dent Student's *t*-test was used for continuous variables. Values of $P < 0.05$ were considered to be significant.

RESULTS

HBsAg seroprevalence in HIV-infected individuals

AMONG 118 HIV infected patients, 15 samples were HBsAg positive by all three assays. Three HBV DNA positive samples were HBsAg positive on the EIA and/or ELISA alone. Overall, 18 patients (15.3%) were considered to be positive for HBsAg.

Prevalence of HBV DNA

Hepatitis B virus DNA was detected in 50 out of 118 HIV-infected patients (42.4%). Of these, 32 patients (64% [27.1% of all patients]) were HBsAg negative, indicating occult HBV infection. Meanwhile, seven patients with occult HBV infection were serologically positive for the anti-HBc antibody,[23] 12 patients were negative for all serological markers, and three patients were positive for anti-HBc and anti-HBs (data not shown). HBV DNA was detected in all HBsAg positive subjects, and the mean HBV viral load was 6.1 ± 1.6 log copies/mL.

Characteristics of patients with HBV and HIV co-infection according to HBsAg status

The virological, serological and clinical characteristics of patients positive for HBsAg and patients with occult HBV infection and HIV co-infection are shown in Table 1. High HBV viral loads (≥5 log copies/mL, $P = 0.0002$) and high aspartate aminotransferase (AST) levels (≥40 IU/L, $P = 0.047$) were significantly more frequent in HBsAg positive carriers than those in occult HBV infected patients.

Serological status and clinical characteristics of HBV DNA negative patients

The serological status and clinical characteristics of HBV DNA negative patients are summarized in Table 2. AST levels were 40 IU/L or more in 15 out of 31 patients (48.4%) positive for anti-HBs and/or anti-HBc but negative for HBV DNA. The prevalence of AST of 40 IU/L or

Table 1 Characteristics of patients with HBV and HIV co-infection according to HBsAg status

Characteristic	HBsAg positive patients ($n = 18$)	Occult HBV-infected patients ($n = 32$)	All patients ($n = 50$)
Males, *n* (%)	17 (94.4)	28 (87.5)	45 (90.0)
Age, years (mean ± SD)	30.9 ± 7.7	33.2 ± 8.3	33.3 ± 8.9
CD4 count <200 cells/mm^3, *n* (%)	11 (61.1)	25 (78.1)	36 (72.0)
CD4 cell count, cells/mm^3 (mean ± SD)	160.9 ± 116.9	141.1 ± 151.0	148.3 ± 138.7
HBV viral load ≥5 log copies/mL, *n* (%)	12 (66.7)*	4 (12.5)*	16 (32.0)
AST ≥40 IU/L, *n* (%)	12 (66.7)**	12 (37.5)**	24 (48.0)
AST level (mean ± SD)	74.3 ± 102.4	38.44 ± 24.9	51.4 ± 65.8
ALT ≥40 IU/L, *n* (%)	12 (66.7)	14 (43.8)	26 (52.0)
ALT, IU/L (mean ± SD)	70.4 ± 71.9	41.7 ± 32.8	52.0 ± 51.7
HBV subgenotypes, *n*	13	29	42
HBV/B3, *n* (%)	11 (84.6)	28 (96.6)	39 (92.9)
HBV/C1, *n* (%)	2 (15.4)	1 (3.4)	3 (7.1)
Transmission route, *n* (%)			
IDU	13 (72.2)	22 (66.8)	35 (70.0)
Sexual contact	5 (27.8)	10 (31.3)	15 (30.0)
Anti-HBs antibody, *n* (%)	1 (5.6)	12 (37.5)	13 (26.0)
Mutation in the *a* determinant region, *n*	17	31	48
T123A, *n* (%)	0	2 (6.5)	2 (4.2)
Q129H, *n* (%)	2 (11.8)	0	2 (4.2)
M133L, *n* (%)	6 (35.3)	19 (61.3)	25 (52.1)

*$P = 0.0002$, **$P = 0.047$. ALT, alanine aminotransferase; anti-HBc, HBV core antibodies; anti-HBs, hepatitis B surface antigen antibodies; AST, aspartate aminotransferase; HBV, hepatitis B virus; IDU, injection drug use; SD, standard deviation.

Table 2 Serological and clinical characteristics of HBV DNA negative patients ($n = 68$)

	Anti-HBs positive patients	Anti-HBc positive patients	Anti-HBs and/or anti-HBc positive patients
Number (%)	18 (26.5)	17 (25.0)	31 (45.6)
Males, *n* (%)	14 (77.8)	15 (88.2)	26 (83.9)
Age, years (mean ± SD)	33.2 ± 6.8	36.3 ± 10.3	35.1 ± 8.7
CD4 cell count <200 cells/mm^3, *n* (%)	13 (72.2)	13 (76.5)	24 (77.4)
CD4 cell count, cells/mm^3 (mean ± SD)	129.1 ± 151.6	131.1 ± 154.8	115.3 ± 137.7
AST ≥40 IU/L, *n* (%)	5 (7.4)	8 (11.9)	15 (48.4)
AST, IU/L (mean ± SD)	42.7 ± 35.4	49.4 ± 27.3	47.4 ± 33.1
ALT ≥40 IU/L, *n* (%)	7 (10.3)	10 (14.7)	18 (26.5)
ALT, IU/L (mean ± SD)	48.9 ± 31.0	58.7 ± 38.3	53.6 ± 35.9

ALT, alanine aminotransferase; anti-HBc, HBV core antibodies; anti-HBs, hepatitis B surface antigen antibodies; AST, aspartate aminotransferase; HBV, hepatitis B virus; SD, standard deviation.

more was not significantly different between HBV DNA negative and HBV DNA positive patients. Previous HBV infection was recorded in 45.6% (31/68) of HBV DNA negative patients.

CD4 cell count

The mean CD4 cell count was 148.7 ± 149.9 cells/mm^3 in all 118 HIV-infected patients, 161.0 ± 116.9 in HBsAg positive patients and 141.1 ± 151.0 in patients with occult HBV infection and HIV co-infection. The CD4 cell count was less than 200 cells/mm^3 in 83 out of 118 HIV-infected patients (70.3%). Among HBV DNA positive patients, 36 out of 50 (72.0%) had a CD4 cell count of less than 200 cells/mm^3. The prevalence of ALT of 40 IU/L or more was much higher in patients with a CD4 cell count of less than 200 cells/mm^3. Among HBsAg positive patients, the mean AST and ALT levels tended to be higher in those with a CD4 cell count of less than 200 cells/mm^3 compared with those with a cell count of 200 cells/mm^3 or more, although this was not statistically significant (see Table S1 in the supplemental material).

Identification of HBV genotypes

Consensus nucleotide sequences were generated and aligned with reference sequences. Based on the pre-S2-S region (nt. 84–465), HBV subgenotypes B3 (HBV/B3) ($n = 7$) and HBV/C1 ($n = 2$) were identified in HBsAg positive patients, while HBV/B3 ($n = 3$) and HBV/C1 ($n = 1$) were identified in patients with occult HBV infection (Fig. 1). In the S region (nt. 406–646), HBV/B3 ($n = 4$) was identified in HBsAg positive patients and HBV/B3 ($n = 25$) was identified in patients with occult HBV infection. Similar to Indonesian patients with HBV infection alone, HBV/B3 was the most prevalent subgenotype in patients with HIV co-infection regardless of HBsAg positivity.

Mutational analysis in relation to HBsAg status and drug resistance

The amino acid substitutions in the *a* determinant region (amino acids 124–147) are shown in Figure 2. A single amino acid substitution (T123A) was found in two patients with occult HBV infection. G145R and K12R, common escape mutations, were not detected in this study. One hundred and nine subjects (92.4%) were on HAART (lamivudine, zidovudine, and nevirapine or efavirenz) to control HIV. Amino acid mutations in the polymerase region were detected in two HBsAg positive patients; one was rtM204I and the other was rtL180M plus rtM204I. The patient with rtM204I had high ALT levels (80 IU/L), while HBV viral loads were relatively high in both of these patients with mutations in the polymerase region.

DISCUSSION

INDONESIA IS A country with a moderate to high prevalence of HBV. It was reported that co-infection with HBV and HIV was prevalent in countries with highly endemic HBV infection because of shared routes of transmission.[24] In this study, 15% of the HIV-infected patients were HBsAg positive, which is extremely high compared with the prevalence of HBsAg carriers in the general population in Indonesia. It is also noteworthy that 42.2% of the HIV-infected patients were considered to have HBV and HIV co-infection, if we included

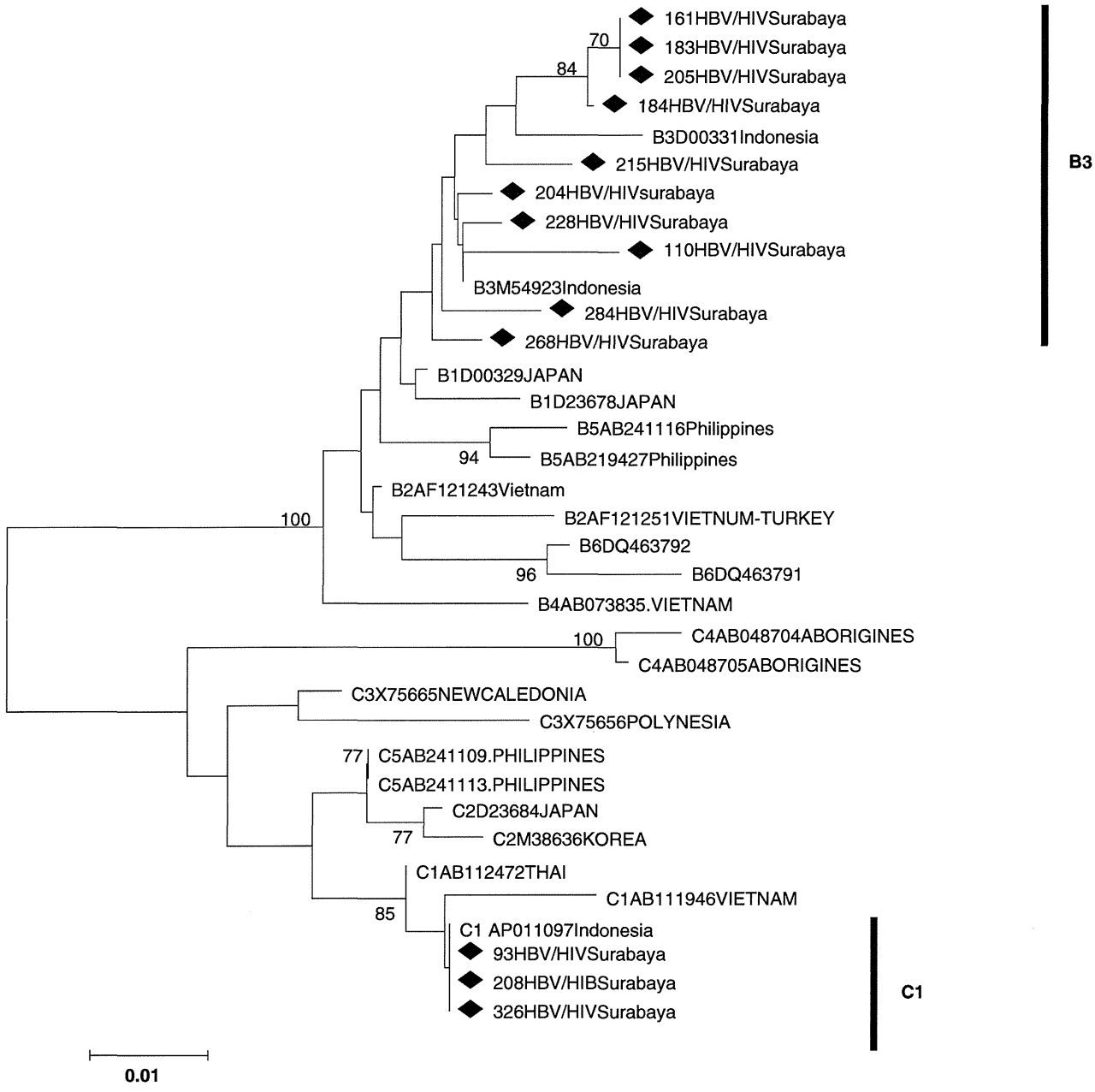

Figure 1 Phylogenetic tree constructed with the neighbor-joining method based on the partial nucleotide sequence of the S region of 22 hepatitis B virus (HBV) reference strains. Reference isolates are indicated with their accession numbers and the country of origin is reported for each HBV/B and HBV/C strain. The number inside the tree indicates the bootstrap reliability.

occult HBV infection. Moreover, 68.6% of the HIV-infected patients in this study had current or previous HBV infection.

