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.

References

1. Bialek SR, Terrault NA. The changing epidemiology and natural history of hepatitis C virus infection. Clin Liver Dis. 2006;10:697–715.
2. Williams R. Global challenges in liver disease. Hepatology. 2006;44:521–6.
3. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med. 2002;347:975–82.
4. Backus LI, Boothroyd DB, Phillips BR, Mole LA. Predictors of response of US veterans to treatment for the hepatitis C virus. Hepatology. 2007;46:37–47.
5. Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. Hepatology. 2003;38:645–52.
6. Kurosaki M, Sakamoto N, Iwasaki M, Sakamoto M, Suzuki Y, Hiramatsu N, et al. Pretreatment prediction of response to peginterferon plus ribavirin therapy in genotype 1 chronic hepatitis C using data mining analysis. J Gastroenterol. 2011;46:401–9.
7. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-α and ribavirin therapy for chronic hepatitis C. Nat Genet. 2009;41:1105–9.
8. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature. 2009;461:399–401.
9. Suppiah V, Moldovan M, Ahlenstiel G, Ber T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet. 2009;41:1105–9.
10. Rauch A, Kutalik Z, Descombes P, Cai T, Di Iulio J, Mueller T, et al. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure—a genome-wide association study. Gastroenterology. 2010;138:1338–45.
11. El-Shamy A, Sasayama M, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, et al. Prediction of efficient virological response to pegylated interferon/ribavirin combination therapy by NS5A sequences of hepatitis C virus and anti-NS5A antibodies in pretreatment sera. Microbiol Immunol. 2007;51:471–82.
12. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology. 2008;48:38–47.
13. El-Shamy A, Kim SR, Ide YH, Sasase N, Imoto S, Deng L, et al. Polymorphisms of hepatitis C virus non-structural protein 5A and core protein and clinical outcome of pegylated-interferon/ribavirin combination therapy. Intervirology. 2012;55:1–11.
14. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med. 1996;11(334):77–81.
15. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol. 2007;6:403–10.
16. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and

non-virological response to interferon–ribavirin combination therapy. Intervirology. 2005;48:372–80.
17. Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, et al. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J Gen Virol. 1992;73:673–9.
18. Ogata S, Nagano-Fujii M, Ku Y, Yoon S, Hotta H. Comparative sequence analysis of the core protein and its frameshift product, the F protein, of hepatitis C virus subtype 1b strains obtained from patients with and without hepatocellular carcinoma. J Clin Microbiol. 2002;40:3625–30.
19. Fukuhara T, Taketomi A, Okano S, Ikegami T, Soejima Y, Shirabe K, et al. Mutations in hepatitis C virus genotype 1b and the sensitivity of interferon–ribavirin therapy after liver transplantation. J Hepatol. 2010;52:672–80.
20. Hayes CN, Kobayashi M, Akuta N, Suzuki F, Kumada H, Abe H, et al. HCV substitutions and IL28B polymorphisms on outcome of peg-interferon plus ribavirin combination therapy. Gut. 2011; 60:261–7.
21. Kurosaki M, Tanaka Y, Nishida N, Sakamoto N, Enomoto N, Honda M, et al. Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in IL28B and viral factors. J Hepatol. 2011;54:439–48.
22. Macdonald A, Harris M. Hepatitis C virus NS5A: tales of a promiscuous protein. J Gen Virol. 2004;85:2485–502.
23. El-Shamy A, Shoji I, Kim SR, Ide Y, Imoto S, Deng L, et al. Sequence heterogeneity in NS5A of hepatitis C virus genotypes 2a and 2b and clinical outcome of pegylated-interferon/ribavirin therapy. PLoS One. 2012;7(2):e30513.
24. Tsai YH, Kuang WF, Lu TY, Kao JH, Lai MY, Liu CJ, et al. The non-structural 5A protein of hepatitis C virus exhibits genotypic differences in interferon antagonism. J Hepatol. 2008;49:899–907.
25. Hughes M, Gretton S, Shelton H, Brown DD, McCormick CJ, Angus AG, et al. A conserved proline between domains II and III of hepatitis C virus NS5A influences both RNA replication and virus assembly. J Virol. 2009;83:10788–96.
26. Tellinghuisen TL, Foss KL, Treadaway J. Regulation of hepatitis C virion production via phosphorylation of the NS5A protein. PLoS Pathog. 2008;4:e1000032.
27. Vlotides G, Sorensen AS, Kopp F, Zitzmann K, Cengic N, Brand S, et al. SOCS-1 and SOCS-3 inhibit IFN-α-induced expression of the antiviral proteins 2,5-OAS and MxA. Biochem Biophys Res Commun. 2004;320:1007–14.
28. Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, et al. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. Nat Immunol. 2003; 4:69–77.
29. Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol. 2003;4:63–8.
30. Marcello T, Grakoui A, Barba-Spaeth G, Machlin ES, Kotenko SV, MacDonald MR, et al. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. Gastroenterology. 2006;131:1887–98.
31. Berg T, von Wagner M, Nasser S, Sarrazin C, Heintges T, Gerlach T, et al. Extended treatment duration for hepatitis C virus type 1: comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. Gastroenterology. 2006;130:1086–97.
32. McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, Kauffman R, et al. Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. N Engl J Med. 2009;360:1827–38.
33. Hézode C, Forestier N, Dusheiko G, Ferenci P, Plo S, Goeser T, et al. Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. N Engl J Med. 2009;360:1839–50.

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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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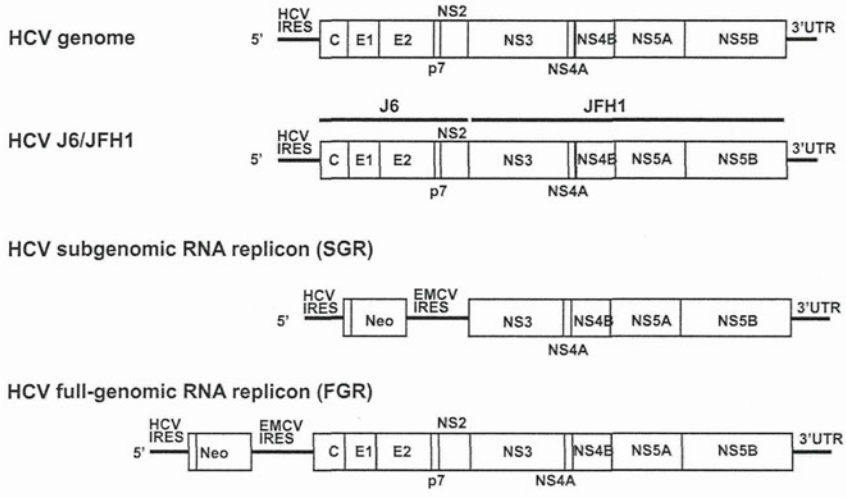

FIG 1 The HCV genome, chimeric HCV J6/JFH1, and the HCV RNA replicons. Schematic diagrams of the HCV genome, the chimeric HCV J6/JFH1 genome, SGR, and FGR are shown. IRES, internal ribosome entry site; EMCV, encephalomyocarditis virus; Neo, neomycin resistance gene.

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′-CCAGTGTGGTGAATTCACCATGGTGAACTATGCAACAGGGAA-3′; antisense primer, 5′-CGAAGGGCCCTCTAGAGATGTACCAGGCAGCACAGA-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′-CCAGTGTGGTGAATTCACCATGGCGCCTATTACGGCCTACTC-3′; antisense primer, 5′-CGAAGGGCCCTCTAGAGCACTCTTCCATCTCATCGAA-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

