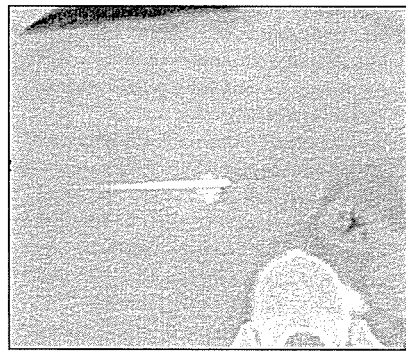
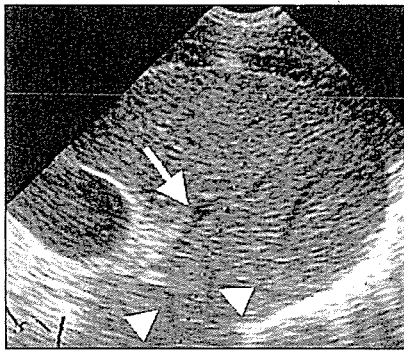
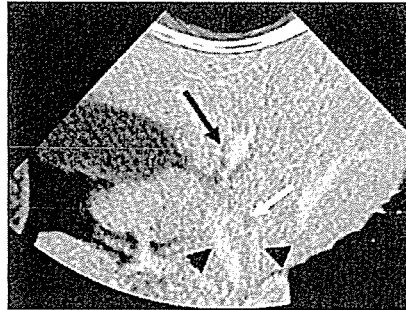
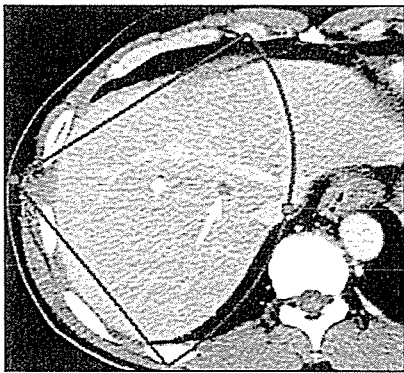
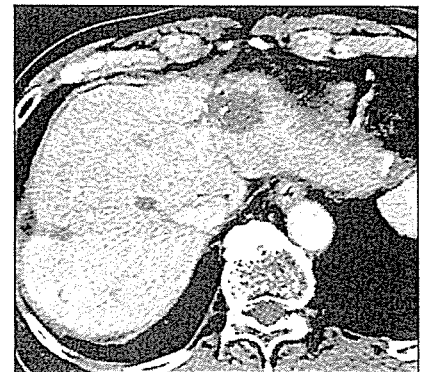
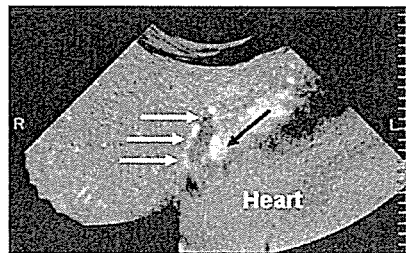
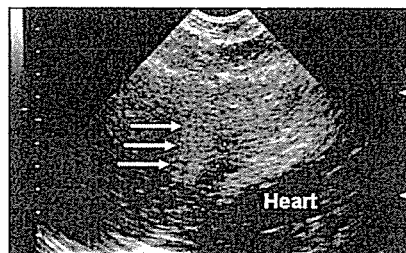
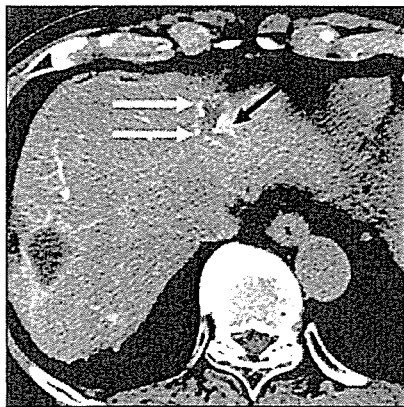


Virtual Sonographic Radiofrequency Ablation



**Fig. 3**—61-year-old man with hepatocellular carcinoma (maximum diameter, 6 mm) in superior anterior segment who received initial treatment. **A**, Contrast-enhanced CT scan obtained before treatment shows low-attenuation area (arrow). **B**, Reconstructed virtual sonographic image shows branches of portal vein (black arrow) and hepatic vein (black arrowheads), in addition to low-attenuation area (white arrow) seen in **A**. **C**, Nodule was not visualized clearly on conventional sonography; thus, same slice as in **B** is revisualized on conventional sonography and nodule is punctured. Arrow indicates portal vein; arrowheads indicate hepatic vein. **D**, After puncture, CT scan shows that needle accurately hits target nodule. **E**, After treatment, dynamic CT scan reveals completely necrotic area.



**Fig. 4**—71-year-old man with hepatocellular carcinoma who received additional treatment. **A**, Contrast-enhanced CT scan obtained before treatment showed high-attenuation area in lateral segment (black arrow). This nodule was adjacent to necrotic area (white arrows), which had previously undergone percutaneous ethanol injection. **B**, Necrotic area is seen before treatment (white arrows), but pulsation of heart prevents clear visualization of nodule. **C**, Reconstructed virtual sonographic image shows necrotic area as hypoattenuating (white arrows) and target nodule as hyperattenuating (black arrow). Puncture is performed at area of hyperattenuation. **D**, After treatment, dynamic CT scan reveals completely necrotic area.

or in deep tissue may not be depicted on contrast-enhanced sonography.

We previously reported that virtual sonographic images reconstructed by MDCT were effective in the treatment of HCC [14]. HCC nodules that are not visualized on conventional sonography but are visualized on dynamic CT can be visualized on virtual sonography. In the present study, slices made by virtual sonography were visualized again on conventional sonography, and then the site of the target nodule on virtual sonography was punctured. First, a phantom including modeled tumors that were not depicted by sonography and modeled vessels, was produced. Referring to the virtual sonographic images, we performed the punctures on the phantom. These punctures were shown to be accurate, prompting us to believe that these images could be used in the clinical setting. Thus, we punctured the HCC nodules in the patients and found that with only one puncture, the needle had advanced with almost 100% accuracy to all target nodules, indicating that this method had good efficacy. The virtual sonographic image revealed an adequate site for puncture, and thus virtual sonographic radiofrequency ablation was performed significantly fewer times than was standard radiofrequency ablation in the initial-treatment ( $p = 0.003$ ) and additional-treatment ( $p = 0.003$ ) groups. Consequently, the length of hospital stay, number of CT examinations required to estimate therapeutic efficacy, and cost are expected to decrease with use of this procedure.

No local recurrence was seen in the virtual sonographic radiofrequency ablation group. Furthermore, no major complications were noted in any patients, indicating that this treatment is safe. In conclusion, in the treatment of nodules not depicted on sonography, puncture assisted by virtual sonography appears efficacious and safe.

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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-fHCV-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-fHCV-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. © 2005 Elsevier B.V. All rights reserved.

**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<sub>GDD→AAG</sub>) 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<sub>GDD→AAG</sub> 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<sup>-</sup>) (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 rTth 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 diethyl 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<sub>2</sub>, 200 µM (each) deoxynucleoside triphosphate, and 5 U of rTth 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 *rTth*, 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<sub>2</sub> 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)<sub>16</sub> 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<sub>2</sub>, 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 [ $\alpha$ -<sup>32</sup>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 *rTth* 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  $\mu$ L of purified cDNA in a reaction mixture containing 1  $\mu$ L of LightCycler Fast Start DNA Master SYBR Green I, 4 mM of MgCl<sub>2</sub>, 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/ $\mu$ L.

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)<sub>16</sub> 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/ $\mu$ L.

