(Calbiochem, San Diego, CA), phorbol 12-myristate 13-acetate (PMA) (100 nM) (Sigma-Aldrich, St. Louis, MO).

Plasmid transfection and luciferase assay

Huh-7 cells were co-transfected with the firefly luciferase plasmid and pGL4-RL-tk, an expression vector of renilla luciferase, which was used as an internal control, using FuGENE HD reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Activities of firefly luciferase and renilla luciferase were measured using the Dual-Glo Luciferase Assay System (Promega, Madison, WI), and then relative luciferase activity was calculated by normalizing firefly luciferase activity to renilla luciferase activity.

RNA extraction

Total RNA was isolated from cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. The isolated RNA was treated with DNase I (Promega, Madison, WI) to avoid contamination with transfected plasmid, and then purified with a mixture of phenol, chloroform, and isoamylalcohol (pH 7.9), followed by ethanol precipitation.

Western blot analysis

Cultured cells were lysed with a lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protein inhibitor cocktail (Nacalai Tesque), in PBS, pH 7.4). Equal amounts of protein were electrophoretically separated by polyacrylamide gel and transferred onto PVDF membrane. For immunodetection, the following antibodies were used: anti-STAT1 antibody, anti-phospho-STAT1 antibody, anti-phospho-PKC-α/β II (Thr 638/641) antibody, anti-phospho-PKC-δ (Thr 505) antibody, anti-C/EBP antibody, anti-RXR antibody, anti-Sp1 antibody, anti-β-actin antibody from Cell Signaling Technology (Beverly, MA), and anti-Mx antibody from Abcam (Cambridge, UK). The signals of phosphorylated proteins such as phospho-PKC-α/β, -δ and phospho-STAT1 were analyzed quantitatively using image analyzing software (ImageJ; version 1.45).

Small RNA interference

Stealth Select RNAi specific for STAT1 (HSS 10273) was purchased from Invitrogen (Carlsbad, CA). Silencer Select siRNA specific for C/EBP (ID: S2890), RXR (ID: S12386) and Sp1 (ID: S13319) were purchased from Ambion (Austin, TX). Stealth RNAi Negative Control Low GC Duplex (Invitrogen, Carlsbad, CA) was used as a control for the off-target effect following Stealth Select RNAi delivery. The transfections were carried out using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the reverse transfection protocol.

Real-time reverse-transcription PCR

For cDNA synthesis, 1 μg of total RNA was reverse-transcribed using High Capacity RNA-to-DNA Master Mix (Applied Biosystems, Foster City, CA). cDNA, equivalent to 20 ng RNA, was used as a template for real-time reverse-transcription PCR (RT-PCR) using Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). mRNA expressions of C/EBP, FTF, HNF1, HNF3, and HNF4 were measured using TaqMan Gene Expression Assays and were corrected with the quantified expressions level of β-actin mRNA. Assay IDs for the genes were as follows: C/EBP (Hs00269972_s1), FTF (Hs00187067_m1), HNF1 (Hs00167041_m1), HNF3 (Hs00232754_m1), and HNF4 (Hs01023298_m1).

For the detection of pgRNA and pre-C mRNA, the primers and the probes were designed as follows according to a previous study (Laras et al., 2002): the sense primer was 5′-TCTTGTACATGTCCCACTGTTCAA-3′ (nt 1843–1866); the anti-sense primer was 5′-AATGCCATGCCCCAAAGC-3′ (nt 1890–1909); the probe was 5′-FAM-CTCCAAGCTGTGCCTT -3′ (nt 1869–1884). Since they were within precore/core coding sequence, only the total abundance of pgRNA and pre-C RNA could be detected.

Statistical analysis

Data were presented as mean ± SD. Differences between two groups were determined using Student's t-test for unpaired observations. $p < 0.05$ was considered statistically significant.

Disclosures

All authors have nothing to disclose.

References

Antonucci, T.K., Rutter, W.J., 1989. Hepatitis B virus (HBV) promoters are regulated by the HBV enhancer in a tissue-specific manner. J. Virol. 63, 579–583.

Azzi, A., Boscoboinik, D., Hensey, C., 1992. The protein kinase C family. Eur. J. Biochem. 208, 547–557.

Beck, J., Nassal, M., 2007. Hepatitis B virus replication. World J. Gastroenterol. 13, 48–64.

Breitkreutz, D., Braiman-Wiksman, L., Daum, N., Denning, M.F., Tennenbaum, T., 2007. Protein kinase C family: on the crossroads of cell signaling in skin and tumor epithelium. J. Cancer Res. Clin. Oncol. 133, 793–808.

Bruss, V., Ganem, D., 1991. The role of envelope proteins in hepatitis B virus assembly. Proc. Nat. Acad. Sci. U.S.A. 88, 1059–1063.

Caraglia, M., Abbruzzese, A., Leardi, A., Pepe, S., Budillon, A., Baldassare, G., Selleri, C., Lorenzo, S.D., Fabbrocini, A., Giuberti, G., Vitale, G., Lupoli, G., Bianco, A.R., Tagliaferri, P., 1999. Interferon-alpha induces apoptosis in human KB cells through a stress-dependent mitogen activated protein kinase pathway that is antagonized by epidermal growth factor. Cell Death Differ. 6, 773–780.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., Nishizuka, Y., 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 257, 7847–7851.

Darnell, J.E., Kerr, I.M., Stark, G.R., 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264, 1415–1421.

David, M., Petricoin, E., Benjamin, C., Pine, R., Weber, M.J., Larner, A.C., 1995. Requirement for MAP kinase (ERK2) activity in interferon alpha- and interferon beta-stimulated gene expression through STAT proteins. Science 269, 1721–1723.

Delmotte, M.H., Tahayato, A., Formstecher, P., Lefebvre, P., 1999. Serine 157, a retinoic acid receptor alpha residue phosphorylated by protein kinase C in vitro, is involved in RXR.RARalpha heterodimerization and transcriptional activity. J. Biol. Chem. 274, 38225–38231.

Der, S.D., Zhou, A., Williams, B.R., Silverman, R.H., 1998. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc. Nat. Acad. Sci. U.S.A. 95, 15623–15628.

Goh, K.C., Haque, S.J., Williams, B.R., 1999. p38 MAP kinase is required for STAT1 serine phosphorylation and transcriptional activation induced by interferons. EMBO J. 18, 5601–5608.

Griner, E.M., Kazanietz, M.G., 2007. Protein kinase C and other diacylglycerol effectors in cancer. Nat. Rev. Cancer 7, 281–294.

Gschwendt, M., Müller, H.J., Kielbassa, K., Zang, R., Kittstein, W., Rincke, G., Marks, F., 1994. Rottlerin, a novel protein kinase inhibitor. Biochem. Biophys. Res. Commun. 199, 93–98.

Guo, W., Chen, M., Yen, T.S., Ou, J.H., 1993. Hepatocyte-specific expression of the hepatitis B virus core promoter depends on both positive and negative regulation. Mol. Cell. Biol. 13, 443–448.

Ishida, H., Ueda, K., Ohkawa, K., Kanazawa, Y., Hosui, A., Nakanishi, F., Mita, E., Kasahara, A., Sasaki, Y., Hori, M., Hayashi, N., 2000. Identification of multiple transcription factors, HLF, FTF, and E4BP4, controlling hepatitis B virus enhancer II. J. Virol. 74, 1241–1251.

Johnson, J.L., Raney, A.K., McLachlan, A., 1995. Characterization of a functional hepatocyte nuclear factor 3 binding site in the hepatitis B virus nucleocapsid promoter. Virology 208, 147–158.

Jonas, M.M., Block, J.M., Haber, B.A., Karpen, S.J., London, W.T., Murray, K.F., Narkewicz, M.R., Rosenthal, P., Schwarz, K.B., McMahon, B.J., Foundation, H.B., 2010. Treatment of children with chronic hepatitis B virus infection in the United States: patient selection and therapeutic options. Hepatology 52, 2192–2205.

Kang, H., Yu, J., Jung, G., 2008. Phosphorylation of hepatitis B virus core C-terminally truncated protein (Cp149) by PKC increases capsid assembly and stability. Biochem. J. 416, 47–54.
Kaur, S., Parmar, S., Smith, J., Katsoulidis, E., Li, Y., Sassano, A., Majchrzak, B., Uddin, S., Tallman, M.S., Fish, E.N., Platanias, L.C., 2005. Role of protein kinase C-delta (PKC-delta) in the generation of the effects of IFN-alpha in chronic myelogenous leukemia cells. Exp. Hematol. 33, 550–557.
Kikkawa, U., Kishimoto, A., Nishizuka, Y., 1989. The protein kinase C family: heterogeneity and its implications. Annu. Rev. Biochem. 58, 31–44.
Laras, A., Koskinas, J., Hadziyannis, S.J., 2002. In vivo suppression of precore mRNA synthesis is associated with mutations in the hepatitis B virus core promoter. Virology 295, 86–96.
Li, M., Xie, Y., Wu, X., Kong, Y., Wang, Y., 1995. HNF3 binds and activates the second enhancer, ENII, of hepatitis B virus. Virology 214, 371–378.
Li, M., Xie, Y.H., Kong, Y.Y., Wu, X., Zhu, L., Wang, Y., 1998. Cloning and characterization of a novel human hepatocyte transcription factor, hB1F, which binds and activates enhancer II of hepatitis B virus. J. Biol. Chem. 273, 29022–29031.
Liaw, Y.F., 2009. HBeAg seroconversion as an important end point in the treatment of chronic hepatitis B. Hepatol. Int.
Lok, A.S., McMahon, B.J., 2009. Chronic hepatitis B: update 2009. Hepatology 50, 661–662.
López-Cabrera, M., Letovsky, J., Hu, K.Q., Siddiqui, A., 1990. Multiple liver-specific factors bind to the hepatitis B virus core/pregenomic promoter: trans-activation and repression by CCAAT/enhancer binding protein. Proc. Nat. Acad. Sci. U.S.A. 87, 5069–5073.
López-Cabrera, M., Letovsky, J., Hu, K.Q., Siddiqui, A., 1991. Transcriptional factor C/EBP binds to and transactivates the enhancer element II of the hepatitis B virus. Virology 183, 825–829.
Mahoney, C.W., Shuman, J., McKnight, S.L., Chen, H.C., Huang, K.P., 1992. Phosphorylation of CCAAT-enhancer binding protein by protein kinase C attenuates site-selective DNA binding. J. Biol. Chem. 267, 19396–19403.
Marte, B.M., Meyer, T., Stabel, S., Standke, G.J., Jaken, S., Fabbro, D., Hynes, N.E., 1994. Protein kinase C and mammary cell differentiation: involvement of protein kinase C alpha in the induction of beta-casein expression. Cell Growth Differ. 5, 239–247.
Martiny-Baron, G., Kazanietz, M.G., Mischak, H., Blumberg, P.M., Kochs, G., Hug, H., Marmé, D., Schächtele, C., 1993. Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. J. Biol. Chem. 268, 9194–9197.
Moolla, N., Kew, M., Arbuthnot, P., 2002. Regulatory elements of hepatitis B virus transcription. J. Viral. Hepat. 9, 323–331.
Nakao, K., Nakata, K., Yamashita, M., Tamada, Y., Hamasaki, K., Ishikawa, H., Kato, Y., Eguchi, K., Ishii, N., 1999. p48 (ISGF-3gamma) is involved in interferon-alpha-induced suppression of hepatitis B virus enhancer-1 activity. J. Biol. Chem. 274, 28075–28078.
Nishizuka, Y., 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334, 661–665.
Pal, S., Claffey, K.P., Cohen, H.T., Mukhopadhyay, D., 1998. Activation of Sp1-mediated vascular permeability factor/vascular endothelial growth factor transcription requires specific interaction with protein kinase C zeta. J. Biol. Chem. 273, 26277–26280.
Pfeffer, L.M., Eisenkraft, B.L., Reich, N.C., Improta, T., Baxter, G., Daniel-Issakani, S., Strulovici, B., 1991. Transmembrane signaling by interferon alpha involves diacylglycerol production and activation of the epsilon isoform of protein kinase C in Daudi cells. Proc. Nat. Acad. Sci. U.S.A. 88, 7988–7992.
Pfeffer, L.M., Strulovici, B., Saltiel, A.R., 1990. Interferon-alpha selectively activates the beta isoform of protein kinase C through phosphatidylcholine hydrolysis. Proc. Nat. Acad. Sci. U.S.A. 87, 6537–6541.
Rafty, L.A., Khachigian, L.M., 2001. Sp1 phosphorylation regulates inducible expression of platelet-derived growth factor B-chain gene via atypical protein kinase C-zeta. Nucleic Acids Res. 29, 1027–1033.
Raney, A.K., Johnson, J.L., Palmer, C.N., McLachlan, A., 1997. Members of the nuclear receptor superfamily regulate transcription from the hepatitis B virus nucleocapsid promoter. J. Virol. 71, 1058–1071.
Rang, A., Heise, T., Will, H., 2001. Lack of a role of the interferon-stimulated response element-like region in interferon alpha -induced suppression of Hepatitis B virus in vitro. J. Biol. Chem. 276, 3531–3535.
Romero, R., Lavine, J.E., 1996. Cytokine inhibition of the hepatitis B virus core promoter. Hepatology 23, 17–23.
Schulte-Frohlinde, E., Seidler, B., Burkard, I., Freilinger, T., Lersch, C., Erfle, V., Foster, G.R., Classen, M., 2002. Different activities of type I interferons on hepatitis B virus core promoter regulated transcription. Cytokine 17, 214–220.
Srivastava, K.K., Batra, S., Sassano, A., Li, Y., Majchrzak, B., Kiyokawa, H., Altman, A., Fish, E.N., Platanias, L.C., 2004. Engagement of protein kinase C-theta in interferon signaling in T-cells. J. Biol. Chem. 279, 29911–29920.
Su, H., Yee, J.K., 1992. Regulation of hepatitis B virus gene expression by its two enhancers. Proc. Nat. Acad. Sci. U.S.A. 89, 2708–2712.
Sureau, C., Romet-Lemonne, J.L., Mullins, J.I., Essex, M., 1986. Production of hepatitis B virus by a differentiated human hepatoma cell line after transfection with cloned circular HBV DNA. Cell 47, 37–47.
Tur-Kaspa, R., Teicher, L., Laub, O., Itin, A., Dagan, D., Bloom, B.R., Shafritz, D.A., 1990. Alpha interferon suppresses hepatitis B virus enhancer activity and reduces viral gene transcription. J. Virol. 64, 1821–1824.
Uddin, S., Sassano, A., Deb, D.K., Verma, A., Majchrzak, B., Rahman, A., Malik, A.B., Fish, E.N., Platanias, L.C., 2002. Protein kinase C-delta (PKC-delta) is activated by type I interferons and mediates phosphorylation of Stat1 on serine 727. J. Biol. Chem. 277, 14408–14416.
Uddin, S., Yenush, L., Sun, X.J., Sweet, M.E., White, M.F., Platanias, L.C., 1995. Interferon-alpha engages the insulin receptor substrate-1 to associate with the phosphatidylinositol 3′-kinase. J. Biol. Chem. 270, 15938–15941.
Vannice, J.L., Levinson, A.D., 1988. Properties of the human hepatitis B virus enhancer: position effects and cell-type nonspecificity. J. Virol. 62, 1305–1313.
von Hahn, T., Schulze, A., Chicano Wust, I., Heidrich, B., Becker, T., Steinmann, E., Helfritz, F.A., Rohrmann, K., Urban, S., Manns, M.P., Pietschmann, T., Ciesek, S., 2011. The novel immunosuppressive protein kinase C inhibitor sotrastaurin has no pro-viral effects on the replication cycle of hepatitis B or C virus. PLoS One 6, e24142.
Wang, W.X., Li, M., Wu, X., Wang, Y., Li, Z.P., 1998. HNF1 is critical for the liver-specific function of HBV enhancer II. Res. Virol. 149, 99–108.
Wang, Y., Chen, P., Wu, X., Sun, A.L., Wang, H., Zhu, Y.A., Li, Z.P., 1990. A new enhancer element, ENII, identified in the X gene of hepatitis B virus. J. Virol. 64, 3977–3981.
Yee, J.K., 1989. A liver-specific enhancer in the core promoter region of human hepatitis B virus. Science 246, 658–661.
Yuh, C.H., Ting, L.P., 1990. The genome of hepatitis B virus contains a second enhancer: cooperation of two elements within this enhancer is required for its function. J. Virol. 64, 4281–4287.
Yuh, C.H., Ting, L.P., 1991. C/EBP-like proteins binding to the functional box-alpha and box-beta of the second enhancer of hepatitis B virus. Mol. Cell. Biol. 11, 5044–5052.