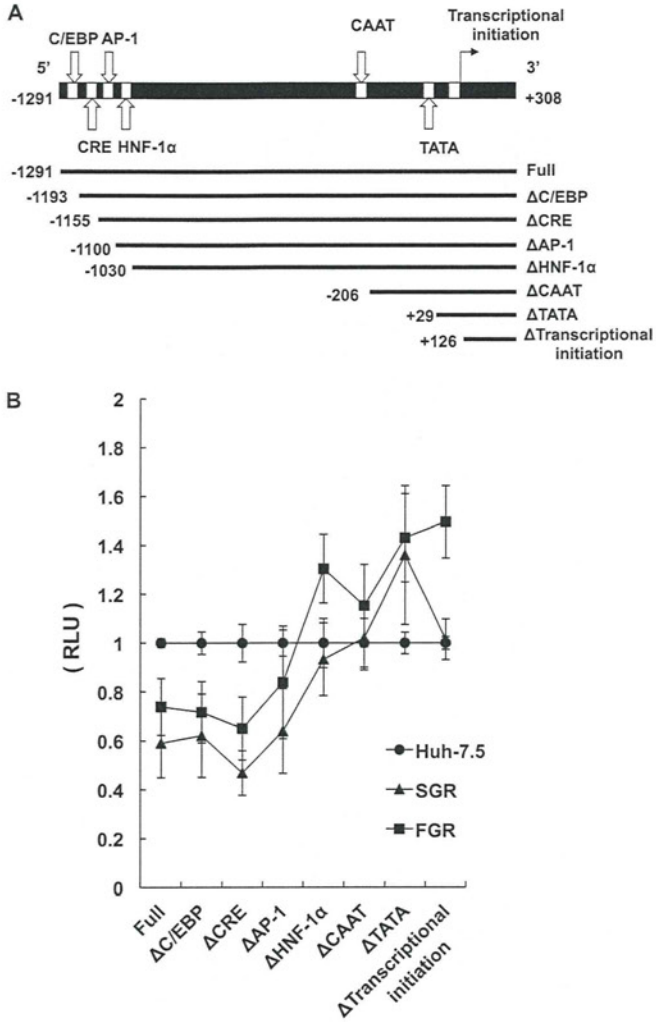

FIG 2 HNF-1α-binding site is important for HCV-induced suppression of GLUT2 promoter. (A) A series of constructs in which genomic GLUT2 promoter DNA fragments were fused to a promoterless firefly luciferase gene of the pGL4 vector were generated with the 3′ end always terminating at bases +308 from transcriptional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

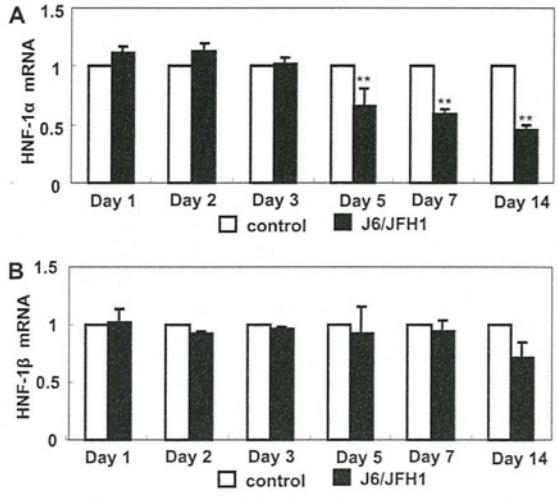

FIG 3 Quantitative RT-PCR analysis of mRNA for HNF-1α and HNF-1β in HCV J6/JFH1-infected cells. Huh-7.5 cells (2.5×10^5 cells/six-well plate) were infected with HCV J6/JFH1 at a multiplicity of infection of 2. Cells were cultured and harvested at the indicated times. Total RNA was extracted, and the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

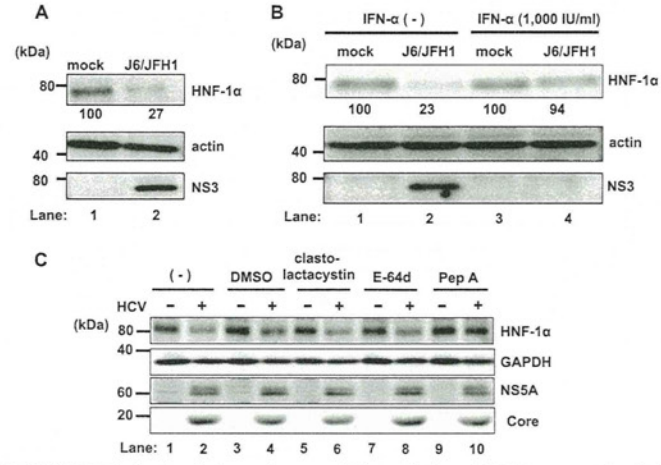

FIG 4 HCV infection induces lysosomal degradation of HNF-1α protein. (A) HCV infection decreased the levels of HNF-1α protein in Huh-7.5 cells. Huh-7.5 cells (2.5×10^5 cells/six-well plate) were infected with HCV J6/JFH1 at a multiplicity of infection of 2. Cells were cultured and harvested at 5 days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

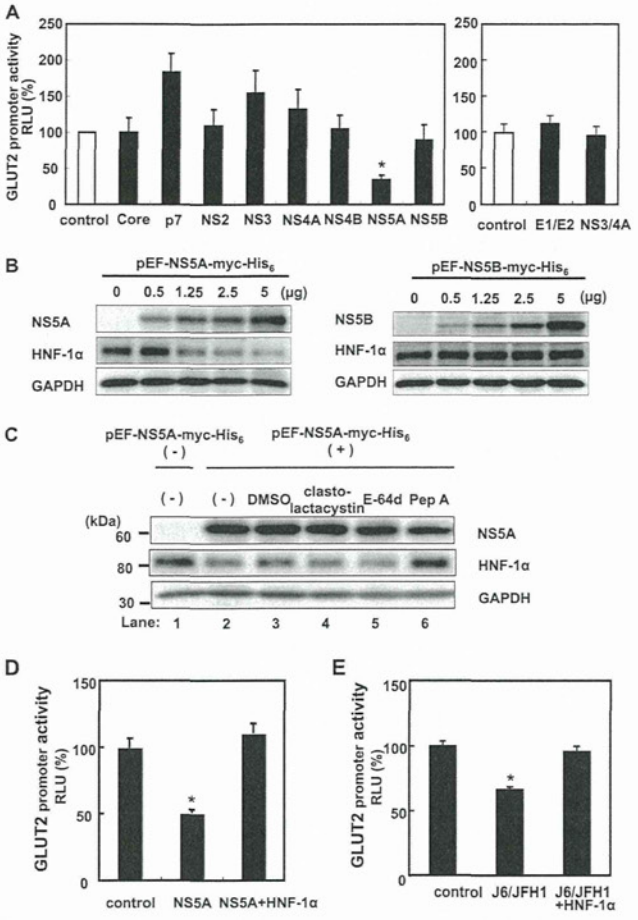

FIG 5 HCV NS5A protein is involved in suppression of GLUT2 promoter activity and lysosomal degradation of HNF-1α protein. (A) Huh-7.5 cells were plated at 1×10^5 cells/12-well plate. After cells were cultured for 12 h, cells were cotransfected with each HCV protein plasmid (0.5 μg), the human GLUT2 promoter 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-

