	35 (17/48)	32 (9/28)	40 (8/20)	NS
Visit B				
Serum positive-strand HCV RNA	91 (43/47)	93 (26/28)	89 (17/19)	NS
PBMC positive-strand HCV RNA	44 (20/45)	29 (8/28)	71 (12/17)	.01
PBMC negative-strand HCV RNA	38 (17/45)	25 (7/28)	59 (10/17)	.03

NOTE. Data are percentage (no. positive/no. tested) of women, unless otherwise noted. $P > .05$); PBMC, peripheral-blood mononuclear cell.

coinfected women than in the HCV-monoinfected women at both time points (visit A, 285 vs. 1139 cells/μL [$P < .0001$]; visit B, 376 vs. 1101 cells/μL [$P < .0001$]). After ART initiation (between visits A and B), median plasma HIV RNA levels decreased, from 4.1 to 2.1 $\log_{10}$ copies/mL, in the HIV/HCV–coinfected women ($P = .0002$), whereas mean CD4 cell counts increased, from 285 to 376 cells/μL ($P = .4$), in these women.

Strand-specific HCV RNA detection rates. At visit A, positive-strand HCV RNA was detected, by a strand-specific rtPCR assay, in serum from 42 (89%) of 47 women, including 23 (82%) of 28 HCV-monoinfected women and 19 (100%) of 19 HIV/HCV–coinfected women (table 2). At visit B, 43 (91%) of 47 women had detectable levels of positive-strand HCV RNA in serum. Rates of detection in the serum compartment were not significantly different between the HCV-monoinfected and the HIV/HCV–coinfected women at either visit. We did not systematically measure levels of negative-strand HCV RNA in the serum compartment. However, of 47 women tested, 34 (72%) had undetectable or negligible levels (<1000 copies/μL) of negative-strand HCV RNA (data not shown). Furthermore, among those women in whom both strands were detected, the ratio of negative-strand:positive-strand HCV RNA in the serum compartment was <1% in both groups, suggesting that there is a vast excess of positive-strand HCV RNA in the serum compartment (data not shown). In contrast, the proportion of negative-strand HCV RNA relative to positive-strand HCV RNA in the PBMC compartment was significantly higher (particularly in the HIV/HCV–coinfected women), a finding that is consistent with higher rates of HCV replication in the PBMC compartment.

Our findings regarding detection of positive- and negative-strand HCV RNA in the PBMC compartment by the strand-specific rtPCR assay were strongly suggestive of extrahepatic HCV replication. At visit A, positive-strand HCV RNA was detected in the PBMC compartments of 20 (42%) of 48 women, including 9 (32%) of 28 HCV-monoinfected women and 11 (55%) of 20 HIV/HCV–coinfected women. Negative-strand HCV RNA was detected in 17 (35%) of 48 women, including 9 (32%) of 28 HCV-monoinfected women and 8 (40%) of 20 HIV/HCV–coinfected women. At visit B, after the HIV-infected women had initiated ART, positive-strand HCV RNA was still more readily detected in the HIV/HCV–coinfected women than in the HCV-monoinfected women (8/28 [29%] vs. 12/17 [71%]; $P = .01$). The negative-strand HCV RNA detection rate was also significantly different in the HCV-monoinfected and the

Table 3. Median strand-specific hepatitis C virus (HCV) RNA levels.

Visit, clinical variable	HCV monoinfected	HIV/HCV coinfected	*P*
Visit A, median (75th percentile)			
Serum positive-strand HCV RNA level	3.6 (5.0)	5.2 (5.6)	.002
PBMC positive-strand HCV RNA level	0 (31.2)	2.5 (30.7)	NS
PBMC negative-strand HCV RNA level	0 (2.3)	0 (20.0)	NS
Visit B, median (75th percentile)			
Serum positive-strand HCV RNA level	4.4 (5.0)	5.5 (5.9)	.003
PBMC positive-strand HCV RNA level	0 (48.0)	5.4 (38.2)	NS
PBMC negative-strand HCV RNA level	0 (2.3)	0.6 (19.3)	NS

NOTE. Data for serum HCV RNA levels, which are $\log_{10}$ transformed, are no. of HCV RNA copies per microliter (extracted from 140 μL of serum); data for PBMC HCV RNA levels, which are untransformed, are no. of HCV RNA copies per molecule of glyceraldehyde-3-phosphate dehydrogenase. The medians for several of the clinical variables are 0 because of low rates of detection. NS, not significant ($P > .05$); PBMC, peripheral-blood mononuclear cell.

HIV/HCV–coinfected women at visit B (7/28 [25%] vs. 10/17 [59%]; $P = .03$). Negative-strand HCV RNA was detected in the PBMC compartment only when positive-strand HCV RNA was also detected.