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  $\mu$ L 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  $\beta$ -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  $\mu$ g 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  $\beta$ -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  $\beta$ -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

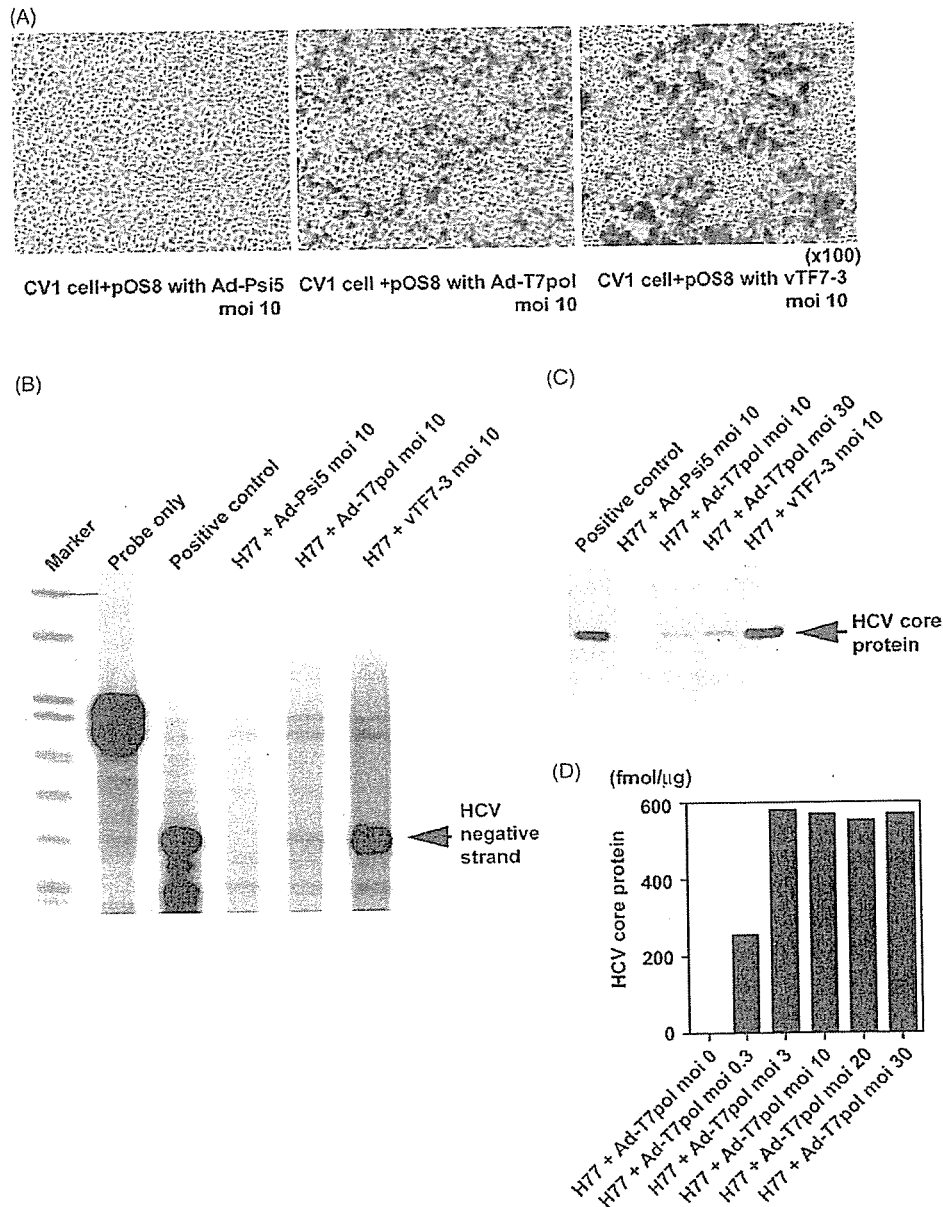


Fig. 1. Comparison of the transfection and infection efficiency of HCV replication system using vaccinia-T7 and adeno-T7 vectors: (A) after transfection with the pOS8 plasmid, cells were infected with virus vectors using control adenovirus (Ad-Psi5), recombinant adeno-T7 polymerase (Ad-T7pol), or vaccinia-T7 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<sup>GDD→AAG</sup> 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 pres-

ence 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<sup>GDD→AAG</sup>) indicates that replication was dependent on an intact polymerase sequence. H77 + Ad-T7pol expressed significantly higher core protein levels compared to H77<sup>GDD→AAG</sup> + 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<sup>GDD→AAG</sup> 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

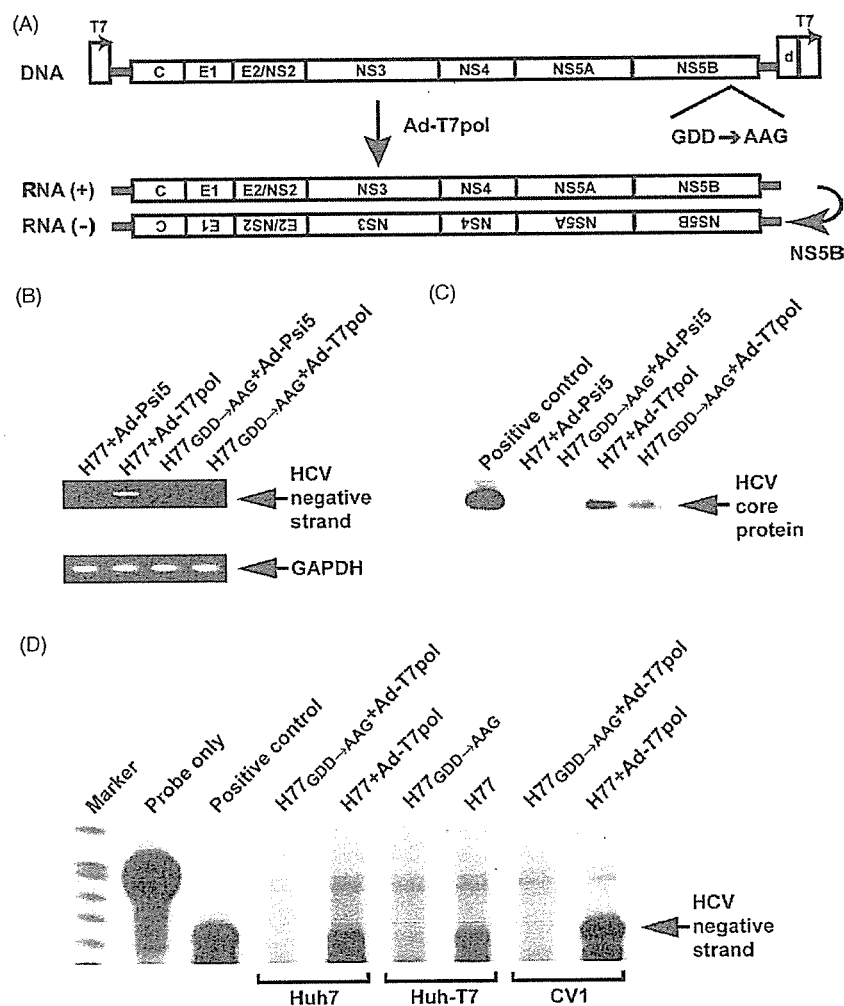


Fig. 2. (A) A control plasmid was prepared by mutating the active site motif from GDD to AAG in the NS5B RNA-dependent RNA polymerase sequence (H77<sup>GDD→AAG</sup>). (B) A qualitative strand-specific RT-PCR for negative-strand HCV RNA was performed as previously described (Lanford et al., 1995). (C) 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.