The clinical significance of occult HBV infection in HIV-infected patients is still controversial.[25,26] Therefore, we evaluated whether occult HBV infection contributes to liver damage in HIV-infected patients. It was previously reported that mutations in a determinant region, such as G145R, may induce HBsAg immune escape.[27] In this study, only two patients had an amino acid substitution (T123A) in this region, which suggests that the escape mutants including G145R and K122R are rarely

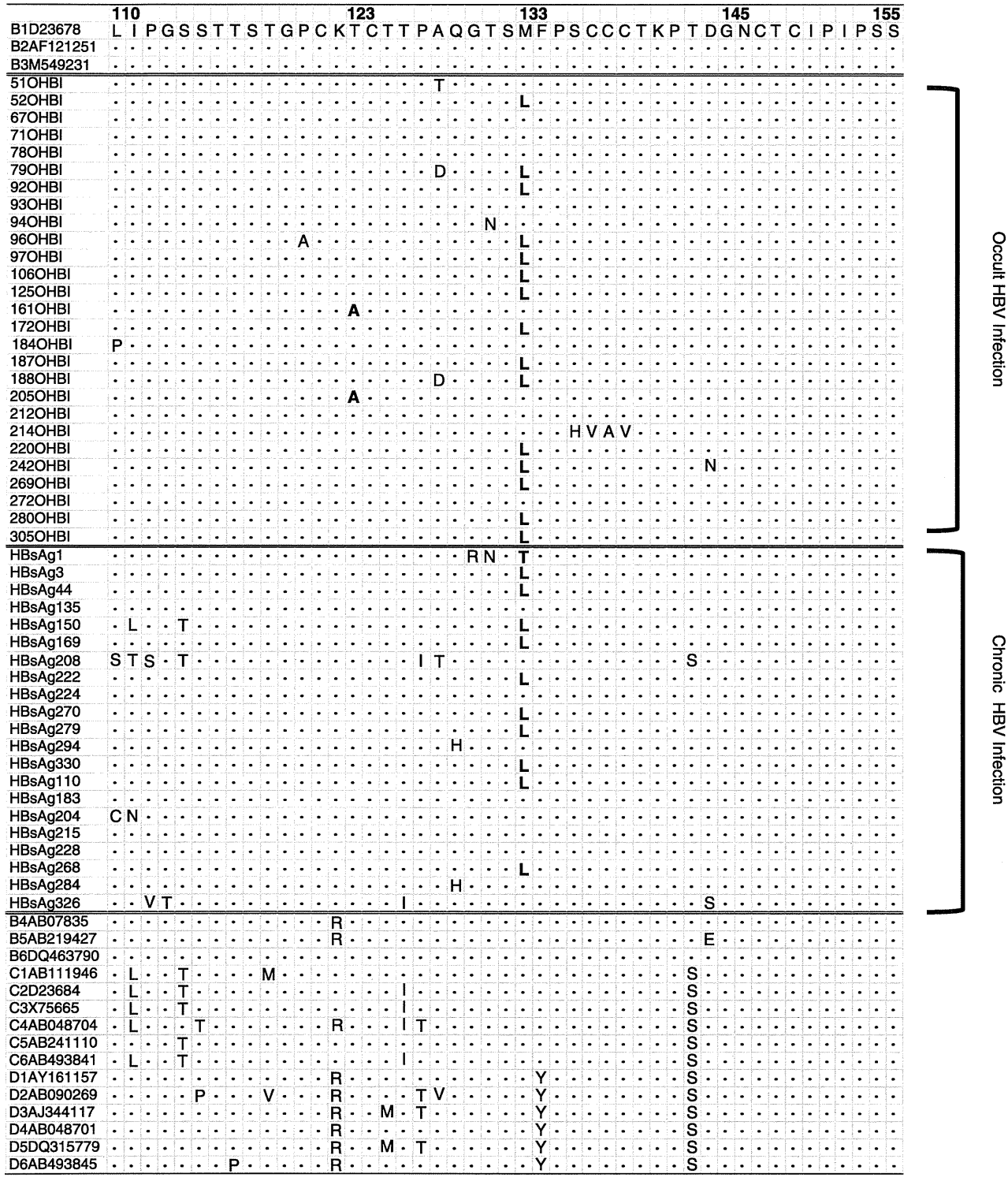

Figure 2 Surface antigen *a* determinant amino acid sequence alignment for hepatitis B virus (HBV). The first-line sequence is the consensus sequence corresponding to the HBV subgenotype (B1; accession no. D23678) reference strain retrieved from the Japan/GenBank database. Dots indicate positions with amino acids identical to the HBV/B1 consensus sequence.

detected in Indonesian HBV patients and it is probably because of the presence of different HBV subgenotypes in Indonesian patients compared with those in other countries. We also found that the AST level and HBV viral load were significantly higher in HBsAg positive patients compared with occult HBV-infected patients, suggesting that hepatic damage is partly dependent on HBV viral factors.

In general, the hepatic damage associated with HBV infection is dependent on viral factors and the host's immune response to HBV-infected hepatocytes. In this study, the AST and the ALT level in HBsAg positive patients tended to be higher in those with a CD4 cell count of less than 200 cells/mm^3 than in patients with a CD4 cell count of 200 cells/mm^3 or more. This also suggests that hepatic damage is partly derived from host immunity in the severe immunosuppressive state.[28] In addition, the prevalence of occult HBV infection detected in this study was significantly higher than that previously found in Indonesian blood donors (8.1%).[29] This observation may be due to an advanced stage of HIV disease (70.3% with a CD4 cell count <200 cells/mm^3 in this study).[30]

In this study, the mode of transmission was mainly IDU, unlike in the USA and Europe where HIV is mainly transmitted by sexual contact followed by IDU.[31] HBV genotyping revealed that several strains were of subgenotype C1, which originated in southeastern Asian countries, including the Philippines, Vietnam and Thailand. These results suggest that HIV also spread through the same mode of transmission. Indonesia is facing one of the most rapidly growing HIV epidemics in Asia, except for Papua, and this increase is mainly driven by IDU.[17] The prevalence of HIV in the general population is still quite low (0.2%), but its prevalence among IDU exceeds 50%,[32] increasing the risk of HIV and HBV co-infection. A significant increase in IDU among young adults was recently reported in Indonesia, and IDU was identified as the main cause of the rapid spread of HIV/AIDS in southeastern Asia.[33]

Highly active antiretroviral therapy is used to treat patients worldwide, and HIV is now well controlled in many countries. Consequently, viral hepatitis caused by HBV and liver toxicity caused by HAART are issues for patients with HBV and HIV co-infection undergoing HAART therapy.[34] Lamivudine frequently induces multidrug resistance in the RT domain of the polymerase region. M204I and L180M are common amino acid mutations that cause multidrug resistance. We found two HBsAg positive patients with high HBV viral loads and lamivudine resistance, even though lamivudine was used for the treatment of HIV. The combination of tenofovir plus emtricitabine or lamivudine should therefore be preferred in this setting.[35] Clearly, studies are needed to examine HBV DNA status and to establish individualized treatment plans for patients with multidrug resistance.

In conclusion, HBV co-infection, including occult HBV infection, was common in Indonesian HIV positive patients. Additional studies are needed to understand how prolonged HIV infection accelerates the course of chronic HBV. Moreover, clinicians and health workers should consider the national guidelines for prophylaxis, screening and treatment.

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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article:

Table S1 HBV viral load, AST, and ALT in HBsAg-positive patients according to CD4 cell counts.

Polymorphisms of the Core, NS3, and NS5a Proteins of Hepatitis C Virus Genotype 1b Associate with Development of Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is one of the common sequels of hepatitis C virus (HCV) infection. It remains controversial, however, whether HCV itself plays a direct role in the development of HCC. Although HCV core, NS3, and NS5A proteins were reported to display tumorigenic activities in cell culture and experimental animal systems, their clinical impact on HCC development in humans is still unclear. In this study we investigated sequence polymorphisms in the core protein, NS3, and NS5A of HCV genotype 1b (HCV-1b) in 49 patients who later developed HCC during a follow-up of an average of 6.5 years and in 100 patients who did not develop HCC after a 15-year follow-up. Sequence analysis revealed that Gln at position 70 of the core protein (core-Gln^{70}), Tyr at position 1082 plus Gln at 1112 of NS3 (NS3-Tyr^{1082}/Gln^{1112}), and six or more mutations in the interferon/ribavirin resistance-determining region of NS5A (NS5A-IRRDR≥6) were significantly associated with development of HCC. Multivariate analysis identified core-Gln^{70}, NS3-Tyr^{1082}/Gln^{1112}, and α-fetoprotein (AFP) levels (>20 ng/L) as independent factors associated with HCC. Kaplan-Meier analysis revealed a higher cumulative incidence of HCC for patients infected with HCV isolates with core-Gln^{70}, NS3-Tyr^{1082}/Gln^{1112} or both than for those with non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}). In most cases, neither the residues at position 70 of the core protein nor positions 1082 and 1112 of the NS3 protein changed during the observation period. *Conclusion*: The present results suggest that HCV isolates with core-Gln^{70} and/or NS3-Tyr^{1082}/Gln^{1112} are more closely associated with HCC development compared to those with non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}). (HEPATOLOGY 2012;00:000–000)

Hepatitis C virus (HCV) is a major etiologic agent of chronic hepatitis worldwide, with the estimated number of infected individuals being more than 180 million. Approximately 15% to 20% of chronically infected individuals undergo liver cirrhosis in a decade or so after infection, with hepatocellular carcinoma (HCC) arising from cirrhosis at an estimated rate of 1% to 4% per year.[1-3] Several host factors such as male gender, older age, elevated α-fetoprotein (AFP) level, advanced liver fibrosis as well as nonresponsiveness to interferon (IFN) therapy have been reported as important predictors of HCC development.[4,5] Recently, a host genetic factor, i.e., the *DEPDC5* locus polymorphism, was reported to be associated with progression to HCC in HCV-infected individuals.[6] On the other hand, it remains controversial as to whether HCV itself plays a direct role in the development of HCC. Experimental data suggest that HCV contributes to HCC by modulating pathways that promote malignant transformation of hepatocytes. HCV core, NS3, and NS5A proteins were shown to be involved in a number of potentially oncogenic pathways in cell culture and experimental animal systems.[7] HCV core protein rendered cultured cells more resistant to apoptosis[8,9] and promoted *ras* oncogene-mediated transformation.[10,11]

Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; IRRDR, interferon/ribavirin resistance-determining region; ISDR, interferon sensitivity-determining region.

