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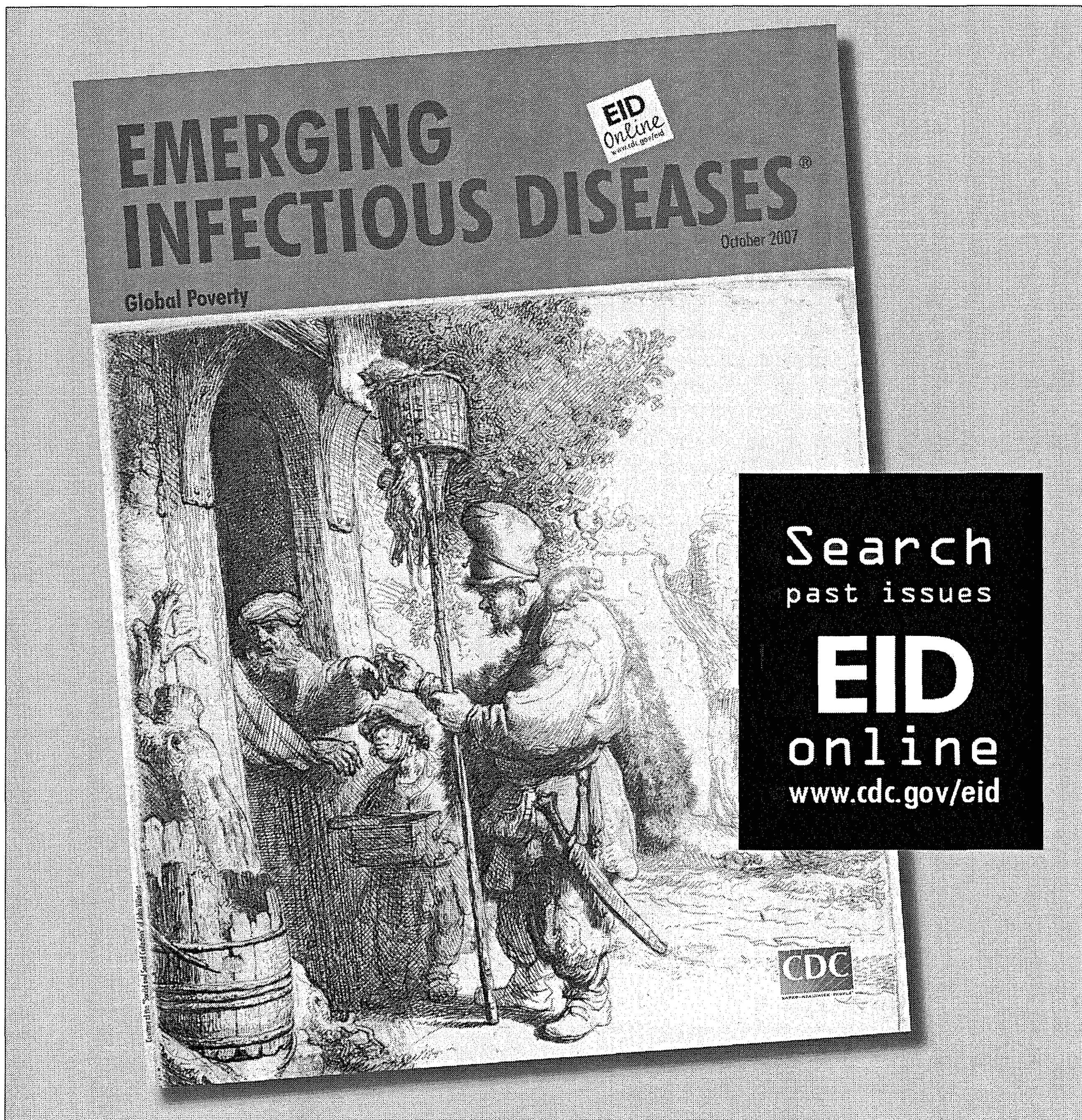

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Hepatitis C Virus Infection Suppresses GLUT2 Gene Expression via Downregulation of Hepatocyte Nuclear Factor 1α

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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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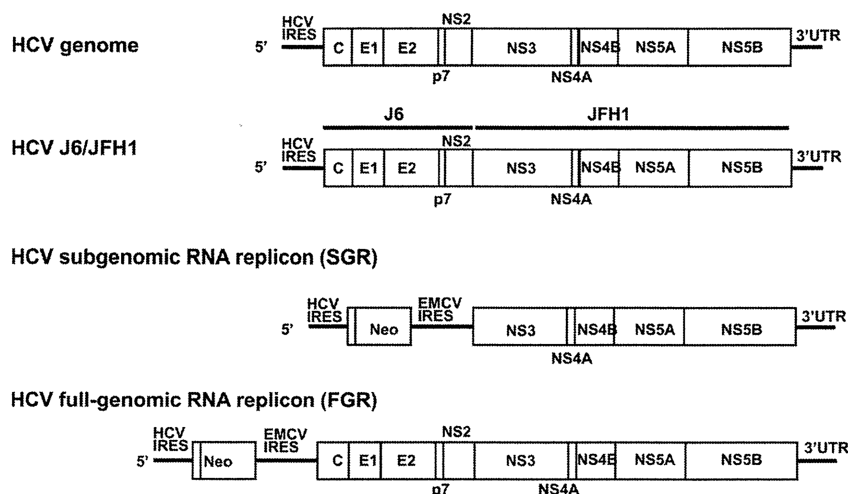

FIG 1 The HCV genome, chimeric HCV J6/JFH1, and the HCV RNA replicons. Schematic diagrams of the HCV genome, the chimeric HCV J6/JFH1 genome, SGR, and FGR are shown. IRES, internal ribosome entry site; EMCV, encephalomyocarditis virus; Neo, neomycin resistance gene.

