Table 1 Univariate analysis of factors associated with SVR, relapse, and NVR

Factor	All	SVR	Relapse	NVR	*p* value		
					SVR versus non-SVR	SVR versus relapse	Response versus NVR
n	75	37	20	18	–	–	–
Age (years)	60 (30–74)	57 (33–70)	63 (30–74)	63 (40–71)	**0.0018**	**0.0071**	0.111
Sex: male/female	43/32	23/14	11/9	9/9	0.486	0.778	0.587
BMI (kg/m^2)	22.2 (15.7–37.6)	22.1 (18.3–37.6)	21.9 (15.7–30.7)	23.0 (16.6–31.3)	0.844	0.357	0.298
HCV RNA (Log IU/mL)	6.2 (5.0–7.1)	6.2 (5.0–7.1)	6.2 (5.3–6.7)	6.2 (5.3–7.1)	0.727	0.913	0.606
ALT (U/L)	38 (8–265)	37 (11–174)	37 (10–265)	41 (8–148)	0.618	0.493	0.896
γ-GTP (U/L)	32 (9–406)	32 (9–406)	25 (9–127)	44 (20–151)	0.614	0.503	**0.07**
Hemoglobin (g/dL)	14.0 (11.0–18.6)	14.4 (11.9–18.6)	14.3 (11.0–16.1)	13.2 (12.0–14.5)	**0.0049**	0.213	**0.0020**
Platelets ($\times 10^4$/mm^3)	16.3 (9.1–30.9)	16.9 (9.1–30.9)	18.9 (9.8–25.2)	12.1 (9.1–21.8)	0.124	0.802	**0.0016**
Total cholesterol (mg/dL)	176 (99–248)	181 (106–248)	164 (100–230)	182 (99–237)	0.572	0.243	0.617
PEG-IFN (μg/kg/week): $<1.4/\geq 1.4$	23/52	14/23	5/15	4/14	0.184	0.326	0.373
Ribavirin (mg/kg/day): $<11.0/\geq 11.0$	46/29	21/16	13/7	12/6	0.422	0.545	0.594
IRRDR mutations: $\leq 5/\geq 6$	45/30	13/24	17/3	15/3	**0.00002**	**0.00035**	**0.027**
ISDR mutations: $\leq 1/\geq 2$	57/18	27/10	18/2	12/6	0.597	0.182	0.346
HCV core aa 70: wild/mutant	51/24	30/7	12/8	9/9	**0.025**	0.117	**0.083**
HCV core aa 91: wild/mutant	53/22	27/10	14/6	12/6	0.801	1.000	0.768
IL28B genotype: major/minor	57/18	34/3	16/4	7/11	**0.0024**	0.226	**0.0000095**

Values in bold are significant

SVR sustained virological response, *NVR* null virological response, *non-SVR* relapse *plus* NVR, *Response* non-NVR (SVR *plus* relapse), *BMI* body mass index, *ALT* alanine aminotransferase, *γ-GTP* gamma-glutamyl transpeptidase, *IRRDR* interferon/ribavirin resistance-determining region, *ISDR* interferon sensitivity-determining region, *IL* interleukin, *HCV* hepatitis C virus, *PEG-IFN* pegylated interferon

by 47 % (27/57), relapse was shown by 32 % (18/57), and NVR was shown by 21 % (12/57). Although a significant correlation was observed between ISDR heterogeneity and early virological response such as RVR ($p = 0.028$) (data not shown), no significant correlation was observed between ISDR heterogeneity and late virological response such as SVR, relapse, and NVR (Table 1). In this connection, ISDR heterogeneity at a cutoff point of one mutation (ISDR ≥ 1 vs. ISDR $= 0$) was also not significantly associated with treatment outcome (data not shown).

Correlation between core mutations and treatment responses

Examination of the possible correlation of either arginine at position 70 (Arg^{70}) or leucine at position 91 (Leu^{91}) of the core protein of HCV with treatment responses [15] revealed that among 51 patients infected with HCV core aa 70 wild (Arg^{70}), SVR was achieved by 59 % (30/51), relapse was shown by 24 % (12/51), and NVR was shown by 18 % (9/51). By contrast, among 24 patients infected with HCV core aa 70 mutant (non-Arg^{70}), SVR was achieved by 29 % (7/24), relapse was shown by 33 % (8/24), and NVR was shown by 38 % (9/24). There was a significant difference in the proportion of HCV core aa 70 wild and mutant between SVR and non-SVR patients ($p = 0.025$), and between response and NVR patients ($p = 0.083$). No significant correlation was observed between HCV core aa 91 heterogeneity and virological responses (Table 1).

Correlation between the genetic variation near the IL28B gene and treatment responses

The frequency of allele rs8099917 among the patients was 76 % for TT (57/75), 4 % for TG (3/75), and 20 % for GG (15/75). Univariate analysis revealed that among patients with genotype TT (IL28B major), SVR was achieved by 60 % (34/57), relapse was shown by 28 % (16/57), and NVR was shown by 12 % (7/57). By contrast, among patients with TG or GG (IL28B minor), SVR was achieved by 17 % (3/18), relapse was shown by 22 % (4/18), and NVR was shown by 61 % (11/18). There was a significant

difference in the proportion of IL28B major and minor between SVR and non-SVR patients ($p = 0.0024$), and between response and NVR patients ($p = 0.0000095$) (Table 1).

Identification of independent predictive factors for SVR, relapse, and NVR by multivariate logistic regression analysis

Factors significantly associated with certain virological responses were identified by multivariate analysis: IRRDR ≥6 [odds ratio (OR) 11.906, $p < 0.0001$] and age <60 years (OR 0.228, $p = 0.015$) were significantly associated with SVR; IRRDR ≤5 (OR 0.070, $p = 0.0008$) and age ≥60 years (OR 5.825, $p = 0.015$) with relapse; and IL28B minor (OR 14.618, $p = 0.0019$) and platelets $<15 \times 10^4/mm^3$ (OR 0.113, $p = 0.0096$) with NVR (Table 2).

Positive predictive values of combinations of IRRDR mutation and age for SVR

As stated above, IRRDR ≥6 predicted SVR with a positive value of 80 % (24/30) (Table 1). Assessment of the predictability of SVR by combinations of IRRDR mutation and age, the two most potent factors identified by multivariate analysis, revealed that IRRDR ≥6 and age <60 years predicted SVR with a positive value of 93.3 % (14/15) and that IRRDR ≤5 and age ≥60 years predicted non-SVR with a value of 84.0 % (21/25) (Table 3).

Positive predictive values of combinations of IL28B and platelets for NVR

Based on their significant correlation with NVR as demonstrated by multivariate analysis, combinations of IL28B genotype and platelets were examined for their positive predictive values for NVR. IL28B minor and platelets $<15 \times 10^4/mm^3$ predicted NVR with a positive value of 85.7 % (6/7). On the other hand, IL28B major and platelets $\geq 15 \times 10^4/mm^3$ predicted viral disappearance either transiently (relapse) or sustainably (SVR), referred to as response, with a value of 97.1 % (34/35) (Table 4).

Positive predictive values of combinations of IRRDR mutation and IL28B for SVR and non-NVR (response)

Significant correlation was observed between IRRDR and IL28B ($p = 0.003768$) (data not shown). The combination of IRRDR ≥6 and IL28B major predicted SVR with a positive value of 82.1 % (23/28), and predicted non-NVR (response) with a value of 92.9 % (26/28). On the other hand, IRRDR ≤5 and IL28B minor predicted non-SVR with a value of 87.5 % (14/16) (Table 5).

Positive predictive values of combinations of IRRDR mutation and HCV core aa 70 for SVR and non-NVR (response)

Combinations of IRRDR ≥6 and HCV core aa 70 wild predicted SVR with a positive value of 82.6 % (19/23), and predicted non-NVR (response) with a value of 91.3 %

Table 2 Multivariate analysis of factors associated with SVR, relapse, and NVR

Factor	Category	SVR		Relapse		NVR	
		Odds ratio (95 % CI)	*p* value	Odds ratio (95 % CI)	*p* value	Odds ratio (95 % CI)	*p* value
IRRDR mutations	≤5	1	**<0.0001**	1	**0.0008**	NA	NA
	≥6	11.906 (3.421–41.440)		0.070 (0.015–0.331)			
Age (years)	<60	1	**0.015**	1	**0.015**	NA	NA
	≥60	0.228 (0.069–0.749)		5.825 (1.415–23.980)			
HCV core aa 70	Wild	1	0.112	NA	NA	NA	NA
	Mutant	0.358 (0.101–1.270)					
IL28B genotype	Major	NA	NA	NA	NA	1	**0.0019**
	Minor					14.618 (2.699–79.173)	
Platelets ($\times 10^4/mm^3$)	<15	NA	NA	NA	NA	1	**0.0096**
	≥15					0.113(0.022–0.588)	
γ-GTP (U/L)	<80	NA	NA	NA	NA	NA	NA
	≥80						
Hemoglobin (g/dL)	<14	NA	NA	NA	NA	1	0.105
	≥14					0.274 (0.057–1.309)	

SVR sustained virological response, *NVR* null virological response, *95 % CI* 95 % confidence interval, *γ-GTP* gamma-glutamyl transpeptidase, *IRRDR* interferon/ribavirin resistance-determining region, *NA* not applicable

Table 3 Positive predictive values of combinations of IRRDR mutation and age for SVR