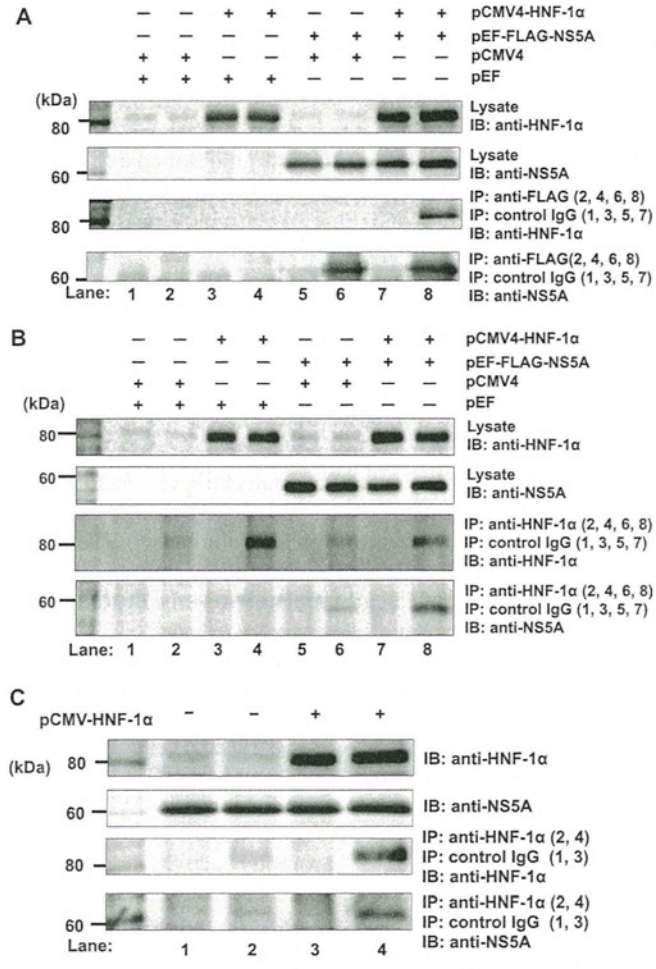

FIG 6 NS5A protein interacts with HNF-1α protein. (A) Huh-7.5 cells were plated at 1.2×10^6 cells/10-cm dish and cultured for 12 h. Cells were transfected with plasmids as indicated. At 48 h after transfection, cells were harvested. Cell lysates were immunoprecipitated with either anti-FLAG mouse MAb (lanes 2, 4, 6, and 8) or control IgG (lanes 1, 3, 5, and 7), and bound proteins were immunoblotted with anti-HNF-1α rabbit PAb (third blot) or anti-NS5A mouse MAb (fourth blot). Protein expression of HNF-1α or FLAG-NS5A was confirmed using the same cell lysates by immunoblotting with either anti-HNF-1α rabbit PAb (first blot) or anti-NS5A mouse MAb (second blot). (B) Cell lysates were immunoprecipitated with either anti-HNF-1α rabbit PAb (lanes 2, 4, 6, and 8) or control IgG (lanes 1, 3, 5, and 7), and bound proteins were immunoblotted with either anti-HNF-1α rabbit PAb (third blot) and anti-NS5A mouse MAb (fourth blot). (C) Full-genome replicon Con1 (RCYM1) cells were plated at 1.2×10^6 cells/10-cm plate and transfected with or without pCMV-HNF-1α plasmid and cultured for 48 h. Cells were harvested and assayed for immunoprecipitation with anti-HNF-1α rabbit PAb (lanes 2 and 4) or control IgG (lanes 1 and 3). Bound proteins were immunoblotted with anti-HNF-1α goat PAb (third blot) or anti-NS5A mouse MAb (fourth blot). Input samples were immunoblotted with either anti-HNF-1α PAb (first blot) or anti-NS5A MAb (second blot). IP, immunoprecipitation; IB, immunoblotting.

1α-binding site on NS5A protein, coimmunoprecipitation analyses were performed. By use of a panel of NS5A deletion mutants (Fig. 7A), FLAG-HNF-1α protein was found to coimmunoprecipitate with all of the HA-NS5A proteins except HA-NS5A consisting of aa 357 to 447 [HA-NS5A(357–447), HA-NS5A(250–447), or HA-NS5A(214–447) (Fig. 7B, lower left panel). These results suggest that domain I of NS5A consisting of aa 1 to 213 is important for HNF-1α binding. FLAG-HNF-1α protein was also found to coimmunoprecipitate with NS5A(1–126)-myc-His_6 and NS5A(1–147)-myc-His_6. These data led to the conclusion that the HNF-1α-binding domain of NS5A protein was aa 1 to 126.

DISCUSSION

In this study, we aimed to clarify molecular mechanisms of HCV-induced suppression of GLUT2 gene expression. The reporter assays of the human GLUT2 promoter suggest that the HNF-1α-binding site is crucial for HCV-induced suppression of GLUT2 promoter activity (Fig. 2). HCV infection significantly reduced the levels of HNF-1α mRNA (Fig. 3A). Moreover, HCV infection remarkably decreased HNF-1α protein levels (Fig. 4A). Our results suggest that HCV infection suppresses GLUT2 gene expression via NS5A-mediated lysosomal degradation of HNF-1α protein (Fig. 5). Immunoprecipitation analyses revealed that NS5A protein physically interacts with HNF-1α protein (Fig. 6) and that domain I of NS5A is important for HNF-1α binding (Fig. 7). Taken together, our results suggest that HCV infection suppresses GLUT2 transcription via downregulation of HNF-1α expression at both transcriptional and translational levels (Fig. 8).

We demonstrated that HNF-1α protein levels were greatly reduced compared to the reduced levels of HNF-1α mRNA. We demonstrated that pepstatin A, but not E64-d, restored the levels of HNF-1α protein, suggesting that an aspartic protease is involved in the degradation of HNF-1α protein. Pepstatin A is widely used for investigation of autophagy and lysosomal degradation. Further studies are needed to elucidate how HCV induces lysosomal degradation of HNF-1α protein and how HNF-1α protein is selectively downregulated by HCV infection. Our data suggest that the HCV NS5A protein is responsible for the HCV-induced degradation of HNF-1α protein. Using a panel of NS5A deletion mutants, we demonstrated that domain I of NS5A is important for association with HNF-1α protein. NS5A domain I is relatively conserved among HCV genotypes compared to domains II and III, suggesting that NS5A–HNF-1α interaction is common to all the HCV genotypes. Domain I coordinates a single zinc atom per protein molecule and is essential for HCV RNA replication (35). The crystal structure of NS5A domain I revealed the presence of a zinc coordination motif and a C-terminal disulfide bond (36). NS5A domain I was found to bind many host proteins, RNA, and membranes (16). It is possible that physical interaction between NS5A protein and HNF-1α protein is important for selective degradation of HNF-1α protein. One possible mechanism is that NS5A protein may recruit HNF-1α protein to the lysosome. Further study is necessary to test this possibility.

We observed that deletion of the GLUT2 transcriptional start site enhances expression of the GLUT2 reporter in FGR cells (Fig. 2B). Cha et al. (7) previously reported that deletion down to nucleotide +73 of the GLUT2 promoter resulted in a marked increase and that further deletion to nucleotide +188 caused a drastic decrease in luciferase activity, indicating the presence of negative- and positive-regulator elements in the 5′ untranslated region. The role of these elements in HCV-infected cells remains to be elucidated.

We demonstrated that HCV J6/JFH1 infection reduced the HNF-1α mRNA level and HNF-1α protein level. Our results contradict an earlier report (32) demonstrating that expression of HNF-1 mRNA was increased in subgenomic replicon Huh.8 cells (3). We observed downregulation of HNF-1α mRNA and

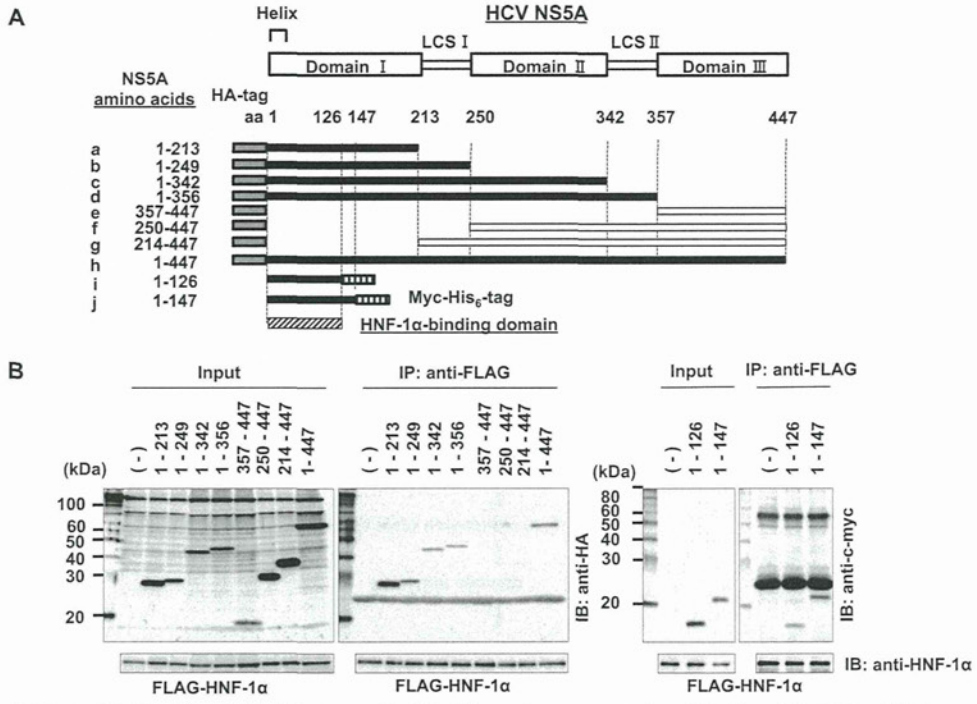

FIG 7 Mapping of the HNF-1α-binding domain for NS5A protein. (A) Schematic representation of the hepatitis C virus NS5A protein. NS5A consists of three domains (domains I, II, and III) with domains separated by low-complexity sequences (LCS I and II). The position of the amino-terminal amphipathic helix membrane anchor is shown (labeled helix). The NS5A deletion mutants (a to j) contain the NS5A amino acids indicated to the left. Each NS5A deletion mutant contains either HA tag in the N terminus (a to h) or myc-His_6 tag in the C terminus (i and j). The gray region of each represents the HA tag sequence. The lattice region of each represents the myc-His_6 tag (i and j). Closed boxes represent proteins that are bound specifically to HNF-1α protein, and open boxes represent those that are not bound. (B) Huh-7.5 cells were transfected with each NS5A mutant plasmid together with a FLAG-HNF-1α expression plasmid. At 48 h posttransfection, cells were harvested, and cell lysates were immunoprecipitated with anti-FLAG beads. Input samples and immunoprecipitated samples were immunoblotted with anti-HA MAb (two left panels, top), anti-c-myc MAb (two right panels, top), or anti-HNF-1α PAb (all panels, bottom).