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Mechanism of the dependence of hepatitis B virus genotype G on co-infection with other genotypes for viral replication

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SUMMARY. Hepatitis B virus (HBV) is classified into several genotypes. Genotype G (HBV/G) is characterised by worldwide dispersion, low intragenotypic diversity and a peculiar sequence of the precore and core region (stop codon and 36-nucleotide insertion). As a rule, HBV/G is detected in co-infection with another genotype, most frequently HBV/A2. In a previous *in vivo* study, viral replication of HBV/G was significantly enhanced by co-infection with HBV/A2. However, the mechanism by which co-infection with HBV/A2 enhances HBV/G replication is not fully understood. In this study, we employed 1.24-fold HBV/A2 clones that selectively expressed each viral protein and revealed that the core protein expressing construct significantly enhanced the replication of HBV/G in Huh7 cells. The introduction of the HBV/A2 core promoter or core protein or both genomic regions into the HBV/G genome showed that both the core promoter and core protein are required for efficient HBV/G replication. The effect of genotype on the interaction between foreign core protein and HBV/G showed that HBV/A2 was the strongest enhancer of HBV/G replication. Furthermore, Western blot analysis of Dane particles isolated from cultures of Huh7 cells co-transfected by HBV/G and a cytomegalovirus (CMV) promoter–driven HBV/A2 core protein expression construct indicated that HBV/G employed HBV/A2 core protein during particle assembly. In conclusion, HBV/G could take advantage of core proteins from other genotypes during co-infection to replicate efficiently and to effectively package HBV DNA into virions.

Keywords: co-transfection, core protein, genotype A, genotype G, hepatitis B virus, replication.

INTRODUCTION

Hepatitis B virus (HBV) infection affects more than 350 million people and is one of the major causes of acute and chronic liver disease. Acute HBV infection in adults is usually self-limiting, while chronic HBV infection can cause chronic hepatitis, liver cirrhosis or hepatocellular carcinoma [1]. As the clinical course in infected individuals depends on a complex interplay among various factors including viral, host and environmental factors, molecular characteristics of HBV including the genotype could become increasingly important in our understanding of HBV clinical implications [2].

Abbreviations: CMV, cytomegalovirus; CP, core promoter; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; SEAP, secreted alkaline phosphatase.

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Eight major HBV genotypes (A–H) have been identified by a sequence divergence >8% in the entire HBV genome [3,4] and have a relatively distinct geographical distribution, which may be associated with anthropological history [5]. Hepatitis B virus genotype G (HBV/G) was first described in 2000 by studies carried out in France [6]. It is usually detected during co-infection with other genotypes, most frequently with HBV/A2 [7,8]. Co-infection with HBV/C and H has also been reported [9–11]. One of the features distinguishing HBV/G from other genotypes is the 36-nucleotide (nt) insertion in its core gene [6,12]. Recent studies indicated that the 36-nt insertion increased core protein translation without enhancing mRNA abundance [13], and insertion of the 36-nt in the core region of genotypes A and D impaired genome replication, despite upregulation of core protein expression, indicating that the 36-nt insertion could alter core protein expression without altering the mRNA expression [14]. The other feature of the HBV/G genome that is unique is the possession of two stop codons in the precore region that prevents the expression of hepatitis B e antigen (HBeAg) [6,12]. Nevertheless, some HBV/G carriers are

HBeAg positive, which is explained by co-infection with an HBeAg-expressing HBV/A strain [7].

As previously reported, HBV/G monoinfection in uPA/SCID mice that had been transplanted with human hepatocytes (hereafter referred to as chimeric mice) resulted in very low level viral replication, but HBV replication increased markedly when the animals were co-infected with HBV/A2, C or H [11,15]. Furthermore, the co-infection induced more pronounced fibrosis, which concurs with findings from studies of immunosuppressed patients [16]. However, as it is still unclear how the interaction between HBV/G and other genotypes enhances the replication of HBV/G and affects the virological and clinical manifestation within an individual, we conducted *in vitro* studies using 1.24-fold HBV clones to elucidate the mechanism of HBV/G replication during co-infection.

MATERIALS AND METHODS

Plasmid constructs of HBV DNA and sequencing

Hepatitis B virus DNA was extracted from 100 μL of serum using the QIAamp DNA blood kit (Qiagen GmbH, Hilden, Germany). Four primer sets were designed to amplify two fragments (A and B) covering the entire HBV/G genome. PCR with nested primers was performed using TaKaRa LA Taq polymerase (Takara Biochemicals, Kyoto, Japan) for 35 cycles (30 s at 95° C, 30 s at 60° C and 2 min at 72° C). The primer pairs and protocols for plasmid construction are outlined in the Supporting Information. As reported previously [17], these fragments were added to the pUC19 vector, which had been deprived of promoters (Invitrogen Corp., Carlsbad, CA, USA), by digestion with *Hin*dIII and *Eco*RI, resulting in the 1.24-fold HBV genome – required to transcribe the oversized pregenome and precore messenger RNA. Cloned HBV DNA sequences were confirmed with Prism BigDye (Applied Biosystems, Foster City, CA, USA) using the ABI 3100 automated sequencer.

HBV DNA mutagenesis and construct design

HBV/A2 and HBV/G clones containing the 1.24-fold HBV genome were constructed using isolates obtained from a co-infected Caucasian patient from the San Francisco cohort described in our previous study (patient #1) [7]. The study design conformed to the 1975 Declaration of Helsinki and was approved by our institutional ethics committee. Written informed consent was obtained from the patient. The HBV/A2 clones isolated from the patient's blood specimen did not possess any precore or core promoter mutations that are known to affect HBeAg expression. To study the interaction between the different genotype isolates, the following viral protein expression constructs were prepared (outlined in Fig. 1) in HBV/A2 recombinant plasmids: HBV/A2-S, HBV/A2-core, HBV/A2-pol and HBV/A2-X and were each able to selectively translate one of the four viral proteins (the large surface, precore/core, polymerase and X proteins, respectively), whereas translation of the other three was prevented by the introduction of point mutations that produced corresponding stop codons (Fig. 1a). The following stop codons were used: (i) for surface protein: change from TTA to TAG in the 15th codon of the S gene (T198A) [18], (ii) for core protein: change from AAG to TAG in the 96th codon of the core gene (A2186T), (iii) for polymerase: change from CAACAA to TAATAA in the 283rd and 284th codons of the pol gene (C2558T/C2592T) and (iv) for X protein: change from CAA to TAA in the 7th codon of the HBx gene (C1395T) [19]. All of the above HBV/A2 recombinant plasmids possessed a TCTG motif after nucleotide position 1876, which abolished genome replication by altering the ε loop (CTGT to TCTG, nt 1877–1880) [20]. The 'HBV/A2-N' clone contained all six mutations and was used as an experimental negative control. All of the mutations in this study (substitutions, insertions and deletions) were created by overlapping PCR extension followed by the exchange of endonuclease enzyme-restricted fragments, as described previously [13,21].

Three cytomegalovirus (CMV) promoter-driven expression clones were constructed containing the whole core genes (not including the precore section) of HBV/G (nt 1901–2488), HBV/A2 (nt 1901–2458) and HBV/C (nt 1901–2452): CMV-HBV/G/core, CMV-HBV/A2/core and CMV-HBV/C/core, respectively (Fig. 1b).

Three replicating recombinant constructs were created by recombination of different genomic sections of HBV/G and HBV/A2 (Fig. 1c). The 'HBV-G/A2-CP' clone was a HBV/G-based construct in which the leading fragment containing the core promoter (CP) region (nt 1413–1806) was replaced with that of HBV/A2. The 'HBV-G/A2-CP +core' clone was also an HBV/G-based construct, in which the leading fragment containing the core promoter (CP), precore and core region (nt 1413–2821) of HBV/G was replaced with those of HBV/A2. The 'HBV-G/A2-core' clone was an HBV/G-based construct in which the fragment of the precore and core region (nt 1806–2821) was replaced with those of HBV/A2.

Cell culture and transfection

After 16 h of culture, Huh7 cells were transfected with 5 μg of DNA construct per 10-cm diameter dish using the Fugene 6 transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol and harvested 3 days later. Transfection efficiency was measured by co-transfection with 0.5 μg of a reporter plasmid expressing secreted alkaline phosphatase (SEAP) and normalised with subsequent SEAP measurement from culture supernatant using a SEAP reporter assay kit (Toyobo, Osaka, Japan) [17]. Three experiments were conducted for each clone.