IRRDR mutations	Age (years)	SVR	Non-SVR	Odds ratio (95 % CI)	*p* value
≥6	**<60**	**14/15 (93.3 %)**	1/15 (6.7 %)	73.481 (7.418–727.850)	0.0002
≥6	≥60	10/15 (66.7 %)	5/15 (33.3 %)	10.500 (2.308–47.777)	0.0024
≤5	<60	9/20 (45.0 %)	11/20 (55.0 %)	4.295 (1.075–17.167)	0.0392
≤5	**≥60**	4/25 (16.0 %)	**21/25 (84.0 %)**	1	–

SVR sustained virological response, *IRRDR* interferon/ribavirin resistance-determining region, *95 % CI* 95 % confidence interval

Table 4 Positive predictive values of combinations of IL28B genotype and baseline platelets for NVR

IL28B genotype	Platelets ($\times 10^4/mm^3$)	NVR	Response	Odds ratio (95 % CI)	*p* value
Major	**≥15**	1/35 (2.8 %)	**34/35 (97.1 %)**	1	–
Major	<15	6/22 (27.3 %)	16/22 (72.7 %)	12.750 (1.414 to 114.931)	0.023
Minor	≥15	5/11 (45.5 %)	6/11 (54.5 %)	28.333 (2.796 to 287.103)	0.0047
Minor	**<15**	**6/7 (85.7 %)**	1/7 (14.3 %)	203.999 (11.174 to >999.999)	0.0003

NVR null virological response, *Response* non-NVR (SVR *plus* relapse), *95 % CI* 95 % confidence interval

(21/23). On the other hand, IRRDR ≤5 and HCV core aa 70 mutant predicted non-SVR with a value of 88.2 % (15/17) (Table 6).

Discussion

Host factors (such as age, sex, ethnicity, platelets, liver fibrosis, and obesity) and viral factors (genotype and viral load) have been associated with the outcome of PEG-IFN/RBV therapy [6]. To date, few studies have compared the impact of viral genetic polymorphisms, such as IRRDR, ISDR, and core mutations, and IL28B polymorphisms as host genetic factors on the clinical outcome of PEG-IFN/RBV therapy. Recently, viral genetic polymorphisms including double-wild in the core region, IRRDR ≥6, and ISDR ≥2 have been described as significant predictors of SVR to PEG-IFN/RBV therapy for 48 weeks [13, 19]. IL28B major genotype (TT) and core aa 70 substitutions are independent predictors of SVR, and IL28B minor genotype is an independent predictor of NVR [20]. Also, IL28B polymorphisms and mutations in the ISDR of HCV are significant pretreatment predictors of response to PEG-IFN/RBV therapy [21]. Nonetheless, IRRDR polymorphism, which is a potent viral determiner of SVR [11–13], was not examined in these studies.

In the present study we compared the impact of IRRDR, ISDR, and core mutations as viral genetic polymorphisms, and IL28B genotype as a host genetic factor, on the clinical outcome of PEG-IFN/RBV therapy—SVR, relapse, and NVR—for CHC-1b with a high viral load. IRRDR ≥6 was identified as a viral genetic polymorphism that independently predicted SVR to PEG-IFN/RBV treatment (Tables 1, 2). Moreover, IRRDR ≤5 was identified as a viral genetic polymorphism that most effectively predicted relapse, and IL28B minor genotype (TG or GG) was identified as a host genetic factor that most effectively predicted NVR.

On the other hand, ISDR ≥2 was not significantly associated with treatment outcome in the present cohort, although it is considered a viral determiner of SVR [19, 21]. ISDR was identified as a factor showing significant correlation with RVR ($p = 0.028$) by univariate analysis (data not shown). In other words, ISDR was a factor related to only early viral dynamics.

The C-terminal region of NS5A such as IRRDR is among the most variable sequences across the different genotypes and subtypes of HCV [22, 23]. The correlation observed between IRRDR heterogeneity and PEG-IFN/RBV responsiveness might be linked to experimental observations that an HCV subgenomic RNA replicon containing NS5A of HCV-1b exerts more profound inhibitory effects on IFN activities than does its original HCV-2a replicon, and that domain swapping of a C-terminal region of NS5A including IRRDR results in a transfer of their anti-IFN activities [24]. Moreover, the C-terminal region of NS5A has been implicated as playing important roles in viral replication and particle formation [25, 26]. These clinical and experimental data thus support our hypothesis that IRRDR is involved, at least partly, in the viral strategy of evading IFN-mediated antiviral host defense mechanisms. Similarly, the aa substitutions in the core region are associated with proteins involved in resistance to IFN monotherapy, such as SOCS, which are

Table 5 Positive predictive values of combinations of IRRDR mutation and IL28B genotype for SVR and non-NVR (response)

IRRDR mutations	IL28B genotype	SVR	Non-SVR	SVR versus non-SVR		NVR	Response	NVR versus response	
				Odds ratio (95 % CI)	*p* value			Odds ratio (95 % CI)	*p* value
≥6	**Major**	**23/28 (82.1 %)**	5/28 (17.9 %)	32.200 (5.489–188.909)	0.0001	2/28 (7.1 %)	**26/28 (92.9 %)**	1	–
≥6	Minor	1/2 (50.0 %)	1/2 (50.0 %)	7.000 (0.302–162.202)	0.225	1/2 (50.0 %)	1/2 (50.0 %)	13.000 (0.572–295.204)	0.107
≤5	Major	11/29 (37.9 %)	18/29 (62.1 %)	4.278 (0.813–22.513)	0.0863	5/29 (17.2 %)	24/29 (82.8 %)	2.708 (0.480–15.294)	0.259
≤5	Minor	2/16 (12.5 %)	**14/16 (87.5 %)**	1	–	10/16 (62.5 %)	6/16 (37.5 %)	21.667 (3.733–125.766)	0.0006

SVR sustained virological response, *NVR* null virological response, *Response* non-NVR (SVR *plus* relapse), *IRRDR* interferon/ribavirin resistance-determining region, *95 % CI* 95 % confidence interval

Table 6 Positive predictive values of combinations of IRRDR mutation and HCV core aa 70 for SVR and non-NVR (response)

IRRDR mutations	HCV core aa 70	SVR	Non-SVR	SVR versus non-SVR		NVR	Response	NVR versus response	
				Odds ratio (95 % CI)	*p* value			Odds ratio (95 % CI)	*p* value
≥6	**Wild**	**19/23 (82.6 %)**	4/23 (17.4 %)	35.625 (5.730–221.504)	0.0001	2/23 (8.7 %)	**21/23 (91.3 %)**	1	–
≥6	Mutant	5/7 (71.4 %)	2/7 (28.6 %)	18.750 (2.065–170.214)	0.0092	1/7 (14.3 %)	6/7 (85.7 %)	1.750 (0.134–22.778)	0.669
≤5	Wild	11/28 (39.3 %)	17/28 (60.7 %)	4.853 (0.924–25.496)	0.062	7/28 (25.0 %)	21/28 (75.0 %)	3.500 (0.650–18.852)	0.145
≤5	**Mutant**	2/17 (11.8 %)	**15/17 (88.2 %)**	1	–	8/17 (47.1 %)	9/17 (52.9 %)	9.333 (1.6346–52.917)	0.012

SVR sustained virological response, *NVR* null virological response, *Response* non-NVR (SVR *plus* relapse), *IRRDR* interferon/ribavirin resistance-determining region, *95 % CI* 95 % confidence interval

known to inhibit IFN-α-induced activation of the Jak-STAT pathway and the expression of the antiviral proteins 2′,5′-OAS and MxA [27].

The IL28B gene encodes a cytokine distantly related to type I (α and β) IFN and to the IL10 family. IL28B, IL28A, and IL29 are three closely related cytokine genes that encode proteins known as type III IFN (IFN-λs) and form a cytokine gene cluster at chromosomal region 19q13 [28]. The three cytokines IFN-λ1, -λ2, and -λ3 are induced by viral infection and have antiviral activities [29, 30]: IFN-λ induces a steady increase in the expression of a subset of IFN-stimulated genes, whereas IFN-α induces the same genes with more rapid and transient kinetics [31].

In the present study, the prediction of response to PEG-IFN/RBV combination therapy based on these concurrent factors was highly positive: SVR was positively predicted in 93.3 % of patients with IRRDR ≥6 and age <60 years (Table 3), in 82.1 % of those with IRRDR ≥6 and IL28B major (Table 5), and in 82.6 % of those with IRRDR ≥6 and HCV core aa 70 wild (Table 6). Relapse was positively predicted in 73.3 % of patients with IRRDR ≤5 and age ≥60 years, and in 77.8 % of those with IRRDR ≤5 and HCV core aa 70 mutant (data not shown). NVR was positively predicted in 85.7 % of patients with IL28B minor and platelets $<15 \times 10^4/mm^3$ (Table 4). On the basis of these observations, new therapeutic strategies could be designed for treating chronic HCV-1b infection: patients predicted to achieve an SVR would be most eligible for standard PEG-IFN/RBV therapy for 48 weeks, those predicted to relapse could be advised to adopt an extended 72-week therapy instead of the 48-week standard therapy [30], and those predicted to have NVR could be advised to wait for a future therapy such as a combination of protease inhibitors [32, 33].

In conclusion, viral genetic polymorphisms in IRRDR (≥6 or ≤5 mutations) and HCV core aa 70 (wild or mutant), host factors such as IL28B genotype (major or minor), age (<60 or ≥60 years), and platelet counts ($\geq 15 \times 10^4/mm^3$ or less), and combinations of these factors could be used to design therapeutic strategies for patients infected with HCV-1b with high viral loads. Further prospective study is needed to verify this hypothesis.