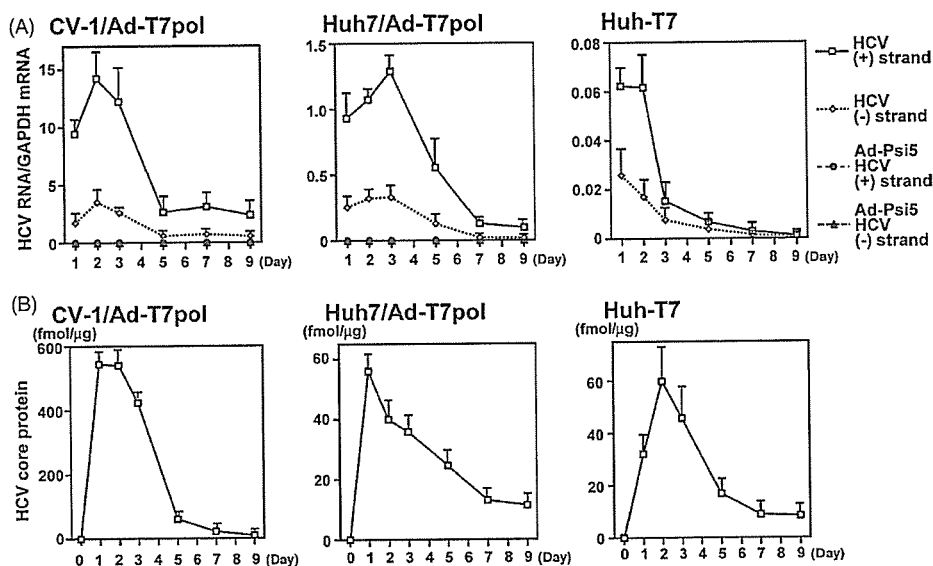


Fig. 3. (A) Real-time PCR of HCV positive- and negative-strand RNA was performed as described. Data are expressed as the strand-specific HCV copy number per molecule of GAPDH. Error bars indicate the mean  $\pm$  standard error (S.E.) of four replicates. (B) A quantitative core ELISA measured HCV protein production in CV-1, Huh7, and Huh-T7 cell lines. Data are expressed as fmol (mean  $\pm$  S.E.) of HCV core per  $\mu$ g 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 ( $14.21 \pm 3.95$  versus  $8.55 \pm 0.61$ ,  $p < 0.05$ ). A trend toward reduced HCV RNA was also observed on day 3 ( $12.23 \pm 5.43$  versus  $4.13 \pm 0.74$ ,  $p < 0.10$ ). In Huh7 cells, a significant decrease of HCV positive-strand was observed on days 2 ( $1.07 \pm 0.07$  versus  $0.63 \pm 0.14$ ,  $p < 0.05$ ) and 3 ( $1.29 \pm 0.13$  versus  $0.44 \pm 0.15$ ,  $p < 0.05$ ). In Huh-T7 cells, a significant decrease was also observed on day 2 ( $0.06 \pm 0.03$  versus  $0.03 \pm 0.007$ ,  $p < 0.05$ ). For HCV negative-strand RNA (Fig. 4B), only day 3 IFN-treated CV-1 cells had significantly decreased levels compared to untreated cells ( $2.60 \pm 0.41$  versus  $0.97 \pm 0.31$ ,  $p < 0.05$ ).

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 ( $423.51 \pm 25.73$  fmol/ $\mu$ g versus  $190.92 \pm 35.25$  fmol/ $\mu$ g,  $p < 0.05$ ) and 5 ( $60.24 \pm 12.89$  fmol/ $\mu$ g versus  $34.15 \pm 0.76$  fmol/ $\mu$ g,  $p < 0.05$ ). For Huh7 and Huh-T7 cells, HCV core expression was significantly reduced when treated with IFN compared to untreated cells at all time points ( $p < 0.05$ ).



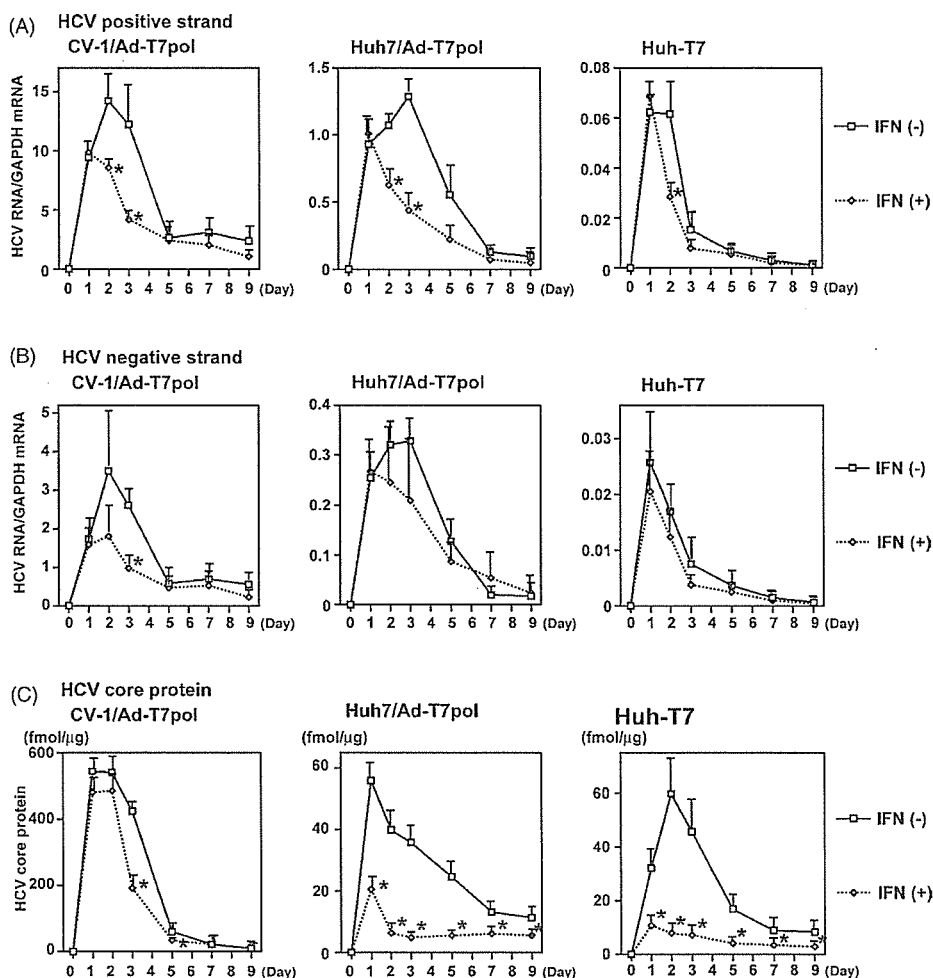


Fig. 4. (A) Real-time PCR of positive-strand HCV RNA was performed in the presence of 1000 IU/mL IFN. Error bar indicates mean  $\pm$  S.E. for four replicates ( $p < 0.05$ ;  $**p < 0.10$ ). (B) Real-time PCR of negative-strand HCV RNA was also performed in the presence of 1000 IU/mL IFN. (C) Quantitative 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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# Influence of Genotypes and Precore Mutations on Fulminant or Chronic Outcome of Acute Hepatitis B Virus Infection