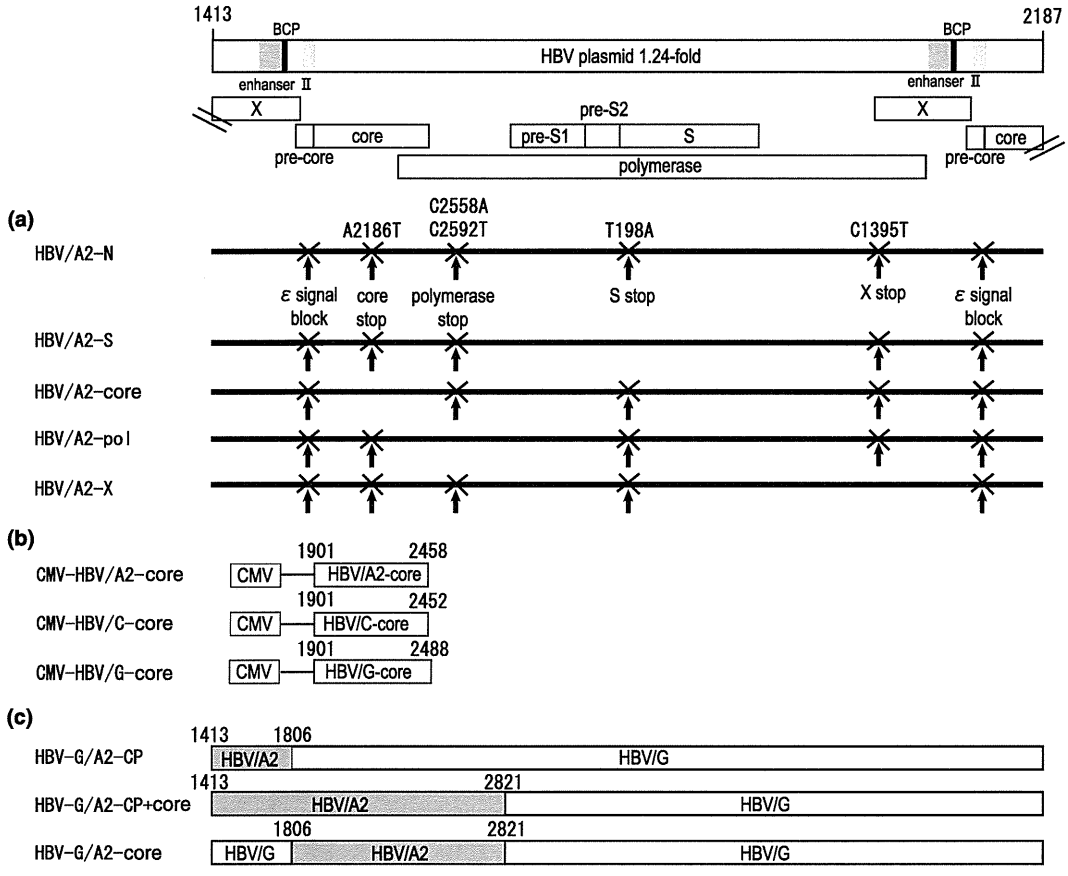

Fig. 1 HBV constructs (1.24-fold) and CMV-driven HBV core protein expression constructs used for the present study. CP, core promoter; BCP, basal core promoter; CMV, cytomegalovirus promoter. Stop codons for the corresponding HBV protein are indicated by crosses and arrows. All HBV/A2 recombinant plasmids consisted of the packaging-negative mutation (ε signal block). In three recombinant constructs between HBV/A2 and HBV/G, the corresponding recombinant genomic parts are shown by the grey bar. CMV-core constructs produce core protein without generating HBeAg in the absence of the preceding ε signal.

Determination of HBV markers

The expression levels of hepatitis B surface antigen (HBsAg) and HBeAg were determined by chemiluminescent enzyme immunoassay using commercial assay kits (Fujirebio Inc., Tokyo, Japan). The detection limit of the HBsAg assay is 0.05 IU/mL. HBV core-related antigen (HBcrAg) was measured in serum using a previously described chemiluminescent enzyme immunoassay [22]. The detection limit of the HBcrAg assay is 1.0 kU/mL.

Southern blot hybridisation

Southern blot hybridisation was performed with full-length probes for each genotype/subgenotype according to previously described methods [23]. In brief, cells were harvested and lysed in 1.5 mL of lysis buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA and 1% NP-40. Half of the cell lysate was treated with 100 μg/mL of RNase A and 200 μg/mL of DNase I for 2 h at 37° C, in the presence of 6 mM Mg acetate. Then, HBV DNA was released by proteinase K digestion, extracted with phenol and precipitated with ethanol after the addition of 20 μg of glycogen. DNA was separated on a 1.2% agarose gel, transferred to a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) and hybridised with an alkaline phosphatase-labelled full-length HBV/G or HBV/A2 fragment generated with a Gene Images AlkPhos direct labelling module (GE Healthcare, Hertfordshire, UK). The detection was performed with CDP-Star, ready-to use (Roche Diagnostics GmbH). The signals were analysed by using a LAS-3000 image analyzer (Fuji Photo Film, Tokyo, Japan).

Western blot analysis

Serum or culture medium samples were subjected to SDS-PAGE under 15–25% polyacrylamide gel electrophoresis conditions. The proteins in the gel were electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA, USA) at 15 V for 45 min. The

membrane was then blocked and probed using alkaline phosphatase-conjugated HB50 (for HBcAg) or HB91 (for HBcrAg) monoclonal antibody [22] at room temperature for 1 h, before being washed and incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solution (KPL, Gaithersburg, MD, USA) for 15 min (for HBcrAg) or 90 min (for HBcAg).

Sucrose density gradient ultracentrifugation

Aliquots (1.7 mL) of 10%, 20%, 30%, 40%, 50% or 60% (w/w) sucrose in 10 mM Tris–HCl, 150 mM NaCl and 1 mM EDTA (pH 7.5) were carefully layered in a 12–mL ultracentrifuge tube and left at room temperature for 6 h. The culture supernatant of Huh7 cells that had been co-transfected with the 1.24-fold HBV genome construct (HBV/G or HBV/A) and/or the CMV-HBV/A2-core plasmid was layered onto this sucrose gradient, and ultracentrifugation was performed at 200 000 × *g* for 15 h at 4° C in a Beckman Sw40Ti rotor (Beckman Coulter, Chaska, MN, USA). Fractions were collected from the top to the bottom of the gradient. The density of each fraction was calculated from its weight and volume. Each fraction was diluted 10-fold and tested for HBcrAg, HBsAg, HBeAg and HBV DNA.

Immunoprecipitation

Immunoprecipitation was carried out using magnetic beads coated with monoclonal anti-HBs from the 'Magnosphere™ MS300/Caboxyl' kit (JSR Corp., Tokyo, Japan) [24]. A 100-μL aliquot of sample was mixed with 100 μL of a magnetic bead suspension. The mixture was then incubated for 1 h at room temperature under gentle agitation and then magnetically separated. The core protein in the precipitate was analysed by Western blotting.

RESULTS

The replication of HBV/G is enhanced by HBV/A2 in co-transfection experiments

In this study, HBV/G and HBV/A2 genome clones (1.24-fold) were constructed from the serum of a HBV carrier that had been co-infected with HBV/G and HBV/A2. The HBV/G-d36 clone is a HBV/G genome-based construct in which the genotype-specific 36-nt insertion was deleted. We performed co-transfection with HBV/A2 and HBV/G clones and assessed virological features. Because of an over 12% sequence divergence between genotype A and G at the nucleotide level [12], the blot was hybridised successively with genotype-specific probes to DNA of each genotype. However, due to the unbiased binding of each probe at lower efficiency in Southern blot analysis [although the replication of HBV/A2 was higher than that of HBV/G, relative value of HBV/A2 with probe G became lower (0.63), as well as the detection of HBV/G with probe A was very weak (0.24)], each probe of genotype G or A was used for hybridisation with the HBV/A2 and HBV/G clones (Fig. 2a). The density of single-strand HBV DNA detected by the genotype-specific probes in Southern blot analysis revealed that co-transfection with HBV/A2 resulted in increased replication of the wild-type HBV/G

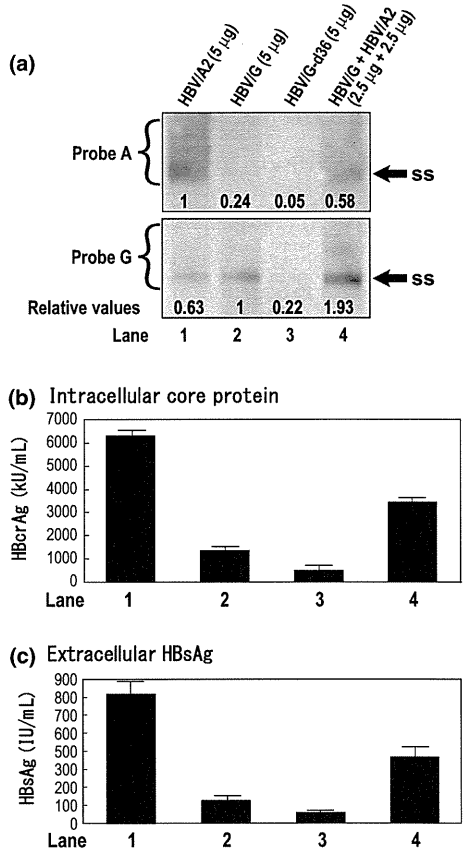

Fig. 2 (a) Southern blot analysis for replicative activity among HBV/G monotransfection, HBV/G-d36 monotransfection, HBV/A2 monotransfection and co-transfection with HBV/A2 and HBV/G (3 days after transfection). HBV/G-d36 clone was a deletion mutant lacking the 36-nt unique insertion in the core gene of the wild-type HBV/G clone. Hybridisation of the blot with genotype-specific probes of genotype A2 (upper) and G (lower). The density values shown at the bottom were measured to the probe-specific DNA sample. Single-stranded (SS) DNA is indicated by arrows. (b) Intracellular expression of core protein was estimated by detecting HBV-core-related antigen (HBcrAg) [22] as measured by a commercial chemiluminescent enzyme immunoassay (mean and standard deviation, $n = 3$). (c) HBsAg levels in the supernatant as detected by a commercial chemiluminescent enzyme immunoassay (mean and standard deviation, $n = 3$). All experiments were tested at least three times.

clone, compared with monotransfection of the wild-type HBV/G (Fig. 2a). The intracellular expression of core protein in the cell lysates and the expression of HBsAg in the culture supernatant were also enhanced by the co-transfection with both HBV/A2 and HBV/G clones (Fig. 2b and 2c). Removing the 36-nt insertion from the wild-type HBV/G genome resulted in a significant reduction in viral replication and core protein expression compared with the wild-type HBV/G clone. These results are in agreement with the observations of a previous study [13].

The core protein of HBV/A2 is essential for efficient replication of HBV/G

To determine how HBV/A2 rescues HBV/G replication during co-transfection, we constructed four HBV/A2 recombinant plasmids that selectively expressed one of the four viral proteins, whereas translation of the other three was prevented by the introduction of stop codons (Fig. 1a). All of these plasmids were prevented from coding for the viral pregenomic RNA containing the 'packaging-negative' mutation in the ε signal loop to abrogate encapsidation (see Materials and methods). Huh7 cells were co-transfected with the wild-type HBV/G clone and one of the four plasmids expressing a single viral protein. According to Southern blot analysis, the expression of intracellular HBV DNA was greatly increased when HBV/G was co-transfected with HBV/A2-core compared with the other three expression plasmids or the experimental control (pUC19 or HBV/A2-N) (Fig. 3a). The intracellular expression of core protein in the cell lysates was also the highest when HBV/G was co-transfected with HBV/A2-core (Fig. 3b). The expression of HBsAg in the culture supernatant was only increased when HBV/G was co-transfected with the HBV/A2-S plasmid (Fig. 3c). These results indicated that the core protein translated from the HBV/A2 recombinant plasmid can enhance HBV/G replication.

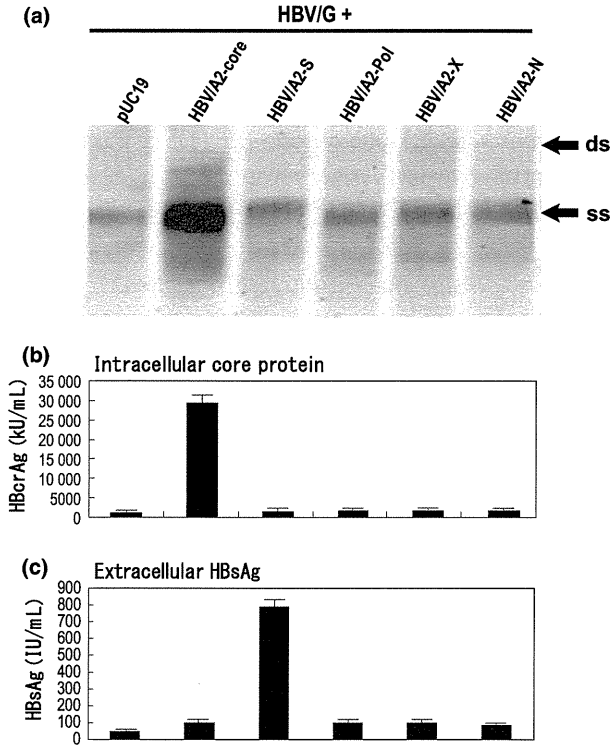

Fig. 3 (a) Southern blot analysis for replication competence of HBV/G clones co-transfected with each of the four HBV/A2 recombinant plasmids: HBV/A2-S, HBV/A2-core, HBV/A2-pol and HBV/A2-X selectively expressing only one of the four viral proteins (large surface, precore/core, polymerase or X protein, respectively). The 'HBV/A2-N' contained all the six mutations to be used as an experimental control. All of the above HBV/A2 recombinant plasmids had the 'packaging-negative' mutation in the ε signal to abrogate encapsidation. (b) Intracellular expression of core protein was measured as described in Fig. 2b. (c) The expression of HBsAg in the culture supernatant was detected as described in Fig. 2c.

The core protein of HBV/A2 is more effective than those of HBV/C and HBV/G at promoting HBV/G replication

To compare the effects of genotype on the ability of the core protein to increase HBV/G replication in co-transfection experiments, we generated three genotype-specific core protein expression constructs (HBV/G, HBV/A2 and HBV/C) driven by the CMV promoter, which produced core protein in the absence of a preceding ε signal (Fig. 1c). Huh7 cells were co-transfected with HBV/G and one of the three core protein expression vectors. Southern blot analysis showed that the level of intracellular HBV DNA was highest during co-transfection with CMV-HBV/A2/core, followed by CMV-HBV/G/core, and was lowest for CMV-HBV/C/core (Fig. 4a), although the expression of core protein in the cell lysates was the highest during co-transfection with CMV-HBV/C/core, followed by CMV-HBV/G/core, and CMV-HBV/A2/core (Fig. 4b). As anticipated, there was no difference in the expression levels of HBsAg in any co-transfection experiment (Fig. 4c).