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

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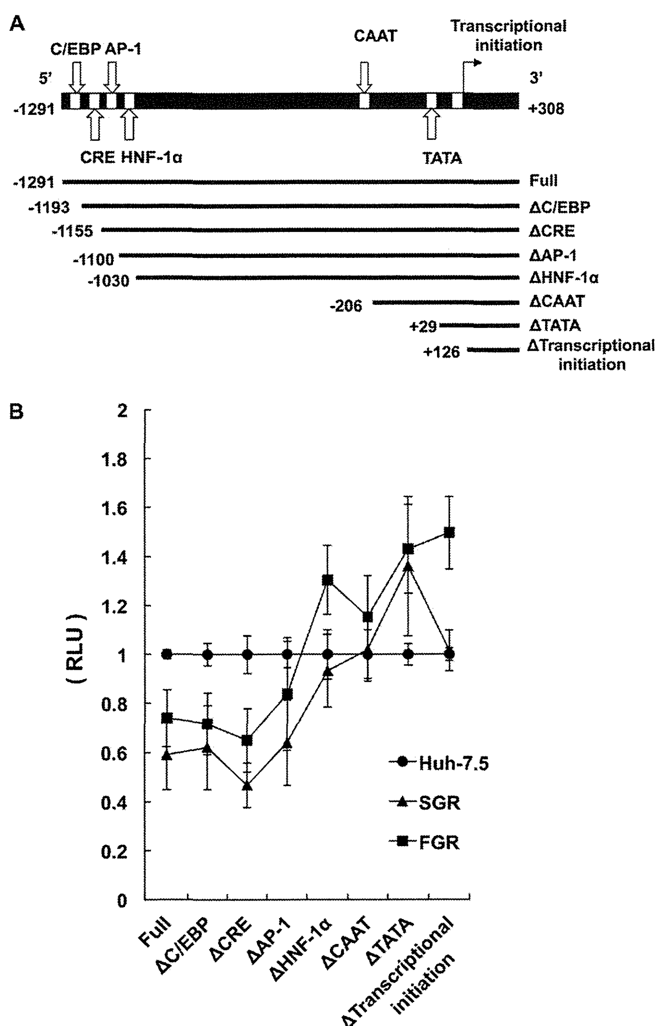

FIG 2 HNF-1α-binding site is important for HCV-induced suppression of GLUT2 promoter. (A) A series of constructs in which genomic GLUT2 promoter DNA fragments were fused to a promoterless firefly luciferase gene of the pGL4 vector were generated with the 3′ end always terminating at bases +308 from transcriptional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

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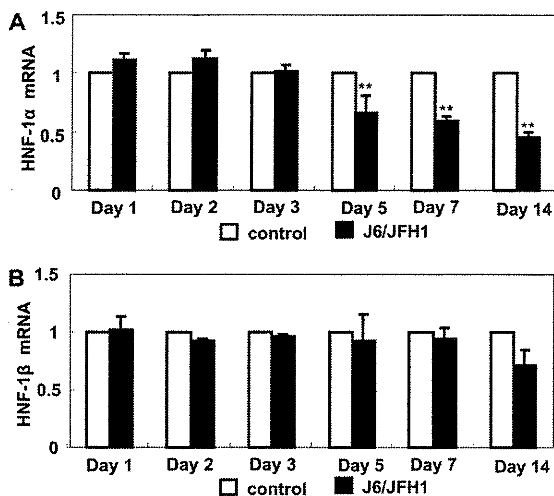

FIG 3 Quantitative RT-PCR analysis of mRNA for HNF-1α and HNF-1β in HCV J6/JFH1-infected cells. Huh-7.5 cells (2.5 × 10^5 cells/six-well plate) were infected with HCV J6/JFH1 at a multiplicity of infection of 2. Cells were cultured and harvested at the indicated times. Total RNA was extracted, and the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

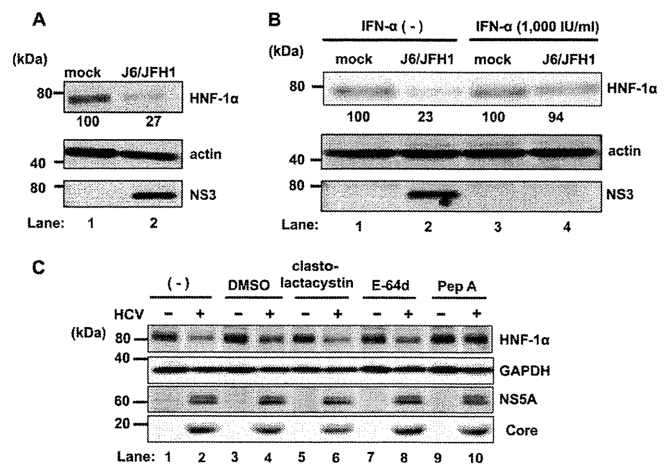

FIG 4 HCV infection induces lysosomal degradation of HNF-1α protein. (A) HCV infection decreased the levels of HNF-1α protein in Huh-7.5 cells. Huh-7.5 cells (2.5 × 10^5 cells/six-well plate) were infected with HCV J6/JFH1 at a multiplicity of infection of 2. Cells were cultured and harvested at 5 days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