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Conflict of interest None of the authors has any conflict of interest.

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Sequence Heterogeneity in NS5A of Hepatitis C Virus Genotypes 2a and 2b and Clinical Outcome of Pegylated-Interferon/Ribavirin Therapy

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Abstract

Pegylated-interferon plus ribavirin (PEG-IFN/RBV) therapy is a current standard treatment for chronic hepatitis C. We previously reported that the viral sequence heterogeneity of part of NS5A, referred to as the IFN/RBV resistance-determining region (IRRDR), and a mutation at position 70 of the core protein of hepatitis C virus genotype 1b (HCV-1b) are significantly correlated with the outcome of PEG-IFN/RBV treatment. Here, we aimed to investigate the impact of viral genetic variations within the NS5A and core regions of other genotypes, HCV-2a and HCV-2b, on PEG-IFN/RBV treatment outcome. Pretreatment sequences of NS5A and core regions were analyzed in 112 patients infected with HCV-2a or HCV-2b, who were treated with PEG-IFN/RBV for 24 weeks and followed up for another 24 weeks. The results demonstrated that HCV-2a isolates with 4 or more mutations in IRRDR (IRRDR[2a]≥4) was significantly associated with rapid virological response at week 4 (RVR) and sustained virological response (SVR). Also, another region of NS5A that corresponds to part of the IFN sensitivity-determining region (ISDR) plus its carboxy-flanking region, which we referred to as ISDR/+C[2a], was significantly associated with SVR in patients infected with HCV-2a. Multivariate analysis revealed that IRRDR[2a]≥4 was the only independent predictive factor for SVR. As for HCV-2b infection, an N-terminal half of IRRDR having two or more mutations (IRRDR[2b]/N≥2) was significantly associated with RVR, but not with SVR. No significant correlation was observed between core protein polymorphism and PEG-IFN/RBV treatment outcome in HCV-2a or HCV-2b infection. ***Conclusion:*** The present results suggest that sequence heterogeneity of NS5A of HCV-2a (IRRDR[2a]≥4 and ISDR/+C[2a]), and that of HCV-2b (IRRDR[2b]/N≥2) to a lesser extent, is involved in determining the viral sensitivity to PEG-IFN/RBV therapy.

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Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease, such as chronic hepatitis, liver cirrhosis and hepatocellular carcinoma, with180 million people being currently infected with HCV worldwide. It is estimated that 70% of acute infections become persistent [1]. As a consequence of the long-term persistence of HCV infection, the number of patients with hepatocellular carcinoma is expected to increase further over the next 20 years. More than two decades have passed since the discovery of HCV, and yet therapeutic options remain limited. Standard regimens for treatment of chronic hepatitis C include pegylated interferon alpha (PEG-IFN) and ribavirin (RBV) [2]. In addition, two protease inhibitors (telaprevir and boceprevir) were approved in May 2011 by the U. S. Food and Drug Administration (FDA) for clinical use in combination with PEG-IFN/RBV to treat chronic hepatitis C patients with HCV genotype 1 [3,4].

In Japan, about 70% of HCV-infected patients are infected with HCV genotype 1b (HCV-1b) and most of the remaining patients are infected with HCV-2a (25%) or HCV-2b (5%) [5]. When treated with PEG-IFN/RBV, the sustained virological response (SVR) rate is ca. 50% in HCV-1b infection, and ca. 80% in HCV-2a and -2b infections [2,6]. The mechanism(s) underlying the different responses among patients with different HCV genotypes and subtypes is still unclear. However, this suggests that viral genetic heterogeneity could affect, at least to some extent, the sensitivity to IFN-based therapy. In this context, sequence heterogeneity of the viral NS5A protein has been widely discussed for its correlation with IFN responsiveness. Sequence variations within a region in NS5A of HCV-1b defined as the IFN sensitivity-determining region (ISDR) is correlated with IFN responsiveness [7]. In HCV-2a infection, the influence of sequence heterogeneity in and around a region corresponding to ISDR on the IFN responsiveness was also suggested [8–10]. Recently, we identified a

new region near the C-terminus of NS5A of HCV-1b, which we refer to as the IFN/RBV resistance-determining region (IRRDR) [11,12]. The degree of sequence variation within IRRDR was significantly correlated with the clinical outcome of PEG-IFN/RBV combination therapy. The significance of IRRDR of other HCV genotypes, however, has not been investigated yet.

In addition to the NS5A sequence variation, HCV core protein polymorphism was also proposed as a pretreatment predictor of poor virological response in HCV-1b-infected patients treated with PEG-IFN/RBV therapy [13]. It is not clear at this stage whether core protein polymorphism could be used to predict the treatment outcome in HCV-2a and -2b infections. In the present study, we investigated the impact of viral genetic heterogeneity in the NS5A and core regions of HCV-2a and -2b on PEG-IFN/RBV treatment outcome. To the best of our knowledge, this is the first report describing the possible correlation between PEG-IFN/RBV responsiveness and NS5A-IRRDR heterogeneity of HCV-2a and -2b.

Materials and Methods

Ethics statement

The study protocol, which conforms to the provisions of the Declaration of Helsinki, was approved beforehand by the Ethic Committees in Kobe Asahi Hospital and Kobe University, and written informed consent was obtained from each patient prior to the treatment.

Patients

A total of 112 patients seen at Kobe Asahi Hospital and Kobe University Hospital, Kobe, Japan, who were chronically infected with HCV-2a (61 patients) or HCV-2b (51 patients), were enrolled in the study. HCV subtype was determined according to the method of Okamoto et al. [14]. The patients were treated with PEG-IFN α-2b (Pegintron®; Schering-Plough, Kenilworth, NJ) (1.5 μg per kilogram body weight, once weekly, subcutaneously) and RBV (Rebetol®; Schering-Plough) (600~800 mg daily, per os), for 24 weeks according to a standard treatment protocol for Japanese patients established by a hepatitis study group of the Ministry of Health, Labour and Welfare, Japan. All patients received >80% of scheduled dosage of PEG-IFN and RBV. Serum samples were collected from the patients at intervals of 4 weeks before, during and after the treatment, and tested for HCV RNA and core antigen titers as reported previously [15].

Sequence analysis of the NS5A and core regions

HCV RNA was extracted from 140 μl of serum using a commercially available kit (QIAmp viral RNA kit; QIAGEN, Tokyo, Japan). The extracted RNA was reverse transcribed and amplified for NS5A and core regions using Super script III one step RT-PCR platinum Taq HiFi (Invitrogen, Tokyo, Japan). The resultant RT-PCR product was subjected to a second-round PCR by using Platinum Taq DNA polymerase high fidelity III (Invitrogen). Primers used for amplification of full-length NS5A of the HCV-2a and -2b genomes and those of the core region of HCV-2a were reported previously [16,17]. Primers for amplification of the core region of HCV-2b are as follows: C-2b/1 (5′-AGCCATAGTGGTCTGCGGAACC-3′; sense, nucleotides [nt] 136 to 157) and C-2b/4 (5′-GGAACARTTGCACTCTTGG-GTG-3′; antisense, nt 1241 to 1262) for one step RT-PCR; C-2b/2 (5′-CCACTCTATGTCCGGTCATTTGG-3′; sense, nt 208 to 230) and C-2b/3 (5′-GAGCTGCCAGGTGATGCTG-3′; antisense, nt 971 to 989) for the second round PCR. RT was performed at 45°C for 30 min and terminated at 94°C for 2 min, followed by the first-round PCR over 35 cycles, with each cycle consisting of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 68°C for 90 sec. The second-round PCR was performed under the same condition. The sequences of the amplified fragments were determined by direct sequencing without subcloning. The amino acid (aa) sequences were deduced and aligned using GENETYX Win software version 7.0 (GENETYX Corp., Tokyo, Japan). The numbering of aa residues for HCV-2a and -2b isolates is according to the polyprotein of HCV-J6 [18] and -J8 [19], respectively.

Statistical analysis

Numerical data were analyzed by *S*tudent's *t* test while categorical data by Fisher's exact probability test [8]. To evaluate the optimal threshold of the number of aa mutations in ISDR and IRRDR for prediction of treatment outcomes, the receiver operating characteristic curve was constructed. Univariate and multivariate logistic regression analyses were performed to identify independent predictors for treatment outcomes. All statistical analyses were performed using the SPSS version 16 software (SPSS Inc., Chicago, IL). Unless otherwise stated, a P value of <0.05 was considered statistically significant.

Nucleotide sequence accession numbers

The sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB600751 through AB600834.

Results

Patients' Responses to PEG-IFN/RBV Combination Therapy in HCV-2a and HCV-2b infections

Of the 61 patients infected with HCV-2a, 46 (75%) patients cleared HCV viremia by week 4 (rapid virological response [RVR]), and all the patients (100%) by week 12 (early virological response [EVR]) and at week 24 (end-of-treatment response [ETR]) (Table 1). Likewise, of 51 patients infected with HCV-2b, 34 (67%), 51 (100%) and 50 (98%) patients achieved RVR, EVR and ETR, respectively. After the end of treatment, 105 patients (58 with HCV-2a and 47 with HCV-2b) could be followed up for another 24 weeks. At the end, SVR was achieved by 49 (84%) patients infected with HCV-2a and by 34 (72%) patients with HCV-2b. Only 9 (16%) and 13 (28%) patients with HCV-2a and -2b, respectively, were non-SVR. There was no case of null-response (continuous viremia throughout the treatment and follow up periods) since all the non-SVR patients once cleared viremia at a certain time point followed by a rebound in viremia either before or after the end of the treatment (relapse).