A comparison of viral replication among HBV/G and recombinant HBV/G clones

To examine the effects of genetic recombination and the roles of the core promoter, precore and core genomic regions in the interaction of HBV/G and HBV/A2 during co-transfection, we employed three HBV/G and HBV/A2 chimaeric replicating constructs (see Materials and methods), which are shown in Fig. 1c. After the transfection experiment, Southern blot analysis of cell lysates indicated an abundant level of DNA expression in HBV/G/A2-CP/core–transfected cells compared with those in the cells transfected with HBV/G-wild type, HBV/G/A2-CP and HBV/G/A2-core (Fig. 5a). As shown in Fig. 5b, the highest

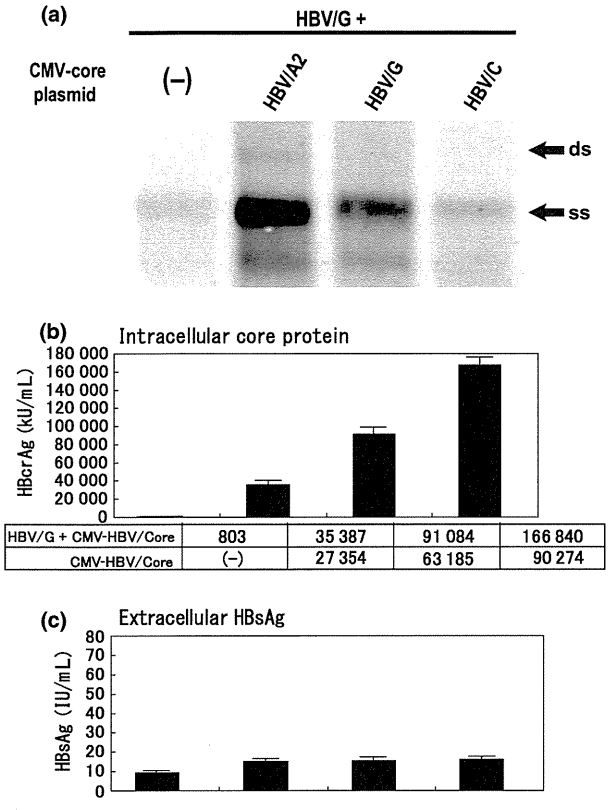

Fig. 4 (a) Southern blot analysis for the expression of intracellular HBV/G DNA during co-transfection with the three core protein expression constructs for each genotype (HBV/G, HBV/A2 and HBV/C) driven by the CMV promoter, which produced core protein in the absence of a preceding ε signal. (b) Intracellular expression of core protein. (c) The expression of HBsAg in the culture supernatant.

levels of core protein (HBcrAg) expression were observed for the HBV/G/A2-CP and HBV/G/A2-CP/core-transfected cultures, which was in sharp contrast with the low levels observed in the HBV/G/A2-core and the wild-type HBV/G cultures. The discrepancy between viral replication and core production of the HBV/G/A2-CP clone might indicate insufficient virion assembly. Figure 5c shows the HBeAg levels measured in culture supernatants. The expression of HBeAg was the highest in the HBV/G/A2-CP/core culture distantly followed by that in the HBV/G/A2-core culture. The HBV/G/A2-CP and wild-type HBV/G clones expressed HBeAg protein at levels close to or below the level of detection. Nevertheless, a high HBcrAg titre was detected in the cell lysate of the HBV/G/A2-CP clone, although its DNA level was as low as that of the wild-type HBV/G clone (Fig. 5a). These results indicated that low replication of HBV/G might be explained by low synthesis of HBV/G core protein due to weak core promoter activity or dysfunction, as well as insufficient virion assembly due to the larger core protein of HBV/G (12-aa unique insertion).

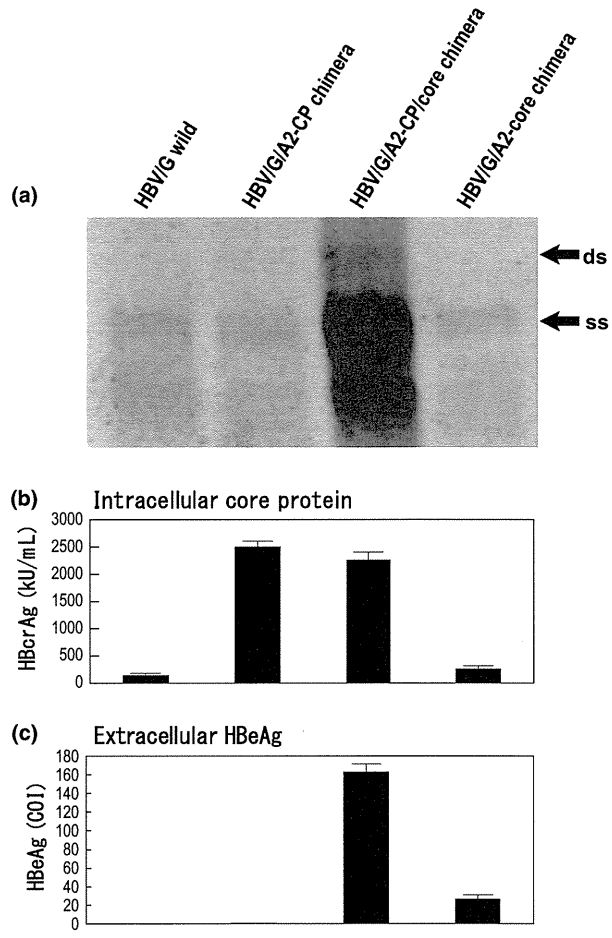

Fig. 5 (a) Southern blot analysis for HBV replication among HBV/G and three chimeric replicating constructs created by recombination of different genomic sections of HBV/G and HBV/A2 (see Materials and methods). The 'HBV/G/A2-CP' clone was a HBV/G-based construct in which the fragment containing the core promoter (CP) region but not the precore or core was replaced by the corresponding sequence from HBV/A2. The 'HBV/G/A2-Core' clone was an HBV/G-based construct in which the section of the precore and core region was replaced with that of HBV/A2. For the 'HBV/G/A2-CP/core' clone, the CP, precore and core region of HBV/G were replaced with that of HBV/A2. (b) Intracellular expression of core protein. (c) Extracellular expression of HBeAg levels detected by a commercial chemiluminescent enzyme immunoassay (mean and standard deviation, $n = 3$).

Dane particles produced by HBV/G during co-transfection with HBV/A2 were packed in HBV/A2 protein

To investigate the effects of HBV/A2 core protein during HBV/G viral assembly, we tried to define whether the Dane particles in B Huh7 cells that had been co-transfected with wild-type HBV/G and the CMV-HBV/A2-core plasmid

(source of the Fig. 4 lane 2) contained HBV/A2 or HBV/G core protein. To extract the Dane particles, we employed ultracentrifugation of the culture media through a 10–60% (w/w) sucrose density gradient followed by immunoprecipitation using anti-HBs-coated magnetic beads. We thereby extracted Dane particles, which were then analysed using Western blotting. The obtained fractions were tested for HBcrAg, HBsAg and HBV DNA (Fig. 6a). HBcrAg appeared in the high-density fractions, and its levels peaked in the same fraction (fraction 22) as HBV DNA. As reported previously, the fraction in which the levels of HBV DNA and HBcrAg peaked contained Dane particles [22]. To eliminate contamination of the Dane particles with 'naked' core particles or core protein, they were specifically retrieved from sucrose high-density fraction 22 by means of immunoprecipitation using anti-HBs-coated magnetic beads. The media supernatant obtained from the culture of cells that had been subjected to CMV-HBV/A2/core clone monotransfection was also subjected to sucrose gradient ultracentrifugation using the same protocol. Sucrose high-density fraction 22, in which the HBcrAg concentration peaked, presumably contained 'naked' core particles or core protein (Fig. 6b). This fraction was collected and processed in the same manner via anti-HBs-coated magnetic bead separation and was used as negative control for this procedure (Fig. 6c, lane 4). To discriminate between HBV/G and HBV/A2 core proteins on Western blot analysis probed with anti-HBc antibody, we employed cell lysates produced from cells that had been transfected with the wild-type HBV/G clone and those produced with the HBV/A2 clone as controls. As can be seen on the Western blotting image (Fig. 6c), HBV/G-transfected cells (lane 1) produced larger proteins than the HBV/A2-transfected cells (lane 2), which can be explained by the 12-aa insertion in the core protein of HBV/G coded by its 36-nt unique insertion. Interestingly, the most saturated band associated with the Dane particles produced by HBV/G that had been co-transfected with CMV-HBV/A2/core (lane 3) was the same size as that for HBV/A2, suggesting that HBV/G competitively produces Dane particles consisting of HBV/A2 core protein during virion assembly.

DISCUSSION

HBV/G was first isolated in 2000 in France and the USA and was later found in Thailand, Japan and Mexico, indicating its global dissemination and association with specific risk groups, such as injection drug users (IDU) and men who had sex with men (MSM) [25]. Studies have also demonstrated that throughout the world, HBV/G strains possess unprecedented genetic homology and are mainly detected during co-infection with another genotype that is endemic in the area. Further studies have suggested that genotype G represents a 'replication-defective' variant of HBV that requires co-infection with another genotype to establish a persistent infection. We and others have reported *in vitro* and *in vivo* experimental evidence of this HBV/G dependence [13–15]. The unique 36-nt insertion within core coding region increases core protein level and genome replication in genotype G but impairs replication, not core protein expression, in other genotypes [14]. These results strongly suggest the 36-nt/12-aa insertion has pleiotropic effects on core protein expression, genome replication and virion secretion [14]. To obtain clues about the mechanism by which genotype G works in combination with genotype A to effect its replication, we performed co-transfection experiments using Huh7 cells.

Using HBV/A2 viral proteins expressing plasmids, we determined that a HBV/A2 plasmid that selectively expressed core protein was capable of increasing the replication of the wild-type HBV/G (Fig. 3a). The replication of HBV/G during co-transfection was not affected by other viral elements of HBV/A2 because of the presence of the 'packaging-negative mutation' in the epsilon-coding region and stop codons preventing the translation of the other three viral proteins (the polymerase, surface and X proteins). The specific role of the core protein was further confirmed in experiments with CMV promoter–driven core expressing constructs, in which the core protein alone enhanced HBV/G replication in the absence of HBV pregenomic RNA. Interestingly, co-transfection of HBV/G with the CMV-HBV/A2/core expression construct produced the highest levels of intracellular DNA, even though this combination produced the lowest intracellular core protein level, compared with the CMV-core constructs of the other two genotypes (HBV/G and HBV/C) (Figs 4a,b). The replication of HBV/G was the highest during co-transfection with the CMV-HBV/A2/core expression construct, which agreed with the results of experiments using other genotype (HBV/D, HBV/B1) CMV-core constructs (data not shown). Thus, the core protein of HBV/A2 was confirmed to play an important role in upregulating HBV/G replication and performed this task more efficiently than the other genotypes. These experimental results might explain why HBV/A is the genotype that is most frequently found in co-infections with HBV/G [12,26].

Moreover, HBV/G core protein overexpression achieved by the co-transfection of HBV/G with CMV-HBV/G/core did not enhance replication, suggesting that HBV/G core protein is functionally defective; that is, results in insufficient viral packaging. To investigate the functional defect in the HBV/G core protein, we exchanged the core gene of the wild-type HBV/G for the corresponding gene of HBV/A2 (HBV/G/A2-core); the introduction of the HBV/A2 core promoter together with core coding region into the HBV/G genome (HBV/G/A2-CP/core) significantly enhanced replication. However, the replication of the recombinant construct (HBV/A2 core coding region; HBV/G/A2-core) did not differ from that of the wild-type HBV/G, suggesting that the replacement of HBV/G/A2-core alone was not