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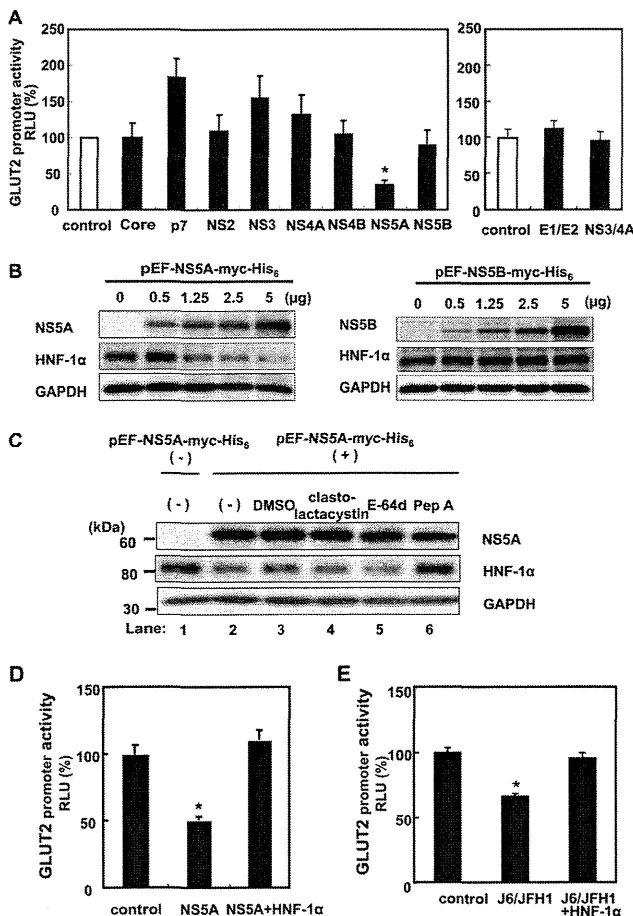

FIG 5 HCV NS5A protein is involved in suppression of GLUT2 promoter activity and lysosomal degradation of HNF-1α protein. (A) Huh-7.5 cells were plated at 1×10^5 cells/12-well plate. After cells were cultured for 12 h, cells were cotransfected with each HCV protein plasmid (0.5 μg), the human GLUT2 promoter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

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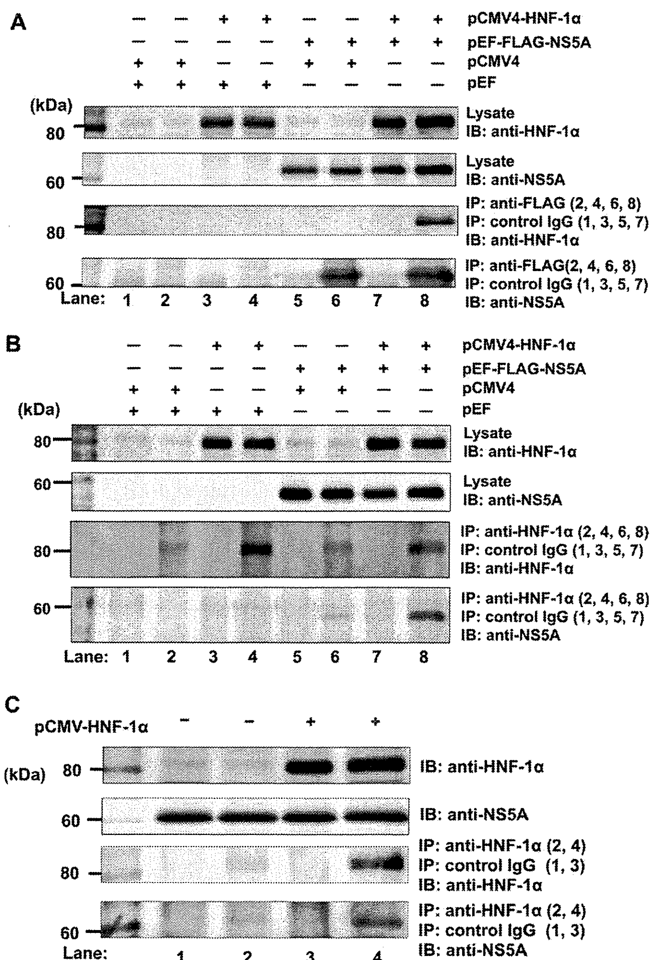

FIG 6 NS5A protein interacts with HNF-1α protein. (A) Huh-7.5 cells were plated at 1.2 × 10^6 cells/10-cm dish and cultured for 12 h. Cells were transfected with plasmids as indicated. At 48 h after transfection, cells were harvested. Cell lysates were immunoprecipitated with either anti-FLAG mouse MAb (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

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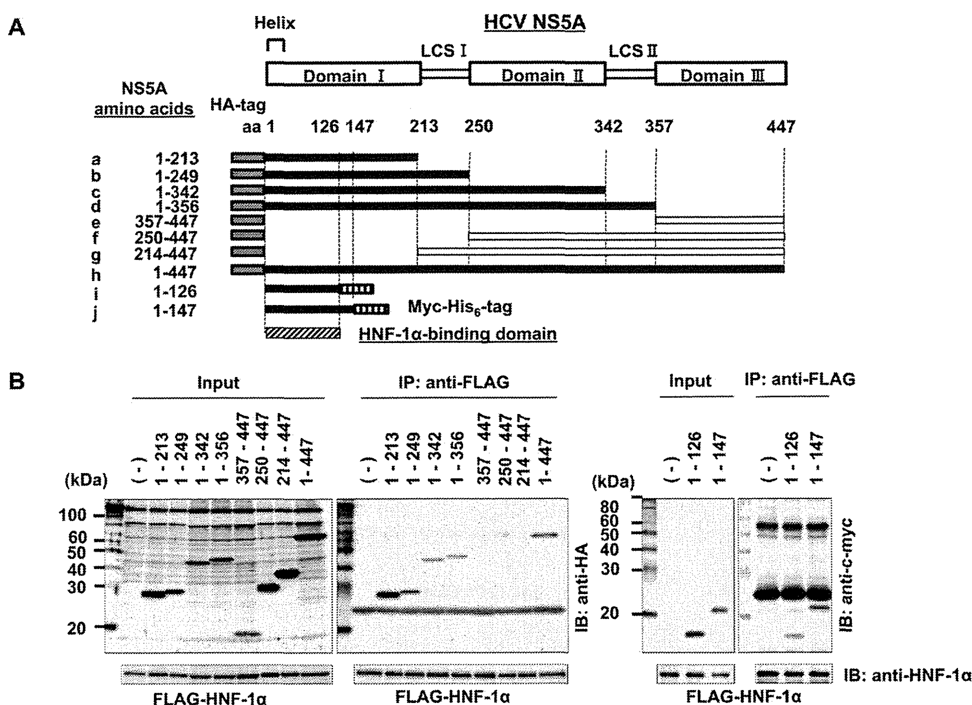

FIG 7 Mapping of the HNF-1α-binding domain for NS5A protein. (A) Schematic representation of the hepatitis C virus NS5A protein. NS5A consists of three domains (domains I, II, and III) with domains separated by low-complexity sequences (LCS I and II). The position of the amino-terminal amphipathic helix 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 posttranslational 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).