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The outcome of acute hepatitis B virus (HBV) infection is variable, influenced by host and viral factors. From 1982 through 2004, 301 patients with acute HBV infection entered a multi-center cross-sectional study in Japan. Patients with fulminant hepatitis (n = 40) were older ( $44.7 \pm 16.3$  vs.  $36.0 \pm 14.3$  years,  $P < .0017$ ), less predominantly male (43% vs. 71%,  $P = .0005$ ), less positive for hepatitis B e antigen (HBeAg) (23% vs. 60%,  $P < .0001$ ), less infected with subgenotype Ae (0% vs. 13%,  $P < .05$ ), and more frequently with Bj (30% vs. 4%,  $P < .0001$ ) than those with acute self-limited hepatitis (n = 261). Precore (G1896A) and core-promoter (A1762T/G1764A) mutations were more frequent in patients with fulminant than acute self-limited hepatitis (53% vs. 9% and 50% vs. 17%,  $P < .0001$  for both). HBV infection persisted in only three (1%) patients, and they represented 2 of the 23 infected with Ae and 1 of the 187 with the other subgenotypes (9% vs. 0.5%,  $P = .032$ ); none of them received antiviral therapy. In multivariate analysis, age 34 years or older, Bj, HBeAg-negative, total bilirubin 10.0 mg/dL or greater, and G1896A mutation were independently associated with the fulminant outcome. In *in vitro* transfection experiments, the replication of Bj clone was markedly enhanced by introducing either G1896A or A1762T/G1764A mutation. **In conclusion**, persistence of HBV was rare (1%) and associated with Ae, whereas fulminant hepatitis was frequent (13%) and associated with Bj and lack of HBeAg as well as high replication due to precore mutation in patients with acute HBV infection. *Supplementary material for this article can be found on the HEPATOLOGY website (<http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html>). (HEPATOLOGY 2006; 44:326-334.)*

Abbreviations: HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; HBe, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; ELA, enzyme immunoassay; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; ALT, alanine aminotransferase.

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Approximately 3 billion people, one half of the world population, have been exposed to hepatitis B virus (HBV), of whom approximately 350 million are persistently infected with it.<sup>1</sup> Acute infection with HBV resolves in the great majority but can induce fulminant hepatitis or go on to become chronic. Host and viral factors may influence fulminant or chronic outcome of acute HBV infection, but they are not fully defined.

Eight genotypes have been detected by a sequence divergence greater than 8% in the entire HBV genome of approximately 3,200 nucleotides (nt), and designated by capital alphabet letters from A (HBV/A) to H in the order of documentation.<sup>2-5</sup> They have distinct geographical distributions associated with severity of liver disease as well as response to antiviral therapies.<sup>6-8</sup> Furthermore, subgenotypes have been reported for HBV/A, B, and C and named Aa/A1 (Asian/African type) and Ae/A2 (European type),<sup>9</sup> Bj/B1 (Japanese type) and Ba/B2 (Asian type),<sup>10</sup> as well as Cs/C1 (Southeast Asian type) and Ce/C2 (East Asian type).<sup>11-13</sup> Increasing lines of evidence indicate that subgenotypes of HBV/A and B influence the replication of HBV and bear clinical relevance.<sup>14-16</sup> Furthermore, genotypes affect mutations in precore region and core promoter, thereby influencing the expression of hepatitis B e antigen (HBeAg).<sup>8,17</sup>

During the 23 years from 1982 to 2004, a multi-center cross-sectional study was conducted throughout Japan on 301 patients with acute hepatitis B. We examined the influence of genotypes/subgenotypes on their fulminant or chronic outcome. Furthermore, the influence of G1896A or A1762T/G1764A on replication of HBV was evaluated in an *in vitro* replication model.

## Patients and Methods

**Patients With Acute Hepatitis B.** During 1982 through 2004, 336 consecutive cases of acute hepatitis B were registered in 16 hospitals throughout Japan. These hospitals were from the following eight areas: Hokkaido (represented by J.-H. K. and S.H.), Tohoku (T.K. and K.S.), Kanto (H.T., Y.A. and K.I.), Koshin (E.T. and S.O), Tokai (A.O., Y.T., E.O., M.S., R.U., M.M., and S.K.), Kinki (T.O.), Honshu/Shikoku (Y.M., K.H., and M.O.), and Kyushu (H.Y. and H.S.). The diagnosis of acute hepatitis B was contingent on a sudden onset of clinical symptoms of hepatitis and detection of high-titered antibody to hepatitis B core antigen (anti-HBc) of IgM class in serum. Patients with initial high-titered anti-HBc ( $\geq 90\%$  inhibition by a 1:200 diluted serum) were excluded; they were diagnosed as exacerbation of chronic hepatitis B. Patients with acute hepatitis A, hepatitis C, or human immunodeficiency virus co-infection, and drug-

or alcohol-induced acute hepatitis also were excluded; hepatitis D virus infection was not examined because of its extreme rarity in Japan.<sup>18</sup> Most of them were followed for clinical outcomes until the disappearance of hepatitis B surface antigen (HBsAg) during 24 weeks or longer after the presentation. The criteria of fulminant hepatitis are based on the report by Trey et al.,<sup>19</sup> with a slight modification in 1981 (Inuyama symposium, Aichi, Japan): coma of grade II or higher and prothrombin time less than 40% developing within 8 weeks after the onset. Serum samples were collected at the presentation and had been stored at  $-80^{\circ}\text{C}$ . HBV genotypes, HBV DNA, and HBeAg were determined, and clinical outcomes of acute hepatitis were analyzed. The study protocol conformed to the 1975 Declaration of Helsinki, and was approved by the Ethics Committees of the institutions. Every patient gave an informed consent for this study.

**Serological Markers of HBV Infection.** HBsAg was determined by hemagglutination (MyCell; Institute of Immunology Co., Ltd., Tokyo, Japan) or enzyme immunoassay (EIA) (AxSYM; Abbott Japan, Tokyo, Japan), and HBeAg by enzyme-linked immunosorbent assay (F-HBe; Kokusai Diagnostic, Kobe, Japan) or chemiluminescent EIA (Fujirebio Inc., Tokyo, Japan). Anti-HBc of IgM and IgG classes were determined by radioimmunoassay (Abbott Japan).

**Genotypes and Subgenotypes of HBV.** The six major HBV genotypes (A-F) were determined serologically by EIA using commercial kits (HBV GENOTYPE EIA; Institute of Immunology). The method depends on the combination of epitopes on preS2-region products detected by monoclonal antibodies, which is specific for each of them.<sup>20</sup> HBV/G was determined by a slight modification of the polymerase chain reaction (PCR) with specific primers.<sup>21</sup>

Subgenotypes of HBV/A designated Ae prevalent in Europe and Aa frequent in Africa as well as Asia,<sup>9</sup> which corresponds to subgroup A' originally reported by Bowyer et al.,<sup>22</sup> were determined by PCR restriction fragment length polymorphism (RFLP) involving nucleotide conversions in an immediate upstream of the precore region that are specific for each of them.<sup>16,23</sup> HBV/Bj (Japanese type) lacking the recombination with C over the precore region and the core gene and Ba (Asian type) with the recombination were determined by its absence or presence on HBV DNA sequences, as well as RFLP based on specific nucleotide substitutions, after the methods described previously.<sup>15,24</sup>

Subgenotypes of HBV/C, Cs (Southeast Asian type) found only in Southeast Asia, including Vietnam, Myanmar, Thailand, Laos, Bangladesh, Hong Kong, and Southern China, and Ce (East Asian type), found in Far

East Asia, including Japan, Korea, and Northern China, were determined by the PCR-RFLP method described previously.<sup>12</sup>

**Quantification of HBV DNA and Sequencing.** HBV DNA sequences spanning the S gene were determined by real-time detection PCR according to the method of Abe et al.,<sup>25</sup> with the detection limit of 100 copies/mL. HBV DNA sequences bearing core promoter, precore region, and the core gene were amplified by PCR with hemi-nested primers by the method described previously.<sup>10</sup> Negative samples were tested by another more sensitive second-round PCR with HB7F and HBV1917R (5'-CTC CAC AGT AGC TCC AAA TTC TTT A-3'). Thereafter, PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer.