Strand-specific HCV RNA levels. Strand-specific HCV RNA levels were determined in the serum and PBMC compartments and, in the latter case, were normalized to the GAPDH copy number (table 3). Using dilutions of serum samples for which HCV RNA levels had previously been determined (by use of the Roche Amplicor Monitor Kit), we determined that the lower level of detection for the strand-specific rtPCR assay was ~260 copies/μL. The HIV/HCV–coinfected women had higher positive-strand HCV RNA levels in serum than did the HCV-monoinfected women, both before and after ART initiation (visit A, 3.6 vs. 5.2 $\log_{10}$ copies/μL [$P = .002$]; visit B, 4.4 vs. 5.5 $\log_{10}$ copies/μL [$P = .003$]). Because of the low rates of detection of HCV RNA in the PBMC compartment, medians could not be defined in several instances; therefore, 75th percentiles are presented in table 3. At visit A, there was no significant difference in either positive- or negative-strand HCV RNA levels in the PBMC compartment between the 2 groups. At visit B, after ART initiation, both positive- and negative-strand HCV RNA levels in the PBMC compartment were higher in the HIV/HCV–coinfected women than in the HCV-monoinfected women, but these differences did not reach statistical significance.

Correlation analyses. We also analyzed potential correlations between specific immunologic parameters and strand-specific HCV RNA levels (table 4). Age and plasma HIV RNA levels were not correlated with either positive- or negative-strand HCV RNA levels in either compartment. CD4 cell count was inversely correlated with positive-strand HCV RNA levels in the serum ($P \leq .0001$), but not in the PBMC, compartment. Positive-strand HCV RNA levels in the serum compartment were consistent over time ($P < .0001$), as were both positive- and negative-strand HCV RNA levels in the PBMC compartment ($P < .0001$). However, in the absence of ART, positive-strand HCV RNA levels in the serum compartment were not correlated with either positive- or negative-strand HCV RNA levels in the PBMC compartment. Between visits, neither positive- nor negative-strand HCV RNA levels in the PBMC compartment were correlated.

Table 4. Correlation between clinical variables and strand-specific hepatitis C virus (HCV) RNA levels.

Comparison	Coefficient[a]	*P*
Serum positive-strand HCV RNA level (A) vs.		
Serum positive-strand HCV RNA level (B)	0.75	<.0001
PBMC positive-strand HCV RNA level (A)	0.02	NS
PBMC negative-strand HCV RNA level (A)	0.01	NS
PBMC positive-strand HCV RNA level (A) vs.		
PBMC negative-strand HCV RNA level (A)	0.89	<.0001
PBMC positive-strand HCV RNA level (B)	0.08	NS
PBMC positive-strand HCV RNA level (B) vs. PBMC negative-strand HCV RNA level (B)	0.90	<.0001
PBMC negative-strand HCV RNA level (A) vs. PBMC negative-strand HCV RNA level (B)	0.06	NS
CD4 cell count vs.		
Serum positive-strand HCV RNA level (A)	−0.54	<.0001
PBMC positive-strand HCV RNA level (A)	−0.10	NS
PBMC negative-strand HCV RNA level (A)	0.02	NS
Plasma HIV RNA level vs.		
Serum positive-strand HCV RNA level (A)	0.11	NS
PBMC positive-strand HCV RNA level (A)	0.31	NS
PBMC negative-strand HCV RNA level (A)	0.18	NS

NOTE. A and B refer to the visit. NS, not significant ($P > .05$); PBMC, peripheral-blood mononuclear cell.

[a] Spearman correlation coefficient for all women.

DISCUSSION

Researchers have long sought to establish whether HCV replicates outside the liver, because detection of HCV RNA in extrahepatic reservoirs has important implications for transmission, disease progression, and effective treatment. Nonetheless, achieving a definitive demonstration of extrahepatic HCV replication has been limited by several biological and technical considerations. Foremost, the lack of a robust cell-culture system has made it exceedingly difficult to compare HCV replication in different cell populations. To date, the dynamics of HCV replication have typically been examined by intensive study of serum-specific or liver-specific HCV RNA; however, viral replication in such extrahepatic reservoirs as PBMCs may not reflect replication in these other compartments. Furthermore, although detection of positive-strand HCV RNA cannot distinguish between nucleic acids participating in replication and those already incorporated into viral particles, detection of replication intermediates, such as negative-strand HCV RNA, is a more biologically relevant measure of active virus replication. Negative-strand HCV RNA is generally present at levels 10–100-fold lower than those of positive-strand HCV RNA [36, 37]; thus, highly sensitive and specific detection assays must be used. Although distinguishing between positive- and negative-strand HCV RNA is critical, not all strand-specific detection methods have high specificity for detection of negative-strand HCV RNA. Here, we have used a validated strand-specific rtPCR assay that includes the *Tth* enzyme. Because this enzyme contains separate reverse transcriptase and DNA-dependent polymerase functions, it is highly specific and is ideal for discriminating between positive- and negative-strand RNA [17, 19].