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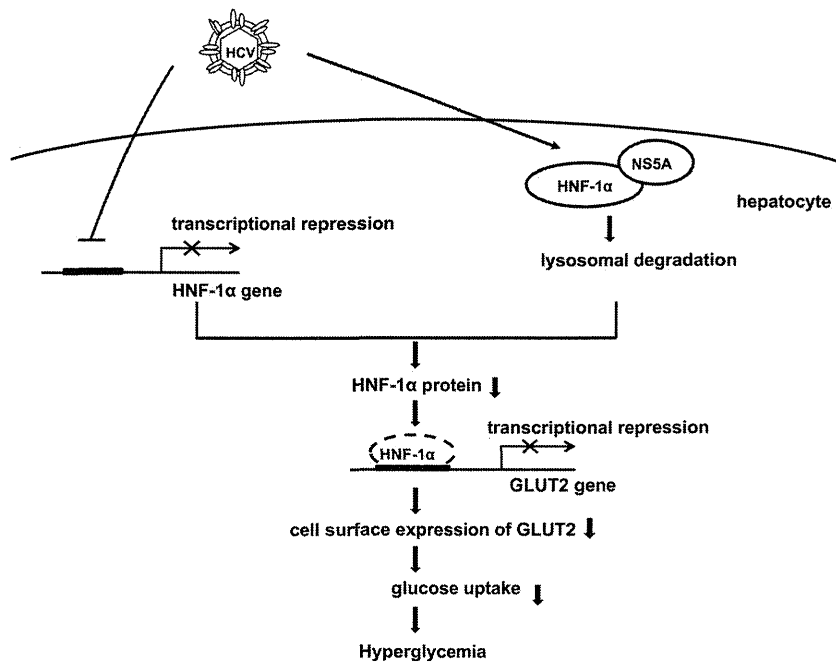

FIG 8 A proposed mechanism of the HCV-induced suppression of GLUT2 via downregulation of HNF-1α. HCV infection downregulates HNF-1α at transcriptional and posttranslational levels, resulting in suppression of GLUT2 gene transcription. HCV NS5A protein physically interacts with HNF-1α protein and enhances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

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EDITORIAL

Roles of the two distinct proteasome pathways in hepatitis C virus infection

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Abstract

Hepatitis C virus (HCV) infection often causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. The development of a HCV cell culture system enabled us to investigate its whole HCV life cycle and develop a better understanding of the pathogenesis of this virus. Post-translational modification plays a crucial role in HCV replication and in the maturation of viral particles. There is growing evidence also suggesting that the ubiquitin-proteasome pathway and the ubiquitin-independent proteasome pathway are involved in the stability control of HCV proteins. Many viruses are known to manipulate the proteasome pathways to modulate the cell cycle, inhibit apoptosis, evade the immune system, and activate cell signaling, thereby contributing to persistent infection and viral carcinogenesis. The identification of functional interactions between HCV and the proteasome pathways will therefore shed new light on the life cycle and pathogenesis of HCV. This review summarizes the current knowledge on the involvement of the ubiquitin-dependent and -independent proteasome pathways in HCV infection and discusses the roles of these two distinct mechanisms in HCV pathogenesis.

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Key words: Hepatitis C virus; Ubiquitin; Proteasome; Degradation; Hepatitis

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INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded, positive-sense RNA virus from the family *Flaviviridae* and is the main cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma[1-5]. More than 170 million people worldwide are chronically infected with HCV[6]. The 9.6-kb HCV genome encodes a unique open reading frame encoding a large precursor polyprotein, which is cleaved co-translationally into at least 10 proteins by two viral proteases and two cellular signalases[4,5,7-10].

The previous establishment of a HCV cell culture system has facilitated studies of the whole viral life cycle[11-13]. The HCV life cycle is tightly regulated by both viral and cellular proteins[5] and evidence is accumulating to show that the stability of HCV proteins is regulated through both the ubiquitin-dependent and ubiquitin-independent proteasome pathways[14-18]. Moreover, HCV infection has been shown to trigger the degradation of host factors[19]. It is well known that many viruses manipulate the ubiquitin-proteasome pathway to promote their propagation by redirecting the cellular ubiquitin machinery to enable replication, egress and evasion of the host immune system[20]. Although the majority of the protein turnover mediated by the proteasome occurs through the canonical ubiquitin-dependent 26S proteasome pathway, a number of viral proteins and host proteins are degraded

through the 20S proteasome without prior polyubiquitylation[21,22]. The functional differences between these two proteasome pathways are poorly understood, although a number of proto-oncogenes and tumor suppressors are degraded through both mechanisms, indicative of a system that tightly regulates the turnover of key cellular proteins[23-28].