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Moreover, transgenic mice expressing the HCV core protein in the liver developed HCC.[12] However, the clinical impact of HCV proteins on HCC development in humans and whether all HCV isolates are equally associated with HCC is yet to be determined. In a clinical setting, HCV core protein mutations at positions 70 (Gln^{70}) and/or 91 (Met^{91}) were closely associated with HCC development.[13-16] Gln^{70} and/or Met^{91} were also linked to resistance to PEG-IFN/ribavirin (RBV) treatment.[17-20] In addition, we and other investigators reported that an N-terminal part of the NS3 protein has the capacity to transform NIH3T3 and rat fibroblast cells[21,22] and to render NIH3T3 cells more resistant to DNA damage-induced apoptosis, which is thought to be a prerequisite for malignant transformation of the cell.[23] Also, the NS5A protein is a pleiotropic protein with key roles in both viral RNA replication and modulation of the host cell functions.[24] In particular, the links between NS5A and the IFN responses have been widely discussed. It was proposed initially that sequence variations within a region in NS5A spanning from amino acids (aa) 2209 to 2248, called the IFN sensitivity-determining region (ISDR), were correlated with IFN responsiveness.[25] Subsequently, in the era of PEG-IFN/RBV combination therapy, we identified a new region near the C-terminus of NS5A spanning from aa 2334 to 2379, which we referred to as the IFN/RBV resistance-determining region (IRRDR).[26,27] The degree of sequence variations within the IRRDR was significantly associated with the clinical outcome of PEG-IFN/RBV therapy. In the context of HCC, several retrospective studies suggested that IFN-based therapy might reduce the risk of HCC development.[4,28-30]

In an attempt to clarify whether viral factors, in particular those within the core, NS3, and NS5A proteins, are involved in HCC development, we carried out a comparative analysis of the aa sequences obtained from HCV patients who developed HCC and those who did not. In addition, we studied the sequence evolution of these genes in the interval between chronic hepatitis C and HCC development over a period of 15 years.

Patients and Methods

Ethics Statement. The study protocol, which conforms to the provisions of the 1975 Declaration of Helsinki, was approved beforehand by the Ethic Committees in Akashi City Hospital and Kobe University Graduate School of Medicine, and written informed consent was obtained from each patient enrolled in this study.

Patients. A total of 49 HCV-infected patients who developed HCC (HCC group) were retrospectively examined. They were followed up (from 1988 to 2003) with an average period until HCC development being 6.5 ± 2.9 years. Paired serum samples at the time of chronic hepatitis C (pre-HCC sample) and HCC development (post-HCC sample) were collected. As a control group, 100 HCV-infected patients who were followed up over a period of 15 years (from 1988 to 2003) without HCC development were retrospectively examined. Serum samples of the control group were available at the time of first visit to the clinic. All patients enrolled in this study were chronically infected with HCV genotype 1b (HCV-1b). HCV subtype was determined as reported previously.[31] Serum HCV RNA titers were quantitated by reverse-transcription polymerase chain reaction (RT-PCR0 with an internal RNA standard derived from the 5′ noncoding region of HCV (Amplicor HCV Monitor test, v. 2.0, Roche Diagnostics, Tokyo, Japan). All patients underwent liver biopsy and were diagnosed as chronic hepatitis. All HCC and 68% (68/100) of non-HCC patients received IFN-monotherapy, either natural IFN alpha (Sumiferon, Dainipponsumitomo Pharmaceutical, Osaka, Japan) at a dose of 6 million units (MU) or recombinant IFN alpha 2b (Intron A; Schering-Plough, Osaka, Japan) at a dose of 10 MU, 3 times a week for 6 months. All HCC patients were nonresponders (NR), who had detectable viremia during the entire course of IFN treatment. On the other hand, 18 (26%) of the 68 non-HCC patients treated with IFN achieved HCV RNA negativity at the end of treatment followed by rebound viremia within 6 months after the treatment and, therefore, they were referred to as relapsers. The other 50 IFN-treated, non-HCC patients were NR. The remaining 32 non-HCC patients did not receive IFN. All patients were seen every 2 months and tested for liver function markers during the follow-up period.

Sequence Analysis of HCV Core, NS3, and NS5A Proteins. HCV RNA was extracted from 140 μL of serum using a commercially available kit (QIAmp viral

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RNA kit; Qiagen, Tokyo, Japan). The core, NS3, and NS5A regions of the HCV genome were amplified as described elsewhere.[26,32-34] The sequences of the amplified fragments were determined by direct sequencing. The aa sequences were deduced and aligned using GENETYX Win software version 7.0 (GENETYX, Tokyo, Japan). The numbering of aa was according to the polyprotein of the prototype of HCV-1b; HCV-J.[35]

Statistical Analysis. Statistical differences in the baseline parameters of HCC and control groups were determined by Student's t test for numerical variables and Fisher's exact probability or chi-square tests for categorical variables. Likewise, statistical differences in viral mutations between HCC and control groups were determined by Fisher's exact probability test. Kaplan-Meier analysis was performed to estimate the cumulative incidence of HCC. The data obtained were evaluated by the log-rank test. Univariate and multivariate logistic analyses were performed to identify variables that independently associated with HCC development. Variables with $P < 0.1$ in univariate analysis were included in a backward stepwise multivariate logistic regression analysis. The odds ratios and 95% confidence intervals (95% CI) were calculated. All statistical analyses were performed using SPSS v. 16 software (Chicago, IL). Unless otherwise stated, $P < 0.05$ was considered statistically significant.

Nucleotide Sequence Accession Numbers. The sequence data reported in this article have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB719460 through AB719842.

Results

Demographic Characteristics of HCC and Control Groups. The clinical characteristics of HCC and control groups are shown in Table 1. The HCC group had significantly higher titers of ALT, AST, and AFP, and higher fibrosis staging score than that of the control group. There was no significant difference in viremia titers between the two groups.

Table 1. Demographic Characteristics of HCC and Control Groups

Factor	HCC	Control	*P*-value
Age	57.3 ± 7.0*	56.4 ± 8.3	0.54
Sex (male/female)	31/18	54/46	0.29
ALT (IU/L)	159.4 ± 79.8	129.7 ± 51.5	0.007
AST (IU/L)	113.0 ± 62.2	91.6 ± 44.1	0.017
AFP (ng/L)	29.1 ± 33.7	18.4 ± 4.4	0.002
Platelets (x 10^4/mm^3)	16.2 ± 2.8	16.2 ± 2.4	0.88
Inflammation grading score	8.7 ± 0.9	8.4 ± 1.2	0.05
Fibrosis staging score	2.4 ± 0.5	2.2 ± 0.5	0.02
HCV-RNA (KIU/mL)	593.4 ± 112.3	618.1 ± 95.9	0.17

*Mean ± SD. HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; AST, aspartate transaminase; AFP; α-fetoprotein.

Correlation Between Core Protein Sequence Polymorphism and HCC Development. HCV core protein sequences were obtained from all (49/49) and 94% (94/100) of pre-HCC and control patients' sera, respectively. Comparative sequence analysis revealed that 22 (45%) of 49 HCV isolates in the pre-HCC sera (pre-HCC isolates) and 59 (63%) of 94 HCV isolates from the control group (control isolates) had wild-core (Arg^{70}/Leu^{91}) (Table 2). The difference between HCC and control groups was hovering at a statistically significant level ($P = 0.05$). When the sequence pattern at position 70 alone was examined, a stronger association with HCC was observed. We found that 21 (43%) of 49 pre-HCC isolates had Gln^{70} while only 13 (14%) of 94 control isolates did ($P = 0.0002$). On the other hand, there was no significant correlation between sequence pattern at position 91 and HCC. Thus, a single mutation at position 70 (Gln^{70}) was the only polymorphic factor within core protein that was significantly associated with HCC development. It should be noted that there was no significant correlation between Gln^{70} and the degree of fibrosis progression (data not shown).

Correlation Between NS3 Protein Sequence Polymorphism and HCC Development. Sequences of NS3 serine protease domain (aa 1027 to 1146) were obtained from 94% (46/49) and 93% (93/100) of pre-HCC and control isolates, respectively. We found that 29 (63%) of 46 pre-HCC isolates had Tyr and Gln at positions 1082 and 1112, respectively (Tyr^{1082}/Gln^{1112}), while 39 (42%) of 93 control isolates did (Table 2). The difference in the proportion between pre-HCC and control isolates was statistically significant ($P = 0.029$). On the other hand, there was no significant correlation between Tyr^{1082}/Gln^{1112} and the degree of fibrosis progression (data not shown).

Correlation Between NS5A Protein Sequence Polymorphism and HCC Development. NS5A protein sequences were obtained from 92% (45/49) and 74% (74/100) of pre-HCC and control isolates, respectively. Twenty-four (53%) of 45 pre-HCC isolates had IRRDR of 6 or more mutations (IRRDR≥6) while only 15 (20%) of 74 control isolates did (Table 2; $P = 0.0003$). We also found that pre-HCC isolates tended to have a higher degree of sequence heterogeneity in ISDR than control isolates, although not statistically significant due probably to the small number of

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Table 2. Correlation Between HCC and Sequence Polymorphic Factors of Core, NS3 and NS5A

HCV Protein	Factor	No. of Subjects / No. of Total* HCC	Control	*P*-value
Core	Wild-core (Arg^{70}/Leu^{91})	22/49 (45%)	59/94 (63%)	0.05
	Non-wild-core	27/49 (55%)	35/94 (37%)	
	Gln^{70}	21/49 (43%)	13/94 (14%)	0.0002
	Non-Gln^{70}	28/49 (57%)	81/94 (86%)	
	Leu^{91}	37/49 (76%)	70/94 (74%)	1.0
	Non- Leu^{91}	12/49 (24%)	24/94 (26%)	
NS3	Tyr^{1082} / Gln^{1112}	29/46 (63%)	39/93 (42%)	0.029
	Non-(Tyr^{1082} / Gln^{1212})	17/46 (37%)	54/93 (58%)	
NS5A	IRRDR≥6	24/45 (53%)	15/74 (20%)	0.0003
	IRRDR≤5	21/45 (47%)	59/74 (80%)	
	ISDR≥3	11/45 (24%)	8/74 (11%)	0.07
	ISDR≤2	34/45 (76%)	66/74 (89%)	
	Asn^{2218}	11/45 (24%)	3/74 (4%)	0.002
	Non-Asn^{2218}	34/45 (76%)	71/74 (96%)	

*Number of subjects with a given factor / total number of HCC or control. HCC, hepatocellular carcinoma; Arg^{70}, arginine at position 70 of the core protein; Leu^{91}, leucine at position 91 of the core protein; Gln^{70}, glutamine at position 70 of the core protein; Tyr^{1082}, tyrosine at position 1082 of NS3; Gln^{1212}, glutamine at position 1212 of NS3; IRRDR, interferon/ribavirin resistance-determining region; ISDR, interferon sensitivity-determining region; Asn^{2218}, asparagine at position 2218 of NS5A-ISDR.

cases examined; 11 (24%) of 45 pre-HCC isolates and 8 (11%) of 74 of control isolates had ISDR with three or more mutations ($P = 0.07$). Moreover, Asn at position 2218 (Asn^{2218}) within the ISDR was found in 24% (11/45) of pre-HCC isolates and only in 4% (3/74) of the control isolates ($P = 0.002$), suggesting that Asn^{2218} is significantly associated with development of HCC.