HNF-1α protein in SGR cells as well as in FGR cells (data not shown). We also demonstrated that the ectopic expression of NS5A protein decreased the endogenous HNF-1α protein level. The reasons for these discrepancies remain to be elucidated.

We along with other groups previously reported that HCV NS5A protein is involved in mitochondrial reactive oxygen species (ROS) production (11, 13, 38). Mitochondrial ROS generation is known to induce the autophagy pathway (22) and lysosomal membrane permeabilization (8). Therefore, it is necessary to determine whether NS5A-induced ROS production enhances autophagic degradation or lysosomal membrane permeabilization. Several groups have reported that autophagy vesicles accumulate in HCV-infected cells and that autophagy proteins can function as proviral factors required for HCV replication (14). Autophagy degrades macromolecules and organelles. Based on the means by which cargo is delivered to the lysosomes, three different autophagy pathways are described: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). At first, autophagy was considered a nonselective bulk degradation process. CMA, however, results in specific degradation of the cytosolic proteins in a molecule-by-molecule fashion. Most known substrates for CMA contain a peptide sequence biochemically related to KFERQ (12). Although the typical KFERQ peptide motif is not found in HNF-1α protein, it is possible that KFERQ-like sequences can be generated by post-translational modifications. It is also possible that HNF-1α protein possesses other degradation motifs. The molecular mechanism underlying NS5A-dependent lysosomal degradation of HNF-1α protein needs to be elucidated.

HNF-1α is a homeodomain-containing transcription factor, which is expressed in the liver, pancreatic β cells, and other tissues (1). Intriguingly, HNF-1α is known to play a crucial role in diabetes. Heterozygous germ line mutations in the gene encoding HNF-1α are responsible for an autosomal dominant form of non-insulin-dependent diabetes, MODY3 (40). Mutations in the HNF-1α gene disrupt GLUT2 function as a glucose sensor in pancreatic β cells, resulting in severe insulin secretory defects (39). It is unclear whether HNF-1α mutations in the liver affect glucose homeostasis in MODY3 patients. Two strains of HNF-1α-deficient mice have been reported. The mice of the first strain, created using standard methods for making knockout mice, are born normally, but most die postnatally around the weaning period after a progressive wasting syndrome (31). Mice of the second strain, created using the Cre-loxP recombination method, had a normal life span (20). The knockout mice of the second strain were dwarfed, diabetic, and infertile. Moreover, the knockout mice had enlarged livers and exhibited progressive liver damage.

HNF-1α was also identified as a tumor suppressor gene involved in human liver tumorigenesis since biallelic inactivating mutations of the HNF-1α gene were found in 50% of hepatocellular adenomas and, in rare cases, of well-differentiated hepatocellular carcinomas developed in the absence of cirrhosis (5).

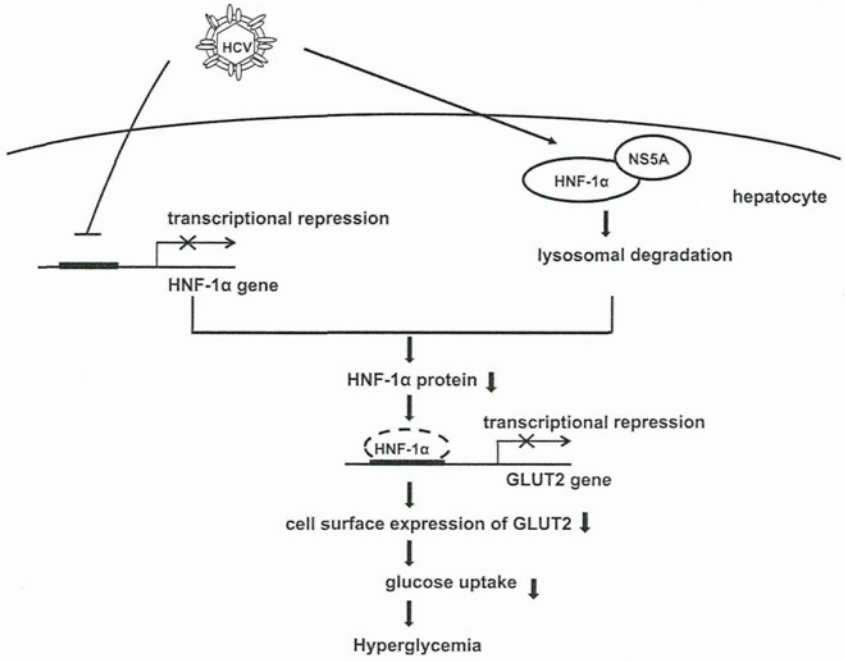

FIG 8 A proposed mechanism of the HCV-induced suppression of GLUT2 via downregulation of HNF-1α. HCV infection downregulates HNF-1α at transcriptional and posttranslational levels, resulting in suppression of GLUT2 gene transcription. HCV NS5A protein physically interacts with HNF-1α protein and enhances lysosomal degradation of HNF-1α protein.

Moreover, HNF-1α has been shown to regulate a large number of genes related to glucose, fatty acid, bile acid, cholesterol, and lipoprotein metabolisms as well as inflammation (1). Therefore, it is possible that HCV-induced downregulation of HNF-1α may play a crucial role in metabolic disorders as well as tumorigenesis.

To determine which HCV protein is involved in the suppression of the GLUT2 promoter, we examined the effects of transient expression of HCV proteins on GLUT2 promoter activity. Overexpression of NS5A suppressed GLUT2 promoter activity, whereas overexpression of p7 enhanced GLUT2 promoter activity (Fig. 5A). SGR cells express NS5A protein but lack p7 protein. FGR cells express both NS5A protein and p7 protein. However, GLUT2 promoter activity was suppressed in both SGR and FGR cells (Fig. 2B). This discrepancy between transient expression system and replicon cells may result from the differences in trafficking of p7 because it is a complex process potentially regulated by both the cleavage from its upstream signal peptides and targeting signals within the protein sequence (15).

We previously reported that HCV infection promotes hepatic gluconeogenesis in HCV J6/JFH1-infected Huh-7.5 cells (11). HCV infection transcriptionally upregulates the genes for phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase), the rate-limiting enzymes for hepatic gluconeogenesis. We demonstrated that gene expression of PEPCK and G6Pase was regulated by the transcription factor forkhead box O1 (FoxO1) in HCV-infected cells. Phosphorylation of the FoxO1 at Ser319 was markedly diminished in HCV-infected cells, resulting in increased nuclear accumulation of FoxO1. HCV NS5A protein was directly linked with FoxO1-dependent increased gluconeogenesis. HCV-induced downregulation of GLUT2 expression and upregulation of gluconeogenesis may cooperatively contribute to development of type 2 diabetes in HCV-infected patients at least to some extent. HCV-induced downregulation of GLUT2 expression and upregulation of gluconeogenesis may result in high concentrations of glucose in HCV-infected hepatocytes. As suggested in a recent study, low glucose concentrations in the hepatocytes inhibit HCV replication (28). Therefore, high glucose levels in the hepatocytes may confer an advantage in efficient replication of HCV.

In conclusion, we provided evidence suggesting that HCV infection downregulates HNF-1α expression at both transcriptional and posttranslational levels. HCV-induced downregulation of HNF-1α may play a crucial role in glucose metabolic disorders caused by HCV infection. Strategies aimed at HCV-induced downregulation of HNF-1α protein may lead to the development of new therapeutic agents for HCV-induced diabetes.

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We have no potential conflicts of interest to report.