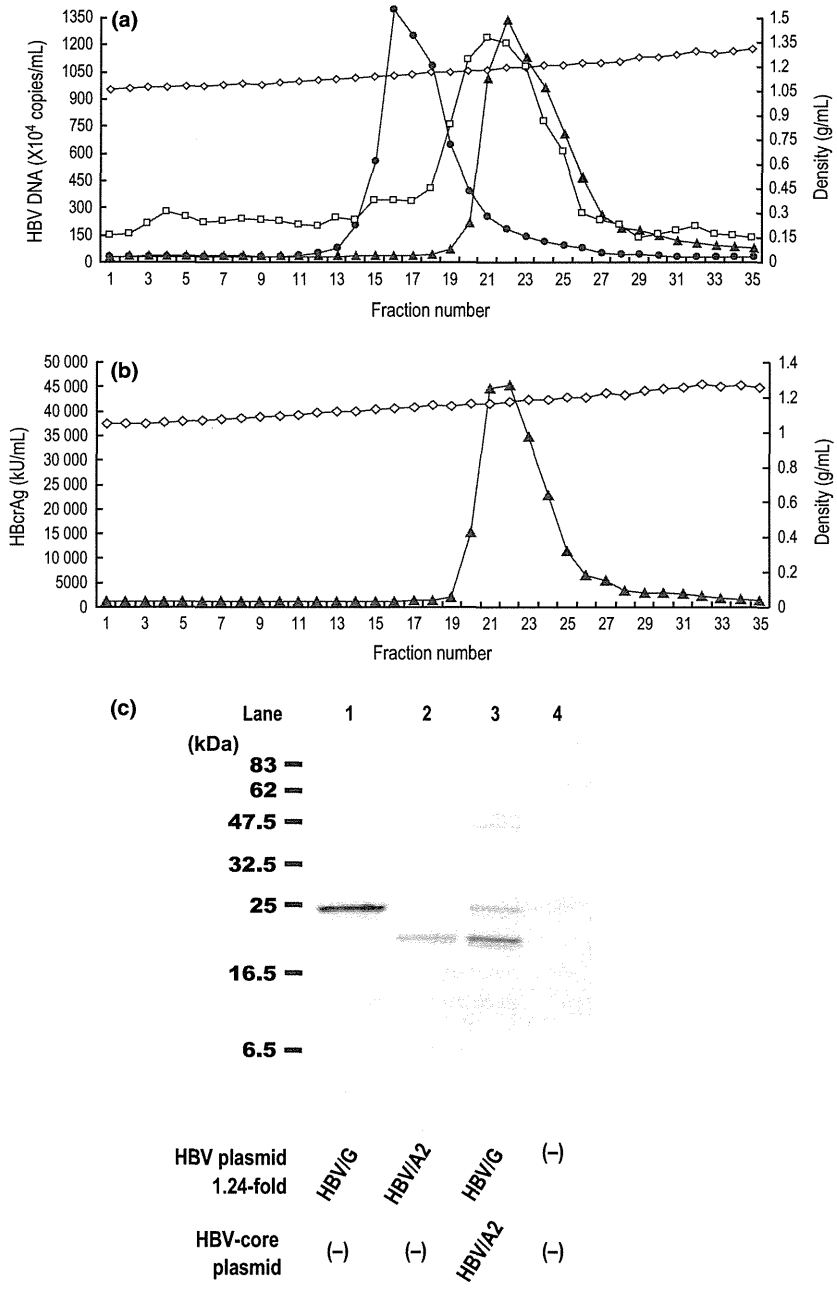

Fig. 6 (a) Sucrose gradient analysis of the culture media of Huh7 cells that had been co-transfected with wild-type HBV/G and the CMV-HBV/A2-core plasmid. It was subjected to ultracentrifugation through a 10–60% (w/w) sucrose density gradient. Density of each fraction is shown as a line with diamond symbols. Fractions were diluted 10-fold and tested for HBsAg (•) (IU/mL), HBcrAg (▲) (KU/mL) and HBV DNA (□) (10^4 copies/mL). (b) Sucrose gradient analysis of culture supernatant obtained from the cells that were subjected to CMV-HBV/A2/core monotransfection using the same protocol. (c) Western blot analysis for HBV core protein was probed by anti-HBc antibody. HBV/G/core and HBV/A2/core were obtained from cell lysates that were transfected with the wild-type HBV/G clone and the wild-type HBV/A2 clone, respectively. The 'HBV/G + CMV-HBV/A2/core' was obtained from sucrose high-density fraction 22 (Fig. 6a) that had been co-transfected with wild-type HBV/G and the CMV-HBV/A2-core plasmid by means of immunoprecipitation using anti-HBs-coated magnetic beads.

enough for viral replication because the core promoter of HBV/G was not capable of generating sufficient amounts of core protein to enhance HBV replication. As well, an HBV/G/A2-CP construct containing the HBV/A core promoter region in the context of the wild-type HBV/G genome did not enhance replication, even though its core protein

production was significantly increased (Figs 5a,b). Although it was previously reported that the 36-nt insertion of the HBV/G core gene was required for both efficient core protein expression and HBV/G replication [13], in this study, the discrepancy between viral replication and core production of the HBV/G/A2-CP clone might indicate insufficient virion assembly due to the larger core protein of HBV/G (12-aa unique insertion). *Trans*-complementation experiments carried out by Gutelius *et al.* [14] demonstrated an association between enhanced core protein level and reduced replication capacity only when the core and polymerase proteins are expressed from the same RNA template. Thus, it was indicated that HBV/G itself could not replicate sufficiently due to a defect in its core protein and/or the core promoter of HBV/G.

Finally, we investigated whether HBV/G utilises the core protein of HBV/A2 for virion packaging. Dane particles obtained from the culture supernatants of cells that had been co-transfected with HBV/G and CMV-HBV/A2/core were assessed by Western blotting, and it was found that the Dane particles of HBV/G contained HBV/A core proteins. Thus, it was implied that HBV/G replication is enhanced by the core protein of HBV/A because it is more suitable for virion packaging than its own core protein, suggesting that the core protein of HBV/A is a key element enhancing the replication of HBV/G during co-infection. Interestingly, our experiments demonstrated that there were large differences in core protein expression among the CMV-core constructs of different genotypes, despite the fact that all of the CMV-core constructs had the same CMV promoter (Fig. 4b). In a previous report, it was speculated that the core protein binds to its own mRNA to influence protein translation [13]. For example, dihydrofolate reductase protein has been found to downregulate its own translation by binding to cognate mRNA [27,28]. Therefore, we predict that the core protein of HBV/A2 enhances HBV/G replication by affecting viral promoters or transcription in addition to its role in virion packaging.

In conclusion, enhanced replication of HBV/G requires the HBV/A2 core protein during co-infection with HBV/A2. Our findings provide a possible explanation that the core protein of HBV/A2 is more suitable for virion packaging rather than that of HBV/G, and the replication of HBV/G occurs at a very low level, which may be due to defects in its core protein functions and/or core promoter activity. Further experiments are warranted to clarify the detailed roles of the enhanced HBV/G replication by co-infection with the other genotype and the clinical manifestation of HBV/G infection.

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CONFLICT OF INTEREST STATEMENT

None declared.

REFERENCES

1 Kao JH, Chen DS. Global control of hepatitis B virus infection. *Lancet Infect Dis* 2002; 2(7): 395–403. Epub 2002/07/20.
2 Kim BK, Revill PA, Ahn SH. HBV genotypes: relevance to natural history, pathogenesis and treatment of chronic hepatitis B. *Antivir Ther* 2011; 16(8): 1169–1186. Epub 2011/12/14.
3 Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y *et al.* Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988; 69 (Pt 10): 2575–2583. Epub 1988/10/01.
4 Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* 1994; 198(2): 489–503. Epub 1994/02/01.
5 Orito E, Ichida T, Sakugawa H, Sata M, Horiike N, Hino K *et al.* Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 2001; 34(3): 590–594. Epub 2001/08/30.
6 Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF *et al.* A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000; 81(Pt 1): 67–74. Epub 2000/01/21.
7 Kato H, Orito E, Gish RG, Bzowej N, Newsom M, Sugauchi F *et al.* Hepatitis B e antigen in sera from individuals infected with hepatitis B virus of genotype G. *Hepatology* 2002; 35(4): 922–929. Epub 2002/03/27.
8 Osiowy C, Gordon D, Borlang J, Giles E, Villeneuve JP. Hepatitis B virus genotype G epidemiology and co-infection with genotype A in Canada. *J Gen Virol* 2008; 89(Pt 12): 3009–3015. Epub 2008/11/15.
9 Suwannakarn K, Tangkijvanich P, Theamboonlers A, Abe K, Poovorawan Y. A novel recombinant of hepatitis B virus genotypes G and C isolated from a Thai patient with hepatocellular carcinoma. *J Gen Virol* 2005; 86(Pt 11): 3027–3030. Epub 2005/10/18.
10 Sanchez LV, Tanaka Y, Maldonado M, Mizokami M, Panduro A. Difference of hepatitis B virus genotype distribution in two groups of Mexican

patients with different risk factors. High prevalence of genotype H and G. *Intervirology* 2007; 50(1): 9–15. Epub 2006/12/14.
11 Tanaka Y, Sanchez LV, Sugiyama M, Sakamoto T, Kurbanov F, Tatematsu K *et al.* Characteristics of hepatitis B virus genotype G coinfected with genotype H in chimeric mice carrying human hepatocytes. *Virology* 2008; 376(2): 408–415. Epub 2008/05/14.
12 Kato H, Orito E, Gish RG, Sugauchi F, Suzuki S, Ueda R *et al.* Characteristics of hepatitis B virus isolates of genotype G and their phylogenetic differences from the other six genotypes (A through F). *J Virol* 2002; 76(12): 6131–6137. Epub 2002/05/22.
13 Li K, Zoulim F, Pichoud C, Kwei K, Villet S, Wands J *et al.* Critical role of the 36-nucleotide insertion in hepatitis B virus genotype G in core protein expression, genome replication, and virion secretion. *J Virol* 2007; 81(17): 9202–9215. Epub 2007/06/15.
14 Gutelius D, Li J, Wands J, Tong S. Characterization of the pleiotropic effects of the genotype G-specific 36-nucleotide insertion in the context of other hepatitis B virus genotypes. *J Virol* 2011; 85(24): 13278–13289. Epub 2011/10/14.
15 Sugiyama M, Tanaka Y, Sakamoto T, Maruyama I, Shimada T, Takahashi S *et al.* Early dynamics of hepatitis B virus in chimeric mice carrying human hepatocytes monoinfected or coinfected with genotype G. *Hepatology* 2007; 45(4): 929–937. Epub 2007/03/30.
16 Lacombe K, Massari V, Girard PM, Serfaty L, Gozlan J, Pialoux G *et al.* Major role of hepatitis B genotypes in liver fibrosis during coinfection with HIV. *AIDS* 2006; 20(3): 419–427. Epub 2006/01/28.
17 Sugiyama M, Tanaka Y, Kato T, Orito E, Ito K, Acharya SK *et al.* Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. *Hepatology* 2006; 44(4): 915–924. Epub 2006/09/29.
18 Bruss V, Ganem D. The role of envelope proteins in hepatitis B virus assembly. *Proc Natl Acad Sci U S A* 1991; 88(3): 1059–1063. Epub 1991/02/01.
19 Tang H, Delgermaa L, Huang F, Oishi N, Liu L, He F *et al.* The transcriptional transactivation function of HBx protein is important for its augmentation role in hepatitis B virus replication. *J Virol* 2005; 79(9): 5548–5556. Epub 2005/04/14.
20 Pollack JR, Ganem D. An RNA stem-loop structure directs hepatitis B virus genomic RNA encapsidation. *J Virol* 1993; 67(6): 3254–3263. Epub 1993/06/01.
21 Ozasa A, Tanaka Y, Orito E, Sugiyama M, Kang JH, Hige S *et al.* Influence of genotypes and precore mutations on fulminant or chronic outcome of acute hepatitis B virus infection. *Hepatology* 2006; 44(2): 326–334. Epub 2006/07/28.
22 Kimura T, Ohno N, Terada N, Rokuhara A, Matsumoto A, Yagi S *et al.* Hepatitis B virus DNA-negative Dane particles lack core protein but contain a 22-kDa precore protein without C-terminal arginine-rich domain. *J Biol Chem* 2005; 280(23): 21713–21719. Epub 2005/04/09.
23 Fujiwara K, Tanaka Y, Paulon E, Orito E, Sugiyama M, Ito K *et al.* Novel type of hepatitis B virus mutation: replacement mutation involving a hepatocyte nuclear factor 1 binding site tandem repeat in chronic hepatitis B virus genotype E. *J Virol* 2005; 79(22): 14404–14410. Epub 2005/10/29.
24 Orchard S, Martens L, Tasman J, Binz PA, Albar JP, Hermjakob H. 6th HUPO Annual World Congress – Proteomics Standards Initiative Workshop 6–10 October 2007, Seoul, Korea. *Proteomics* 2008; 8(7): 1331–1333. Epub 2008/03/05.
25 Kurbanov F, Tanaka Y, Mizokami M. Geographical and genetic diversity of the human hepatitis B virus. *Hepatol Res* 2010; 40(1): 14–30. Epub 2010/02/17.
26 Osiowy C, Giles E. Evaluation of the INNO-LiPA HBV genotyping assay for determination of hepatitis B virus genotype. *J Clin Microbiol* 2003; 41(12): 5473–5477. Epub 2003/12/10.
27 Chu E, Takimoto CH, Voeller D, Grem JL, Allegra CJ. Specific binding of human dihydrofolate reductase protein to dihydrofolate reductase messenger RNA in vitro. *Biochemistry* 1993; 32(18): 4756–4760. Epub 1993/05/11.
28 Ercikan-Abali EA, Banerjee D, Waltham MC, Skacel N, Scotto KW, Bertino JR. Dihydrofolate reductase protein inhibits its own translation by binding to dihydrofolate reductase mRNA sequences within the coding region. *Biochemistry* 1997; 36(40): 12317–12322. Epub 1997/10/07.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1. Plasmid construct of HBV/G.

Gut Online First, published on November 7, 2012 as 10.1136/gutjnl-2012-302553

OPEN ACCESS

ORIGINAL ARTICLE

Hepatitis C virus kinetics by administration of pegylated interferon-α in human and chimeric mice carrying human hepatocytes with variants of the *IL28B* gene

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► Additional supplementary files are published online only. To view these files please visit the journal online (http://dx.doi.org/10.1136/gutjnl-2012-302553).

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ABSTRACT

Objective Recent studies have demonstrated that genetic polymorphisms near the *IL28B* gene are associated with the clinical outcome of pegylated interferon α (peg-IFN-α) plus ribavirin therapy for patients with chronic hepatitis C virus (HCV). However, it is unclear whether genetic variations near the *IL28B* gene influence hepatic interferon (IFN)-stimulated gene (ISG) induction or cellular immune responses, lead to the viral reduction during IFN treatment.