Cumulative HCC Incidence on the Basis of Core-Gln^{70}, NS3-Tyr^{1082}/Gln^{1112}, NS5A-IRRDR≥6, and NS5A-Asn^{2218}. Follow-up study revealed that the cumulative HCC incidence in patients infected with HCV-1b isolates with core protein of Gln^{70} and those of non-Gln^{70}, respectively, was 29% and 5% at the end of 5 years, 56% and 23% at the end of 10 years, and 63% and 26% at the end of 15 years (Fig. 1A), with the differences between the two groups being statistically significant ($P < 0.0001$; Log-rank test). Likewise, the cumulative HCC incidence in patients infected with HCV-1b isolates with NS3 of Tyr^{1082}/Gln^{1112} and those of non-(Tyr^{1082}/Gln^{1112}), respectively, was 15% and 7% at the end of 5 years, 37% and 24% at the end of 10 years, and 45% and 24% at the end of 15 years ($P = 0.02$) (Fig. 1B). Also, the cumulative HCC incidence in patients infected with HCV-1b isolates of IRRDR≥6 and those of IRRDR≤5, respectively, was 18% and 10% at the end of 5 years, 59% and 22% at the end of 10 years, and 63% and 27% at the end of 15 years ($P = 0.0002$) (Fig. 1C). Similarly, the cumulative HCC incidence in patients infected with HCV-1b isolates of Asn^{2218} and those of non-Asn^{2218}, respectively, was 31% and 9% at the end of 5 years, 77% and 28% at the end of 10 years, and 77% and 33% at the end of 15 years ($P = 0.0003$) (Fig. 1D).

Identification of Independent Factors Correlated with HCC Development by Univariate and Multivariate Logistic Regression Analyses. In order to identify significant independent factors associated with HCC development, all available data of baseline patients' parameters and core, NS3, and NS5A polymorphic factors were first analyzed by univariate logistic analysis. This analysis yielded eight factors that were significantly associated with HCC development: core-Gln^{70}, NS3-(Tyr^{1082}/Gln^{1112}), NS5A-IRRDR≥6, NS5A-Asn^{2218}, increased levels of ALT (>165 IU/L), AST (>65 IU/L), and AFP (>20 ng/L), and fibrosis staging score (≥3). Subsequently, those eight factors were entered in multivariate logistic regression analysis. This analysis identified two viral factors, core-Gln^{70} and NS3-(Tyr^{1082}/Gln^{1112}), and a host factor, AFP levels (>20 ng/L), as independent factors associated with HCC development (Table 3). T3

The vast majority of pre-HCC isolates (85%; 39/46) had core-Gln^{70} and/or NS3-Tyr^{1082}/Gln^{1112} and only 15% (7/46) had non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}). By contrast, about a half of control isolates (52%; 46/89) had non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}) (Fig. 2A). The difference in the proportion between HCC and control groups was statistically significant ($P < 0.0001$). Furthermore, the cumulative HCC incidence after 15-year follow-up was highest (63%) among patients with core-Gln^{70} plus NS3-(Tyr^{1082}/Gln^{1112}), whereas it was lowest (11%) among patients with non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}) (Fig. 2B), with the difference being statistically significant ($P < 0.0001$; Log-rank test). F2

Evolution of the Sequences of the Core, NS3, and NS5A Proteins During the Follow-up Period from Chronic Hepatitis to HCC Development. Finally, we investigated sequence evolution of the core protein, NS3 and NS5A (IRRDR and ISDR) during the follow-up period from chronic hepatitis to HCC development by comparing the sequences between pre- and post-HCC isolates. The residue at position 70 of the core protein was conserved in 91% (41/45) of sequence pairs analyzed. The substitutions observed at this position were from Arg^{70} and His^{70} each to Gln^{70} in two cases and from Gln^{70} to Arg^{70} in the other two cases. The residues at positions 1082 and 1112 of

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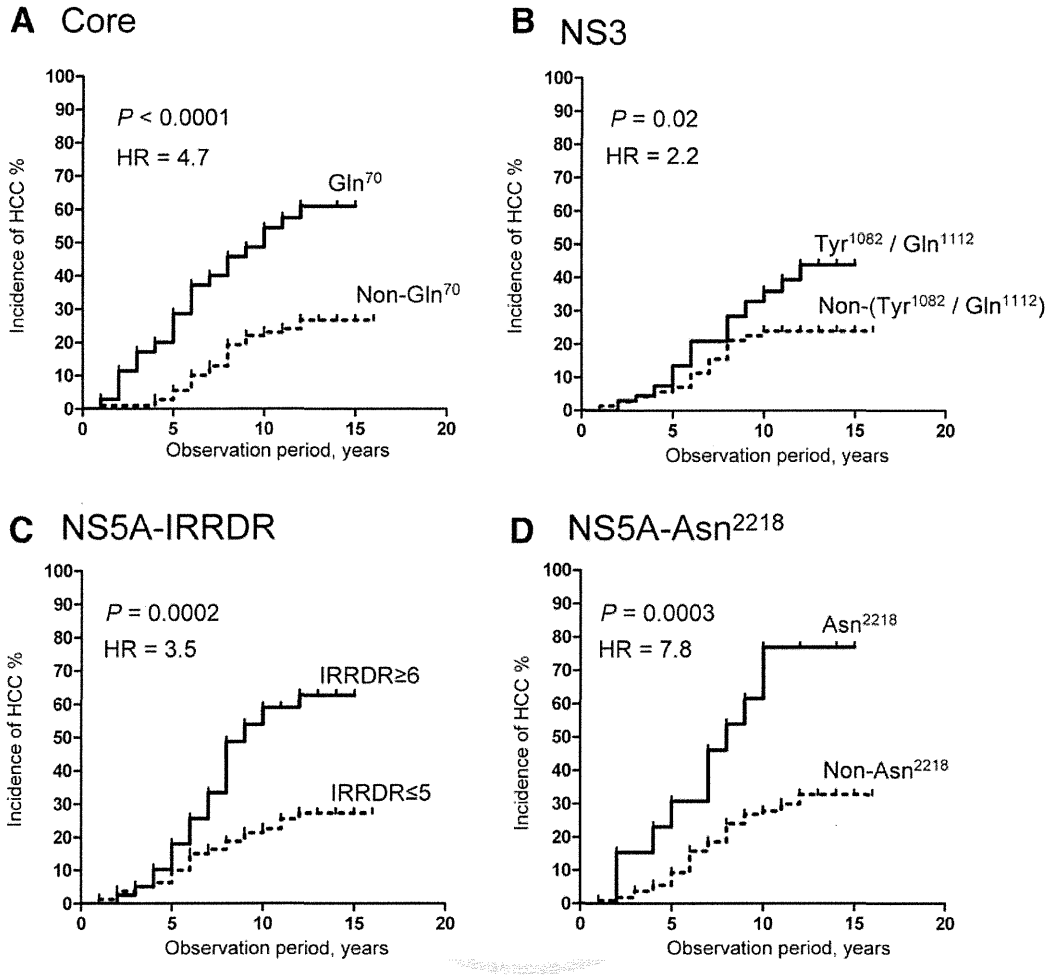

Fig. 1. Cumulative HCC incidence on the basis of HCV-1b sequence patterns. (A) Position 70 of the core protein. The numbers of core-Gln^{70} and non-Gln^{70} analyzed were 34 and 109, respectively. (B) Positions 1082 and 1112 of NS3. The numbers of NS3-(Tyr^{1082}/Gln^{1112}) and non-(Tyr^{1082}/Gln^{1112}) analyzed were 68 and 71, respectively. (C) NS5A-IRRDR. The numbers of NS5A-IRRDR≥6 and IRRDR≤5 analyzed were 39 and 80, respectively. (D) NS5A-Asn^{2218}. The numbers of NS5A-Asn^{2218} and non-Asn^{2218} analyzed were 14 and 105, respectively.

NS3 were conserved in 95% (41/43) and 100% (43/43), respectively, of the sequence pairs analyzed.

IRRDR and ISDR showed a high degree of sequence evolution. IRRDR sequences were different between pre- and post-HCC isolates in 66% (25/38) of cases analyzed (Fig. 3). IRRDR sequences tended to be more polymorphic at the time of HCC occurrence. Frequency of HCV isolates with IRRDR≥6 was

Table 3. Univariate and Multivariate Regression Analyses to Identify Independent Factors Associated with HCC

	Univariate		Multivariate	
Variable	Odds Ratio (95% CI)	*P*-value	Odds Ratio (95% CI)	*P*-value
Core-Gln^{70}	0.23 (0.10 - 0.52)	0.0004	6.8 (2.1 - 23.0)	0.001
NS3-Tyr^{1082} / Gln^{1212}	2.4 (1.1 - 4.9)	0.029	3.4 (1.1 - 10.0)	0.03
NS5A-IRRDR≥6	4.5 (2.0 - 10.0)	0.0003		
NS5A-Asn^{2218}	7.7 (2.0 - 29.0)	0.002		
AFP (>20 ng/L)	12 (5.1 - 30.0)	0.0001	19.5 (4.7 - 80.0)	0.0001
ALT (>165 IU/L)	4.0 (1.8 - 8.6)	0.0006		
AST (>65 IU/L)	3.9 (1.5 - 10.0)	0.003		
Fibrosis staging score (≥3)	2.4 (1.1 - 4.9)	0.02		

Gln^{70}, glutamine at position 70 of the core protein; Tyr^{1082}, tyrosine at position 1082 of NS3; Gln^{1212}, glutamine at position 1212 of NS3; IRRDR, interferon/ribavirin resistance-determining region; Asn^{2218}, asparagine at position 2218 of NS5A-ISDR, ALT, alanine aminotransferase; AST, aspartate transaminase; AFP; α-fetoprotein; IFN; interferon.

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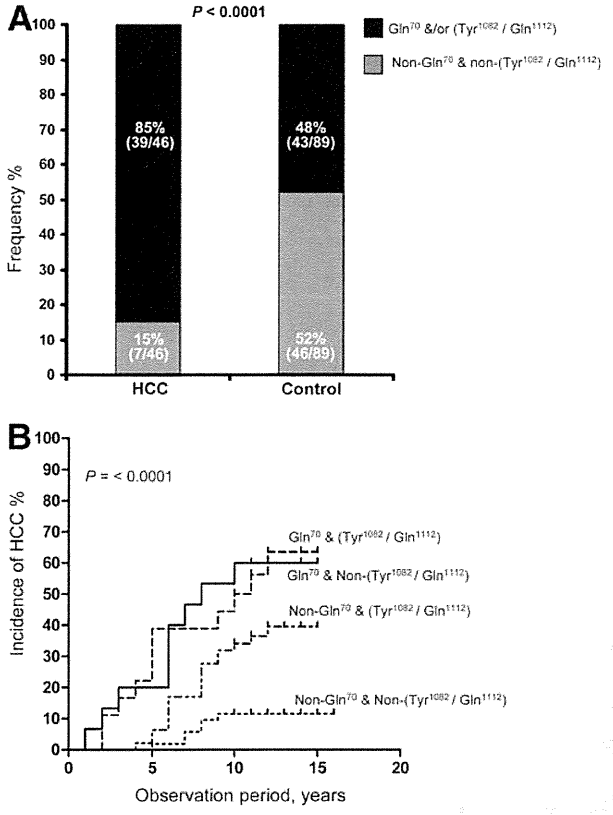

Fig. 2. (A) Proportions of HCV-1b isolates of the HCC high-risk group (core-Gln^{70} and/or NS3-[Tyr^{1082}/Gln^{1112}]) and the low-risk group (non-Gln^{70} and non-[Tyr^{1082}/Gln^{1112}]) among HCC and control groups. (B) Cumulative HCC incidence on the basis of different combined sequence patterns of position 70 of the core protein and positions 1082 and 1112 of NS3. Core-Gln^{70} and NS3-(Tyr^{1082}/Gln^{1112}), n = 18; core-Gln^{70} and non-(Tyr^{1082}/Gln^{1112}), n = 16; non-Gln^{70} and NS3-(Tyr^{1082}/Gln^{1112}), n = 48; non-Gln^{70}/non-(Tyr^{1082}/Gln^{1112}), n = 53.

significantly higher in post-HCC isolates than in pre-HCC isolates; IRRDR≥6 was found in 47% (18/38) of post-HCC isolates compared to 24% (9/38) of pre-HCC isolates ($P = 0.03$). On the other hand, ISDR≥3 was found in 21% (8/38) of post-HCC isolates compared to 11% (4/38) of pre-HCC isolates, with the difference between the two groups being not statistically significant ($P = 0.3$).