**Construction of Plasmid and Site-Directed Mutagenesis of HBV DNA.** Serum samples were obtained from two patients infected with HBV/Bj and a patient with Ce. HBV DNA was extracted from 100  $\mu$ L serum using QIAamp DNA blood kit (QIAGEN, GmbH, Hilden, Germany). Four primer sets were designed to amplify two fragments covering the entire HBV genome. Amplified fragments were inserted into pGEM-T Easy Vector (Promega, Madison, WI) and cloned in DH5a competent cells (TOYOBO, Osaka, Japan). At least five clones of each fragment were sequenced and the consensus sequence determined. Among them, those containing the consensus sequence were identified and adopted as templates for further construction. Finally, 1.24-fold the HBV genome (nt 1413-3215/1-2185), just enough to transcribe oversized pregenome and precore mRNA, was constructed into pUC19 vector (Invitrogen Corp., Carlsbad, CA). For site-directed mutagenesis, the wild-type HBV was digested by *HindIII* and *EcoO65I* and ligated with the fragment carrying T1762/A1764 to produce 1.24-fold the genome carrying the core-promoter double mutation. Similarly, 1.24-fold the HBV genome with the precore stop-codon mutation (1896A) was generated. Further details are available online at: <http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html>.

**Cell Culture and DNA Transfection.** For the standard replication assay, 10-cm-diameter dishes were seeded with  $1 \times 10^6$  Huh7 cells each. After 16 hours of culture, cells were transfected with 5  $\mu$ g DNA construct using the FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) and harvested 3 days later. Transfection efficiency was measured by cotransfection with 1  $\mu$ g reporter plasmid expressing secreted alkaline phosphatase and estimating its enzymatic activity in the culture supernatant.

**Southern Blot Hybridization.** HBV DNA samples

from cells at day 3 in culture were separated on 1.2% (wt/vol) agarose gel, transferred to a positive-charged nylon membrane (Roche Diagnostics), and hybridized with full-length HBV DNA labeled with alkaline phosphatase. Detection was performed with CDP-star (Amersham Biosciences, Piscataway, NJ), and signals were analyzed in the LAS-1000 image analyzer (Fuji Photo Film, Tokyo, Japan).

**Statistical Analysis.** Categorical variables were compared between groups by the chi-squared test and non-categorical variables by the Mann-Whitney *U*-test. A *P* value less than .05 was considered significant. Multivariate analyses with logistic regression were used to determine independent factors for fulminant hepatitis. STATA Software (StataCorp LP, College Station, TX) version 8.0 was employed for analyses.

## Results

### *Demographic and Clinical Differences in Patients Infected With Various HBV Genotypes/Subgenotypes.*

Genotypes of HBV were not classifiable in 28 (8%), and sufficient clinical data were not available in 7 (2%) of the 336 patients with acute hepatitis B. Exclusive of these 35 patients, 301 (90%) were left for evaluation of HBV genotypes in reference to clinical outcome.

HBV genotypes/subgenotypes were Aa in 10 (3%), Ae in 33 (11%), Ba in 22 (7%), Bj in 22 (7%), Cs in 11 (4%), Ce in 192 (64%), D in 5 (2%), and G in 6 (2%); none of them were infected with F or H (Table 1). All six patients with HBV/G were co-infected with another genotype; Ae in two, Ba in two, and Ce in the remaining two. The mean age was lower in the patients with HBV/Ae than Ba ( $P = .0001$ ), Aa ( $P < .01$ ), Bj or Cs ( $P < .05$  for each) and Ce than Ba ( $P < .05$ ). Men predominated in HBV infections with foreign (Ae and Ba) compared with domestic genotypes (Bj and Ce) ( $P < .05$ ).

HBeAg was detected in 79% of patients with HBV/Ae at a frequency much higher than that with Bj ( $P < .005$ ), Ce ( $P < .001$ ) or Ba ( $P < .05$ ). HBeAg in four of the six (67%) patients with HBV/G was coded for by HBV of the other genotypes co-infecting them, because it has two stop codons and an insertion in the core gene that prohibit encoding HBeAg.<sup>21</sup> HBV DNA levels as well as HBeAg-positive rates at the presentation were higher in HBV/Ae than Ce ( $P < .005$ ) or Bj ( $P < .05$ ) infection.

The peak alanine aminotransferase (ALT) level was higher in HBV/Bj than Ae infection ( $P < .05$ ). Fulminant hepatitis was significantly more frequent in patients infected with HBV/Bj (55%) than the other genotypes ( $P < .05$ ); it occurred in two of the five (40%) patients with HBV/D, also. In reflection of severe clinical course,

**Table 1. Clinical Characteristics of Patients Acutely Infected With HBV of Distinct Genotypes/Subgenotypes**

Features	Genotypes/Subgenotypes							
	Aa (n = 10)	Ae (n = 33)	Ba (n = 22)	Bj (n = 22)	Cs (n = 11)	Ce (n = 192)	D <sup>a</sup> (n = 5)	G <sup>a,b</sup> (n = 6)
Age (years)	42.2 ± 13.1	31.2 ± 10.3 <sup>d</sup>	41.5 ± 10.7 <sup>e</sup>	43.5 ± 19.1	38.5 ± 11.1	36.3 ± 15.0	38.6 ± 20.8	42.7 ± 17.5
Men	8 (80%)	30 (91%) <sup>f</sup>	19 (86%) <sup>g</sup>	9 (41%)	7 (64%)	122 (64%)	2 (40%)	6 (100%)
HBeAg positive	7 (70%)	26 (79%) <sup>h</sup>	11 (50%)	8 (36%)	8 (73%)	101 (53%)	1 (20%)	4 (67%)
ALT (IU/L)	1875 ± 759	2070 ± 1113 <sup>i</sup>	2523 ± 1185	3472 ± 2720	2269 ± 995	2610 ± 1719	2559 ± 1672	2142 ± 722
Duration of elevated ALT (weeks) <sup>c</sup>	7.9 ± 5.8	9.5 ± 6.2	8.8 ± 3.7 <sup>j</sup>	6.0 ± 2.5	10.1 ± 7.5	7.7 ± 5.1	5.7 ± 2.1	9.8 ± 1.5
Total bilirubin (mg/dL)	14.1 ± 10.3	9.0 ± 7.2	9.3 ± 5.9	10.9 ± 9.0	11.0 ± 13.8	9.8 ± 10.7	8.2 ± 2.2	13.0 ± 7.8
HBV DNA (log copies/mL)								
Median	4.76	6.08 <sup>k</sup>	5.15	4.93	5.61	4.94	5.91	5.97
(range)	(2.90-8.08)	(2.00-8.46)	(2.00-8.19)	(2.00-8.44)	(2.00-8.50)	(2.00-9.06)	(2.00-8.37)	(3.35-7.11)
<2.00 (undetectable)	0 (0%)	1 (3%)	2 (9%)	3 (14%)	2 (18%)	28 (15%)	1 (20%)	0 (0%)
Medication with								
Lamivudine	1 (10%)	9 (27%)	2 (9%)	5 (23%)	2 (18%)	28 (15%)	4 (80%)	2 (33%)
Steroid	0	3 (9%)	0	5 (23%)	1 (9%)	16 (8%)	0	0

<sup>a</sup>Patients with HBV genotype D or G were not included in the analysis.

<sup>b</sup>All patients with HBV genotype G were co-infected with HBV of another genotype; Ae in two, Ba in two, and Ce in two.