Comparison of the base line demographic characteristics between SVR and non-SVR patients revealed that, in HCV-2a infection, SVR patients had a significantly lower average age than that of non-SVR (Table 2). In HCV-2b infection, on the other hand, SVR patients had significantly γ-GTP levels than those of non-SVR. There was no significant difference in viremia titers between SVR and non-SVR in patients infected with HCV-2a or -2b.

Sequence Analysis of NS5A of HCV-2a and HCV-2b

The entire NS5A region of the HCV-2a and -2b genomes in pretreatment sera were sequenced, and aa sequences deduced. All the sequences obtained were aligned and the consensus sequences for HCV-2a and -2b were inferred. An N-terminal half (aa 1977 to 2196) of the consensus sequences of HCV-2a and -2b isolates were each identical to the prototype sequences, HCV-J6 [18] and

Table 1. Proportions of various virological responses of HCV-2a- and HCV-2b-infected patients treated with PEG-IFN/RBV.

Response	Proportion		
	HCV-2a	HCV-2b	All
RVR	46/61* (75%)	34/51 (67%)	80/112 (71%)
Non-RVR	15/61 (25%)	17/51 (33%)	32/112 (29%)
EVR	61/61 (100%)	51/51 (100%)	112/112 (100%)
ETR	61/61 (100%)	50/51 (98%)	111/112 (99%)
SVR	49/58 (84%)	34/47 (72%)	83/105 (79%)
Non-SVR	9/58 (16%)	13/47 (28%)	22/105 (21%)

*No. of patients/no. of total.
Abbreviations: RVR, rapid virological response; EVR, early virological response; ETR, end-of-treatment response; SVR, sustained virological response.
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HCV-J8 [19], respectively. The remaining C-terminal half (aa 2197 to 2442) of the consensus sequences were identical to those reported by Murakami et al. [8] except that His at position 2358 in the HCV-2b sequence was replaced with Cys, which was more conserved (59% of the isolates tested) than His (22%).

To investigate the impact of NS5A heterogeneity on the clinical outcome of PEG-IFN/RBV therapy, we first performed a sliding window analysis with a window size of 20 residues over the full-length NS5A sequences obtained from 23 RVR and 7 non-RVR patients infected with HCV-2a along with the consensus sequence, as described previously [8]. This analysis revealed that the number of aa mutations differed significantly between RVR and non-RVR isolates in two regions within the C-terminal half of NS5A (data not shown). The more C-terminally located one exactly matched the region that corresponded to IRRDR of HCV-1b, ranging from aa 2332 to 2387, thus being referred to as IRRDR[2a] (see Figure 1). The other region composed of a part of ISDR plus its carboxy-flanking region, ranging from aa 2232 to 2262, thus being referred to as ISDR/+C[2a] (see Figure 2). It was confirmed that the average numbers of aa mutations in IRRDR[2a] and ISDR/+C[2a] were each significantly larger in isolates from RVR than those from non-RVR patients (Table 3). More importantly, the average numbers of aa mutations in IRRDR[2a] and ISDR/+C[2a] were each significantly larger in SVR than in non-SVR. Sequences of IRRDR[2a] and ISDR/+C[2a] obtained from SVR and non-SVR patients and the number of mutations of each isolate are shown in Figures 1 and 2.

Likewise, a sliding window analysis on HCV-2b isolates (16 RVR and 6 non-RVR) identified an N-terminal part of IRRDR (aa 2332 to 2357), referred to as IRRDR/N[2b], that showed a significant difference in the number of aa mutations between RVR and non-RVR (data not shown). The average numbers of aa mutations in IRRDR/N[2b] were significantly larger in RVR than in non-RVR (Table 3). However, they did not differ significantly between SVR and non-SVR. Sequences of IRRDR[2b]/N obtained from RVR and non-RVR patients are shown in Figure 3.

Correlation between NS5A Sequence Heterogeneity and SVR or RVR in HCV-2a and HCV-2b infections

The receiver operating characteristic analysis identified the optimal thresholds of the numbers of aa mutations in IRRDR[2a] and ISDR/+C[2a] for the prediction of RVR and SVR in HCV-2a infection; four and one for IRRDR[2a] and ISDR/+C[2a], respectively (data not shown). Accordingly, we found that 86% (42/49) of SVR patients, and only 22% (2/9) of non-SVR, were infected with HCV-2a isolates having IRRDR with 4 or more mutations (IRRDR[2a]≥4) (Table 4). On the other hand, 14% (7/49) of SVR, and 78% (7/9) of non-SVR patients, were infected with isolates having IRRDR with 3 or less mutations (IRRDR[2a]≤3). These results suggested that IRRDR[2a]≥4 was significantly associated with SVR ($P=0.0003$). Similarly, 93% (42/46) of RVR patients, and only 33% (5/15) of non-RVR, were infected with HCV-2a isolates of IRRDR[2a]≥4 while 7% (4/46) of RVR patients, and 67% (10/15) of non-RVR, were infected with HCV-2a isolates of IRRDR[2a]≤3, with the results suggesting that IRRDR[2a]≥4 was significantly associated with RVR as well ($P<0.0001$).

As for ISDR/+C[2a] heterogeneity, 71% (35/49) of SVR, and 22% (2/9) of the non-SVR patients, were infected with HCV-2a isolates with ISDR/+C having one or more mutation (ISDR/+C[2a]≥1) (Table 4). On the other hand, 29% (14/49) of SVR patients, and 78% (7/9) of the non-SVR, were infected with isolates with ISDR/+C without mutation (ISDR/+C[2a] = 0). Thus, ISDR/+C[2a]≥1 was significantly associated with SVR ($P=0.008$).

Table 2. Demographic characteristics of HCV-2a- and HCV-2b-infected patients with SVR and non-SVR.

Factor	HCV-2a			HCV-2b		
	SVR	Non-SVR	*P* value	SVR	Non-SVR	*P* value
Age	49.78±13.67*	62.89±7.01	**0.007**	50.03±15.03	55.08±11.22	0.28
Sex (male/female)	22/27	3/6	0.72	17/17	8/5	0.53
Body weight (kg)	60.39±11.00	54.67±10.51	0.15	57.72±13.46	65.08±7.26	0.06
Platelets ($\times10^4$/mm^3)	18.54±5.71	19.43±10.78	0.72	17.57±5.65	15.20±7.281	0.27
Hemoglobin (g/dl)	14.38±6.07	14.0±1.56	0.88	14.19±1.59	13.78±1.5	0.49
γ-GTP (IU/L)	37.66±53.25	36.83±24.82	0.97	39.68±34.33	81.30±69.11	**0.02**
ALT (IU/L)	64.75±52.45	94.38±141.3	0.28	86.35±91.95	86.85±118.7	0.98
HCV-RNA (KIU/ml)	1350±1424	1598±1464	0.63	5543±7643	7905±14210	0.47
HCV core antigen (fmol/L)	6543±6927	6105±8290	0.91	9054±6743	9390±8723	0.92

*Mean ± S.D.
Abbreviations: SVR, sustained virological response; γ-GTP, gamma glutamyl transpeptidase; ALT, alanine aminotransferase.
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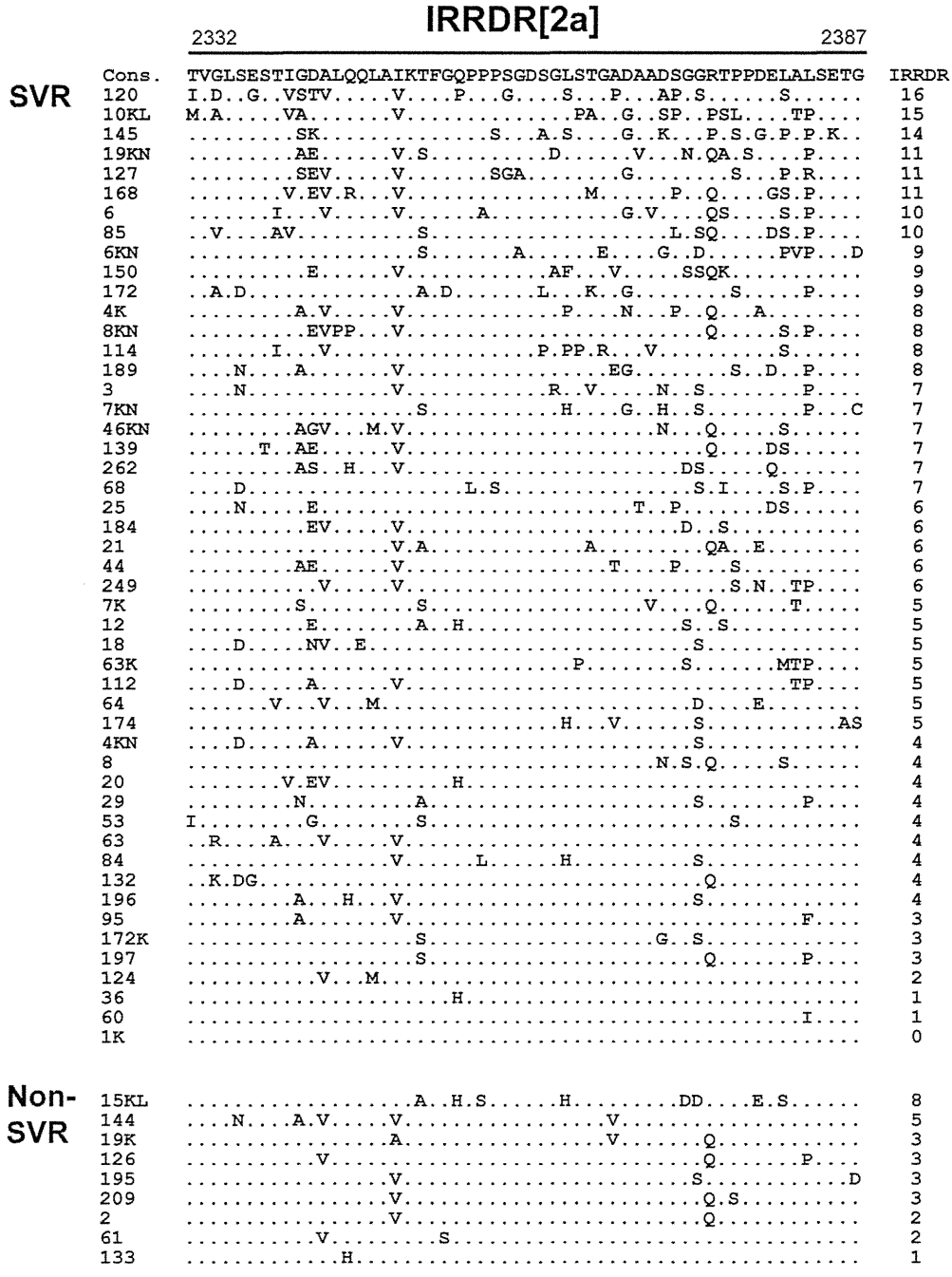