Design Changes in HCV-RNA levels before therapy, at day 1 and weeks 1, 2, 4, 8 and 12 after administering peg-IFN-α plus ribavirin were measured in 54 patients infected with HCV genotype 1. Furthermore, we prepared four lines of chimeric mice having four different lots of human hepatocytes containing various single nucleotide polymorphisms (SNP) around the *IL28B* gene. HCV infecting chimeric mice were subcutaneously administered with peg-IFN-α for 2 weeks.

Results There were significant differences in the reduction of HCV-RNA levels after peg-IFN-α plus ribavirin therapy based on the *IL28B* SNP rs8099917 between TT (favourable) and TG/GG (unfavourable) genotypes in patients; the first-phase viral decline slope per day and second-phase slope per week in TT genotype were significantly higher than in TG/GG genotype. On peg-IFN-α administration to chimeric mice, however, no significant difference in the median reduction of HCV-RNA levels and the induction of antiviral ISG was observed between favourable and unfavourable human hepatocyte genotypes.

Conclusions As chimeric mice have the characteristic of immunodeficiency, the response to peg-IFN-α associated with the variation in *IL28B* alleles in chronic HCV patients would be composed of the intact immune system.

Significance of this study

What is already known on this subject?

- Genetic polymorphisms near the *IL28B* gene are associated with a chronic HCV treatment response.
- HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline in HCV kinetics in the first and second phases by peg-IFN-α-based therapy.
- During the acute phase of HCV infection, a strong immune response among patients with the *IL28B* favourable genotype could induce more frequent spontaneous clearance of HCV.

What are the new findings?

- In chronically HCV genotype 1b-infected chimeric mice that have the characteristic of immunodeficiency, no significant difference in the reduction in serum HCV-RNA levels and the induction of antiviral hepatic ISG by the administration of peg-IFN-α was observed between favourable and unfavourable human hepatocyte *IL28B* genotypes.
- By comparison of serum HCV kinetics between human and chimeric mice, the viral decline in both the first and second phases by peg-IFN-α treatment was affected by the variation in *IL28B* genotypes only in chronic hepatitis C patients.

How might it impact on clinical practice in the foreseeable future?

- The immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN-α-based therapy.

INTRODUCTION

Hepatitis C is a global health problem that affects a significant portion of the world's population. The WHO estimated that, in 1999, 170 million hepatitis C virus (HCV)-infected patients were present worldwide, with 3–4 million new cases appearing per year.[1]

The standard therapy for hepatitis C still consists of pegylated interferon-α (peg-IFN-α), administered once weekly, plus daily oral ribavirin for 24–48 weeks in countries where protease inhibitors are not available.[2] This combination therapy is quite successful in patients with HCV genotype 2 or 3 infection, leading to a sustained virological response (SVR) in approximately 80–90% of patients treated; however, in patients infected with HCV genotype 1 or 4, only approximately half of all treated individuals achieved a SVR.[3 4]

Table 1 Characteristics of 54 patients infected HCV genotype 1

	IL28B SNP rs8099917		
	TT (n=34)	TG (n=19) + GG (n=1)	p Value
Age (years)	55.6±10.1	54.7±11.3	0.746
Gender (male %)	70	50	0.199
Body mass index (kg/m^2)	24.6±3.1	24.7±3.3	0.870
Viral load at therapy (log IU/ml)	6.0±0.7	5.8±0.8	0.357
SVR rate (%)	50	11	0.012
Serum ALT level (IU/l)	100.3±80.8	79.3±45.0	0.226
Platelet count (×10^4/μl)	17.1±9.0	16.5±5.8	0.771
Fibrosis (F3+4 %)	42	40	0.877

HCV, hepatitis C virus; SNP, single nucleotide polymorphism; SVR, sustained virological response.

Host factors were shown to be associated with the outcome of the therapy, including age, sex, race, liver fibrosis and obesity.[5] Genome-wide association studies have demonstrated that genetic variations in the region near the interleukin-28B (*IL28B*) gene, which encodes interferon (IFN)-λ3, are associated with a chronic HCV treatment response.[6–10] Furthermore, it was demonstrated that genetic variations in the *IL28B* gene region are also associated with spontaneous HCV clearance.[11–12]

Interestingly, a recent report showed the effect of genetic polymorphisms near the *IL28B* gene on the dynamics of HCV during peg-IFN-α plus ribavirin therapy in Caucasian, African American and Hispanic individuals;[13] HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline of HCV in the first phase, which is associated with the inhibition of viral replication as well as the second phase associated with immuno-destruction of viral-infected hepatocytes.[14] However, it is unknown how a direct effect by the *IL28B* genetic variation, such as the induction of IFN-stimulated genes (ISG) or cellular immune responses, would influence the viral kinetics during IFN treatment. Over recent periods, engineered severe combined immunodeficient (SCID) mice transgenic for urokinase-type plasminogen activator (uPA) received human hepatocyte transplants (hereafter referred to as chimeric mice)[15–17] and are suitable for experiments with hepatitis viruses in vivo.[18 19] We have also reported that these chimeric mice carrying human hepatocytes are a robust animal model to evaluate the efficacy of IFN and other anti-HCV agents.[20 21]

The purpose of this study was to reveal the association between genetic variations in the *IL28B* gene region and viral decline during peg-IFN-α treatment in patients with HCV, and to clarify the association between different *IL28B* alleles of human hepatocytes in chimeric mice and the response to peg-IFN-α without immune response. These studies will elucidate whether the immune response by the *IL28B* genetic variation affects the viral kinetics during peg-IFN-α treatment.

MATERIALS AND METHODS

Patients

Fifty-four Japanese patients with chronic HCV genotype 1 infection at Nagasaki Medical Center and Nagoya City University were enrolled in this study (table 1). Patients received peg-IFN-α2a (180 μg) or 2b (1.5 μg/kg) subcutaneously every week and were administered a weight-adjusted dose of ribavirin (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg daily), which is the recommended dosage in Japan. Patients with other hepatitis virus infection or HIV coinfection were not included in the study. The study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki as reflected by earlier approval by the institutions' human research committees.

Laboratory tests

Blood samples were obtained before therapy, as well as on day 1 and at weeks 1, 2, 4, 8 and 12 after the start of therapy and were analysed for the HCV-RNA level by the commercial Abbott Real-Time HCV test with a lower limit of detection of 12 IU/ml (Abbott Molecular Inc., Des Plains, Illinois, USA). Genetic polymorphism in the *IL28B* gene (rs8099917), a single nucleotide polymorphism (SNP) recently identified to be associated with treatment response,[6–8] was tested by the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, California, USA).

HCV infection of chimeric mice with the liver repopulated for human hepatocytes

SCID mice carrying the uPA transgene controlled by an albumin promoter were injected with 5.0–7.5×10^5 viable hepatocytes through a small left-flank incision into the inferior splenic pole, thereafter chimeric mice were generated. The chimeric mice were purchased from PhoenixBio Co, Ltd (Hiroshima, Japan).[17] Human hepatocytes with the *IL28B* homozygous favourable allele, heterozygous allele or homozygous unfavourable allele were imported from BD Biosciences (San Jose, California, USA) (table 2). Murine serum levels of human albumin and the body weight were not significantly different among four chimeric mice groups, providing a reliable comparison for anti-HCV agents.[22] Three different serum samples were obtained from three chronic HCV patients (genotype 1b).[21 22] Each mouse was intravenously infected with serum sample containing 10^5 copies of HCV genotype 1b. Administration of peg-IFN-α2a (Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) at the dose formulation (30 μg/kg) was consecutively applied to each mouse on days 0, 3, 7 and 10 (table 3).

HCV-RNA quantification

HCV-RNA in mice sera (days 0, 1, 3, 7 and 14) was quantified by an in-house real-time detection PCR assay with a lower quantitative limit of detection of 10 copies/assay, as previously reported.[21]

Quantification of IFN-stimulated gene-expression levels

For analysis of endogenous ISG levels, total RNA was isolated from the liver using the RNeasy RNA extraction kit (Qiagen, Valencia, California, USA) and complementary DNA synthesis

Table 2 Four lines of uPA/SCID mice from four different lots of human hepatocytes (donor) containing various SNP around the *IL28B* gene

uPA/SCID mice	Donor	Race	Age	Gender	rs8103142	rs12979860	rs8099917
PXB mice	A	African American	5 Years	Male	CC	TT	TG
	B	Caucasian	10 Years	Female	CC	TT	TG
	C	Hispanic	2 Years	Female	TT	CC	TT
	D	Caucasian	2 Years	Male	TT	CC	TT

PXB mice; urokinase-type plasminogen activator/severe combined immunodeficiency (uPA/SCID) mice repopulated with approximately 80% human hepatocytes.
SCID, severe combined immunodeficient; SNP, single nucleotide polymorphism.

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Table 3 Dosage and time schedule of pegIFN-α2a* treatment for HCV genotype 1b infected chimeric mice

				Dose			
Donor hepatocytes†	No of chimeric mice	Inoculum	Test compound	Level (μg/kg)	Concentrtion (μg/ml)	Volume (ml/kg)	Frequency
A	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
B	4	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
D	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10

*Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.
†The *IL28B* genetic variation of the donor hepatocytes was indicated in table 2.
HCV, hepatitis C virus; peg-IFN-α, pegylated interferon α.

was performed using 2.0 μg of total RNA (High Capacity RNA-to-cDNA kit; Applied Biosystems). Fluorescence real-time PCR analysis was performed using an ABI 7500 instrument (Applied Biosystems) and TaqMan Fast Advanced gene expression assay (Applied Biosystems). TaqMan Gene Expression Assay primer and probe sets (Applied Biosystems) are shown in the supplementary information (available online only). Relative amounts of messenger RNA, determined using a FAM-Labeled TaqMan probe, were normalised to the endogenous RNA levels of the housekeeping reference gene, glyceraldehyde-3-phosphate dehydrogenase. The delta Ct method ($2^{-(\text{delta Ct})}$) was used for quantitation of relative mRNA levels and fold induction.[23 24]

Statistical analyses

Statistical differences were evaluated by Fisher's exact test or the χ^2 test with the Yates correction. Mice serum HCV-RNA and intrahepatic ISG expression levels were compared using the Mann–Whitney U test. Differences were considered significant if p values were less than 0.05.

RESULTS

Characteristics of the study patients

Genotypes (rs8099917) TT, TG and GG were detected in 34, 19 and one patient infected with HCV genotype 1, respectively. SVR rates were significantly higher in HCV patients with genotype TT than in those with genotype TG/GG (50% vs 11%, p=0.012). The initial HCV serum load was comparable between genotypes TT and TG/GG (6.0±0.7 vs 5.8±0.8 log IU/ml). There were no significant differences in sex (male%, 70% vs 50%), age (55.6±10.1 vs 54.7±11.3 years), serum alanine aminotransferase level (100.3±80.8 vs 79.3±45.0 IU/L), platelet count (17.1±9.0 vs 16.5±5.8×10^4/μl) and fibrosis stages (F3/4%, 42% vs 40%) between HCV patients with the favourable (rs8099917 TT) and unfavourable (rs8099917 TG/GG) *IL28B* genotypes (table 1).

Changes in serum HCV-RNA levels in patients treated by peg-IFN-α plus ribavirin

Figure 1 shows the initial change in the serum HCV-RNA level for 14 days after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1 based on the genetic polymorphism near the *IL28B* gene. The immediate antiviral response (viral drop 24 h after the first IFN injection) was significantly higher in HCV patients with genotype TT than genotype TG/GG (−1.08 vs −0.39 log IU/ml, p<0.001). Figure 2 also shows the subsequent change in the serum HCV-RNA reduction after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1. Similarly, during peg-IFN-α plus ribavirin therapy, a statistically significant difference in the median reduction in serum HCV-RNA levels was noted according to the genotype (TT vs TG/GG). The median reduction in the serum HCV-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between genotypes TT and TG/GG was as follows: −1.58 vs −0.62, p<0.001; −2.35 vs −0.91, p<0.001;

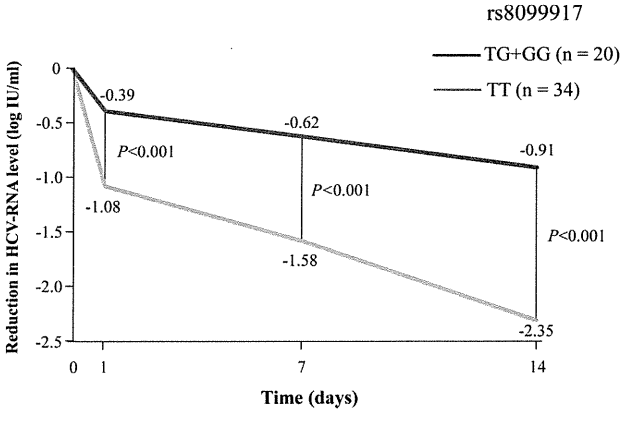

Figure 1 Rapid reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 7 and 14 days between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with peg-IFN-α plus ribavirin.