<sup>c</sup>Exclusive of the 16 patients who died of fulminant hepatitis, 3 receiving liver transplantation and 10 without clinical data available.

<sup>d</sup> $P = .0001$ , Ae vs. Ba.  $P < .01$ , Ae vs. Aa.  $P < .05$ , Ae vs. Bj or Cs.

<sup>e</sup> $P < .05$ , Ba vs. Ce.

<sup>f</sup> $P = .0001$ , Ae vs. Bj.  $P < .005$ , Ae vs. Ce.

<sup>g</sup> $P < .005$ , Ba vs. Bj.  $P < .05$ , Ba vs. Ce.

<sup>h</sup> $P < .005$ , Ae vs. Bj.  $P < .01$ , Ae vs. Ce.  $P < .05$ , Ae vs. Ba.

<sup>i</sup> $P < .05$ , Ae vs. Bj.

<sup>j</sup> $P < .01$ , Ba vs. Bj.  $P < .05$ , Ba vs. Ce.

<sup>k</sup> $P < .005$ , Ae vs. Ce.  $P < .05$ , Ae vs. Bj.

the peak ALT level tended to be high in patients with HBV/Bj.

Presumed infection routes of 301 patients were sexual transmission in 172 (57%), blood transfusion in 4 (1%), medical accidents in 17 (6%), and unknown in the remaining 108 (36%).

#### Clinical Outcome of Patients With Acute Hepatitis

**B.** Fulminant hepatitis developed in 40 (13%) patients. To cope with severe acute liver disease, lamivudine and steroid were administered to 53 (18%) and 25 (8%) patients, respectively. Fulminant hepatitis led to death in 16 (5%) patients, and three (1%) received liver transplantation. Exclusive of the 40 patients with fulminant hepatitis who received various treatments and five without clinical data, 256 (85%) were followed for the chronic outcome (Fig. 1). Serum ALT levels stayed elevated for longer than 24 weeks for the diagnosis of chronic hepatitis in eight (3%) of them. Among them, five had cleared HBsAg from serum until then, and therefore, their liver function abnormality was not attributed to persistent HBV infection. Table 2 summarizes persistence of HBV infection in the 256 patients with acute hepatitis; 253 (99%) lost serum HBsAg by 6 months. Hence, HBV infection evolved into chronicity in only 3 of the 256 (1%) patients, representing 2 of the 32 (6%) infected with HBV/Ae and 1 of the 21 (5%) with Ba. All of the three with chronic outcome had low-titered IgG anti-HBc at the presentation, and

two of them had been negative for HBsAg before the presentation. None of them had received lamivudine or steroid treatment during their acute phase of illness. Of the patients without antiviral therapy, chronic outcome was significantly more frequent in those infected with HBV/Ae than non-Ae genotypes (9%  $\frac{2}{23}$  vs. 0.5%  $\frac{1}{187}$ ,  $P = .032$ ).

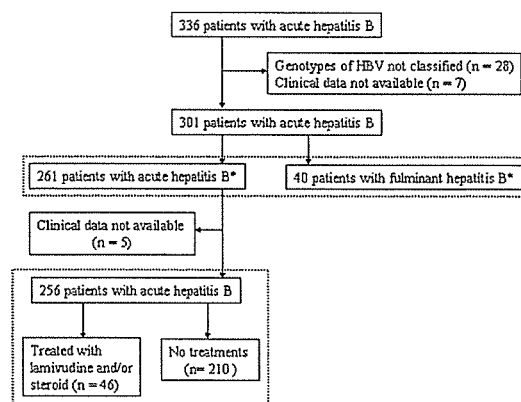


Fig. 1. A flow diagram of 336 patients studied. Comparison was made between patients with fulminant and acute self-limited hepatitis (upper dotted area), and the chronicity was compared between patients with and without treatments (lower dotted area). \*Of 301 patients, 37 were negative for HBV DNA, including 27 with acute and 10 with fulminant hepatitis.

**Table 2. Persistence of HBV Infection in the Patients With Acute Hepatitis Who Did or Did Not Receive Lamivudine or Steroid**

Treatment	Total	Genotypes/Subgenotypes							
		Aa (n = 8) <sup>a</sup>	Ae (n = 32) <sup>a</sup>	Ba (n = 21) <sup>a</sup>	Bj (n = 10) <sup>a</sup>	Cs (n = 10) <sup>a</sup>	Ce (n = 167) <sup>a</sup>	D (n = 3) <sup>a</sup>	G (n = 5) <sup>a</sup>
Total (n = 256)	3/256 (1.2%)	0	2/32 (6%) <sup>c</sup>	1/21 (5%)	0	0	0	0	0
Lamivudine (n = 36) <sup>b</sup>	0/36 (0%)	0/1 (0%)	0/9 (0%)	0/2 (0%)	0	0/1 (0%)	0/19 (0%)	0/2 (0%)	0/2 (0%)
Steroid (n = 16) <sup>b</sup>	0/16 (0%)	0	0/3 (0%)	0	0	0/1 (0%)	0/12 (0%)	0	0
Neither	3/210 (1.4%)	0/7 (0%)	2/23 (9%) <sup>c</sup>	1/19 (5%)	0/10 (0%)	0/8 (0%)	0/139 (0%)	0/1 (0%)	0/3 (0%)

<sup>a</sup>Exclusive of 40 patients with fulminant hepatitis and 5 without clinical data available.

<sup>b</sup>Six patients received steroid along with lamivudine.

<sup>c</sup> $P < .05$ , Ae vs. non-Ae.

**Comparison Between Patients With Fulminant and Acute Self-Limited Hepatitis.** Table 3 compares demographic, clinical, and virological characteristics between the 40 patients with fulminant and the 261 with acute self-limited hepatitis for whom analysis was feasible. Patients with fulminant hepatitis were significantly older ( $44.7 \pm 16.3$  vs.  $36.0 \pm 14.3$  years,  $P = .0017$ ), less predominantly male (43% vs. 71%,  $P = .0005$ ) and less often positive for HBeAg (23% vs. 60%,  $P < .0001$ ) than those with acute hepatitis. Peak ALT and total bilirubin levels were higher for fulminant than acute hepatitis ( $P < .0001$ ), reflecting severe hepatic lesions. Notably, the median HBV DNA level was lower in patients with fulminant than acute hepatitis (4.89 vs. 5.19 log copies/mL,  $P = .0178$ ); the frequency of unde-

etectable HBV DNA at the presentation was higher in fulminant hepatitis (25% vs. 10%,  $P = .0086$ ). Lamivudine or steroid was given significantly more often to patients with fulminant hepatitis.

There were marked differences in the distribution of genotypes between patients with fulminant and acute hepatitis. HBV/Ae was less frequent (0% vs. 13%,  $P = .0121$ ), whereas Bj was more often (30% vs. 4%,  $P < .0001$ ) in patients with fulminant than acute hepatitis. Although HBV/Ce tended to be less frequent in patients with fulminant than acute hepatitis (55% vs. 65%), the difference fell short of being significant.