Discussion

HCC is one of the common long-term complications of HCV infection. However, whether HCV itself plays a direct role in the development of HCC and whether all HCV isolates are equally associated with HCC development remain to be determined. HCV core, NS3, and NS5A proteins have been reported to affect a wide variety of potentially oncogenic pathways in cell culture and experimental animal systems.[7] In the present study, we demonstrated that HCV isolates with core-Gln^{70}, NS3-Tyr^{1082}/Gln^{1112} or NS5A-IRRDR≥6 were closely associated with HCC development. In addition, a follow-up study revealed that sequence patterns at position 70 of the core protein and positions 1082 and 1112 of NS3 did not significantly alter during the progression from chronic hepatitis to HCC while NS5A-IRRDR showed a significantly higher degree of sequence heterogeneity in post-HCC than in pre-HCC isolates.

Correlation between polymorphisms at positions 70 and 91 of HCV-1b core protein and IFN-based treatment outcome was extensively studied, especially in a Japanese population.[17-20] Interestingly, the same mutations were also associated with progression to HCC in the Japanese population with HCV-1b infection.[13] Results obtained in the present study confirmed and emphasized the significant association between the mutation at position 70 (core-Gln^{70}), but not at position 91, and HCC development (Tables 2, 3; Fig. 1A). Despite the clinical evidence that strongly supports the correlation between core-Gln^{70} and HCC development, the molecular mechanism underlying this correlation is still obscure. Delhem et al.[36] found that tumor-derived HCV core proteins, but not nontumor-derived ones, interact with and activate double-stranded RNA-dependent protein kinase (protein kinase R or PKR), which might modulate viral persistence and carcinogenesis. Gln^{70} was found in two of the three tumor-derived sequences, whereas Arg^{70} was found in two of the three nontumor-derived ones.

As for the NS3 protein of HCV, the possible link between an N-terminal portion of NS3 encoding viral serine protease (aa 1027 to 1146) and hepatocarcinogenesis was reported.[21,22] However, information about the relationship between NS3 sequence diversity and HCC development is still limited. We previously reported a significant correlation between predicted secondary structure of an N-terminal portion of NS3 and HCC development.[34] In the present study, we demonstrated that HCV patients infected with HCV isolates with NS3-(Tyr^{1082}/Gln^{1112}) were at a higher risk to develop HCC than those infected with HCV isolates with non-Tyr^{1082}/Gln^{1112} (Tables 2, 3; Fig. 2B). Computer-assisted secondary structure analysis of NS3 revealed that Tyr^{1082} was associated with the presence of a turn structure at around position 1083 while Phe^{1082} was associated with the absence of the turn structure.[34] Notably, the catalytic triad of NS3 serine protease consists of His^{1083}, Asp^{1107}, and Ser^{1165}.[37] Since positions 1082 and 1112 are in close vicinity of the catalytic triad, sequences diversity at these positions

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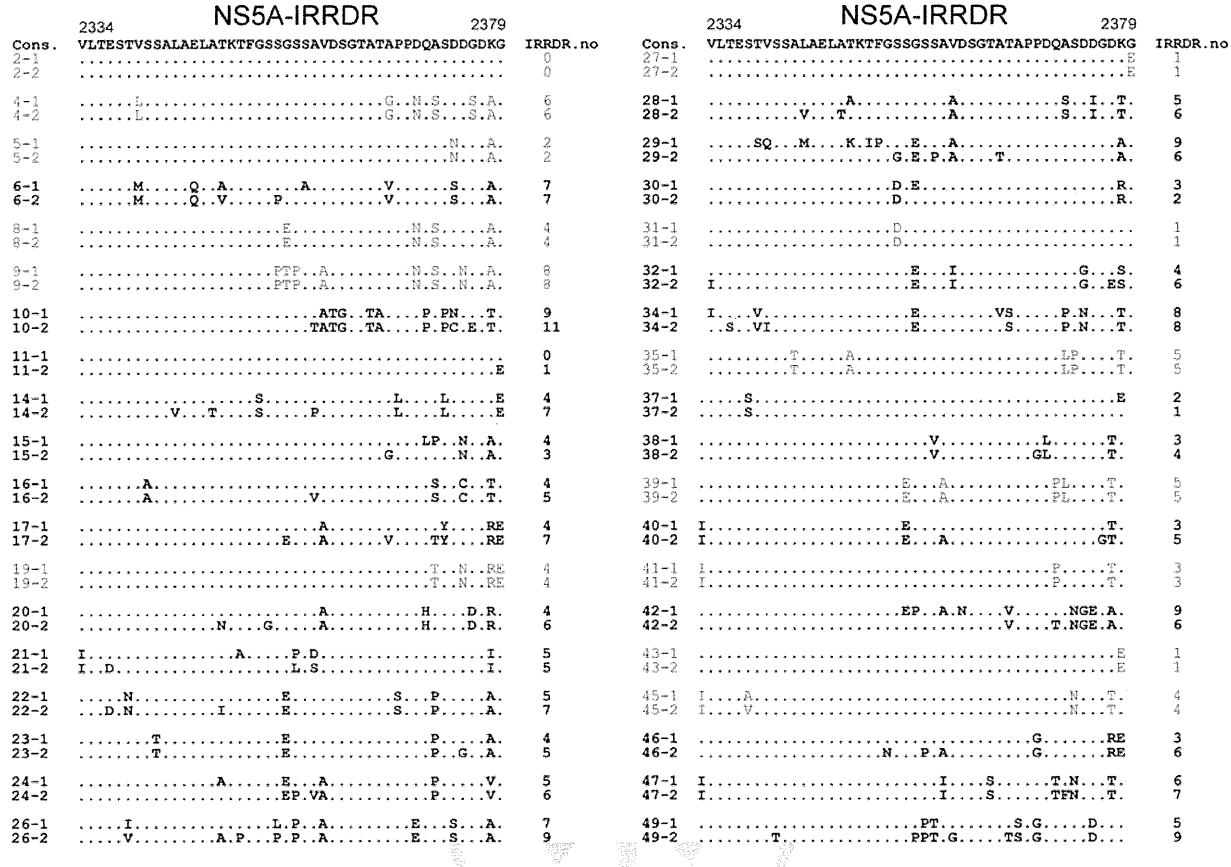

Fig. 3. Pairwise comparison of IRRDR sequences of HCV-1b during the follow-up period between chronic hepatitis and HCC development. Sequence pairs that differ between pre- (numbered with -1) and post-HCC isolates (numbered with -2) are shown. The consensus sequence (Cons.) is shown at the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the Cons. sequence. The numbers of IRRDR mutations are shown on the right.

might influence the serine protease activity and also pathogenicity of HCV. Large-scale, multicenter clinical studies as well as more detailed experimental studies at the molecular and cellular levels are needed to clarify the importance of sequence diversity at positions 1082 and 1112 of NS3 in HCV-mediated hepatocarcinogenesis.

HCV heterogeneity in NS5A-ISDR and NS5A-IRRDR are correlated with IFN-responsiveness.[17,18,25,26] As IFN-based therapy reduces the risk of HCC development,[4,28-30] we were interested to investigate whether there is a correlation between sequence heterogeneity in NS5A and development of HCC. Our present results revealed that a high degree of sequence heterogeneity in IRRDR (IRRDR≥6) was closely associated with HCC development (Table 2). We previously reported that IRRDR≥6 was significantly associated with good responses to PEG-IFN/RBV combination therapy.[26,27] These results collectively suggest that oncogenic properties and PEG-IFN/RBV responsiveness are independent viral characteristics and that PEG-IFN/RBV therapy helps eliminate oncogenic HCV isolates, thus reducing the risk of HCC development.

Position 2218 of NS5A, located within ISDR, appears to tolerate a wide range of aa substitutions as observed in different HCV-1b isolates.[25,38,39] Interestingly, Asn at position 2218 (Asn^{2218}) was detected significantly more frequently in pre-HCC isolates than in the control isolates. Further studies are needed to determine the possible importance of this residue in hepatocarcinogenesis.

Another focus of attention is how the sequences of the core protein, NS3, and NS5A-IRRDR evolve during the interval between chronic hepatitis and HCC development. One of the significant advantages of the present study was that we could conduct a longitudinal investigation by analyzing the target sequences of pre- and post-HCC isolates. We found that core-Gln^{70} and NS3-(Tyr^{1082}/Gln^{1112}) were well conserved in each paired sample. This indicates that core-Gln^{70} and NS3-(Tyr^{1082}/Gln^{1112}) were already present before the

development of HCC. Non-Gln^{70} of the core protein and non-Tyr^{1082} and non-Gln^{1112} of NS3 were also well conserved in each paired sample. These results imply the possibility that these sequence patterns were not a result of HCC but, rather, they were a possible causative factor for the development of HCC. We hypothesize, therefore, that HCV isolates with core-Gln^{70} and/or NS3-(Tyr^{1082}/Gln^{1112}) are highly oncogenic, whereas those with non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}) are less oncogenic. It is not clear yet as to whether these oncogenic mutations were present from the very beginning of HCV infection or if they emerged at a certain timepoint (before the initiation of follow-up) during the long-term persistence through an adaptive viral evolution in the host. More comprehensive follow-up study is needed to address this issue. In any case, the core-Gln^{70} and NS3-(Tyr^{1082}/Gln^{1112}) would be considered an index for prediction of HCC development. On the other hand, IRRDR in NS5A is more tolerant for sequence evolution. IRRDR in post-HCC isolates showed a significantly higher degree of sequence heterogeneity compared with that in pre-HCC isolates. This observation suggests that IRRDR is under strong selective pressure during the course of HCV infection and that the high degree of IRRDR heterogeneity (IRRDR≥6) in HCV isolates from patients with HCC may not be a causative factor for development of HCC.

In conclusion, the present results suggest the possibility that patients infected with HCV isolates with core-Gln^{70} and/or NS3-(Tyr^{1082}/Gln^{1112}) are at a higher risk to develop HCC compared to those with non-(Gln^{70} plus NS3-Tyr^{1082}/Gln^{1112}).

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Hepatitis C Virus Infection Suppresses GLUT2 Gene Expression via Downregulation of Hepatocyte Nuclear Factor 1α

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Hepatitis C virus (HCV) infection causes not only intrahepatic diseases but also extrahepatic manifestations, including type 2 diabetes. We previously reported that HCV replication suppresses cellular glucose uptake by downregulation of cell surface expression of glucose transporter 2 (GLUT2) (D. Kasai et al., J. Hepatol. 50:883–894, 2009). GLUT2 mRNA levels were decreased in both HCV RNA replicon cells and HCV J6/JFH1-infected cells. To elucidate molecular mechanisms of HCV-induced suppression of GLUT2 gene expression, we analyzed transcriptional regulation of the GLUT2 promoter using a series of GLUT2 promoter-luciferase reporter plasmids. HCV-induced suppression of GLUT2 promoter activity was abrogated when the hepatocyte nuclear factor 1α (HNF-1α)-binding motif was deleted from the GLUT2 promoter. HNF-1α mRNA levels were significantly reduced in HCV J6/JFH1-infected cells. Furthermore, HCV infection remarkably decreased HNF-1α protein levels. We assessed the effects of proteasome inhibitor or lysosomal protease inhibitors on the HCV-induced reduction of HNF-1α protein levels. Treatment of HCV-infected cells with a lysosomal protease inhibitor, but not with a proteasome inhibitor, restored HNF-1α protein levels, suggesting that HCV infection promotes lysosomal degradation of HNF-1α protein. Overexpression of NS5A protein enhanced lysosomal degradation of HNF-1α protein and suppressed GLUT2 promoter activity. Immunoprecipitation analyses revealed that the region from amino acids 1 to 126 of the NS5A domain I physically interacts with HNF-1α protein. Taken together, our results suggest that HCV infection suppresses GLUT2 gene expression via downregulation of HNF-1α expression at transcriptional and posttranslational levels. HCV-induced downregulation of HNF-1α expression may play a crucial role in glucose metabolic disorders caused by HCV.