REFERENCES

1. **Armendariz AD, Krauss RM.** 2009. Hepatic nuclear factor 1-alpha: inflammation, genetics, and atherosclerosis. Curr. Opin. Lipidol. **20**:106–111.
2. **Ban N, et al.** 2002. Hepatocyte nuclear factor-1α recruits the transcriptional co-activator p300 on the GLUT2 gene promoter. Diabetes **51**:1409–1418.
3. **Blight KJ, Kolykhalov AA, Rice CM.** 2000. Efficient initiation of HCV RNA replication in cell culture. Science **290**:1972–1974.
4. **Blight KJ, McKeating JA, Rice CM.** 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J. Virol. **76**: 13001–13014.
5. **Bluteau O, et al.** 2002. Bi-allelic inactivation of TCF1 in hepatic adenomas. Nat. Genet. **32**:312–315.
6. **Bungyoku Y, et al.** 2009. Efficient production of infectious hepatitis C virus with adaptive mutations in cultured hepatoma cells. J. Gen. Virol. **90**:1681–1691.
7. **Cha JY, Kim H, Kim KS, Hur MW, Ahn Y.** 2000. Identification of transacting factors responsible for the tissue-specific expression of human glucose transporter type 2 isoform gene. Cooperative role of hepatocyte nuclear factors 1α and 3β. J. Biol. Chem. **275**:18358–18365.
8. **Denamur S, et al.** 2011. Role of oxidative stress in lysosomal membrane permeabilization and apoptosis induced by gentamicin, an aminoglycoside antibiotic. Free Radic. Biol. Med. **51**:1656–1665.
9. **Deng L, et al.** 2008. Hepatitis C virus infection induces apoptosis through a Bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway. J. Virol. **82**:10375–10385.
10. **Deng L, et al.** 2006. NS3 protein of Hepatitis C virus associates with the tumour suppressor p53 and inhibits its function in an NS3 sequence-dependent manner. J. Gen. Virol. **87**:1703–1713.
11. **Deng L, et al.** 2011. Hepatitis C virus infection promotes hepatic gluconeogenesis through an NS5A-mediated, FoxO1-dependent pathway. J. Virol. **85**:8556–8568.
12. **Dice JF.** 2007. Chaperone-mediated autophagy. Autophagy **3**:295–299.
13. **Dionisio N, et al.** 2009. Hepatitis C virus NS5A and core proteins induce oxidative stress-mediated calcium signalling alterations in hepatocytes. J. Hepatol. **50**:872–882.
14. **Dreux M, Chisari FV.** 2011. Impact of the autophagy machinery on hepatitis C virus infection. Viruses **3**:1342–1357.
15. **Griffin S, Clarke D, McCormick C, Rowlands D, Harris M.** 2005. Signal peptide cleavage and internal targeting signals direct the hepatitis C virus p7 protein to distinct intracellular membranes. J. Virol. **79**:15525–15536.
16. **He Y, Staschke KA, Tan SL.** 2006. HCV NS5A: a multifunctional regulator of cellular pathways and virus replication. *In* Tan SL (ed), Hepatitis C viruses: genomes and molecular biology. Horizon Bioscience, Norfolk, United Kingdom. http://www.ncbi.nlm.nih.gov/books/NBK1621/.
17. **Ikeda M, et al.** 2005. Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. Biochem. Biophys. Res. Commun. **329**:1350–1359.
18. **Inubushi S, et al.** 2008. Hepatitis C virus NS5A protein interacts with and negatively regulates the non-receptor protein tyrosine kinase Syk. J. Gen. Virol. **89**:1231–1242.
19. **Kasai D, et al.** 2009. HCV replication suppresses cellular glucose uptake through down-regulation of cell surface expression of glucose transporters. J. Hepatol. **50**:883–894.
20. **Lee YH, Sauer B, Gonzalez FJ.** 1998. Laron dwarfism and non-insulin-dependent diabetes mellitus in the Hnf-1α knockout mouse. Mol. Cell Biol. **18**:3059–3068.
21. **Lemon SM, Walker C, Alter MJ, Yi M.** 2007. Hepatitis C virus, p 1291–1304. *In* Knipe DM, et al (ed), Fields virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
22. **Li ZY, Yang Y, Ming M, Liu B.** 2011. Mitochondrial ROS generation for regulation of autophagic pathways in cancer. Biochem. Biophys. Res. Commun. **414**:5–8.
23. **Lindenbach BD, et al.** 2005. Complete replication of hepatitis C virus in cell culture. Science **309**:623–626.
24. **Macheda ML, Rogers S, Best JD.** 2005. Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. J. Cell Physiol. **202**: 654–662.
25. **Malecki MT, Mlynarski W.** 2008. Monogenic diabetes: implications for therapy of rare types of disease. Diabetes Obes. Metab. **10**:607–616.
26. **Mason AL, et al.** 1999. Association of diabetes mellitus and chronic hepatitis C virus infection. Hepatology **29**:328–333.
27. **Murakami K, et al.** 2006. Production of infectious hepatitis C virus particles in three-dimensional cultures of the cell line carrying the genome-length dicistronic viral RNA of genotype 1b. Virology **351**:381–392.
28. **Nakashima K, Takeuchi K, Chihara K, Hotta H, Sada K.** 2011. Inhibition of hepatitis C virus replication through adenosine monophosphate-activated protein kinase-dependent and -independent pathways. Microbiol. Immunol. **55**:774–782.
29. **Negro F.** 2011. Mechanisms of hepatitis C virus-related insulin resistance. Clin. Res. Hepatol Gastroenterol. **35**:358–363.
30. **Negro F, Alaei M.** 2009. Hepatitis C virus and type 2 diabetes. World J. Gastroenterol. **15**:1537–1547.
31. **Pontoglio M, et al.** 1996. Hepatocyte nuclear factor 1 inactivation results in hepatic dysfunction, phenylketonuria, and renal Fanconi syndrome. Cell **84**:575–585.
32. **Qadri I, et al.** 2004. Induced oxidative stress and activated expression of manganese superoxide dismutase during hepatitis C virus replication: role of JNK, p38 MAPK and AP-1. Biochem. J. **378**:919–928.
33. **Shirakura M, et al.** 2007. E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein. J. Virol. **81**:1174–1185.
34. **Takeda J, Kayano T, Fukomoto H, Bell GI.** 1993. Organization of the human GLUT2 (pancreatic beta-cell and hepatocyte) glucose transporter gene. Diabetes **42**:773–777.
35. **Tellinghuisen TL, Marcotrigiano J, Gorbalenya AE, Rice CM.** 2004. The NS5A protein of hepatitis C virus is a zinc metalloprotein. J. Biol. Chem. **279**:48576–48587.
36. **Tellinghuisen TL, Marcotrigiano J, Rice CM.** 2005. Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. Nature **435**:374–379.
37. **Wakita T, et al.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. **11**:791–796.
38. **Wang AG, et al.** 2009. Non-structural 5A protein of hepatitis C virus induces a range of liver pathology in transgenic mice. J. Pathol. **219**:253–262.
39. **Wang H, Maechler P, Hagenfeldt KA, Wollheim CB.** 1998. Dominant-negative suppression of HNF-1alpha function results in defective insulin gene transcription and impaired metabolism-secretion coupling in a pancreatic beta-cell line. EMBO J. **17**:6701–6713.
40. **Yamagata K, et al.** 1996. Mutations in the hepatocyte nuclear factor-1α gene in maturity-onset diabetes of the young (MODY3). Nature **384**:455–458.

Journal of Medical Virology 84:229–234 (2012)

A Point Mutation at Asn-534 That Disrupts a Conserved *N*-Glycosylation Motif of the E2 Glycoprotein of Hepatitis C Virus Markedly Enhances the Sensitivity to Antibody Neutralization

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The molecular basis of antibody neutralization against hepatitis C virus (HCV) is poorly understood. The E2 glycoprotein of HCV is critically involved in viral infectivity through specific binding to the principal virus receptor component CD81, and is targeted by anti-HCV neutralizing antibodies. A previous study showed that a mutation at position 534 (N534H) within the sixth *N*-glycosylation motif of E2 of the J6/JFH1 strain of HCV genotype 2a (HCV-2a) was responsible for more efficient access of E2 to CD81 so that the mutant virus could infect the target cells more efficiently. The purpose of this study was to analyze the sensitivity of the parental J6/JFH1, its cell culture-adapted variant P-47 possessing 10 amino acid mutations and recombinant viruses with the adaptive mutations to neutralization by anti-HCV antibodies in sera of HCV-infected patients. The J6/JFH1 virus was neutralized by antibodies in sera of patients infected with HCV-2a and -1b, with mean 50% neutralization titers being 1:670 and 1:200, respectively ($P < 0.00001$). On the other hand, the P-47 variant showed 50- to 200-times higher sensitivity to antibody neutralization than the parental J6/JFH1 without genotype specificity. The N534H mutation, and another one at position 416 (T416A) near the first *N*-glycosylation motif to a lesser extent, were shown to be responsible for the enhanced sensitivity to antibody neutralization. The present results suggest that the residues 534, and 416 to a lesser extent, of the E2 glycoprotein are critically involved in the HCV infectivity and antibody neutralization. ***J. Med. Virol. 84:229–234, 2012.*** © 2011 Wiley Periodicals, Inc.