Precore stop-codon mutation (G1896A) and core-promoter double mutation (A1762T/G1764A) were more

**Table 3. Comparison Between Patients With Fulminant and Acute Self-Limited Hepatitis Who Were Infected With HBV**

Features	Fulminant (n = 40)	Acute (n = 261)	P Value
Age (years)	44.7 ± 16.3	36.0 ± 14.3	.0017
Men	17 (43%)	186 (71%)	.0005
HBeAg positive	9 (23%)	157 (60%)	<.0001
ALT (IU/L)	4315 ± 2889	2284 ± 1221	<.0001
Total bilirubin (mg/dL)	20.5 ± 16.4	8.3 ± 7.3	<.0001
HBV DNA (log copies/mL)			
Median	4.89	5.19	.0178
(range)	(2.00-8.44)	(2.00-9.06)	
<2.00 (undetectable)	10 (25%)	27 (10%)	.0086
Treatment			
Lamivudine	16 (40%)	37 (14%)	.0003
Steroid	9 (23%)	16 (6%)	.0022
Genotypes/subgenotypes			
Aa	1 (2.5%)	9 (3%)	NS
Ae	0 (0%)	33 (13%)	.0121
Ba	1 (2.5%)	21 (8%)	NS
Bj	12 (30%)	10 (4%)	<.0001
Cs	1 (2.5%)	10 (4%)	NS
Ce	22 (55%)	170 (65%)	NS
D	2 (5%)	3 (1%)	NS
G	1 (2.5%)	5 (2%)	NS
Mutations <sup>a</sup>			
nt 1753 and/or nt1754 <sup>b</sup>	11/30 (37%)	28/234 (12%)	.0003
A1762T/G1764A	15/30 (50%)	39/234 (17%)	<.0001
G1896A	16/30 (53%)	21/234 (9%)	<.0001
G1899A	7/30 (23%)	8/234 (3%)	<.0001

<sup>a</sup>Exclusive of 37 patients in whom precore region and core-promoter could not be amplified by PCR.

<sup>b</sup>T1753C/A/G and/or T1754C/A/G.



**Table 4. Multivariate Analysis for Factors Independently Associated With Fulminant Hepatitis**

Factors	Odds Ratio	95% Confidence Interval	P Value
Age (yr)			
<34 <sup>a</sup>	1		
≥34	3.472	1.094-11.023	.0347
Sex			
Male	1		
Female	2.272	0.780-6.613	.1323
HBeAg			
Positive	1		
Negative	3.344	1.065-10.506	.0387
ALT (IU/L)			
<2200 <sup>a</sup>	1		
≥2200	2.094	0.683-6.414	.1957
Total bilirubin (mg/dL)			
<10.0 <sup>a</sup>	1		
≥10.0	18.818	4.320-81.980	<.0001
HBVDNA (log copies/mL)			
<5.00 <sup>a</sup>	1		
≥5.00	1.042	0.367-2.961	.9383
Treatment			
Lamivudine (-)	1		
Lamivudine (+)	2.650	0.814-8.625	.1056
Steroid (-)	1		
Steroid (+)	2.515	0.668-9.472	.1728
Genotypes/Subgenotypes			
Non-Bj	1		
Bj	7.001	1.737-28.228	.0062
Mutations			
nt 1753 and/or 1754 <sup>b</sup>			
Absent	1		
Present	2.316	0.698-7.683	.1700
A1762T/G1764A			
Absent	1		
Present	1.013	0.295-3.478	.9841
G1896A			
Absent	1		
Present	4.157	1.265-13.657	.0189
G1899A			
Absent	1		
Present	2.525	0.534-11.949	.2427

<sup>a</sup>Median values.<sup>b</sup>T1753C/A/G or T1754C/A/G.

frequent in patients with fulminant than acute hepatitis (53% vs. 9% and 50% vs. 17%, respectively,  $P < .0001$  for each). Likewise, mutations in core-promoter at nt 1753 or nt 1754, and G1899A mutation were more frequent in patients with fulminant than acute hepatitis ( $P = .0003$  and  $P < .0001$ , respectively).

**Factors Independently Associated With the Development of Fulminant Hepatitis.** Various factors found in association with fulminant hepatitis were evaluated for the independence in multivariate analysis (Table 4). Age 34 years or older (odds ratio 3.47 [95% confidence interval 1.09-11.02],  $P = .035$ ), HBV/Bj (7.00 [1.74-28.23],  $P = .006$ ), HBeAg-negative (3.34 [1.07-10.51],  $P = .039$ ), total bilirubin  $\geq 10.0$  mg/dL (18.82 [4.32-81.98],  $P < .0001$ ) and G1896A (4.16 [1.27-13.66],  $P = .019$ )

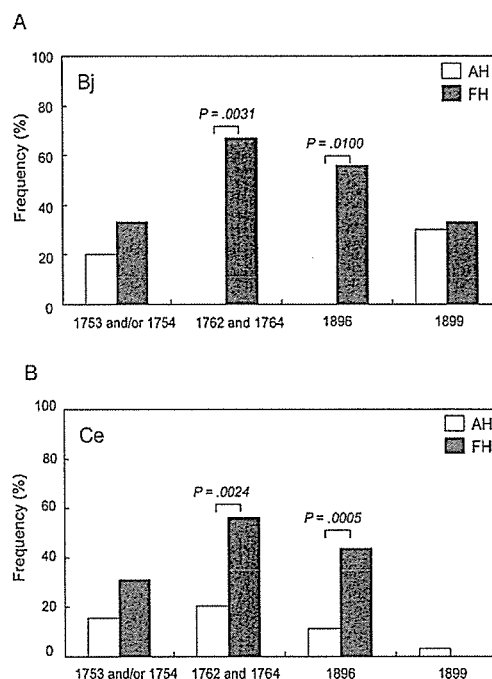


Fig. 2. Frequencies of precore and core-promoter mutations compared between patients with fulminant and acute self-limited hepatitis who were infected with HBV/Bj (A) or Ce (B).

were independent risk factors for the development of fulminant hepatitis.

In view of the majority of Japanese patients who were infected with Bj or Ce, mutations in the precore region and core-promoter were compared between those with fulminant and acute self-limited hepatitis for each subgenotype (Fig. 2). G1896A and A1762T/G1764A were significantly more frequent in patients with fulminant than acute hepatitis infected with either HBV/Bj or Ce (56% vs. 0% and 67% vs. 0% for Bj or 44% vs. 11% and 56% vs. 22% for Ce, respectively,  $P \leq .01$  for all). For the patients infected with HBV/Bj, in particular, precore and core-promoter mutations were highly frequent in those with fulminant hepatitis (56% and 67%, respectively), whereas they occurred in none of those with acute hepatitis. G1899A was equally frequent in both patients with fulminant and acute hepatitis infected with HBV/Bj; it was rarely seen in those with Ce. Mutations involving nt 1753 or nt 1754 tended to be more frequent in patients with fulminant than acute hepatitis.

**Replication of the Wild-Type HBV as Well as Precore and Core-Promoter Mutants In Vitro.** Full-length HBV DNA of the wild-type HBV/Bj from a patient with chronic hepatitis B was incorporated with G1896A or A1762T/G1764A mutation *in vitro*. Another plasmid was constructed with HBV/Bj\_58 carrying G1896A from a fulminant patient. Figure 3 compares

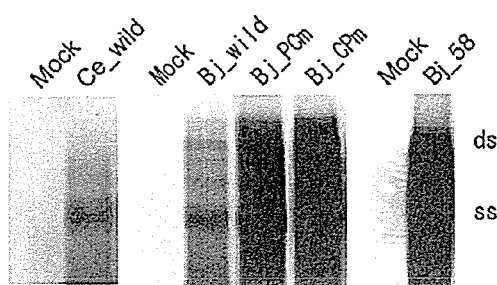


Fig. 3. Southern blot analysis for replicative activity of the wild-type HBV clones (HBV/Ce\_wild and Bj\_wild), as well as mutants with precore (Bj\_PCm) or core-promoter (Bj\_CPm) mutation, and Bj\_58 with precore stop-codon mutation obtained from a patient with fulminant hepatitis.

densities of migration patterns of the wild-type, precore, and core-promoter mutants in Southern blotting analysis. The wild-type HBV/Bj displayed a band for single-stranded (ss) HBV DNA and an additional band for double-stranded (ds) HBV DNA. Of note, the densities of these bands were far greater for HBV/Bj mutants incorporated with precore or core-promoter mutation, as well as Bj\_58 with the precore mutation, thereby indicating much enhanced replicative activity of precore or core-promoter mutant *in vitro*. Although the intracellular HBV DNA level for the wild-type HBV/Bj was comparable with that for the wild-type Ce (Fig. 3), the extracellular HBV DNA level in culture media was approximately threefold higher for Bj than Ce ( $P < .01$ ) (Sugiyama M et al., manuscript in submission).