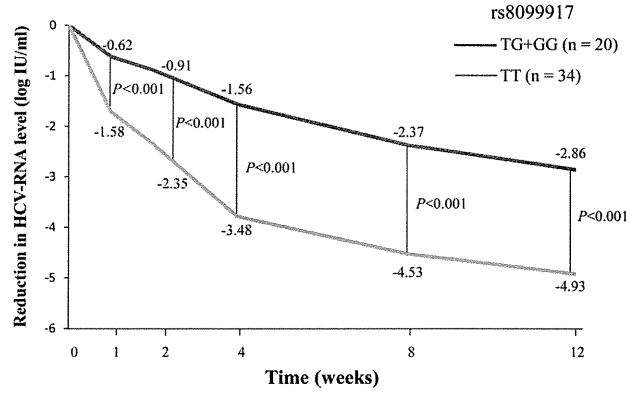

Figure 2 Weekly reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with pegylated interferon α plus ribavirin.

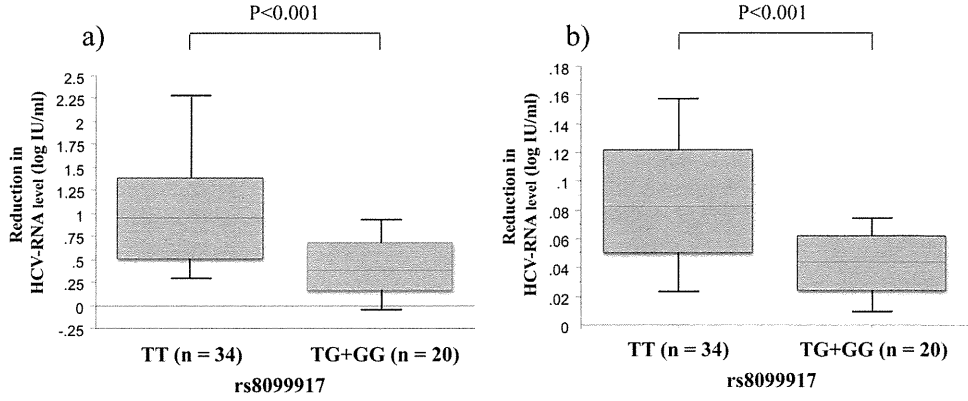

Figure 3 (A) The first-phase viral decline slope per day (Ph1/day) and (B) second-phase viral decline slope per week (Ph2/week) in hepatitis C virus (HCV) genotype 1-infected patients treated with pegylated interferon α plus ribavirin. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively.

−3.48 vs −1.56, p<0.001; −4.53 vs −2.37, p<0.01; −4.93 vs −2.86, p<0.001. Furthermore, the initial first-phase viral decline slope per day (Ph1/day) and subsequent second-phase viral decline slope per week (Ph2/week) in TT genotype were significantly higher than in genotype TG/GG (Ph1/day 0.94 ±0.83 vs 0.38±0.40 log IU/ml, p<0.001; Ph2/week 0.08±0.06 vs 0.04±0.03 log IU/ml, p<0.001) (figure 3).

Changes in serum HCV-RNA levels in chimeric mice treated by peg-IFN-α

In order to clarify the association between *IL28B* alleles of human hepatocytes and the response to peg-IFN-α, we prepared four lines of uPA/SCID mice and four different lots of human hepatocytes containing various rs8099917, rs8103142 and rs12979860 SNPs around the *IL28B* gene (table 2). The chimeric mice were inoculated with serum samples from each HCV-1b patient, and then HCV-RNA levels had increased and reached more than 10^6 copies/ml in all chimeric mice sera at 2 weeks after inoculation. After confirming the peak of HCV-RNA in all chimeric mice, they were subcutaneously administered with four times injections of the bolus dose of peg-IFN-α2a for 2 weeks (table 3). Figure 4 shows the change in the serum HCV-RNA levels for 14 days during IFN injection into chimeric mice transplanted with *IL28B* favourable or unfavourable human hepatocyte genotypes. On peg-IFN-α administration, no significant difference in the median reduction in HCV-RNA levels in the serum A-infected[22] chimeric mice sera was observed between favourable (n=7) and unfavourable

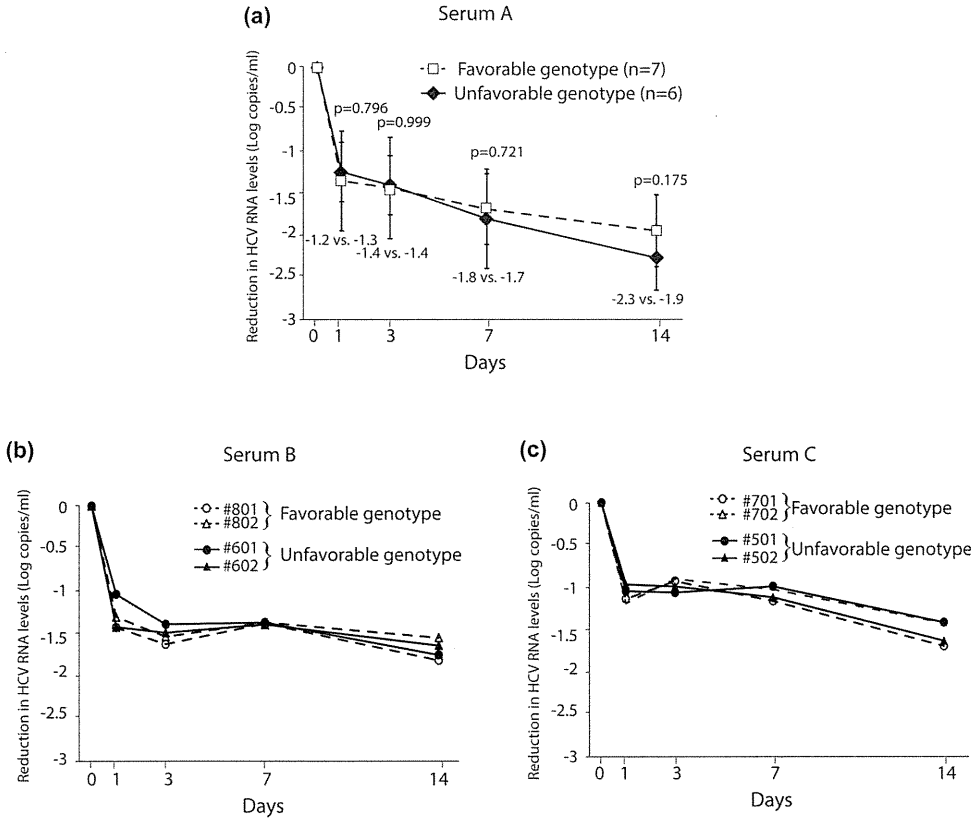

Figure 4 Median reduction of hepatitis C virus (HCV)-RNA levels (log copies/ml) after administering pegylated interferon α to chimeric mice having human hepatocytes containing various single nucleotide polymorphisms around the *IL28B* gene as favourable (rs8099917 TT) and unfavourable (rs8099917 TG) genotypes. Data are represented as mean+SD. Chimeric mice infected with a) serum A (n=7; favourable genotype, n=6; unfavourable genotype), (B) serum B (n=2, each genotype), and (C) serum C (n=2, each genotype). All serum samples were obtained from HCV-1b patients.

(n=6) *IL28B* genotypes on days 1, 3, 7 and 14 (−1.2 vs −1.3, −1.4 vs −1.4, −1.8 vs −1.7, and −2.3 vs −1.9 log copies/ml) (figure 4A). Moreover, we prepared two additional serum samples from the other HCV-1b patients (serum B and C)[21] to confirm the influence of *IL28B* genotype in early viral kinetics during IFN treatment. After establishing persistent infection with new HCV-1b strains in all chimeric mice, they were also administered four times injections of the bolus dose of peg-IFN-α2a for 2 weeks (figure 4B,C). In a similar fashion, no significant difference in HCV-RNA reduction in chimeric mice sera was observed between favourable and unfavourable *IL28B* genotypes.

Expression levels of ISG in chimeric mice livers

Because chimeric mice have the characteristic of severe combined immunodeficiency, the viral kinetics in chimeric mice sera during IFN treatment could be contributed by the innate immune response of HCV-infected human hepatocytes. Therefore, ISG expression levels in mice livers transplanted with human hepatocytes were compared between favourable and unfavourable *IL28B* genotypes (figure 5).

As shown in figure 5A, ISG expression levels in mice livers were measured at 8 h and 24 h after IFN treatment. The levels of representative antiviral ISG (eg, myxovirus resistance protein A, oligoadenylate synthetase 1, RNA-dependent protein kinase) and other ISG for promoting antiviral signalling (eg, Toll-like receptor 3, retinoic acid-inducible gene 1) were significantly induced at least 8 h after treatment, and prolonged at 24 h. No significant difference in ISG expression levels in HCV-infected livers was observed between favourable and unfavourable *IL28B* genotypes. The other inoculum for persistent infection of HCV-1b also demonstrated no significant difference in ISG

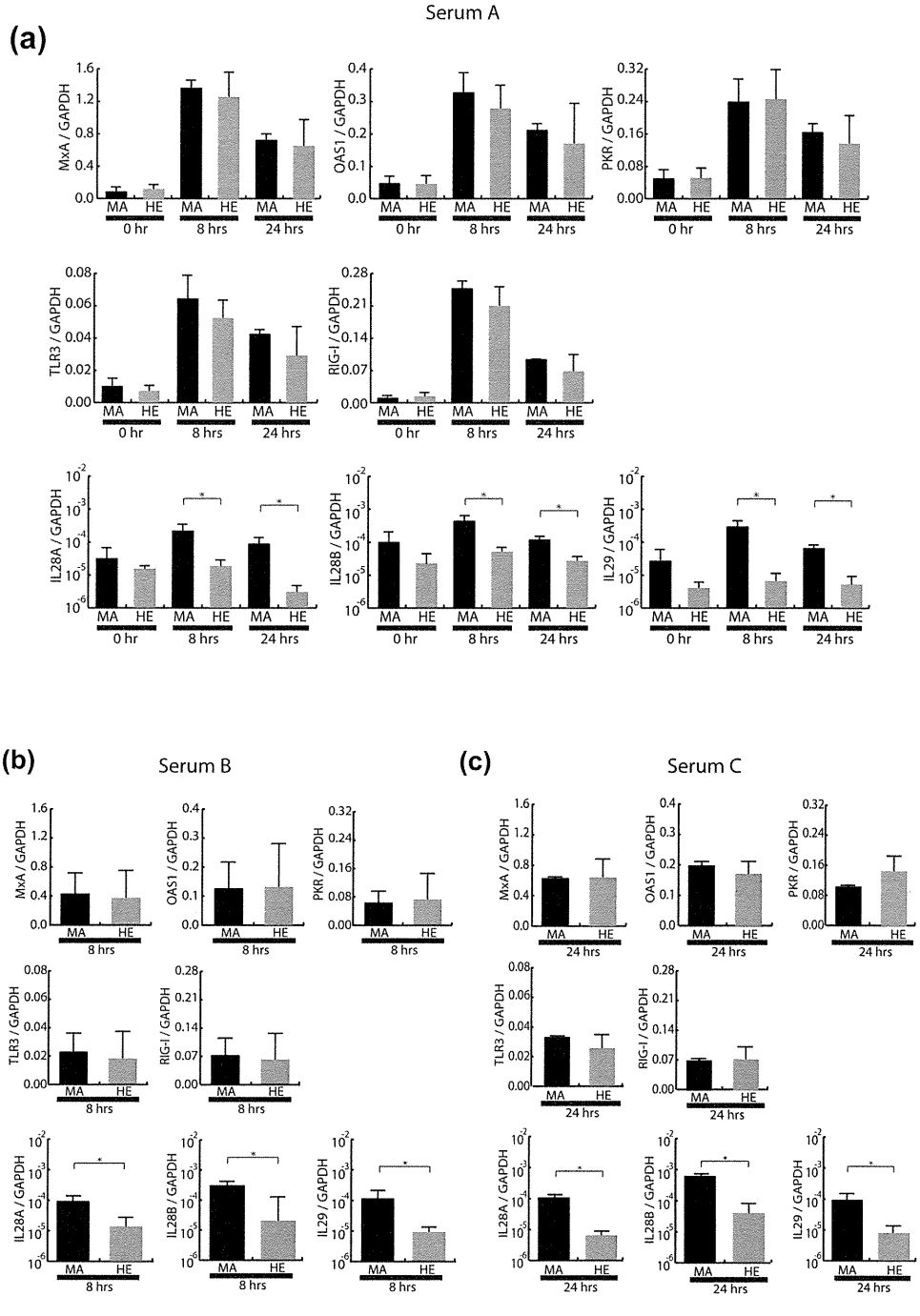

Figure 5 Intrahepatic interferon (IFN)-stimulated gene (ISG) expression levels in the pegylated interferon α (peg-IFN-α)-treated chimeric mice having human hepatocytes containing homozygous favourable allele (rs8099917 TT; MA) and heterozygous unfavourable allele (rs8099917 TG; HE) were measured and expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA. Data are represented as mean+SD. (A) Time kinetics of ISG after administration of the peg-IFN-α in serum A-infected chimeric mice (n=3, each genotype). Comparison of ISG expression levels at (B) 8 h in serum B-infected mice and (C) 24 h in serum C-infected mice after administering peg-IFN-α (n=3, each genotype). Predesigned real-time PCR assay of IL28B transcript purchased from Applied Biosystems can be cross-reactive to IL28A transcript. *p<0.05. MxA, myxovirus resistance protein A; OAS1, oligoadenylate synthetase 1; PKR, RNA-dependent protein kinase; RIG-1, retinoic acid-inducible gene 1; TLR3, Toll-like receptor 3.

expression levels between favourable and unfavourable *IL28B* genotypes (figure 5B,C). Interestingly, IFN-λ expression levels by treatment of peg-IFN-α were significantly induced in HCV-infected human hepatocytes harbouring the favourable *IL28B* genotype (figure 5 A–C).