KEY WORDS: humoral immune mechanism; evasion; glycan

INTRODUCTION

Hepatitis C virus (HCV), a member of the family *Flaviviridae*, the genus *Hepacivirus*, is an enveloped, positive-stranded RNA virus that infects an estimated 170 million people worldwide. The virus evades the

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host immune system to establish chronic infection, which often leads to serious liver diseases, such as cirrhosis and hepatocellular carcinoma. Even with a current standard treatment with pegylated interferon *plus* ribavirin, sustained viral clearance is obtained for only approximately 50% of patients infected with HCV genotype 1b (HCV-1b). Neither antibody-based prophylaxis nor an effective vaccine is currently available.

A better understanding of the interplay between viral and host factors that determine HCV clearance or persistence is needed for the design of effective passive immunotherapy and effective vaccines. A growing body of evidence from studies in humans and chimpanzees suggests that HCV-specific T-cell immunity plays an important role in the viral clearance [Bowen and Walker, 2005]. Also, several studies have indicated a role for humoral immunity in HCV infection [Bartosch et al., 2003; Logvinoff et al., 2004; Lavillette et al., 2005; Netski et al., 2005; Pestka et al., 2007; Dowd et al., 2009]. However, this aspect remains poorly characterized.

The E2 glycoprotein of HCV plays an important role in viral attachment and, therefore, becomes a major target of anti-HCV neutralizing antibodies. Identification of protective epitopes in E2 conserved among different HCV strains is a major challenge in vaccine design [Tarr et al., 2006; Helle et al., 2007; Gal-Tanamy et al., 2008; Keck et al., 2008]. The development of infectious retroviral pseudoparticles (HCVpp) bearing HCV envelope glycoproteins helps us study interactions between E2 epitopes and the virus receptor CD81 or neutralizing antibodies [Bartosch et al., 2003; Logvinoff et al., 2004; Lavillette et al., 2005; Pestka et al., 2007; Dowd et al., 2009]. More significantly, authentic HCV particles produced by the HCV cell culture system (HCVcc) are currently available for this purpose [Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005; Fournier et al., 2007].

Recently, it was demonstrated using HCVcc that a mutation at position 534 from Asn to His (N534H) in the E2 glycoprotein of the HCV J6/JFH1 strain confers an advantage to the mutant viruses at the entry level probably through more efficient access to CD81 [Bungyoku et al., 2009]. The Asn-534 is located in the sixth of 11 *N*-linked glycosylation sites and the N534H mutation is predicted to remove this glycosylation. The present study has shown that the N534H mutation in the E2 glycoprotein of HCV J6/JFH1 markedly enhances the sensitivity of the virus to neutralization by specific neutralizing antibodies in sera of patients infected with HCV.

MATERIALS AND METHODS

Cells and Viruses

Huh-7.5 cells [Blight et al., 2002] and pFL-J6/JFH1 [Lindenbach et al., 2005] were kindly provided by Dr. C. M. Rice (Rockefeller University, New York, NY, USA). Huh-7.5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (Biowest, Nuaille, France), 0.1 mM non-essential amino acids (Invitrogen, Carlsbad, CA), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (Invitrogen) at 37°C in a CO_2 incubator. Propagation of HCV J6/JFH1, its cell culture-adapted mutant P-47 and recombinant viruses possessing each of the adaptive mutations was described previously [Deng et al., 2008; Bungyoku et al., 2009].

Human Sera and Anti-HCV Neutralization Test

Sera were collected from 89 patients infected chronically with HCV-1b or HCV-2a, who were treated with pegylated interferon α-2b and ribavirin, as described previously [El-Shamy et al., 2007, 2008]. Sera were also collected from 11 patients with acute HCV-1b infection, either severe acute hepatitis or mild self-resolving hepatitis. The study protocol was approved by the Ethic Committees in Kobe University and Yamagata University and informed written consent provided by patients and volunteers. Sera collected from healthy volunteers who were negative for anti-HCV antibodies served as a control. The sera were inactivated at 56°C for 30 min before being used for the virus neutralization test.

An HCV neutralization test was performed as described previously [Sasayama et al., 2010]. In brief, serially diluted serum samples were mixed with the same amount of HCV solution containing 1×10^4 cell-infecting units. After incubation at 37°C for 1 hr, the mixtures were inoculated to Huh-7.5 cells (2×10^5 cells per well in 24-well plates) and incubated in a 5% CO_2 incubator. After 3 hr, the inocula were removed and fresh complete DMEM were added to the cells. At 24 hr postinfection, cells were fixed with ice-cold methanol, blocked with 5% goat serum in phosphate-buffered saline and subjected to immunofluorescence analysis using mouse monoclonal antibody against HCV core antigen (2H9) [Wakita et al., 2005] and Alexa Fluor 488-conjugated goat anti-mouse IgG (H + L) (Molecular Probes, Eugene, OR). The immunostained cells were counterstained with Hoechst 33342 (Molecular Probes) at room temperature for 5 min and observed under a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). The number of HCV-infected cells in each well was counted by using a software BZ-H1C (Keyence). The serum dilutions that neutralized 50% of the virus infectivity was calculated by curvilinear regression analysis [Abe et al., 2003]. Titers were expressed as 50% neutralization titers (NT_{50}).

Statistical Analysis

Student's *t*-test was used to compare the data between different groups. A *P*-value of <0.05 was considered to be significant.

RESULTS

Anti-HCV Neutralizing Antibodies in Sera of Patients Infected With HCV

Sera were obtained from patients chronically infected with HCV-1b or -2a, and tested for anti-HCV neutralizing activities. Representative results of neutralization curves using the parental J6/JFH1 and the P-47 mutant as challenge viruses are shown in Figure 1. When measured against J6/JFH1, NT_{50} titers of sera of patients infected with HCV-1b ranged from 1:10 to 1:700, with the mean NT_{50} titer being 1:197, whereas those of patients infected with HCV-2a ranged from 1:100 to 1:1,500, with the mean value being 1:670 (Table I). The difference in NT_{50} between patients infected with HCV-1b and -2a was statistically significant ($P < 0.00001$). When measured against P-47, on the other hand, unexpectedly high NT_{50} titers were obtained ranging from 1:4,000 to 1:182,000, with the mean values being 1:40,500 and 1:32,900 for patients infected with HCV-1b and -2a, respectively. These results suggest the possibility that an adaptive mutation(s) of P-47, most probably present in the envelope glycoproteins, confers higher sensitivity to neutralization by anti-HCV antibodies.

Unlike the case with J6/JFH1, when P-47 was used as a challenge virus, no significant difference in NT_{50} titers was observed between patients infected with HCV-1b and -2a (Table I). This result suggests the possible presence of a genotype-dominant neutralization epitope(s) on the envelope glycoproteins of J6/JFH1 although anti-HCV neutralizing antibodies in patients' sera are reactive to both HCV-1b and -2a. The broad reactivity of the neutralizing antibodies in patients' sera across different HCV genotypes is consistent with previous observations by other researchers [Logvinoff et al., 2004; Meunier et al., 2005; Fournier et al., 2007; Pestka et al., 2007; Scheel et al., 2008].

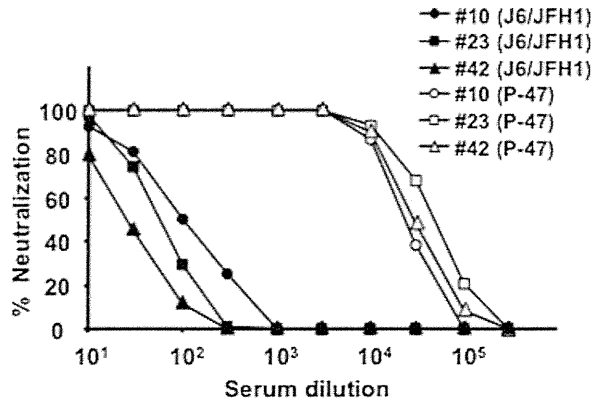

Fig. 1. Neutralization curves (NT_{50} assay) of sera obtained from HCV-infected patients against HCV J6/JFH1 and its adaptive mutant P-47. J6/JFH1 or P-47 was incubated with serial dilutions of HCV-infected patients (nos. 10, 23, and 42; all infected with HCV-1b) and tested for neutralization activities. The neutralization rates at each dilution were plotted. Filled and open symbols indicate data obtained with J6/JFH1 and P-47, respectively.