Hepatitis C virus (HCV) is the main cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. HCV is a single-stranded, positive-sense RNA virus that is classified into the *Flaviviridae* family, *Hepacivirus* genus (21). More than 170 million people worldwide are chronically infected with HCV. The 9.6-kb HCV genome encodes a polyprotein of approximately 3,010 amino acids (aa). The polyprotein is cleaved co- and posttranslationally into at least 10 proteins by viral proteases and cellular signalases: the structural proteins core, E1, E2, and p7 and the nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B (21).

Persistent HCV infection causes not only intrahepatic diseases but also extrahepatic manifestations, such as type 2 diabetes. Clinical and experimental data suggest that HCV infection is an additional risk factor for the development of diabetes (26, 29, 30). HCV-related glucose metabolic changes and insulin resistance have significant clinical consequences, such as accelerated fibrogenesis, reduced virological response to alpha interferon (IFN-α)-based therapy, and increased incidence of hepatocellular carcinoma (29). Therefore, the molecular mechanism of HCV-related diabetes needs to be clarified.

We have sought to identify a novel mechanism of HCV-induced diabetes. We previously demonstrated that HCV suppresses hepatocytic glucose uptake through downregulation of cell surface expression of glucose transporter 2 (GLUT2) in a human hepatoma cell line (19). The uptake of glucose into cells is conducted by facilitative glucose carriers, i.e., glucose transporters (GLUTs). GLUTs are integral membrane proteins that contain 12 membrane-spanning helices. To date, a total of 14 isoforms have been identified in the GLUT family (24). GLUT2 is expressed in the liver, pancreatic β-cells, hypothalamic glial cells, retina, and enterocytes. Glucose is transported into hepatocytes by GLUT2 (34). We previously reported that GLUT2 expression was reduced in hepatocytes obtained from HCV-infected patients (19). We also demonstrated that GLUT2 mRNA levels were lower in HCV replicon cells and in HCV J6/JFH1-infected cells than in the control cells. GLUT2 promoter activity was suppressed in HCV-replicating cells. However, the molecular mechanism of HCV-induced suppression of GLUT2 gene expression remains to be elucidated.

In the present study, we aimed to clarify molecular mechanisms of HCV-induced suppression of GLUT2 gene expression. We analyzed transcriptional regulation of the GLUT2 promoter in HCV replicon cells. We demonstrate that HCV infection downregulates hepatocyte nuclear factor 1α (HNF-1α) expression at both transcriptional and posttranslational levels, resulting in suppression of GLUT2 promoter. We propose that HCV-induced downregulation of HNF-1α may play a crucial role in glucose metabolic disorders caused by HCV.

MATERIALS AND METHODS

Cell culture. The human hepatoma cell line Huh-7.5 (4) was kindly provided by Charles M. Rice (The Rockefeller University, New York, NY).

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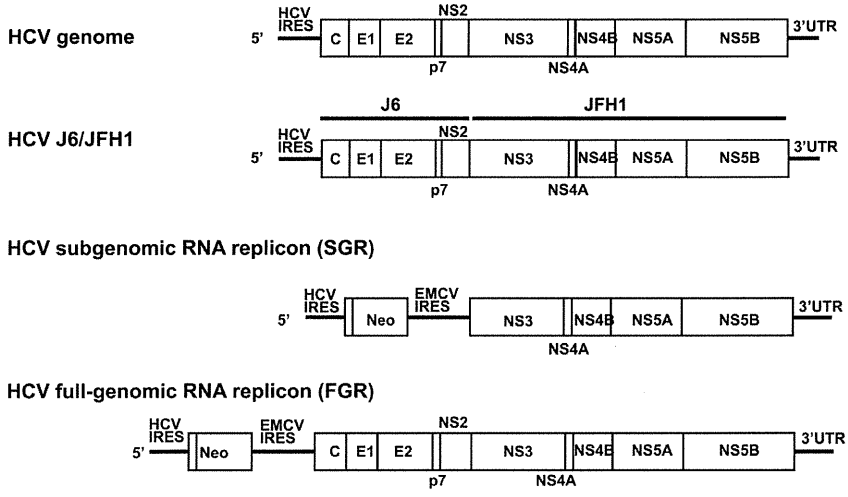

FIG 1 The HCV genome, chimeric HCV J6/JFH1, and the HCV RNA replicons. Schematic diagrams of the HCV genome, the chimeric HCV J6/JFH1 genome, SGR, and FGR are shown. IRES, internal ribosome entry site; EMCV, encephalomyocarditis virus; Neo, neomycin resistance gene.

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (high glucose) with L-glutamine (Wako, Osaka, Japan) supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin (Gibco, NY), 10% heat-inactivated fetal bovine serum (Biowest, France), and 0.1 mM nonessential amino acids (Invitrogen, NY) at 37°C in a 5% CO_2 incubator. Cells were transfected with plasmid DNA using FuGENE 6 transfection reagents (Promega, Madison, WI).

Huh-7.5 cells stably harboring an HCV-1b subgenomic RNA replicon (SGR) were prepared as described previously (18), using pFK5B/2884Gly (a kind gift from R. Bartenschlager, University of Heidelberg, Heidelberg, Germany). The SGR cells express the genomic region from NS3 to NS5B of the HCV Con1 strain (19) (Fig. 1). Cells harboring a full-genome HCV-1b RNA replicon (FGR) derived from Con1 (27) or pON/C-5B (17, 19) (a kind gift from N. Kato, Okayama University, Okayama, Japan) were also used. The FGR cells express all of the HCV proteins (the region ranging from the core protein to NS5B).

The pFL-J6/JFH1 plasmid that encodes the entire viral genome of a chimeric strain of HCV-2a, J6/JFH1 (23), was kindly provided by Charles M. Rice. The HCV genome RNA was synthesized *in vitro* using pFL-J6/JFH1 as a template and was transfected into Huh-7.5 cells by electroporation (6, 9, 23, 37). The virus produced in the culture supernatant was used for infection experiments (6).

Cells were treated with 1,000 IU/ml of IFN-α (Sigma, St. Louis, MO) for 10 days to eliminate HCV replication (19).

Luciferase reporter assay. We constructed the human GLUT2 promoter-luciferase reporter plasmid by cloning a 1.6-kb genomic fragment that encompasses the human GLUT2 promoter region from −1291 to +308, yielding pGLUT2(−1291/+308)-Luc (2, 19), into the pGL4 vector plasmid (Promega). The pGLUT2(−1291/+308)-Luc construct contains a 1,291-bp fragment of the human GLUT2 promoter upstream of the minimal promoter and the coding sequence of the *Photinus pyralis* (firefly) luciferase. We also used seven different GLUT2 promoter-luciferase reporter plasmids, i.e., pGLUT2(−1193/+308)-Luc, pGLUT2 (−1155/+308)-Luc, pGLUT2(−1100/+308)-Luc, pGLUT2(−1030/+308)-Luc, pGLUT2(−206/+308)-Luc, pGLUT2(+29/+308)-Luc, and pGLUT2(+126/+308)-Luc, which lack the binding sequence of the CCAAT/enhancer binding site (C/EBP), cyclic AMP (cAMP) response element (CRE), AP-1 binding site, HNF-1α binding site, CAAT box, TATA-like motif, and transcriptional initiation, respectively (Fig. 2A). The reporter plasmid pRL-CMV-*Renilla* (where CMV is cytomegalovirus) (Promega) was used as an internal control. Cells were transfected with each pGLUT2-Luc construct together with pRL-CMV-*Renilla*. At 48 h after transfection, samples were harvested and assayed for luciferase activity. The luciferase assays were performed using a dual-luciferase reporter assay system (Promega). Luciferase activity was measured by a Lumat LB 9501 instrument (Berthold Technologies GmbH & Co., Bad Wildbad, Germany). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each sample. The number of relative light units (RLU) of the SGR cells or FGR cells transfected with each reporter plasmid is expressed as a ratio of the number of Huh-7.5 cells transfected with each reporter plasmid.

Expression plasmids. Expression plasmids for core protein, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B were described previously (9, 10, 18). To express E1 and E2 (E1/E2), the cDNA fragment of nucleotides (nt) 825 to 2676 derived from the HCV Con1 strain was amplified by PCR using the plasmid pFKI389neo/core-3′/Con1 (a kind gift from R. Bartenschlager) as a template. Specific primers used for PCR were as follows: sense primer, 5′-CCAGTGTGGTGAATTCAC CATGGTGAACTATGCAACAGGGAA-3′; antisense primer, 5′-CGAAG GGCCCTCTAGAGATGTACCAGGCAGCACAGA-3′. To express NS3 and NS4A (NS3/4A), the cDNA fragment of nt 3420 to 5474 derived from the HCV Con1 strain was amplified by PCR. Specific primers were as follows: sense primer, 5′-CCAGTGTGGTGAATTCACCATGGCGCCTA TTACGGCCTACTC-3′; antisense primer, 5′-CGAAGGGCCCTCTAGA GCACTCTTCCATCTCATCGAA-3′. These amplified PCR products were purified, and each of them was inserted into the EcoRI-XbaI site of pEF1/myc-His A (Invitrogen) using an In-Fusion HD-Cloning kit (Clontech, Mountain View, CA). To express a series of NS5A deletion mutants as hemagglutinin (HA)-tagged proteins, each fragment was amplified by PCR and cloned into the NotI site of pCAG-HA. pEF1A-NS5A (Con1)-myc-His was used as a template (18). The primer sequences used in this study are available from the authors upon request. The sequences of the inserts were extensively verified by sequencing (Operon biotechnology, Tokyo, Japan). The plasmids pEF1A-NS5A(1–126)-myc-His, consisting of residues 1 to 126 in NS5A, and pEF1A-NS5A(1–147)-myc-His were described previously (18).