Ubiquitin is a 76 amino acid polypeptide that is highly conserved among eukaryotic organisms. The ubiquitin/26S proteasome pathway is composed of an enzymatic cascade that ubiquitylates proteins to target them for proteasomal degradation. The E1 ubiquitin-activating enzyme binds ubiquitin through a thioester linkage in an ATP-dependent manner[29,30]. The activated ubiquitin is then transferred to the E2 ubiquitin-conjugating enzyme which works in conjunction with the E3 ubiquitin ligase, which is responsible for conferring substrate specificity[31]. E3 mediates the transfer of ubiquitin to the target protein which is then rapidly degraded by the 26S proteasome[32,33]. A number of studies have revealed the existence of a proteasome-dependent but ubiquitin-independent pathway for protein degradation. Several key molecules, such as p53, p73, c-fos, p21, SRC-3, and the hepatitis B virus X protein are targeted by two distinct degradation pathways that function in a ubiquitin-dependent and ubiquitin-independent manner, respectively[21-28,34,35]. Although the pathophysiological significance of the proteasomal degradation of the HCV proteins and HCV-induced proteasomal degradation of host proteins remains to be elucidated, evidence is accumulating that the proteasome plays an essential role in propagation of HCV[14,15]. The roles of the proteasome pathways in HCV life cycle as well as in viral pathogenesis are further discussed below.

UBIQUITIN-DEPENDENT DEGRADATION OF HCV PROTEINS BY THE PROTEASOME

HCV core protein

The HCV core protein is a major component of the viral nucleocapsid and is a multifunctional factor involved in both the pathogenesis and hepatocarcinogenesis of HCV and is degraded through the ubiquitin-proteasome pathway[5,16,36]. The cellular ubiquitin ligase E6AP was identified as a HCV core-binding protein in our laboratory and shown to mediate the polyubiquitylation of the core protein and thereby target it for proteasomal degradation[14]. E6AP was first identified as the cellular factor that mediates the ubiquitin-dependent degradation of the tumor suppressor p53 in conjunction with the E6 protein of the cancer-associated human papillomavirus types 16 and 18[37,38]. The region between amino acids 58 and 71 of the HCV core protein is responsible for the interaction with E6AP. These 14 amino acids are highly conserved, with the first nine amino acids (PRGRRQPIP) present in the core proteins of all HCV genotypes. This suggests that the E6AP-dependent degradation of HCV core protein is also conserved. Indeed, a knockdown of endogenous E6AP by siRNA increases the production of infectious HCV particles, further suggesting that E6AP negatively regulates HCV propagation[14].

E2 protein

The HCV envelope proteins comprise two glycoproteins, E1 and E2. HCV infection requires the interaction between these proteins and the host cell membrane. HCV attachment and entry into host cells is a multistep process, involving several cell surface molecules, including CD81[39], the LDL receptor[40], scavenger receptor BI[41], claudin-1[42-44], and occludin[43,45]. Several E2 domains also play crucial roles in virus entry[46]. In addition, HCV E2 has been implicated in conferring resistance to interferon (IFN)-α. E2 contains a region homologous to the double stranded RNA-activated protein kinase (PKR) and its substrate, subunit α of the translation initiation factor eIF2α[27]. The unglycosylated form of the E2 protein (E2-p38) is retained in the cytosol and is degraded through the ubiquitin-proteasome pathway[47]. E2-p38, but not the glycosylated form of E2, interacts with PKR and is stabilized by treatment with IFN-α, suggesting that it contributes to the resistance of HCV to IFN-α. The ubiquitin ligase that targets E2-p38 remains to be identified.

NS5A protein

NS5A protein is a major component of the HCV replication complexes and can be found in basally phosphorylated (56 kDa) and hyperphosphorylated (58 kDa) forms. NS5A harbors an amphipathic α-helix at its amino terminus that promotes membrane association. In addition to this helix region, NS5A contains three domains (Ⅰ-Ⅲ). The N-terminal domain (domain Ⅰ) coordinates a single zinc atom per protein molecule[48]. Zinc mesoporphyrin (ZnMP) is a non-heme metalloporphyrin and a synthetic heme analog of the central zinc in the mesoporphyrin macrocycle. ZnMP enhances the polyubiquitylation and proteasomal degradation of NS5A and suppresses HCV RNA replication[49]. The physiological role of the ubiquitin-dependent proteasomal degradation of NS5A protein is still unclear and the ubiquitin ligase that targets NS5A also remains to be identified.

NS5B protein

The HCV NS5B protein functions as an RNA-dependent RNA polymerase. NS5B binds to a ubiquitin-like protein, hPLIC1[50], which contains 589 amino acids and belongs to a family of type 2 ubiquitin-like (ubl) proteins. hPLIC1 harbors a non-cleavable ubiquitin-like (ubl) domain in its amino terminus and a ubiquitin-associated (uba) domain in its carboxyl terminus[51] and physically associates with the proteasome and at least two ubiquitin ligases (E6AP and βTRCP). NS5B binds to the uba domain of hPLIC1, an interaction which enhances the polyubiquitylation and proteasomal degradation of NS5B, suggesting that