## Discussion

A nationwide survey of genotypes/subgenotypes in patients with acute HBV infection from Japan during the past 2 decades has examined their influence on fulminant and chronic outcomes. The study was feasible in a country where mass vaccination has not been performed because of an extremely high efficacy of immunoprophylaxis on babies born to carrier mothers; it has decreased the persistent HBV carrier rate from 1.4% to 0.3%.<sup>26</sup> Acute HBV infection keeps increasing, however, predominantly through promiscuous sexual contacts in Japan.

Fulminant hepatitis developed rather frequently in 40 of the 301 (13%) patients. This is likely due to selection bias because the study included only patients who were hospitalized for acute hepatitis B. Exclusion of subclinical cases of acute HBV infection would have overestimated the incidence of fulminant hepatitis. Regardless of such a selection bias, influence of HBV genotypes/subgenotypes was evident in comparison with the 40 patients with fulminant and the 261 with acute self-limited hepatitis. Remarkably, none of the 33 patients infected with HBV/Ae

developed fulminant hepatitis. In sharp contrast, 12 of the 22 (55%) patients infected with HBV/Bj developed it. Furthermore, both precore (G1896A) and core-promoter (A1762T/G1764A) mutations were detected significantly more frequently in patients with fulminant than acute self-limited hepatitis. In infection with HBV/Bj, in particular, the frequency of core-promoter mutation was much higher in the patients with fulminant (67%) than that reported in those with chronic hepatitis (16%).<sup>27</sup> Precore and core-promoter mutations are very frequent in patients with fulminant hepatitis from Asia<sup>28-30</sup> and the Middle East.<sup>31</sup> The failure in detecting these mutations in Western countries<sup>32-35</sup> could be attributed to frequent HBV/Ae and rare Bj there. In multivariate analysis, HBeAg-negative, HBV/Bj, and the precore stop-codon mutation for G1896A were independent risk factors for the development of fulminant hepatitis (Table 4). Various mutations at nt 1753 for enhanced HBV replication,<sup>36</sup> as well as those adjacent at nt 1754 prevailing in patients with fulminant hepatitis,<sup>37</sup> occurred more frequently in patients with fulminant than acute self-limited hepatitis. Host factors, such as age and total bilirubin, contributed to the development of fulminant hepatitis as well (Table 4).

*In vitro* replication analysis demonstrated the intracellular HBV DNA level of the wild-type HBV/Bj comparable with that of the wild-type Ce (Fig. 3). The extracellular HBV DNA level of HBV/Bj-clone, however, was much higher than those of the other genotypes, indicating its strong inclination to be secreted from cells (Sugiyama et al., manuscript in submission). Such a high concentration of HBV/Bj in the circulation of patients would rapidly and extensively promote infection of hepatocytes.

Enhanced replication capacities of precore (G1896A) and core-promoter (A1762T/G1764A) mutants for HBeAg-minus and -reduced phenotypes, respectively, were demonstrated in a replication model *in vitro* (Fig. 3). These observations were concordant with those in previous reports<sup>38,39</sup>; however no data are available on the replication of HBV/Bj *in vitro*, either of the wild-type or variants with these mutations. Extremely high intracellular and extracellular expressions of viral DNA were observed for the HBV/Bj clone with precore stop-codon mutation from a patient with fulminant hepatitis. These results might implicate high replication due to mutations of precore region and core-promoter in the induction of fulminant hepatitis. In support of this view, Bocharov et al.<sup>40</sup> have proposed that enhanced HBV replication would efficiently stimulate immune reactions, represented by the cytotoxic T lymphocyte response, suggesting that enhanced replication by HBV/Bj or precore/

core-promoter mutation might lead to fulminant hepatitis.

That HBV DNA levels were lower in patients with fulminant than acute hepatitis, despite a high replication capacity of HBV/Bj incriminated in the development of fulminant hepatic failure, may seem surprising. Because destruction of hepatocytes proceeds swiftly in patients with fulminant hepatitis, hepatic mass for HBV to thrive would have been extremely reduced in them at presentation. As a consequence, some patients with fulminant hepatitis B are without serum HBsAg; they are diagnosed by high-titered IgM anti-HBc.<sup>41</sup> On the contrary, HBV DNA levels were higher in the patients with HBV/Ae than Bj (Table 1); those with Ae tend to delay reducing HBV DNA, some of whom have chronic outcome. Combined, correlating HBV DNA levels with the clinical outcome in acute HBV infection would be difficult.

A wide variation has been seen in the rate of persistence after acute HBV infection in adulthood. No chronic outcomes of acute hepatitis B were seen in female recipients of red blood cells contaminated with HBV (0/28)<sup>42</sup> or patients in an acupuncture-associated outbreak (0/35).<sup>43</sup> In marked contrast, they ranged from 0.2% (14/715) in Greece<sup>44</sup> through 2.7% (1/37) in university students in Taiwan<sup>45</sup> to 10.4% (5/8) in Alaskan Eskimos<sup>46</sup> and 12.1% (7/58) in Germany.<sup>47</sup> HBV genotypes are implicated in a high rate of persistence in European countries where HBV/A is predominant.<sup>48</sup> In Japan, also, adulthood infection tends to persist longer with HBV/A than B or C (23%  $\frac{3}{13}$  vs. 13%  $\frac{1}{8}$  or 12%  $\frac{3}{25}$ ).<sup>49</sup> In the current series on 256 patients with acute hepatitis B in Japan who were followed rigorously, HBV infection persisted in only three (1%), representing 2 of the 32 (6%) with HBV/Ae and 1 of the 21 (5%) with Ba. Hence, 99% of patients lost their HBsAg by 6 months. Persistence of HBV observed in the patients with HBV/Ae (6%) is less frequent than that in 4 of the 31 (13%) patients with Ae from a hospital in metropolitan Tokyo.<sup>49</sup> The difference would be ascribable, at least in part, to lamivudine given to some patients in this study (18%). All patients treated with lamivudine recovered from acute hepatitis, whereas none of the three patients with chronic outcome had received antiviral treatment during their acute phase of illness, indicating that lamivudine might be able to prevent the chronic outcome. Likewise, some patients from metropolitan Tokyo, in whom HBV persisted,<sup>49,50</sup> had received immunosuppressants in the acute phase of infection before referral to their hospital.

Using cell culture and chimeric mice models for the replication system of different genotype/subgenotype clones, we have observed that the replication of HBV is the highest for HBV/Bj or C and the lowest for Aa/Ae

(Sugiyama M et al., manuscript in submission). It is probable that the propensity of HBV/A infection to chronicity would be due to less intensive immune response against its slow viral dynamics. Taken together, the infection with HBV/A appears to persist longer than those with the other genotypes; this needs to be confirmed by further investigation in patients from various countries.

In conclusion, persistence of HBV after acute infection is rare and occurs more often in patients infected with HBV/Ae than others. Fulminant outcome is frequent in hospitalized patients and associated with HBV/Bj accompanied by the lack of serum HBeAg as well as high replication due to precore stop-codon mutation (G1896A), a finding supported by an *in vitro* replication model.

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