DISCUSSION

Several recent studies have demonstrated a marked association between the chronic hepatitis C treatment response[6–9] and SNP (rs8099917, rs8103142 and rs12979860) near or within the region of the *IL28B* gene, which affected the viral dynamics during peg-IFN-α plus ribavirin therapy in Caucasian, African American and Hispanic individuals.[13]

It has been reported that when patients with chronic hepatitis C are treated by IFN-α or peg-IFN-α plus ribavirin, HCV-RNA generally declines after a 7–10 h delay.[25] The typical decline is biphasic and consists of a rapid first phase lasting for approximately 1–2 days during which HCV-RNA may fall 1–2 logs in patients infected with genotype 1, and subsequently a slower second phase of HCV-RNA decline.[26] The viral kinetics had a predictive value in evaluating antiviral efficacy.[14] In this study, biphasic decline of the HCV-RNA level during peg-IFN-α treatment was observed in both patients and chimeric mice infected with HCV genotype 1; however, in the first and second phases of viral kinetics, a difference between *IL28B* genotypes was observed only in HCV-infected patients; a more rapid decline in serum HCV-RNA levels after administering peg-IFN-α plus ribavirin was confirmed in patients with the TT genotype of rs8099917 compared to those with the TG/GG genotype.

On the other hand, in-vivo data using the chimeric mouse model showed no significant difference in the reduction of HCV-RNA titers in mouse serum among four different lots of human hepatocytes containing *IL28B* favourable (rs8099917 TT) or unfavourable (rs8099917 TG) genotypes, which was confirmed by the inoculation of two additional HCV strains. These results indicated that variants of the *IL28B* gene in donor hepatocytes had no influence on the response to peg-IFN-α under immunosuppressive conditions, suggesting that the immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN-α-based therapy.

Two recent studies indeed revealed an association between the *IL28B* genotype and the expression level of hepatic ISG in human studies.[27 28] Quiescent hepatic ISG before treatment among patients with the *IL28B* favourable genotype have been associated with sensitivity to exogenous IFN treatment and viral eradication; however, it is difficult to establish whether the hepatic ISG expression level contributes to viral clearance independently or appears as a direct consequence of the *IL28B* genotype. Another recent study addressed this question and the results suggested that there is no absolute correlation with the *IL28B* genotype and hepatic expression of ISG.[29] Our results on the hepatic ISG expression level in immunodeficient chimeric mice also suggested that no significant difference in ISG expression levels was observed between favourable and unfavourable *IL28B* genotypes. However, these results were not consistent with a previous report using chimeric mice that the favourable *IL28B* genotype was associated with an early reduction in HCV-RNA by ISG induction.[30] The reasons for the discrepancy might depend on the dose and type of IFN treatment, as well as the time point when ISG expression was examined in the liver. In addition, although IFN-λ transcript levels measured in peripheral blood mononuclear cells or liver revealed inconsistent results in the context of an association with the *IL28B* genotype,[7 8] our preliminary assay on the IL28A, IL28B and IL29 transcripts in the liver first indicated that the induction of IFN-λ on peg-IFN-α administration could be associated with the *IL28B* genotype. Therefore, the induction of IFN-λ followed by immune response might contribute to different viral kinetics and treatment outcomes in HCV-infected patients, because no difference was found in chimeric mice without immune response.

It has also been reported that the mechanism of the association of genetic variations in the *IL28B* gene and spontaneous clearance of HCV may be related to the host innate immune response.[11] Interestingly, participants with seroconversion illness with jaundice were more frequently rs8099917 homozygous favourable allele (TT) than other genotypes (32% vs 5%, p=0.047). This suggests that a stronger immune response during the acute phase of HCV infection among patients with the *IL28B* favourable genotype would induce more frequent spontaneous clearance of HCV.

Taking into account both the above results in acute HCV infection and our results conducted on chimeric mice that have the characteristic of immunodeficiency, it is suggested that the response to peg-IFN-α associated with the variation in *IL28B* alleles in chronic hepatitis C patients would be composed of the intact immune system.

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Contributors YT and MM conceived the study. TW and FS and YT conducted the study equally. TW and FS coordinated the analysis and manuscript preparation. All the authors had input into the study design, patient recruitment and management or mouse management and critical revision of the manuscript for intellectual content. TW, FS and YT contributed equally.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of each ethics committee at the Nagoya City University and Nagasaki Medical Center (see supplementary information, available online only).

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REFERENCES

1. **Ray Kim W.** Global epidemiology and burden of hepatitis C. *Microbes Infect* 2002;**4**:1219–25.
2. **Foster GR.** Past, present, and future hepatitis C treatments. *Semin Liver Dis* 2004;**24**(Suppl. 2):97–104.
3. **Fried MW,** Shiffman ML, Reddy KR, *et al*. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002;**347**:975–82.
4. **Manns MP,** McHutchison JG, Gordon SC, *et al*. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001;**358**:958–65.
5. **Mihm U,** Herrmann E, Sarrazin C, *et al*. Review article: predicting response in hepatitis C virus therapy. *Aliment Pharmacol Ther* 2006;**23**:1043–54.
6. **Ge D,** Fellay J, Thompson AJ, *et al*. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009;**461**:399–401.
7. **Suppiah V,** Moldovan M, Ahlenstiel G, *et al*. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 2009;**41**:1100–4.
8. **Tanaka Y,** Nishida N, Sugiyama M,, *et al* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009;**41**:1105–9.
9. **Rauch A,** Kutalik Z, Descombes P, *et al*. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. *Gastroenterology* 2010;**138**:1338–45.

10. **Tanaka Y,** Nishida N, Sugiyama M, *et al*. lambda-Interferons and the single nucleotide polymorphisms: a milestone to tailor-made therapy for chronic hepatitis C. *Hepatol Res* 2010;**40**:449–60.
11. **Thomas DL,** Thio CL, Martin MP, *et al*. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009;**461**:798–801.
12. **Grebely J,** Petoumenos K, Hellard M, *et al*. Potential role for interleukin-28B genotype in treatment decision-making in recent hepatitis C virus infection. *Hepatology* 2010;**52**:1216–24.
13. **Thompson AJ,** Muir AJ, Sulkowski MS, *et al*. Interleukin-28B polymorphism improves viral kinetics and is the strongest pretreatment predictor of sustained virologic response in genotype 1 hepatitis C virus. *Gastroenterology* 2010;**139**:120–29.
14. **Layden-Almer JE,** Layden TJ. Viral kinetics in hepatitis C virus: special patient populations. *Semin Liver Dis* 2003;**23**(Suppl. 1):29–33.
15. **Heckel JL,** Sandgren EP, Degen JL, *et al*. Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. *Cell* 1990;**62**:447–56.
16. **Rhim JA,** Sandgren EP, Degen JL, *et al*. Replacement of diseased mouse liver by hepatic cell transplantation. *Science* 1994;**263**:1149–52.
17. **Tateno C,** Yoshizane Y, Saito N, *et al*. Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* 2004;**165**:901–12.
18. **Mercer DF,** Schiller DE, Elliott JF, *et al*. Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* 2001;**7**:927–33.
19. **Tsuge M,** Hiraga N, Takaishi H, *et al*. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. *Hepatology* 2005;**42**:1046–54.
20. **Kurbanov F,** Tanaka Y, Chub E, *et al*. Molecular epidemiology and interferon susceptibility of the natural recombinant hepatitis C virus strain RF1_2k/1b. *J Infect Dis* 2008;**198**:1448–56.
21. **Kurbanov F,** Tanaka Y, Matsuura K, *et al*. Positive selection of core 70Q variant genotype 1b hepatitis C virus strains induced by pegylated interferon and ribavirin. *J Infect Dis* 2010;**201**:1663–71.
22. **Inoue K,** Umehara T, Ruegg UT, *et al*. Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice in vivo. *Hepatology* 2007;**45**:921–8.
23. **Livak KJ,** Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;**25**:402–8.
24. **Silver N,** Best S, Jiang J, *et al*. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Mol Biol* 2006;**7**:33.
25. **Dahari H,** Layden-Almer JE, Perelson AS, *et al*. Hepatitis C viral kinetics in special populations. *Curr Hepat Rep* 2008;**7**:97–105.
26. **Neumann AU,** Lam NP, Dahari H, *et al*. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science* 1998;**282**:103–7.
27. **Honda M,** Sakai A, Yamashita T, *et al*. Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. *Gastroenterology* 2010;**139**:499–509.
28. **Urban TJ,** Thompson AJ, Bradrick SS, *et al*. IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes in patients with chronic hepatitis C. *Hepatology* 2010;**52**:1888–96.
29. **Dill MT,** Duong FH, Vogt JE, *et al*. Interferon-induced gene expression is a stronger predictor of treatment response than IL28B genotype in patients with hepatitis C. *Gastroenterology* 2011;**140**:1021–31.
30. **Hiraga N,** Abe H, Imamura M, *et al*. Impact of viral amino acid substitutions and host interleukin-28b polymorphism on replication and susceptibility to interferon of hepatitis C virus. *Hepatology* 2011;**54**:764–71.

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Hepatitis C Virus Translation Preferentially Depends on Active RNA Replication

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Abstract

Hepatitis C virus (HCV) RNA initiates its replication on a detergent-resistant membrane structure derived from the endoplasmic reticulum (ER) in the HCV replicon cells. By performing a pulse-chase study of BrU-labeled HCV RNA, we found that the newly-synthesized HCV RNA traveled along the anterograde-membrane traffic and moved away from the ER. Presumably, the RNA moved to the site of translation or virion assembly in the later steps of viral life cycle. In this study, we further addressed how HCV RNA translation was regulated by HCV RNA trafficking. When the movement of HCV RNA from the site of RNA synthesis to the Golgi complex was blocked by nocodazole, an inhibitor of ER-Golgi transport, HCV protein translation was surprisingly enhanced, suggesting that the translation of viral proteins occurred near the site of RNA synthesis. We also found that the translation of HCV proteins was dependent on active RNA synthesis: inhibition of viral RNA synthesis by an NS5B inhibitor resulted in decreased HCV viral protein synthesis even when the total amount of intracellular HCV RNA remained unchanged. Furthermore, the translation activity of the replication-defective HCV replicons or viral RNA with an NS5B mutation was greatly reduced as compared to that of the corresponding wildtype RNA. By performing live cell labeling of newly synthesized HCV RNA and proteins, we further showed that the newly synthesized HCV proteins colocalized with the newly synthesized viral RNA, suggesting that HCV RNA replication and protein translation take place at or near the same site. Our findings together indicate that the translation of HCV RNA is coupled to RNA replication and that the both processes may occur at the same subcellular membrane compartments, which we term the replicasome.

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Introduction

Hepatitis C virus (HCV) is a positive-sense RNA virus that is estimated to chronically infect as many as 3% of the world's population. As a member of Flaviviridae, HCV is an enveloped virus with a single, positive-stranded RNA around 9.6 kb in length [1]. The viral genome encodes a large viral polyprotein, which is proteolytically processed by cellular signal peptidases and viral proteases into structural (C, E1, E2, and p7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins [2]. Membrane association of the viral proteins is essential for HCV replication, at both steps of RNA transcription and translation [3–5]. To decipher the mechanisms by which HCV navigates these steps may necessitate an understanding of the cell biological processes as diverse as cytoplasmic organelle structure and membrane biogenesis and trafficking in the secretory pathway.

Using the HCV subgenomic replicon system as well as infectious virus system, many host factors have been identified to be involved in HCV RNA replication, including the human homologue of the 33-kDa vesicle-associated membrane protein-associated protein (hVAP-33) [6], Golgi-specific brefeldin A resistant guanine nucleotide exchange factor 1(GBF1) [7], Endocytic Rab proteins [8], polypyrimidine-tract-binding protein (PTB) [9,10], La autoantigen [10], SYNCRIP [11], and host geranylgeranylated proteins and fatty acids [12]. These host proteins that are identified to be in the HCV RNA replication complexes are important in either membrane sorting and trafficking or RNA binding and processing. Some of these host factors, such as PTB and La autoantigen, have been found to regulate HCV translation as well_ENREF_13 by virtue of their binding to the 5′ or 3′ UTR of HCV RNA [13–15]. The identification of host proteins with dual-functions in regulating both translation and transcription implies the possibility of coupled transcription/translation of HCV RNA.

The balance between viral RNA transcription and translation is critical for the replication of positive-stranded RNA viruses, since the same RNA is used both for translation and as the template for negative-strand RNA synthesis. Transcription of poliovirus has been reported to be dependent on the translational activity of the viral RNA [16]. On the other hand, the translation of Sindbis virus and vesicular stomatitis virus has been reported to be transcriptionally dependent [17]. Such coupling of transcription-translation has been well documented to confer advantage in maintaining the stability of the RNA molecule in bacteria [18,19] and also to respond to regulatory signals coordinately.

In this study, we observed that HCV RNA exit from the site of RNA synthesis to the Golgi complex, a process that can be blocked by nocodazole, an inhibitor of the ER-Golgi transport pathway. Surprisingly, HCV protein translation was enhanced when HCV RNA movement was blocked, suggesting that the translation of