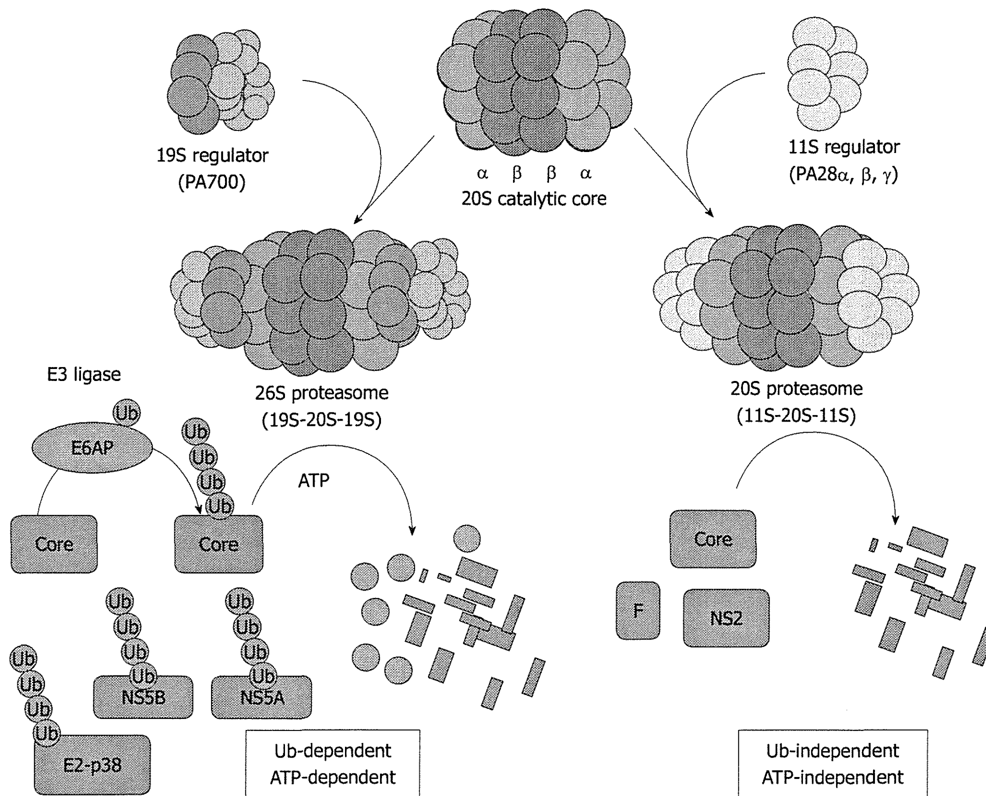

Figure 1 Two distinct proteasome pathways target hepatitis C virus proteins for degradation. The 20S catalytic core is composed of α and β subunits that form a barrel-like structure. The 19S regulator (PA700) can associate with either or both ends of the 20S catalytic core. The combination of one 20S catalytic core and one or two 19S regulator generates the 26S proteasome that is responsible for ubiquitin-dependent ATP-dependent degradation of specific target substrates. E6AP mediates the polyubiquitylation of the hepatitis C virus (HCV) core protein and thereby targets it for ubiquitin-dependent degradation. E2-p38, NS5A, and NS5B are degraded through this ubiquitin-dependent and ATP-dependent proteasome pathway. The proteasome activator, PA28γ, forms a homoheptamer and is implicated in the ubiquitin-independent turnover of the HCV core protein. The F and NS2 proteins are also degraded through the ubiquitin-independent pathway.

hPLIC1 regulates HCV RNA replication by affecting NS5B turnover[50]. The responsible E3 ligase for NS5B ubiquitylation again remains to be identified.

UBIQUITIN-INDEPENDENT DEGRADATION OF HCV PROTEINS *VIA* THE PROTEASOME

Core protein

The HCV core protein specifically interacts with a proteasome activator PA28γ/REGγ in the nucleus and is degraded through a PA28γ-dependent proteasome pathway[18]. *In vivo* experiments in a mouse model have suggested that PA28γ plays a critical role in HCV-associated insulin-resistance, steatogenesis, and hepatocarcinogenesis[52,53]. The proteasomal turnover of the HCV core protein is regulated by two distinct mechanisms, the E6AP-mediated ubiquitin-dependent pathway and the PA28γ-mediated ubiquitin-independent pathway[14,17] (Figure 1). E6AP enhances the ubiquitylation and degradation of the wild-type HCV core protein, but not a lysine-less mutant counterpart, whereas PA28γ enhances the degradation of both the wild type and lysine-less HCV core protein. A knockdown of either E6AP or PA28γ results in the stabilization of the wild-type core protein. However, the knockdown of PA28γ but not E6AP stabilizes the lysine-less mutant core protein, strongly suggesting that PA28γ enhances the ubiquitin-independent pathway. Knockdown of PA28γ in cells at pre-infection or post-infection with the HCV JFH1 strain impaired viral particle production but exhibited no effect on viral RNA replication[15]. The knockdown of PA28γ enhances the polyubiquitylation of the core protein and impairs HCV production, whereas a knockdown of E6AP reduces polyubiquitylation of core protein and enhances virus production. These findings suggest that HCV production is positively regulated by PA28γ and negatively regulated by E6AP through the degradation of the core protein.

F protein

The HCV F protein is encoded by the +1/-2 reading frame encompassed in the 5' end of the polyprotein coding sequence[54]. The F protein is also known as ARFP (alternative reading frame protein) or as core+1 (which indicates the position of the new ORF)[55]. Translation of the F protein is mediated by a ribosomal frameshift at core protein codons 9-11 (HCV-1a strain). The biological role of the F protein remains to be clarified. The F protein is also highly unstable. It has been suggested that the F protein may bind to the α3 subunit of the 20S proteasome[56]. The α3 subunit facilitates the degrada-

tion of the F protein in a dose-dependent manner and a knockdown of the α3 subunit results in the stabilization of the F protein, even in the presence of a replicating HCV genome. The α3-binding domain within the F protein was mapped to the region between amino acids 40 and 60. There are currently three lines of evidence suggesting that the degradation of the F protein is ubiquitin-independent. First, an F protein mutant lacking lysine residues, which therefore cannot be ubiquitylated, is no more stable than the wild-type F protein. Second, F protein expressed in ts85 cells, which harbor a temperature-sensitive E1 ubiquitin-activating enzyme, is not stabilized when the cells are incubated at the non-permissive temperature. Third, the F protein can be degraded by the 20S proteasome *in vitro* in the absence of any ubiquitylation machinery.

NS2 protein

HCV NS2 protein is a transmembrane protein, composed of a highly hydrophobic N-terminal membrane binding domain and a C-terminal globular and cytosolic protease subdomain. NS2 protease cleaves off the N-terminus of NS3 protein and is involved in the assembly of HCV particles[57,58]. NS2 protein is also a short-lived protein that is rapidly degraded by the proteasome in a phosphorylation-dependent manner through the activity of casein kinase 2 (CK2). NS2 is phosphorylated by CK2 on a serine residue at position 168, which is a part of a consensus CK2 phosphorylation sequence motif (S/TXXE)[59]. This CK2 phosphoacceptor motif is highly conserved among NS2 proteins from all HCV genotypes. No ubiquitin conjugation of NS2 has been detected[59] and lysine mutagenesis has been reported to have no effect on NS2 levels[60]. These results suggest that the degradation of the HCV NS2 protein is ubiquitin-independent but proteasome-dependent.