Sera obtained from patients with acute hepatitis C contained much lower titers of anti-HCV neutralizing antibodies compared to those in sera from chronic hepatitis patients, with the average NT_{50} titers against J6/JFH1 and the adaptive mutant P-47 being 1:15 and 1:126, respectively (Table I). Two patients with severe acute hepatitis C with elevated serum alanine aminotransferase levels of >1,000 IU/ml [Saito et al., 2004; unpublished], possessed relatively high NT_{50} titers against P-47 (1:150 and 1:1,100) compared to the remaining nine patients who experienced mild self-resolving hepatitis (<1:10 to 1:50).

A Single-Point Mutation (N534H or T416A) of the HCV E2 Glycoprotein Increases Sensitivity to Neutralization by Anti-HCV Antibodies

Neutralization of virus infectivity by antibodies usually involves their interaction with viral envelope glycoproteins. It has been reported that the cell culture-adapted mutant P-47 possesses 10 amino acid mutations, including four mutations in E2, compared to the parental J6/JFH1 [Bungyoku et al., 2009]. To examine which mutation(s) in E2 is responsible for the increased sensitivity of P-47 to neutralization by antibodies in patients' sera, recombinant viruses possessing each one of the four mutations in E2 were used (Fig. 2A). The result obtained revealed that a recombinant virus possessing a single-point mutation at position 534 from Asn to His (N534H) and another one possessing four mutations (E2) were as sensitive as P-47 to neutralization by sera of chronic hepatitis patients (Fig. 2B) and the two patients with acute hepatitis C (data not shown). The T416A and T396A mutants were also significantly more sensitive than J6/JFH1, but less sensitive than P-47, N534H, and E2 mutants, to neutralization by antibodies in patients' sera. In this connection, it was recently reported that a JFH1 virus-based T416A mutant showed increased sensitivity to antibody neutralization [Dhillon et al., 2010].

DISCUSSION

The present results revealed that sera of patients infected with HCV-1b possessed cross-genotypic neutralizing antibodies against the J6/JFH1 strain of HCV-2a, albeit with significantly lower titers (ca. one-third) compared to the homotypic neutralization titers observed for patients infected with HCV-2a (Table I). When measured against the adaptive mutant P-47 derived from J6/JFH1, neutralizing antibody titers of the patients sera increased markedly to the level 50- to 200-times higher than that measured against J6/JFH1. Also, the partial genotype-specificity observed with J6/JFH1 was no longer evident when measured against P-47. The marked increase in the sensitivity of P-47 to antibody neutralization was assigned to a mutation at position 534 (N534H), and another one at position 416 (T416A) to a lesser extent, of the E2 glycoprotein (Fig. 2).

TABLE I. NT_{50} Titers in Sera of HCV-Infected Patients With Chronic or Acute Hepatitis C

		NT_{50} titer[a] measured against	
CH/AH	Genotype	J6/JFH1	P-47
CH	HCV-1b (n = 69)	197 ± 164 (1)	40,500 ± 31,800 (206)
CH	HCV-2a (n = 20)	670 ± 652[b] (3.4)	32,900 ± 26,500[c] (167)
AH	HCV-1b (n = 11)	15 ± 28 (0.08) (<10–100)	126 ± 326 (0.6) (<10–1,100)

CH, chronic hepatitis; AH, acute hepatitis.
[a]Mean ± SD. The number in the parenthesis means the ratio when compared to the mean titer that was obtained with sera of HCV-1b-infected CH patients against J6/JFH1.
[b]$P < 0.00001$, compared to the mean titer obtained with sera of HCV-1b-infected patients against J6/JFH1 (Student's *t*-test).
[c]$P = 0.33$, compared to the mean titer obtained with sera of HCV-1b-infected patients against P-47 (Student's *t*-test).

The N534H and T416A mutations are located at the sixth, and in close proximity to the first, respectively, of the conserved 11 *N*-linked glycosylation sites of the HCV E2 glycoprotein [Helle et al., 2007; Bungyoku et al., 2009]. It was recently reported that the positions 416 and 534 are conformationally located in the former and the latter halves of the central domain 1 (DIa and DIb), respectively, of E2 and that the two parts of DI domain interact to form the CD81-binding region [Helle et al., 2010; Krey et al., 2010; Albecka et al., 2011]. This region is, therefore, considered as the possible target for neutralizing antibodies that inhibit E2-CD81 interactions [Helle and Dubuisson, 2008; Law et al., 2008; Owsianka et al., 2008; Perotti et al., 2008].

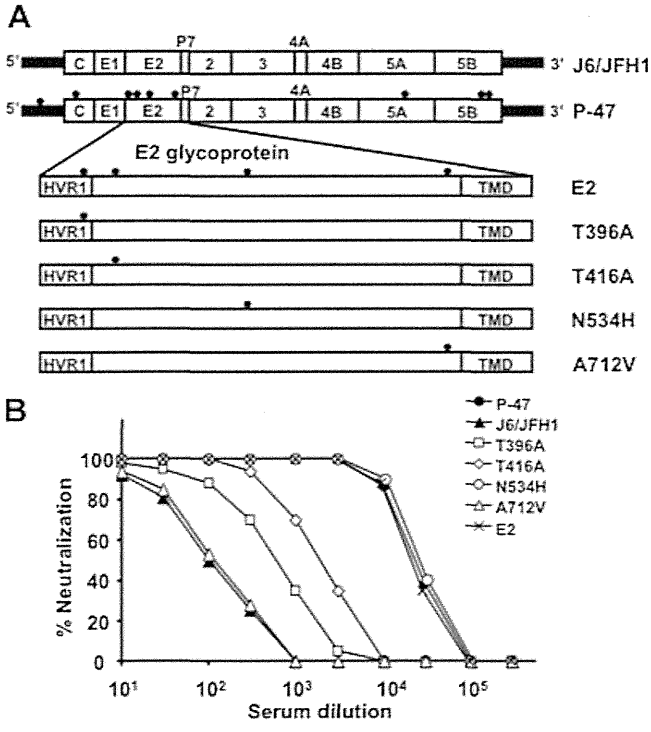

Fig. 2. Effects of amino acid mutations at positions 396, 416, 534, and 712 of the HCV E2 glycoprotein on neutralization by anti-HCV antibodies in patients' sera. **A**: A schematic diagram of the mutations seen in the adaptive mutant P-47 and recombinant viruses carrying each (T396A, T416A, N534H, and A712V) and all (E2) of the four mutations in E2. Filled circles indicate the positions of the mutations. **B**: A representative result of virus neutralization by anti-HCV antibodies in an HCV-infected patient (no. 10; HCV-1b).

The N534H mutation removes glycans at this position as it disrupts the consensus sequence for *N*-linked glycosylation. The removal of glycans at positions 417, 532, and 645 (the first, sixth, and eleventh glycosylation site, respectively) of the H77 isolate (HCV-1a) was shown to increase the sensitivity of HCVpp to neutralizing antibodies and to enhance the access of CD81 to its binding site on E2 [Falkowska et al., 2007; Helle et al., 2007]. It should be noted, however, that the HCVpp system relies on retroviral pseudoparticles bearing HCV envelope glycoproteins that assemble at the plasma membrane or in multivesicular bodies whereas HCV virions assemble on the endoplasmic reticulum membranes that are closely associated with lipid droplet [Miyanari et al., 2007; Helle and Dubuisson, 2008]. Therefore, the virus neutralization data obtained with HCVpp should be verified using the HCVcc system in which virion assembly and maturation take place through the authentic process.

By using the HCVcc system, it was shown that a variant virus possessing the N534K mutation spread faster than the parental JFH1 virus [Delgrange et al., 2007], with the result suggesting the possibility that removal of glycans on residue 534 resulted in more efficient access of E2 to CD81. It is also possible that removal of glycans on this residue might allow more efficient access of neutralizing antibodies to the CD81-binding region of E2, resulting in increased sensitivity to antibody neutralization. In fact, Helle et al. [2010] recently reported that removal of glycans at five (the first, second, fourth, sixth, and eleventh) *N*-linked glycosylation sites in E2 markedly increased the sensitivity of JFH1 virus to antibody neutralization, suggesting that the glycans interfere with the access of neutralizing antibodies to a determinant crucial for virus infectivity. It was also reported that mutations at positions 415 (N415D) and 416 (T416A) near the first glycosylation site of JFH1 virus increased the sensitivity to neutralizing antibodies in patients' sera [Dhillon et al., 2010]. Also, a mutation at position 451 (G451R), which is located in the domain 2 (DII) but still in close proximity to DI [Helle et al., 2010; Krey et al., 2010; Albecka et al., 2011],

increased the sensitivity of JFH1 virus to antibody neutralization [Grove et al., 2008].