Antibodies. The mouse monoclonal antibodies (MAbs) used in this study were anti-FLAG (M2) MAb (F-3165; Sigma), anti-NS5A MAb (MAB8694; Millipore), anti-core protein MAb (2H9) (37), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) MAb (MAB374; Millipore). Polyclonal antibodies (PAbs) used in this study were anti-HNF-1α rabbit PAb (sc-8986; Santa Cruz Biotechnology), anti-HNF-1α goat PAb (sc-6548; Santa Cruz Biotechnology), anti-NS5B goat PAb (sc-17532; Santa Cruz Biotechnology), anti-NS3 rabbit PAb (described elsewhere), and anti-actin goat PAb (C-11; Santa Cruz Biotechnology). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody

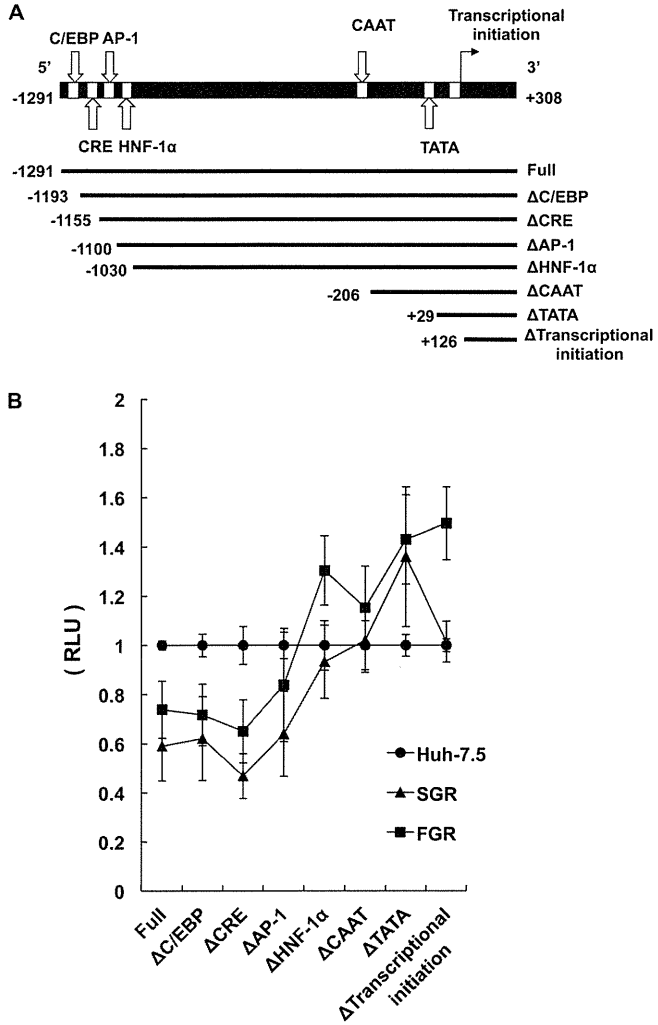

FIG 2 HNF-1α-binding site is important for HCV-induced suppression of GLUT2 promoter. (A) A series of constructs in which genomic GLUT2 promoter DNA fragments were fused to a promoterless firefly luciferase gene of the pGL4 vector were generated with the 3′ end always terminating at bases +308 from transcriptional start site. The 5′ ends began at bases −1291, −1193, −1155, −1100, −1030, −206, +29, and +126 The regions that represent potential binding sites for transcription factors are shown, including a CCAAT/enhancer binding site (C/EBP), cAMP response element (CRE), AP-1 binding site, HNF-1α binding site, CAAT box, and TATA-like motif. The nucleotide at the beginning of the construct is indicated. (B) Huh-7.5 cells, SGR cells, and FGR cells (2.5×10^5 cells/six-well plate) were transfected with each GLUT2 plasmid (0.5 μg) together with pRL-CMV-*Renilla* (25 ng). pRL-CMV-*Renilla* was used as an internal control. At 48 h posttransfection, cells were harvested and assayed for luciferase activities using a dual-luciferase reporter assay system. RLU is expressed as a ratio of the Huh-7.5 cells transfected with each reporter plasmid.

(Cell signaling), HRP-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology), and HRP-conjugated anti-rabbit IgG (Cell signaling) were used as secondary antibodies.

Real-time quantitative reverse transcription-PCR (RT-PCR). Total cellular RNA was isolated using RNAiso reagent (TaKaRa Bio, Kyoto, Japan), and cDNA was generated using a QuantiTect Reverse Transcription system (Qiagen, Valencia, CA). Real-time quantitative PCR was performed using SYBR Premix *Ex Taq* (TaKaRa Bio) with SYBR green chemistry on an ABI Prism 7000 system (Applied Biosystems, Foster, CA), as described previously (11, 19). The β-glucronidase (GUS) gene was used as an internal control. The primers used for real-time PCR are as follows: for HNF-1α (NM_000545), 5′-AGCTACCAACCAAGAAGGGGC-3′ (nt 601 to 621) and 5′-TGACGAGGTTGGAGCCCAGCC-3′ (nt 801 to 781); HNF-1β (NM_000458), 5′-GTTACATGCAGCAACACAACA-3′ (nt 600 to 620) and 5′-TCATATTTCCAGAACTCTGGA-3′ (nt 801 to 782); GUS (NM_000181), 5′-ATCAAAAACGCAGAAAATACG-3′ (nt 1797 to 1817) and 5′-ACGCAGGTGGTATCAGTCTTG-3′ (nt 2034 to 2014).

Immunoblot analysis. Immunoblot analysis was performed essentially as described previously (9, 33). The cell lysates were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA). The membranes were incubated with primary antibody, followed by incubation with peroxidase-conjugated secondary antibody. The positive bands were visualized using ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, United Kingdom). To detect endogenous HNF-1α protein, ECL Plus Western blotting detection reagents were used (GE Healthcare).

Immunoprecipitation. Cultured cells were lysed with a buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 0.1% SDS, 1% NP-40, and Complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The lysate was centrifuged at 12,000 × *g* for 20 min at 4°C, and the supernatant was immunoprecipitated with appropriate antibodies. Immunoprecipitation was performed as described previously (10). Briefly, the cell lysates were immunoprecipitated with control IgG and Dynabeads protein A (Invitrogen) and incubated with appropriate antibodies at 4°C overnight. After being washed with the washing buffer (0.1 M Na-phosphate buffer, pH 7.4) five times, the immunoprecipitates were analyzed by immunoblotting.

Statistical analysis. Results were expressed as means ± standard errors of the means (SEM). Statistical significance was evaluated by analysis of variance (ANOVA), and statistical significance was defined as a P value of <0.05.

RESULTS

HNF-1α-binding site is important for HCV-induced suppression of GLUT2 promoter. To gain an insight into potential regulatory sequences involved in HCV-induced suppression of GLUT2 gene transcription, a 1.6-kb genomic fragment that encompasses the human GLUT2 promoter (−1291 to +308) and a series of deletion mutants were analyzed (Fig. 2A). The ability of the upstream region of the GLUT2 gene to function as a promoter was assessed by its capacity to drive the expression of a luciferase reporter gene. GLUT2 promoter activity was assessed by measuring luciferase activity of the cell extracts derived from transiently transfected Huh-7.5 cells, SGR cells, and FGR cells. As shown in Fig. 2B, a deletion of the promoter sequence to −1100 [pGLUT2(−1100/+308)-Luc [ΔAP-1]] showed lower luciferase activities in HCV replicon cells than in the control cells. Successive removal of nucleotides from −1100 to −1030 completely or almost completely abolished the suppression of the luciferase activity in both FGR and SGR cells, suggesting that the HNF-1α-binding site is important for HCV-induced suppression of GLUT2 promoter.

HCV infection reduces HNF-1α mRNA levels. It is worth noting that HNF-1α is known to play a crucial role in diabetes. Mutations in the HNF-1α gene have been reported to cause a monogenic form of diabetes mellitus with autosomal dominant inheritance, termed maturity onset diabetes of the young 3 (MODY3) (25, 40). Cha et al. (7) reported that HNF-1α functions as a transcriptional transactivator in human GLUT2 gene expression in a human hepatoma cell line. These findings motivated us to further investigate a role of HNF-1α in HCV-induced glucose metabolic disorders in a human hepatoma cell line. To determine

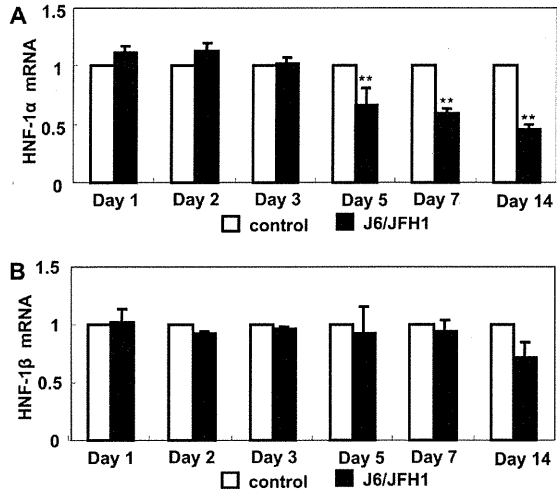

FIG 3 Quantitative RT-PCR analysis of mRNA for HNF-1α and HNF-1β in HCV J6/JFH1-infected cells. Huh-7.5 cells (2.5×10^5 cells/six-well plate) were infected with HCV J6/JFH1 at a multiplicity of infection of 2. Cells were cultured and harvested at the indicated times. Total RNA was extracted, and the levels of HNF-1α mRNA and HNF-1β mRNA were determined by quantitative RT-PCR. Mock-infected cells served as negative controls. **, $P < 0.01$, compared with mock-infected cells.

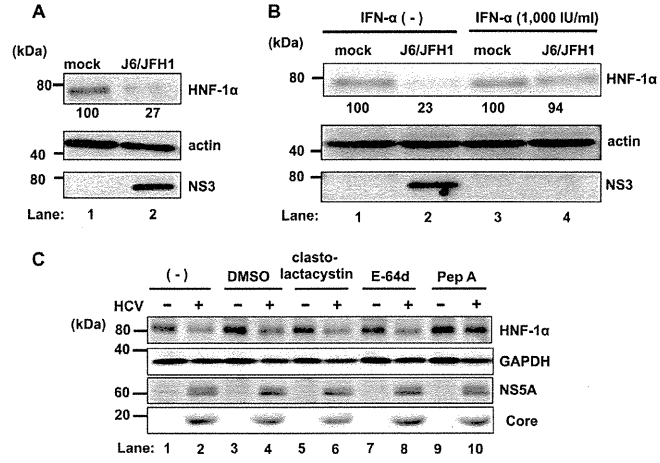

FIG 4 HCV infection induces lysosomal degradation of HNF-1α protein. (A) HCV infection decreased the levels of HNF-1α protein in Huh-7.5 cells. Huh-7.5 cells (2.5×10^5 cells/six-well plate) were infected with HCV J6/JFH1 at a multiplicity of infection of 2. Cells were cultured and harvested at 5 days postinfection. Cells were analyzed by immunoblotting with anti-HNF-1α, anti-NS3, and anti-actin antibodies. The level of actin served as a loading control. The relative levels of protein expression were quantitated by densitometry and are indicated below the respective lanes. (B) HCV-induced downregulation of HNF-1α protein was restored by treatment of the cells with IFN-α. Huh-7.5 cells were plated at 2.5×10^5 cells/six-well plate and cultured for 12 h. The cells were infected with HCV J6/JFH1 at a multiplicity of infection of 2 and cultured for 5 days. The cells were replated at 2.5×10^5 cells/six-well plate and cultured in complete DMEM with or without 1,000 IU/ml IFN-α for 10 days to eliminate HCV. The cells cultured in DMEM without IFN-α served as negative controls. (C) HCV-induced reduction of HNF-1α protein was restored by treatment of the cells with lysosomal protease inhibitor. Huh-7.5 cells were plated at 2.0×10^5 cells/six-well plate and cultured for 12 h. At 5 days postinfection, proteasome inhibitor (30 μM clasto-lactacystin β-lactone) or lysosomal protease inhibitors (40 μM E-64d and 20 μM pepstatin A) were administered to the cells. Cells were cultured for 12 h, harvested, and analyzed by immunoblotting as indicated. The level of GAPDH served as a loading control. DMSO, dimethyl sulfoxide; PepA, pepstatin A.

whether HCV infection suppresses HNF-1α mRNA expression, we quantified mRNA levels of HNF-1α and HNF-1β in HCV J6/JFH1-infected cells and in mock-infected cells by real-time RT-PCR. HNF-1α mRNA levels were significantly reduced in HCV J6/JFH1-infected cells from 5 days postinfection (dpi) to 14 dpi (Fig. 3A). On the other hand, HNF-1β mRNA levels remained unchanged until 14 dpi (Fig. 3B). These results suggest that HCV infection specifically downregulates HNF-1α mRNA expression.