Although several studies have measured HCV replication in the PBMC compartment, only a subset have used a bona fide *Tth*-based amplification assay to distinguish between positive- and negative-strand HCV RNA [4, 9, 17, 29, 38–41]. Our rate of detection of negative-strand HCV RNA in the PBMC compartment was somewhat elevated, compared with the results of these previous studies. Such differences could reflect minor dis-

crepancies in the amplification assay, study populations, HCV antiviral receipt, and/or sample preparation. However, it has previously been demonstrated that both HIV coinfection and testing of multiple extrahepatic samples are associated with an increased likelihood of detection of negative-strand HCV RNA [29, 38, 41]. For example, Laskus et al. demonstrated the presence of negative-strand HCV RNA in 5 of 14 PBMC samples from HIV/HCV–coinfected patients [29]. The authors also suggested that factors governing HCV replication at hepatic and extrahepatic sites may differ. Thus, one might anticipate increased detection of negative-strand HCV RNA in a population such as ours, because no participant received HCV antiviral therapy, a high prevalence of HIV coinfection existed, and we tested multiple samples for each participant. It is also theoretically possible that our use of an all-female cohort is responsible for increased detection of negative-strand HCV RNA, although sex-specific detection rates have not been reported to date.

Our study design has several distinct advantages over those of previously published studies. First, to date, most studies of extrahepatic replication have been restricted to a small population analyzed in a cross-sectional, rather than a longitudinal, manner. Second, paired serum and PBMC samples have not usually been analyzed, making intercompartment comparisons difficult. Third, despite clinical data suggesting that HIV adversely affects HCV replication, disease progression, and treatment response rates, HCV-monoinfected and HIV/HCV–coinfected persons have not typically been analyzed as distinct groups. Fourth, not all previously published studies used a strand-specific rtPCR assay that had high strand specificity.

The present study design does have several limitations. First, very low levels of negative-strand HCV RNA were detected in the serum compartments of a subset of women. Because "naked" negative-strand HCV RNAs are not known to circulate outside of cells, we suggest that these very low levels of negative-strand RNA likely represent a small amount of contaminating RNA from residual PBMCs that were not completely removed during the initial processing of whole blood. Second, given the limited number of PBMCs available, we were not able to more precisely define the cell population(s) within PBMCs that are responsible for HCV replication. Nonetheless, there is growing evidence that HCV may infect several peripheral-blood cell types, including B lymphocytes, granulocytes, monocytes/macrophages, and dendritic cells [8, 12, 40]; it is, however, important to note that each of these previous studies either excluded persons coinfected with HIV or did not report HIV status.

Results from our pilot study should be interpreted with caution, given its limited sample size. Nonetheless, we here report several novel findings regarding extrahepatic HCV replication. First, rates of detection of HCV RNA in the PBMC compartment were higher for HIV/HCV–coinfected women than for HCV-monoinfected women. Previous studies have suggested that serum HCV RNA levels are higher in HIV/HCV–coinfected persons [21]; however, this phenomenon has not been investigated in the PBMC compartment until now. Importantly, negative-strand HCV RNA, indicative of active viral replication, was detected at higher rates in the PBMC compartments of HIV/HCV–coinfected women, highlighting an important interaction between these 2 viruses in this compartment. Second, there was no correlation between plasma HIV RNA levels and positive- or negative-strand HCV RNA levels in either the serum or PBMC compartment. Moreover, ART initiation appeared to have a minimal effect on HCV detection rates and HCV RNA levels, although, because of the limited number of HIV/HCV–coinfected persons included in the present study, we cannot rule out a possible association. The finding of elevated HCV RNA levels in the serum and PBMC compartments even after ART initiation may suggest that immune reconstitution after suppression of HIV is not sufficient to control HCV replication. Third, there was an inverse correlation between CD4 cell counts and positive-strand HCV RNA levels in the serum, but not the PBMC, compartment (i.e., as the CD4 cell count increased, the serum, but not the PBMC, HCV RNA level decreased). Given that several components of PBMCs may support HCV replication [8, 12, 40], it is provocative to speculate that the PBMC compartment may be a site in which HCV is partially protected from adaptive and/or innate immune responses. Fourth, there was a positive correlation between positive- and negative-strand HCV RNA levels in the PBMC compartment. However, serum and PBMC HCV RNA levels did not correlate with each other. Thus, HCV RNA may be regulated differently in these compartments.

The precise mechanisms by which HIV influences extrahepatic HCV replication have yet to be determined. It is possible that HIV-induced immunosuppression results in less immunologic control of HCV replication, although reproducible correlations between HCV RNA levels in the serum compartment and CD4 cell counts have not been confirmed [30]. Moreover, the presence of replicative viral forms in extrahepatic sites does not correlate with CD4 cell count [29]. Interestingly, in the present study, HCV RNA levels in the PBMC compartment did not correlate with CD4 cell counts, although positive-strand HCV RNA levels in the serum compartment and CD4 cell counts were inversely correlated. These data imply that immunosuppression alone is not the sole driving force behind increased detection of HCV RNA in the PBMC compartment. It is also possible that HIV, through the induction of interferon antagonists, blunts host innate antiviral responses that would otherwise inhibit HCV replication. HIV may also render specific types of PBMCs more susceptible to HCV infection and replication [41].

In summary, low-level HCV replication in the PBMC compartment, as indicated by detection of negative-strand HCV RNA, may adversely influence the effectiveness of HCV anti-