In conclusion, the present study using J6/JFH1 virus, another HCVcc strain, has demonstrated that the N534H mutation within the sixth *N*-glycosylation site of the E2 glycoprotein, and the T416A mutation near the first *N*-glycosylation site to a lesser extent, markedly enhances sensitivity to neutralization by antibodies in sera of HCV-infected patients. These results suggest that glycans on Asn-534 of the HCV E2 glycoprotein plays an important role in protecting the virus from humoral immune mechanisms of the host.

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REFERENCES

Abe M, Kuzuhara S, Kino Y. 2003. Establishment of an analyzing method for a Japanese encephalitis virus neutralization test in vero cells. Vaccine 21:1989–1994.

Albecka A, Monserret R, Krey T, Tarr AW, Diesis E, Ball JK, Descamps V, Duverlie G, Rey F, Penin F, Dubuisson J. 2011. Identification of new functional regions in hepatitis C virus envelope glycoprotein E2. J Virol JVI accepts, 85:1777–1792.

Bartosch B, Bukh J, Meunier JC, Granier C, Engle RE, Blackwelder WC, Emerson SU, Cosset FL, Purcell RH. 2003. In vitro assay for neutralizing antibody to hepatitis C virus: Evidence for broadly conserved neutralization epitopes. Proc Natl Acad Sci USA 100:14199–14204.

Blight KJ, McKeating JA, Rice CM. 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J Virol 76:13001–13014.

Bowen DG, Walker CM. 2005. Adaptive immune responses in acute and chronic hepatitis C virus infection. Nature 436:946–952.

Bungyoku Y, Shoji I, Makine T, Adachi T, Hayashida K, Nagano-Fujii M, Ide YH, Deng L, Hotta H. 2009. Efficient production of infectious hepatitis C virus with adaptive mutations in cultured hepatoma cells. J Gen Virol 90:1681–1691.

Delgrange D, Pillez A, Castelain S, Cocquerel L, Rouillé Y, Dubuisson J, Wakita T, Duverlie G, Wychowski C. 2007. Robust production of infectious viral particles in Huh-7 cells by introducing mutations in hepatitis C virus structural proteins. J Gen Virol 88:2495–2503.

Deng L, Adachi T, Kitayama K, Bungyoku Y, Kitazawa S, Ishido S, Shoji I, Hotta H. 2008. Hepatitis C virus infection induces apoptosis through a Bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway. J Virol 82:10375–10385.

Dhillon S, Witteveldt J, Gatherer D, Owsianka AM, Zeisel MB, Zahid MN, Rychłowska M, Foung SK, Baumert TF, Angus AG, Patel AH. 2010. Mutations within a conserved region of the hepatitis C virus glycprotein that influence virus-receptor interaction and sensitivity to neutralizing antibodies. J Virol 84: 5494–5507.

Dowd KA, Netski DM, Wang XH, Cox AL, Ray SC. 2009. Selection pressure from neutralizing antibodies drives sequence evolution during acute infection with hepatitis C virus. Gastroenterology 136:2377–2386.

El-Shamy A, Sasayama M, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. 2007. Prediction of efficient virological response to pegylated interferon/ribavirin combination therapy by NS5A sequences of hepatitis C virus and anti-NS5A antibodies in pretreatment sera. Microbiol Immunol 51:471–482.

El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. 2008. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology 48:38–47.

Falkowska E, Kajumo F, Garcia E, Reinus J, Dragic T. 2007. Hepatitis C virus envelope glycoprotein E2 glycans modulate entry, CD81 binding, and neutralization. J Virol 81:8072–8079.

Fournier C, Duverlie G, François C, Schnuriger A, Dedeurwaerder S, Brochot E, Capron D, Wychowski C, Thibault V, Castelain S. 2007. A focus reduction neutralization assay for hepatitis C virus neutralizing antibodies. Virol J 4:35.

Gal-Tanamy M, Keck ZY, Yi M, McKeating JA, Patel AH, Foung SK, Lemon SM. 2008. In vitro selection of a neutralization-resistant hepatitis C virus escape mutant. Proc Natl Acad Sci USA 105:19450–19455.

Grove J, Nielsen S, Zhong J, Bassendine MF, Drummer HE, Balfe P, McKeating JA. 2008. Identification of a residue in hepatitis C virus E2 glycoprotein that determines scavenger receptor BI and CD81 receptor dependency and sensitivity to neutralizing antibodies. J Virol 82:12020–12029.

Helle F, Dubuisson J. 2008. Hepatitis C virus entry into host cells. Cell Mol Life Sci 65:100–112.

Helle F, Goffard A, Morel V, Duverlie G, McKeating J, Keck ZY, Foung S, Penin F, Dubuisson J, Voisset C. 2007. The neutralizing activity of anti-hepatitis C virus antibodies is modulated by specific glycans on the E2 envelope protein. J Virol 81:8101–8111.

Helle F, Vieyres G, Elkrief L, Popescu CI, Wychowski C, Descamps V, Castelain S, Roingeard P, Duverlie G, Dubuisson J. 2010. Role of N-linked glycans in the functions of hepatitis C virus envelope proteins incorporated into infectious virions. J Virol 84:11905–11915.

Keck ZY, Olson O, Gal-Tanamy M, Xia J, Patel AH, Dreux M, Cosset FL, Lemon SM, Foung SK. 2008. A point mutation leading to hepatitis C virus escape from neutralization by a monoclonal antibody to a conserved conformational epitope. J Virol 82:6067–6072.

Krey T, d'Alayer J, Kikuti CM, Saulnier A, Damier-Piolle L, Petitpas I, Johansson DX, Tawar RG, Baron B, Robert B, England P, Persson MA, Martin A, Rey FA. 2010. The disulfide bonds in glycoprotein E2 of hepatitis C virus reveal the tertiary organization of the molecule. PLoS Pathog 6:e1000762.

Lavillette D, Morice Y, Germanidis G, Donot P, Soulier A, Pagkalos E, Sakellariou G, Intrator L, Bartosch B, Pawlotsky JM, Cosset FL. 2005. Human serum facilitates hepatitis C virus infection, and neutralizing responses inversely correlate with viral replication kinetics at the acute phase of hepatitis C virus infection. J Virol 79:6023–6034.

Law M, Maruyama T, Lewis J, Giang E, Tarr AW, Stamataki Z, Gastaminza P, Chisari FV, Jones IM, Fox RI, Ball JK, McKeating JA, Kneteman NM, Burton DR. 2008. Broadly neutralizing antibodies protect against hepatitis C virus quasispecies challenge. Nat Med 14:25–27.

Lindenbach BD, Evans MJ, Syder AJ, Wölk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM. 2005. Complete replication of hepatitis C virus in cell culture. Science 309:623–626.

Logvinoff C, Major ME, Oldach D, Heyward S, Talal A, Balfe P, Feinstone SM, Alter H, Rice CM, McKeating JA. 2004. Neutralizing antibody response during acute and chronic hepatitis C virus infection. Proc Natl Acad Sci USA 101:10149–10154.

Meunier JC, Engle RE, Faulk K, Zhao M, Bartosch B, Alter H, Emerson SU, Cosset FL, Purcell RH, Bukh J. 2005. Evidence for cross-genotype neutralization of hepatitis C virus pseudoparticles and enhancement of infectivity by apolipoprotein C1. Proc Natl Acad Sci USA 102:4560–4565.

Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, Bartenschlager R, Wakita T, Hijikata M, Shimotohno K. 2007. The lipid droplet is an important organelle for hepatitis C virus production. Nat Cell Biol 9:1089–1097.

Netski DM, Mosbruger T, Depla E, Maertens G, Ray SC, Hamilton RG, Roundtree S, Thomas DL, McKeating J, Cox A. 2005. Humoral immune response in acute hepatitis C virus infection. Clin Infect Dis 41:667–675.

Owsianka AM, Tarr AW, Keck ZY, Li TK, Witteveldt J, Adair R, Foung SK, Ball JK, Patel AH. 2008. Broadly neutralizing human monoclonal antibodies to the hepatitis C virus E2 glycoprotein. J Gen Virol 89:653–659.

Perotti M, Mancini N, Diotti RA, Tarr AW, Ball JK, Owsianka A, Adair R, Patel AH, Clementi M, Burioni R. 2008. Identification of a broadly cross-reacting and neutralizing human monoclonal