HCV INFECTION-INDUCED UBIQUITIN-DEPENDENT DEGRADATION OF CELLULAR PROTEINS *VIA* THE PROTEASOME

Retinoblastoma tumor-suppressor protein

The abundance of the retinoblastoma tumor-suppressor protein (pRb) is negatively regulated in HCV RNA replicon cells[61] and HCVcc-infected cells[19]. The HCV RNA-dependent RNA polymerase NS5B protein forms a complex with pRb, targeting it for degradation, resulting in a reduction of pRb, the activation of the E2F-responsive promoter, and the promotion of cell proliferation[61]. NS5B contains a Leu-x-Cys/Asn-x-Asp motif that is homologous to the Rb-binding domains in the oncoproteins of DNA viruses and interacts with pRb through this motif. The ectopic expression of NS5B induces the polyubiquitylation of pRb, the abundance of which is restored by the siRNA knockdown of E6AP or by the overexpression of a dominant-negative E6AP mutant in HCV RNA replicon cells. This suggests the involvement of E6AP in pRb degradation, induced by HCV. However, it has been reported previously in an *in vitro* assay that the ubiquitylation of pRb is not promoted by E6AP, either in the presence or absence of NS5B[19]. The precise mechanism by which NS5B-dependent pRb ubiquitylation occurs thus remains to be clarified.

Suppressor of cytokine signaling 3

Suppressor of cytokine signaling 3 (SOCS3) is one of the negative regulators of cytokine signaling that function *via* the JAK-STAT pathway[62,63]. The SOCS3 protein levels have been found to be decreased in OR6 cells harboring a HCV genotype 1b replicon and also in Huh 7.5.1 cells infected with the HCV genotype 2a strain JFH1[64]. Treatment with the proteasome inhibitor MG132 blocked the inhibitory effects of HCV on the SOCS3 protein levels in both the replicon-harboring OR6 cells and JFH1-infected cells. JFH1 infection increased the ubiquitylation of SOCS3 compared with the mock infected cells. These results have suggested that HCV infection promotes the degradation of SOCS3 through the ubiquitin-dependent proteasome pathway. The underlying mechanism remains to be elucidated.

HCV INFECTION AFFECTS THE IMMUNOPROTEASOME

Proteasomal epitope processing

The induction of $CD8^+$ T cells is dependent on the generation of MHC class I ligands by the proteasome. Whereas the amino-terminus of each epitope can be further defined by post-proteasomal aminoexopeptidases, the carboxyl terminus needs to be defined precisely by the first cleavage. Through the study of a single source outbreak of HCV, Seifert *et al*[65] have previously identified a mutation at a conserved tyrosine on the HCV NS3 protein, which was a tyrosine to phenylalanine substitution. This mutation was found to impair the correct carboxyl-terminal cleavage of an immune-dominant, HLA-A2 restrictive HCV $NS3_{1073-1081}$ epitope from its mutated polypeptide precursor, not only by the constitutive proteasomes, but also by the immunoproteasome. These mutations impair the induction of HCV-specific $CD8^+$ T cells by affecting the proteasomal antigen-processing machinery.

MHC class I -restricted HCV antigen presentation and the effects of ethanol on this process

In Huh-7 cells co-expressing the HCV core protein and CYP2E1, the core protein slightly enhances 20S proteasome activity through a direct interaction and *via* the induction of low CYP2E1-dependent oxidative stress[66,67]. This proteasome activation event is, however, reversed after ethanol exposure which considerably reduces proteasome function due to the induction of high oxidative stress[66]. Ethanol-elicited suppression of the proteasome in the liver ultimately results in a reduced generation of

Table 1 The ubiquitin-dependent and -independent pathways that target hepatitis C virus proteins for degradation

HCV protein	E3 ligase	Function	Ref.
Ubiquitin-dependent, ATP-dependent, 26S proteasome pathway			
Core	E6AP[1]	Inhibit virus production	[14-17]
E2-p38	Unknown[1]	Immune avoidance	[47]
NS5A	Unknown[1]	Inhibit replication	[49]
NS5B	Unknown[1]	Inhibit replication	[50]
Ubiquitin-independent, ATP-independent, 20S proteasome pathway			
Core	PA28γ[2]	Enhance virus production, steatosis	[15,18,52,53]
F	-[2]	Unknown	[56]
NS2	CK2[2]	Unknown	[57,60]

[1]E3 ligase; [2]Host factor. HCV: Hepatitis C virus; CK2: Casein kinase 2.

antigenic peptides and reduced MHC class I -restricted antigen presentation on hepatocytes[68].

Low-molecular-mass protein 7

The HCV NS3 protein interacts with low-molecular-mass protein 7 (LMP7), a component of the immunoproteasome[69]. The minimal binding domain required for this interaction is located between the pro-sequence region of LMP7 (aa 1-40) and the protease domain of NS3. LMP7 has no effects on NS3 protease activity *in vitro*. The peptidase activities of LMP7 immunoproteasome, however, are markedly reduced in a HCV RNA subgenomic replicon. These findings suggest that the downregulation of proteasome peptidase activities could interfere with the processing of viral antigens for presentation by MHC class I molecules, thereby contributing to persistent infection by HCV.