Figure 1. Sequence alignment of IRRDR[2a]. Sequences of IRRDR[2a] (interferon/ribavirin resistance-determining region of HCV-2a) obtained from SVR and non-SVR patients are aligned. The consensus sequence (Cons) is shown on the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the Cons sequence. The numbers of the mutations in IRRDR[2a] are shown on the right.
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As for HCV-2b infection, the receiver operating characteristic analysis identified "two" as the optimal threshold of the number of mutations in IRRDR/N[2b] by which to predict RVR (data not shown). Accordingly, we found that 65% (22/34) of RVR, and 18% (3/17) of non-RVR patients, were infected with HCV-2b isolates of IRRDR/N[2b]≥2 (Table 4). On the other hand, 35% (12/34) of RVR, and 82% (14/17) of the non-RVR patients, were infected with IRRDR/N[2b]≤1. These results suggested that IRRDR/N[2b]≥2 was significantly associated with RVR ($P = 0.0025$). However, no correlation, or even no tendency toward significant correlation, was observed between IRRDR/N[2b]≥2 and SVR in HCV-2b infection.

Correlation between NS5A Sequence Heterogeneity and Viremia Titers in the Serum of patients infected with HCV-2a and HCV-2b before PEG-IFN/RBV Therapy

Next, we examined the impact of IRRDR sequence heterogeneity on HCV titers in the serum before the initiation of the treatment. As shown in Figure 4A, patients infected with IRRDR[2a]≥4 had significantly lower pretreatment serum

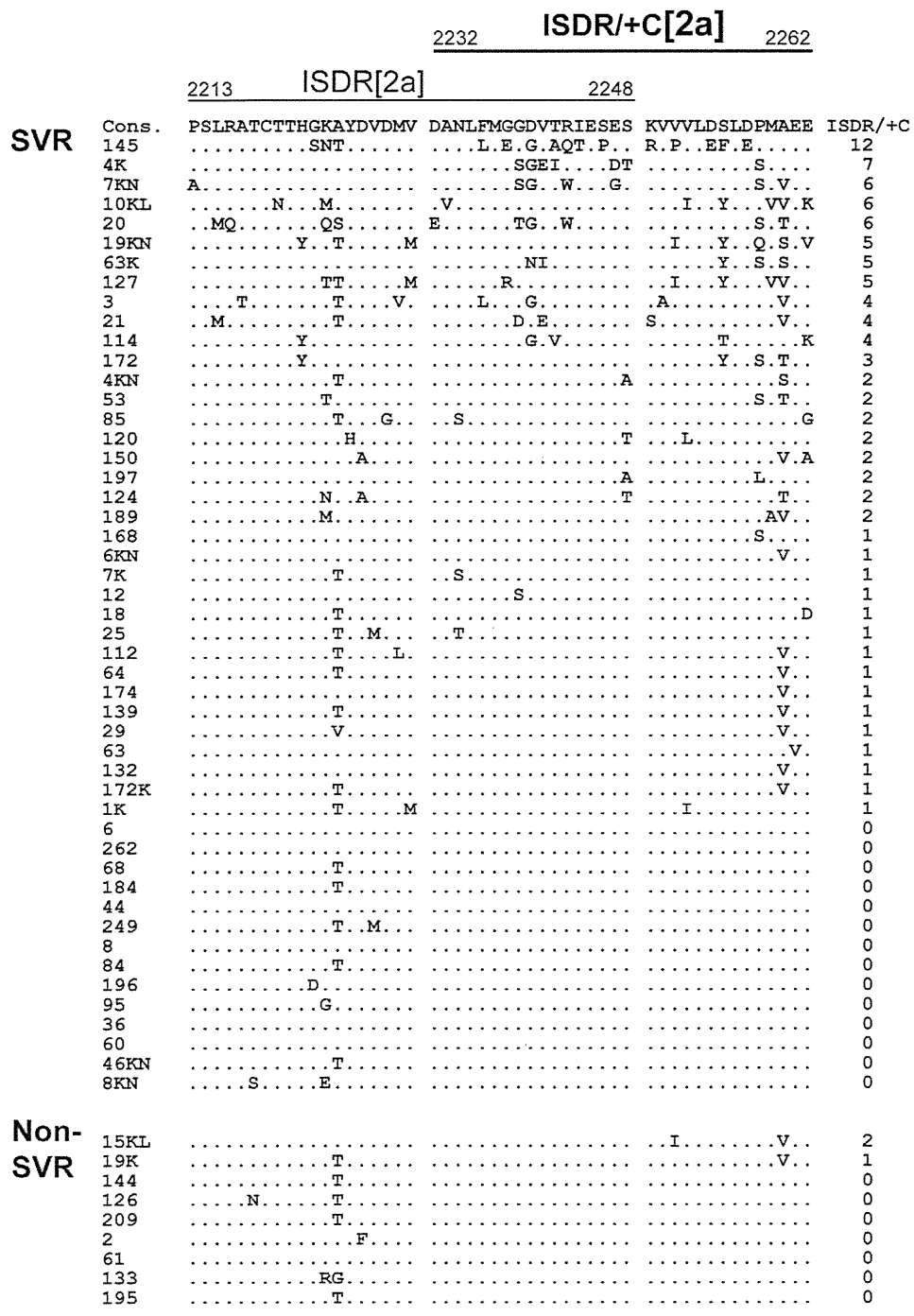

Figure 2. Sequence alignment of ISDR/+C[2a]. Sequences of ISDR/+C[2a] (part of interferon sensitivity determining-region plus its carboxy-flanking region of HCV-2a) obtained from SVR and non-SVR patients are aligned. The consensus sequence (Cons) is shown on the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the Cons sequence. The numbers of the mutations in ISDR/+C[2a] are shown on the right.
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HCV core antigen titers than those infected with IRRDR[2a]≤3. On the other hand, there was no significant difference in HCV viremia titers between ISDR/+C[2a]≥1 and ISDR/+C[2a] = 0 (Figure 4B). Also, in HCV-2b infection, there was no significant difference in pretreatment HCV viremia titers between IRRDR/N[2b]≥2 and IRRDR/N[2b]≤1 (Figure 4C).

Correlation between Core Protein Sequence Heterogeneity and RVR or SVR

A close correlation between core protein sequence patterns and treatment outcome has been proposed in HCV-1b infection [12,13]. To examine this hypothesis in HCV-2a and -2b infections, core regions of the virus genome were amplified from the pretreated sera, and the aa sequences deduced and aligned

Table 3. Average numbers of aa mutations within IRRDR[2a], ISDR/+C[2a] and IRRDR/N[2b] of HCV NS5A obtained form pre-treated sera of HCV-2a and -2b-infected patients with SVR, non-SVR, RVR and non-RVR.

NS5A region	No. of mutations			No. of mutations		
	SVR	Non-SVR	*P* value	RVR	Non-RVR	*P* value
IRRDR[2a] (aa 2332–2387)	6.4±3.4*	3.3±2.1	**0.01**	6.8±3.3	3.3±1.9	0.0003
ISDR/+C[2a] (aa 2232–2262)	2.0±2.4	0.3±0.7	**0.047**	2.1±2.5	0.6±0.7	0.025
IRRDR/N[2b] (aa 2332–2357)	1.8±1.5	1.4±1.3	0.45	2.0±1.4	1.0±1.2	0.01

*Mean ± S.D.

Abbreviations: SVR, sustained virological response; RVR, rapid virological response; IRRDR[2a], interferon/ribavirin resistance-determining region of HCV-2a; ISDR/+C[2a], part of interferon sensitivity determining-region plus its carboxy-flanking region of HCV-2a; IRRDR/N[2b], an N-terminal part of interferon/ribavirin resistance-determining region of HCV-2b.