HCV infection reduces HNF-1α protein levels. To determine whether HCV infection reduces HNF-1α protein levels, endogenous HNF-1α protein levels were examined by immunoblot analysis. The HNF-1α protein level was much lower in J6/JFH1-infected cells than in the mock-infected control (Fig. 4A, upper panel, lane 2). To determine whether HCV infection is specifically involved in reduction of HNF-1α protein, we eliminated HCV by treatment of the cells with IFN-α (Fig. 4B, lower panel, compare lane 2 with lane 4). Upon elimination of HCV, the HNF-1α protein expression level recovered to the level of the mock-infected control (Fig. 4B, upper panel, compare lane 2 with lane 4). These results suggest that HCV infection specifically reduces HNF-1α protein levels.

HCV-induced reduction of HNF-1α protein is restored by treatment of the cells with a lysosomal protease inhibitor. As shown in Fig. 3A, HNF-1α mRNA levels in HCV J6/JFH1-infected cells decreased slowly at day 5 postinfection. One possible explanation is that suppression of HNF-1α mRNA is an indirect effect caused by HCV infection. The degree of the reduction of the HNF-1α protein was larger than that of HNF-1α mRNA (Fig. 4A), suggesting the involvement of protein degradation in reduction of HNF-1α protein levels. To determine whether protein degradation is involved in HCV-induced reduction of HNF-1α protein, we assessed the role of proteasome or lysosome proteases in the reduction of HNF-1α protein. We treated the cells with a proteasome inhibitor, clasto-lactacystin β-lactone, or lysosome protease inhibitors E-64d and pepstatin A. Clasto-lactacystin β-lactone had no effect on the levels of HNF-1α protein (Fig. 4C, upper panel, lane 6). This result suggests that proteasome is not involved in the reduction of HNF-1α protein. E-64d is a cysteine protease inhibitor, and pepstatin A is an aspartic protease inhibitor. Pepstatin A, but not E-64d, restored the levels of HNF-1α protein (Fig. 4C, upper panel, lanes 10 and 8). These results suggest that a lysosomal protease, such as an aspartic protease, is involved in HCV-induced reduction of HNF-1α protein.

Overexpression of NS5A protein suppresses GLUT2 promoter activity. To determine which HCV protein is involved in the suppression of GLUT2 promoter, we examined the effects of transient expression of HCV proteins on GLUT2 promoter activity. Huh-7.5 cells were cotransfected with each HCV protein expression plasmid together with the GLUT2 promoter-luciferase plasmid. The pRL-CMV-*Renilla* plasmid was cotransfected as an internal control. At 48 h posttransfection, cells were harvested and assayed for luciferase activity. As shown in Fig. 5A, overexpression of the NS5A expression plasmid significantly reduced GLUT2 promoter activity. On the other hand, other HCV protein expression plasmids failed to suppress GLUT2 promoter activity (Fig. 5A, left and right panels). These results suggest that NS5A protein is involved in the suppression of GLUT2 promoter activity.

Overexpression of NS5A protein reduces the levels of endogenous HNF-1α protein. To investigate a role of NS5A in the sup-

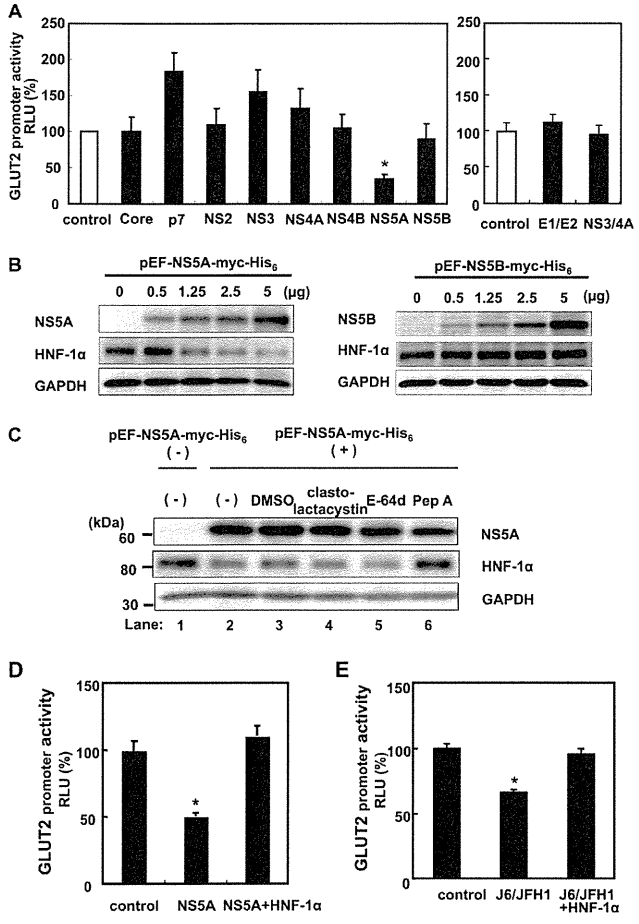

FIG 5 HCV NS5A protein is involved in suppression of GLUT2 promoter activity and lysosomal degradation of HNF-1α protein. (A) Huh-7.5 cells were plated at 1×10^5 cells/12-well plate. After cells were cultured for 12 h, cells were cotransfected with each HCV protein plasmid (0.5 μg), the human GLUT2 promoter reporter plasmid (0.5 μg), and pRL-CMV-*Renilla* (25 ng). pRL-CMV-*Renilla* was used as an internal control. At 48 h posttransfection, cells were harvested. Luciferase assays were performed by using a dual-luciferase reporter assay system. (B) Huh-7.5 cells were plated at 4×10^5 cells/six-well plate and cultured for 12 h. Cells were transfected with increasing amounts of either NS5A plasmid or NS5B plasmid as indicated. At 48 h posttransfection, cells were harvested. Whole-cell lysates were analyzed by immunoblotting with anti-HNF-1α, anti-NS5A, and anti-NS5B antibodies. The level of GAPDH served as a loading control. (C) Huh-7.5 cells (2.5×10^5 cells/six-well plate) were transfected with pEF1A-NS5A-myc-His_6. At 2 days posttransfection, proteasome inhibitor (30 μM clasto-lactacystin β-lactone) or lysosomal enzyme inhibitors (40 μM E-64d and 20 μM pepstatin A) were administered to the cells. Cells were cultured for 12 h and harvested, and the levels of endogenous HNF-1α protein were analyzed by immunoblotting with anti-HNF-1α goat PAb. The level of GAPDH served as a loading control. (D) Huh-7.5 cells (1.0×10^5 cells/12-well plate) were transfected with the human GLUT2 promoter reporter plasmid (0.5 μg) and pRL-CMV-*Renilla* (25 ng). The plasmid pEF1A/myc-His (0.5 μg) was cotransfected to the control cells. Cells were transfected with the plasmid pEF1A-NS5A-myc-His (0.5 μg) together with either empty plasmid pCMV4 (10 ng) or pCMV-HNF-1α (10 ng). At 48 h posttransfection, cells were harvested. Luciferase assays were performed by using a dual-luciferase reporter assay system. *, $P < 0.05$, compared with control. (E) Huh-7.5 cells (1.2×10^6 cells /10 cm-dish) were infected with HCV J6/JFH1 at a multiplicity of infection of 2 and cultured for 5 days. At day 5 postinfection, cells were plated at 1.0×10^5 cells/12-well plate and cultured for 12 h. Mock-infected cells were plated similarly. Cells were transfected with the human GLUT2 promoter reporter plasmid (0.5 μg) and pRL-CMV-*Renilla* (25 ng) together with either empty plasmid pCMV4 or pCMV-HNF-1α, cultured for 48 h, and harvested. Luciferase assays were performed by using a dual-luciferase reporter assay system. *, $P < 0.05$, compared with control.

pression of the GLUT2 promoter, we examined the effect of NS5A protein on the levels of endogenous HNF-1α protein. Huh-7.5 cells were transfected with increasing amounts of either an NS5A expression plasmid or NS5B expression plasmid. At 48 h posttransfection, cells were harvested, and the levels of endogenous HNF-1α protein were analyzed by immunoblot analysis. To detect endogenous HNF-1α protein, highly sensitive Western blotting detection reagents (ECL Plus Western blotting detection reagents) were used. Overexpression of NS5A (Fig. 5B, left panel) but not NS5B (Fig. 5B, right panel) significantly reduced endogenous HNF-1α protein. These results suggest that NS5A protein specifically reduces endogenous HNF-1α protein levels.

To determine if NS5A-dependent reduction of HNF-1α protein is due to lysosomal degradation, we treated the cells with lysosome protease inhibitors. Pepstatin A, but not E-64d, recovered the levels of HNF-1α protein (Fig. 5C, middle panel, lanes 5 and 6), which is consistent with the results found in HCV-infected cells. These results suggest that NS5A is responsible for HCV-induced lysosomal degradation of HNF-1α protein. Taken together, our results suggest that HCV infection suppresses GLUT2 promoter activity via NS5A-dependent lysosomal degradation of HNF-1α protein.

To verify a role of HNF-1α in the HCV-induced suppression of GLUT2 promoter activity, we examined the effects of ectopic expression of HNF-1α on GLUT2 promoter activity in NS5A-transfected cells as well as in HCV J6/JFH1-infected cells. As shown in Fig. 5D, overexpression of NS5A decreased GLUT2 promoter activity, and ectopic expression of HNF-1α restored GLUT2 promoter activity (Fig. 5D). Moreover, HCV J6/JFH1 infection significantly decreased GLUT2 promoter activity, and ectopic expression of HNF-1α restored GLUT2 promoter activity (Fig. 5E). These results are consistent with the notion that HNF-1α protein is a key regulator for HCV-induced suppression of GLUT2 promoter activity.

NS5A protein interacts with HNF-1α protein in Huh-7.5 cells and in FGR Con1 cells. It was previously reported that *in vitro* translated HNF-1 protein was pulled down with glutathione *S*-transferase (GST)-NS5A protein (32). To determine whether NS5A physically interacts with HNF-1α protein in cultured cells, Huh-7.5 cells were cotransfected with each FLAG-tagged NS5A expression plasmid together with the HNF-1α expression plasmid. Immunoprecipitation analysis revealed that HNF-1α protein was coimmunoprecipitated with FLAG-NS5A protein using anti-FLAG MAb (Fig. 6A, third blot, lane 8). No band was detected using control IgG for immunoprecipitation (Fig. 6A, third blot, lane 7). Conversely, immunoprecipitation analysis revealed that NS5A protein was coimmunoprecipitated with HNF-1α protein using anti-HNF-1α rabbit PAb (Fig. 6B, fourth blot, lane 8). Moreover, NS5A protein was coimmunoprecipitated with endogenous HNF-1α protein (Fig. 6B, fourth blot, lane 6), suggesting that NS5A protein indeed interacts with HNF-1α protein.

To confirm that HCV NS5A protein can interact with HNF-1α protein in HCV-replicating cells, we performed immunoprecipitation analysis using FGR Con1 (RCYM1) cells. NS5A protein was coimmunoprecipitated with endogenous HNF-1α protein (Fig. 6C, fourth blot, lane 2). Transfection of HNF-1α protein increased the level of coimmunoprecipitated NS5A protein (Fig. 6C, fourth blot, lane 4), suggesting that HCV NS5A protein indeed interacts with HNF-1α protein in HCV-replicating cells.

HNF-1α binds domain I of NS5A protein. To map the HNF-