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IRRDR[2b] 2387

2332 **IRRDR/N[2b]** 2357

	Cons.	AKVLTQDNVEGVLREMADKVLSPLQD	CNDSGHSTGVDTGGDSVQQPSDETAASEAG	IRRDR/N(2b)	Final response
RVR	73	.....S...I.K.....P..	S....R.....NI...P.....	4	SVR
	138	.....FK...G..F.....	H....R...A.....S.	4	SVR
	143	T.....D...R...S...	H.....AEA.....TA	4	SVR
	166	.R.....K.....SF..	Y.....A.....D..	4	Non-SVR
	183	.....T...I.K.....P..	S....R.....NI..L...A.V....	4	SVR
	231	.....FN.PR.	.....A.....S.D..	4	SVR
	2KL	.....KE.....P..	.....A.....T....	3	SVR
	7	.R.....I.....A.....	.....L...A.....D..	3	SVR
	21KN	.....I....VE.....	HT.....A.....	3	SVR
	116	.....GE..K.....	.....T.	3	SVR
	185	.....I.....I..E.	.....A.....D..	3	Non-SVR
	193	.....E...G.....F.....	.....A.....T.	3	SVR
	205	.....G....F.....F.....	Q.....A.....	3	Non-SVR
	233	.T.....R...K.....	.....T.	3	SVR
	4	V.....S.....	.Q.....T.	2	Non-SVR
	5KN	.R.....K.....	H.....A.....	2	SVR
	9KN	.....K.P..	N....L...AE.....D..	2	SVR
	94	.....F..P..	.....A.....T.	2	SVR
	106K	.....V.....G	.D...R.....	2	SVR
	212	.....E.....A.....	.....D..	2	SVR
	147	.R.....K.....	H.....A.....	2	Unknown
	229	.R.....I....	H.....E.....	2	Non-SVR
	11KL	.....I.....	.H.....M.....V..T.	1	SVR
	55KN	.R.....	H.....A.....A.....	1	SVR
	87	.....K.....	.....T....	1	SVR
	103	..I.....	.....AD.....	1	SVR
	165	.....F.....	H.....A.....S.	1	SVR
	1K	.....	.....A..A....T...T.	0	SVR
	10K	.....	.....I.L.....	0	SVR
	46	.....	.....	0	SVR
	99	.....	.....S..T.	0	SVR
	113	.....	.....T...T.	0	SVR
	179	.....	H.....A....N.A.....T...S.	0	SVR
	187	.....	Y.....T..T.	0	SVR
Non-RVR	18KN	.....G.....F.LP.E	.D.....T.	5	SVR
	13K	.....E...K.....	.D.....E....K.....	2	Non-SVR
	110	.....H...E.....	.H.....N.I..A.....T.	2	Unknown
	11K	.....N.....	.....C...A.....C.....G..T.	1	SVR
	23KN	.....E.....	.D.....M.....A.....S.	1	Non-SVR
	40KN	.....P..	HS...R.....D.E	1	SVR
	89	.....F.....	.....TE	1	Non-SVR
	157	.....N.....	YS.....P.....E	1	SVR
	164	.R.....	S.....I.....D.....T.	1	SVR
	170	.....E.....	SD.....N....I.....T.	1	Unknown
	265KL	.....I.....	.....E.	1	Non-SVR
	1	.....	YH.....A.....I...P.....	0	Non-SVR
	32KN	.....	.....G....	0	Non-SVR
	52	.....	H.....A.....T..T.	0	SVR
	105	.....	Y.....	0	Non-SVR
	107	.....	.....R.....	0	Non-SVR
	134	.....	.....G..T.	0	Unknown

Figure 3. Sequence alignment of NS5A of HCV-2b isolates. Sequences of IRRDR/N[2b] (an N-terminal part of interferon/ribavirin resistance-determining region of HCV-2b) obtained from RVR and non-RVR patients are aligned. The consensus sequence (Cons) is shown on the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the Cons sequence. The numbers of the mutations in IRRDR/N[2b] and the final treatment outcome of each patient are shown on the right.

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Table 4. Correlation between NS5A sequence heterogeneity and SVR or RVR in HCV-2a and HCV-2b infections.

Factor	SVR	Non-SVR	*P* value	RVR	Non-RVR	*P* value
IRRDR[2a]≥4	42/49* (86%)	2/9 (22%)	**0.0003**	42/46 (93%)	5/15 (33%)	<0.0001
IRRDR[2a]≤3	7/49 (14%)	7/9 (78%)		4/46 (7%)	10/15 (67%)	
ISDR/+C[2a]≥1	35/49 (71%)	2/9 (22%)	**0.008**	32/46 (70%)	7/15 (47%)	0.1
ISDR/+C[2a] = 0	14/49 (29%)	7/9 (78%)		14/46 (30%)	8/15 (53%)	
IRRDR/N[2b]≥2	17/34 (50%)	6/13 (46%)	1.0	22/34 (65%)	3/17 (18%)	0.0025
IRRDR/N[2b]≤1	17/34 (50%)	7/13 (54%)		12/34 (35%)	14/17 (82%)	

*No. of isolates with a given factor/total no. of SVR or RVR.
Abbreviations: SVR, sustained virological response; RVR, rapid virological response; IRRDR[2a], interferon/ribavirin resistance-determining region of HCV-2a; ISDR/+C[2a], part of interferon sensitivity determining-region plus its carboxy-flanking region of HCV-2a; IRRDR/N[2b], an N-terminal part of interferon/ribavirin resistance-determining region of HCV-2b.
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with the prototype sequences (HCV-J6 [18] and HCV-J8 [19]). The residues at positions 70 and 91, which were reported to be associated with the treatment outcome in HCV-1b infection [13], were both well conserved among HCV-2a and -2b isolates and, therefore, no correlation with treatment outcome was expected for these residues (Figures S1 and S2). In this connection, the residues at positions 48 and 110 of HCV-2a isolates showed certain degrees of variation. However, there was no significant correlation between the sequence patterns and the treatment outcome.

Identification of Independent Predictive Factors for SVR and RVR in HCV-2a and HCV-2b infections

In order to identify significant independent predictors of SVR in HCV-2a and HCV-2b infections, univariate and multivariate logistic regression analyses were carried out using all available data of baseline patients' parameters and viral genetic polymorphic factors. Univariate analysis identified 3 factors that were significantly associated with SVR in HCV-2a infection; the heterogeneity of IRRDR[2a] (≥4 vs. ≤3), ISDR/+C[2a] (≥1 vs. = 0) and patients' age (<55 years) (Table 5). Subsequently, these factors were entered in multivariate regression analysis. The result obtained revealed that the IRRDR[2a] heterogeneity was the only independent predictive factor for SVR in HCV-2a infection ($P = 0.001$). The IRRDR[2a] heterogeneity was also the independent predictive factor for RVR (Table S1).

As for HCV-2b infection, univariate analysis identified two host factors that were significantly, or almost significantly, associated with SVR; γ-GTP levels (<30 IU/L) and body weight (<65 kg) (Table 5). No viral factor was identified in this analysis. In subsequent multivariate analysis, γ-GTP levels was identified as an independent predictive factor for SVR in HCV-2b infection. In this connection, the heterogeneity of IRRDR/N[2b], a viral factor, was identified to be significantly associated with RVR in HCV-2b infection (Table S1).

Discussion

The clinical outcome of PEG-IFN/RBV therapy for HCV infection is influenced by a number of host and viral factors [20]. It has recently been reported that host genetic polymorphisms near or within the IL28B gene on the chromosome 19 show a critical impact on the treatment outcome of patients infected with HCV-1a and -1b [21–23]. Also, HCV genetic polymorphisms have been known to contribute to differences in the treatment outcome, as demonstrated by the observations that SVR rates for patients infected with HCV genotypes 2 and 3 are higher than those for patients infected with HCV genotype 1 [2,6]. Moreover,

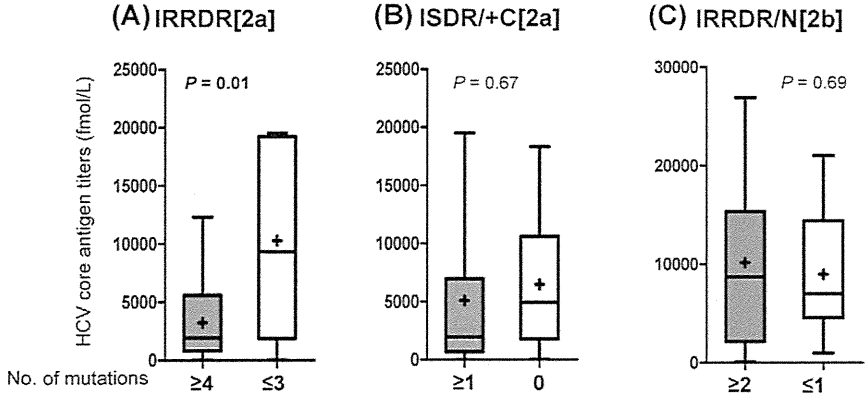

Figure 4. Correlation between NS5A sequence heterogeneity and pretreatment serum HCV core antigen titers in HCV-2a and HCV-2b infections. Pretreatment serum HCV core antigen titers of patients classified on the basis of the number of mutations in IRRDR[2a] (interferon/ribavirin resistance-determining region of HCV-2a) (≥4 vs. ≤3) (A), ISDR/+C[2a] (part of interferon sensitivity determining-region plus its carboxy-flanking region of HCV-2a) (≥1 vs. = 0) (B) and IRRDR/N[2b] (≥2 vs. ≤1) (an N-terminal part of interferon/ribavirin resistance-determining region of HCV-2b) (C) are depicted. Maximum and minimum values are indicated by the upper and lower bars, respectively. Distribution ranges are displayed as boxes. Mean and median values are also indicated inside the boxes as+and horizontal bars, respectively.
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Table 5. Univariate and multivariate analyses for identification of independent predictive factors for SVR in HCV-2a- and -2b-infected patients treated with PEG-IFN/RBV therapy.

Genotype	Variable	Univariate		Multivariate	
		Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
HCV-2a	IRRDR[2a] mutations	21.0 (3.6–122.5)	0.0003	21.0 (3.6–122.5)	**0.001**
	ISDR/+C[2a] mutations	8.8 (1.6–47.4)	0.008		
	Age (<55 years)	9.8 (1.1–84.7)	0.026		
HCV-2b	γ-GTP (<30 IU/L)	26.0 (1.3–504.7)	0.004	6.2 (1.1–36.2)	0.04
	Body weight (<65 kg)	3.8 (1.0–13.9)	0.06		

Abbreviations: SVR, sustained virological response; IRRDR[2a], interferon/ribavirin resistance-determining region of HCV-2a; ISDR/+C[2a], part of interferon sensitivity determining-region plus its carboxy-flanking region of HCV-2a; γ-GTP, gamma glutamyl transpeptidase.
doi:10.1371/journal.pone.0030513.t005

polymorphisms of NS5A and core regions of a given HCV genotype, in particular HCV-1b, have been linked to the difference in SVR rates [7,8,11–13,17]. It should be noted that the significant link between polymorphisms of NS5A and core regions of HCV-1b and treatment outcome was inferred mostly from studies carried out on patients in Asian countries, in particular Japan, and that somewhat controversial results were obtained from studies carried out on patients infected with HCV-1a or -1b in non-Asian countries [24–31]. However, we would like to point out that most of these publications focused mainly on ISDR and core mutations, but not on IRRDR. In addition, the impact of viral genetic variation on treatment outcome in non-HCV-1 infection, either in Asian or non-Asian countries, is still unclear.

In our previous study, we identified IRRDR in NS5A of HCV-1b as a significant determinant for PEG-IFN/RBV treatment outcome; EVR and, more importantly, SVR [11,12]. Consistent with the previous observation, we have demonstrated in the present study that sequence heterogeneity within IRRDR is closely correlated with the treatment responses in HCV-2a and -2b infections. In HCV-2a infection, IRRDR[2a]≥4 was closely associated with RVR (Table S1) and SVR (Table 5). In HCV-2b infection, the sequence heterogeneity within an N-terminal part of IRRDR (IRRDR/N[2b]) was significantly associated with RVR (Table S1). Furthermore, both IRRDR[2a]≥4 and ISDR/+C[2a]≥1 showed remarkable positive predictive values (95%) for SVR prediction (Table S2), suggesting the clinical usefulness of these markers to encourage those patients to receive PEG-IFN/RBV treatment. On the other hand, their negative predictive values for non-SVR were rather low (50% and 33%). This suggests the possible involvement of another factor(s) that determines non-SVR and may limit the clinical usefulness of these markers to accurately predict non-SVR.

The present results were dependent upon the small number of non-SVR patients due to the high response rates of HCV-2a and -2b. In spite of this, the parallels between the RVR/non-RVR and the SVR/non-SVR analyses, especially in HCV-2a infection, support the possibility that the sequences presented in this study are truly representative of the viruses in general circulation.

The clinical correlation between IRRDR sequence heterogeneity and virological responses of IFN-based therapy in HCV infection can be linked to a recent experimental observation by Tsai et al. [32] that an HCV subgenomic RNA replicon containing NS5A of HCV-1b exerted more profound inhibitory effects on IFN activities than the original HCV-2a replicon, and that domain swapping between NS5A sequences of HCV-1b and -2a in the V3 and/or a C-terminal region including IRRDR resulted in a transfer of their anti-IFN activities. Also, it is worthy to note that IRRDR is among the most variable sequences across the different genotypes and subtypes of HCV [33] whereas its upstream and downstream sequences show a higher degree of sequence conservation (Figure 5). This may suggest that whereas the upstream and downstream sequences have a conserved function(s) across all the HCV genotypes, IRRDR sequences have a genotype-dependent or even a strain-dependent function(s). Indeed, the upstream sequences, especially a Pro-rich motif, play key roles in multiple stages of viral replication [34] while the downstream sequence in viral particle assembly and production [35]. Therefore, the sequence heterogeneity of IRRDR and its significant correlation with IFN-responsiveness imply the possibility that IRRDR is involved, at least partly, in the viral strategy to evade IFN-mediated antiviral host defense mechanisms. Its possible molecular mechanism, however, is yet to be elucidated. The IRRDR sequence heterogeneity also suggests genetic flexibility of this region and, indeed, the C-terminal portion of NS5A was shown to tolerate sequence insertions and deletions [36]. This flexibility might play an important role in modulating the interaction with various host systems, including IFN-induced antiviral machineries. It is also possible that the genetic flexibility of IRRDR is accompanied by compensatory changes elsewhere in the viral genome and that these compensatory changes affect overall viral fitness and responses to IFN-based therapy [37].

The relapse rate was higher in HCV-2b infection than in HCV-2a (Table 1). It should be noted that while the sequence heterogeneity within IRRDR[2a] was significantly correlated with both RVR and SVR in HCV-2a infection, IRRDR/N[2b] was correlated only with RVR in HCV-2b infection. These observations might be linked to an intrinsic difference in IFN- and/or RBV-sensitivity between HCV-2a and -2b isolates [8,38]. We assume that HCV-2b is considered between HCV-1b and HCV-2a in terms of resistance to PEG-IFN/RBV treatment and that an extended treatment for a total of 36~48 weeks would be needed to prevent relapse in HCV-2b infection, especially for patients who have risk factors that do not fit the SVR or RVR prediction criteria (Table 5 and Table S1).

A mutation at position 70 of the core protein of HCV-1b has been reported to be correlated with PEG-IFN/RBV treatment outcome [12,13]. In the present study, however, we found no significant correlation between core protein polymorphism and treatment outcome in HCV-2a or -2b infections. The residue at position 70 of the core protein of HCV-2a and -2b isolates was Arg, which is known to be associated with SVR in HCV-1b infection [12,13], and was well conserved in all the isolates tested in the present study (Figures S1 and S2). The observed sequence

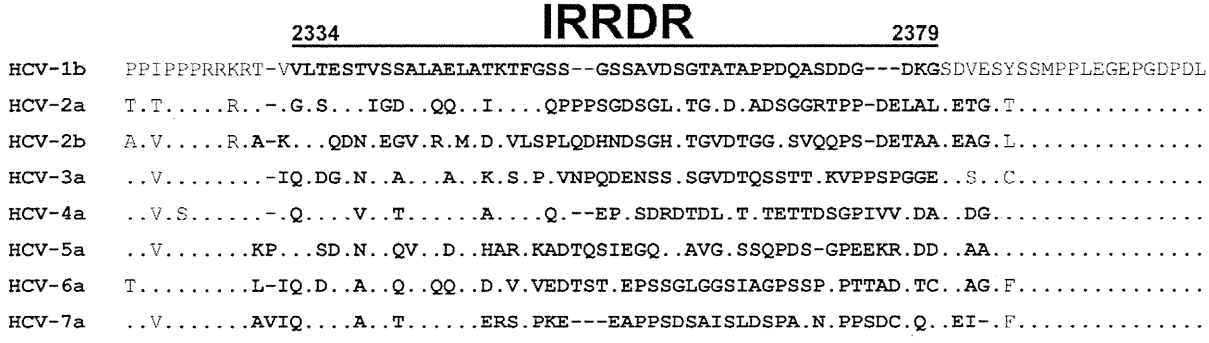

Figure 5. Sequence alignment of IRRDR (interferon/ribavirin resistance-determining region) and its upstream and downstream sequences of different HCV genotypes. The residues in the region that corresponds to IRRDR of HCV-1b [11] are written in boldface letters. Dots indicate residues identical to the HCV-1b sequence. References of aligned sequences are: HCV-1b, El-Shamy et al. [11]; HCV-2a and -2b, Murakami et al. [8]; HCV-3a, X76918; HCV-4a, Y11604; HCV-5a, AF064490; HCV-6a, D84262; HCV-7a, EF108306.
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conservation at position 70 might be the reason for the lack of significant correlation between core protein polymorphism and treatment outcome in HCV-2a or -2b infections. On the other hand, Thr at position 110 of the core protein of HCV-2a has recently been reported to be significantly associated with SVR [10]. In the present study, Thr at position 110 was found in 35% (14/40) and 14% (1/6) of SVR and non-SVR cases, respectively (Figure S1). Similarly, Thr at position 48 was found in 35% (14/40) of SVR cases, but not in non-SVR cases (0/6). The observed differences between SVR and non-SVR, however, were not statistically significant due possibly to the small number of samples tested. A larger-scale study would be needed to determine the possible importance of those residues.

We preliminarily analyzed a host genetic factor, the single nucleotide polymorphism (SNP) at rs8099917 near the IL28B gene [21–23], of a portion of the patients examined in the present study. The result showed that the minor genotypes (T/G and G/G) were found in 5.1% (2/39) and 15.4% (2/13) of RVR and non-RVR patients, respectively, and 2.8% (1/36) and 20.0% (2/10) of SVR and non-SVR patients, respectively (Kim et al., unpublished observation). Although the differences were not statistically significant due probably to the small number of the patients tested, the minor genotypes showed a trend toward being associated with non-SVR, and with non-RVR to a lesser extent, in HCV-2a and -2b infections, as has been reported for HCV-1a and -1b infections [21–23]. The impact of the IL28B SNP, however, appeared to be weaker in HCV-2a and -2b infections than that seen in HCV-1a and -1b infections, and also weaker than that of the most powerful viral factor, IRRDR[2a]≥4, in HCV-2a infection. In this context, we found that, of the four patients with the minor IL28B genotypes, two patients (nos. 2 and 105), who underwent unfavorable treatment response (non-RVR and non-SVR), were infected with HCV isolates of IRRDR[2a]≤3 or IRRDR/N[2b]≤1 while the other two patients (no. 63 and 106), who achieved favorable treatment response (SVR and/or RVR), were infected with HCV isolates of IRRDR[2a]≥4. This might imply the possibility that, in HCV-2 infection, the combination of the minor IL28B genotypes and a low degree of IRRDR sequence heterogeneity has a strong power to predict unfavorable treatment responses whereas a high degree of IRRDR sequence heterogeneity has a dominant predictive power for favorable treatment responses regardless the IL28B genotype. Analysis in a large-scale multicenter study is needed to clarify this issue.

In conclusion, our data suggest that the sequence heterogeneity of NS5A, i.e., IRRDR[2a]≥4, and ISDR/+C[2a]≥1 to a lesser extent, would be a useful predictive marker for SVR in HCV-2a infection. Also, IRRDR/N[2b]≥2 is significantly associated with RVR in HCV-2b infection. These results further emphasize the importance of NS5A, a viral factor, in determining the responsiveness to PEG-IFN/RBV therapy.

Supporting Information

Figure S1 Sequence alignment of the core protein of HCV-2a isolates. Core protein sequences (aa 1 to 120) of HCV-2a obtained from SVR and non-SVR patients are aligned. Prototype sequence of HCV-J6 [18] is shown on the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the prototype sequence.
(TIF)

Figure S2 Sequence alignment of the core protein of HCV-2b isolates. Core protein sequences (aa 1 to 120) of HCV-2b obtained from SVR and non-SVR patients are aligned. Prototype sequence of HCV-J8 [19] is shown on the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the prototype sequence.
(TIF)

Table S1 Univariate and multivariate analyses for identification of independent predictive factors for RVR in HCV-2a- and -2b-infected patients treated with PEG-IFN/RBV therapy.
(DOC)

Table S2 Positive and negative predictive values (PPV and NPV) of NS5A polymorphic factors for SVR prediction.
(DOC)

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Author Contributions

Conceived and designed the experiments: AE SRK HH. Performed the experiments: AE IS YI LD. Analyzed the data: AE IS YI LD SI SY TF ST YY YS TA HH. Contributed reagents/materials/analysis tools: SRK SI SY TF ST YY YS TA. Wrote the paper: AE SRK HH. Obtained permissions from the Ethics Committees: AE SRK HH.

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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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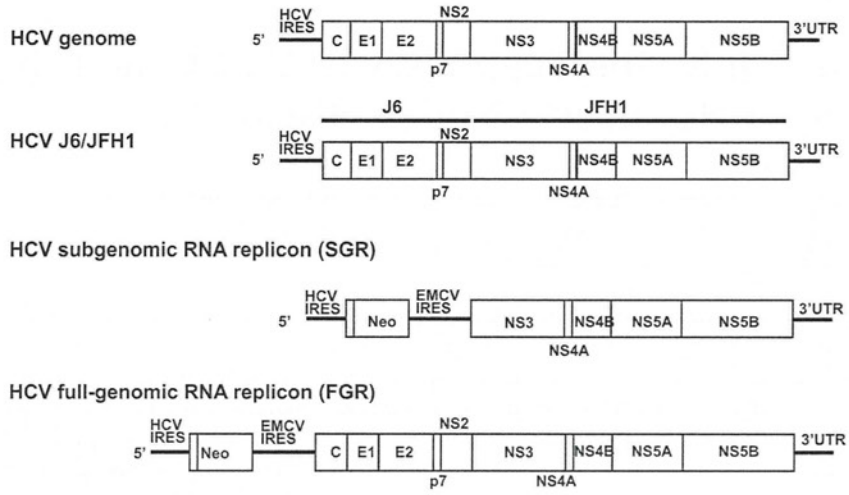

FIG 1 The HCV genome, chimeric HCV J6/JFH1, and the HCV RNA replicons. Schematic diagrams of the HCV genome, the chimeric HCV J6/JFH1 genome, SGR, and FGR are shown. IRES, internal ribosome entry site; EMCV, encephalomyocarditis virus; Neo, neomycin resistance gene.

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

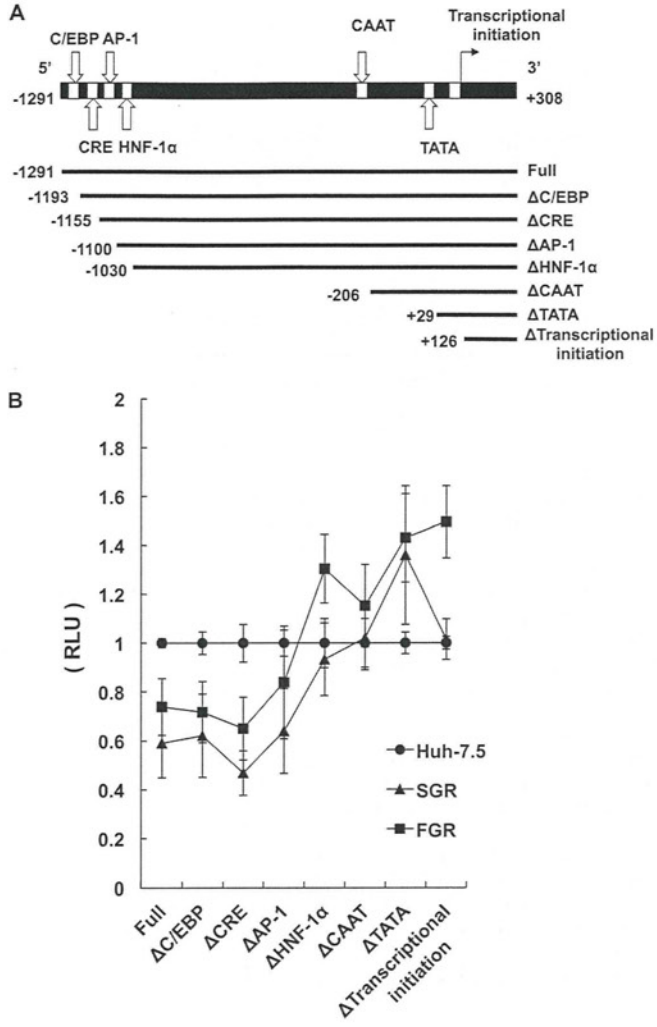

FIG 2 HNF-1α-binding site is important for HCV-induced suppression of GLUT2 promoter. (A) A series of constructs in which genomic GLUT2 promoter DNA fragments were fused to a promoterless firefly luciferase gene of the pGL4 vector were generated with the 3′ end always terminating at bases +308 from transcriptional 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

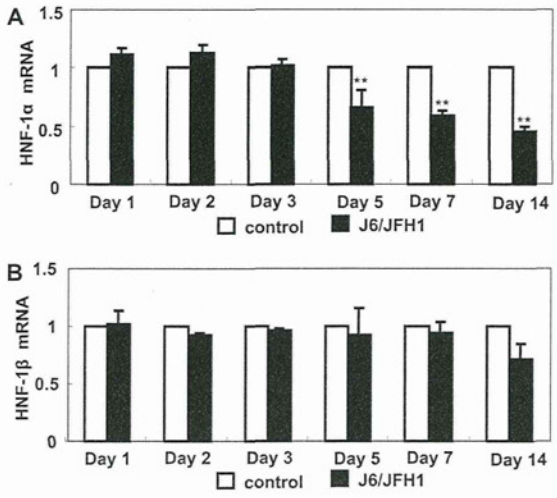

FIG 3 Quantitative RT-PCR analysis of mRNA for HNF-1α and HNF-1β in HCV J6/JFH1-infected cells. Huh-7.5 cells (2.5×10^5 cells/six-well plate) were infected with HCV J6/JFH1 at a multiplicity of infection of 2. Cells were cultured and harvested at the indicated times. Total RNA was extracted, and the 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.

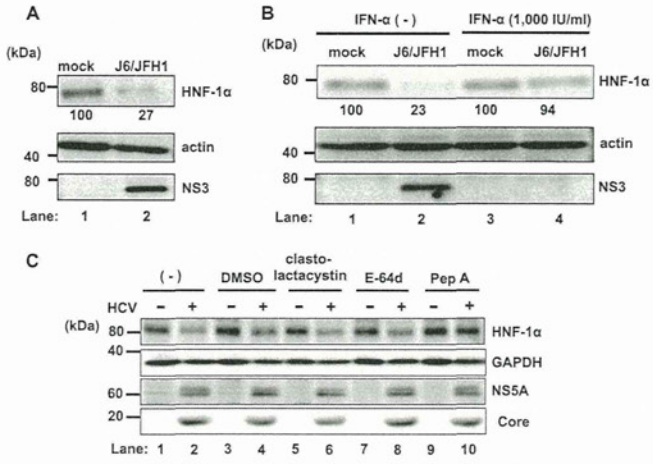

FIG 4 HCV infection induces lysosomal degradation of HNF-1α protein. (A) HCV infection decreased the levels of HNF-1α protein in Huh-7.5 cells. Huh-7.5 cells (2.5×10^5 cells/six-well plate) were infected with HCV J6/JFH1 at a multiplicity of infection of 2. Cells were cultured and harvested at 5 days 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-