CONCLUSION

In the present review, the current knowledge on the involvement of the ubiquitin-proteasome pathway and ubiquitin-independent proteasome pathway on HCV infection is summarized (Figure 1 and Table 1). As is the case with many other virus types, HCV may manipulate the ubiquitin system and the proteasome system to favor its propagation and contribute to viral pathogenesis. The body of knowledge regarding the ubiquitin-system and the proteasome system has markedly grown in recent years[21,22] and it has now been demonstrated that canonical Lys 48-linked polyubiquitin chains are not the only signals that initiate proteasome-mediated degradation. Monoubiquitylation[70], Lys 63-linked chains[71], Lys 11-linked chains[72], and linear chains[73-75] have been reported to have various functions, including the activation of signaling pathways and cell-cycle progression. Nothing is known however about the involvement of de-ubiquitylating enzymes in the HCV life cycle. The future identification of key molecules in the ubiquitin and proteasome systems will likely provide new insights and a better understanding of the life cycle and pathogenesis of HCV, knowledge which will be essential for the design of novel anti-HCV therapeutics.

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NS5A Sequence Heterogeneity of Hepatitis C Virus Genotype 4a Predicts Clinical Outcome of Pegylated-Interferon–Ribavirin Therapy in Egyptian Patients

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Hepatitis C virus genotype 4 (HCV-4) is the cause of approximately 20% of the 180 million cases of chronic hepatitis C in the world. HCV-4 infection is common in the Middle East and Africa, with an extraordinarily high prevalence in Egypt. Viral genetic polymorphisms, especially within core and NS5A regions, have been implicated in influencing the response to pegylated-interferon and ribavirin (PEG-IFN/RBV) combination therapy in HCV-1 infection. However, this has not been confirmed in HCV-4 infection. Here, we investigated the impact of heterogeneity of NS5A and core proteins of HCV-4, mostly subtype HCV-4a, on the clinical outcomes of 43 Egyptian patients treated with PEG-IFN/RBV. Sliding window analysis over the carboxy terminus of NS5A protein identified the IFN/RBV resistance-determining region (IRRDR) as the most prominent region associated with sustained virological response (SVR). Indeed, 21 (84%) of 25 patients with SVR, but only 5 (28%) of 18 patients with non-SVR, were infected with HCV having IRRDR with 4 or more mutations (IRRDR $\geq$ 4) ($P = 0.0004$). Multivariate analysis identified IRRDR $\geq$ 4 as an independent SVR predictor. The positive predictive value of IRRDR $\geq$ 4 for SVR was 81% (21/26; $P = 0.002$), while its negative predictive value for non-SVR was 76% (13/17; $P = 0.02$). On the other hand, there was no significant correlation between core protein polymorphisms, either at residue 70 or at residue 91, and treatment outcome. In conclusion, the present results demonstrate for the first time that IRRDR $\geq$ 4, a viral genetic heterogeneity, would be a useful predictive marker for SVR in HCV-4 infection when treated with PEG-IFN/RBV.

Hepatitis C virus (HCV) is a major cause of chronic liver disease, hepatocellular carcinoma, and deaths from liver disease and is the most common indication for liver transplantation (7, 26–28, 38). HCV has been classified into seven major genotypes and a series of subtypes (35, 36). In general, HCV genotype 4 (HCV-4) is common in the Middle East and Africa, where it is responsible for more than 80% of HCV infections (23). Although HCV-4 is the cause of approximately 20% of the 180 million cases of chronic hepatitis C in the world, it has not been a major subject of research.

Egypt has the highest prevalence of HCV worldwide (15%) and the highest prevalence of HCV-4, which is responsible for 90% of the total HCV infections, with a predominance of the subtype 4a (HCV-4a) (1, 32). This extraordinarily high prevalence results in an increasing incidence of hepatocellular carcinoma in Egypt, which is now the second most frequent cause of cancer and cancer mortality among men (17, 21). More than 2 decades have passed since the discovery of HCV, and yet therapeutic options remain limited. Up to 2011, the standard treatment for chronic hepatitis C consisted of pegylated alpha interferon (PEG-IFN) and ribavirin (RBV) (19); however, by May 2011 two protease inhibitors (telaprevir and boceprevir) were approved by the Food and Drug Administration (FDA) for use in combination with PEG-IFN/RBV for adult chronic hepatitis C patients with HCV genotype 1 (24, 34). Since the approval of these new protease inhibitors for treatment of HCV-1 infection, the response of HCV-4 to the standard regimen of treatment (PEG-IFN/RBV) has lagged behind other genotypes and HCV-4 has become the most resistant genotype to treat. As PEG-IFN/RBV still remains to be used to treat HCV-4-infected patients, exploring the factors that predict the outcome of PEG-IFN/RBV treatment, such as sustained virological response (SVR), for HCV-4 infections is needed to assess more accurately the likelihood of SVR and thus to make more informed treatment decisions.

While the SVR rate for PEG-IFN/RBV treatment hovers at 50 to 60% in HCV-1 and -4 infection, it is up to 80% in HCV-2 and -3 infections (19, 33). This difference in responses among patients infected with different HCV genotypes suggests that viral genetic heterogeneity could affect, at least to some extent, the sensitivity to IFN-based therapy. In this context, the correlation between IFN-based therapy outcome and sequence polymorphisms within the viral core and NS5A proteins has been widely discussed, in particular in regard to Japanese patients with HCV-1b infection. Initially, in the era of IFN monotherapy, it was proposed that sequence variations within a region in NS5A of HCV-1b, called the IFN sensitivity-determining region (ISDR), were correlated with IFN responsiveness (18). Subsequently, in the era of PEG-IFN/

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RBV combination therapy, we identified a new region near the C terminus of NS5A, referred to as the IFN/RBV resistance-determining region (IRRDR) (13). Recently, we also demonstrated the correlation between IRRDR polymorphism and PEG-IFN/RBV treatment outcome in HCV-2a and -2b infections (15). In addition, HCV core protein polymorphism, in particular at positions 70 and 91, was also proposed as a pretreatment predictor of poor virological response in patients infected with HCV-1b (4–6). To the best of our knowledge, there is no information regarding the correlation between sequence heterogeneity in the NS5A and core proteins of HCV-4 and PEG-IFN/RBV treatment outcome. In the present study, we aimed to investigate this issue in Egyptian patients infected with HCV-4.

MATERIALS AND METHODS

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

Patients. A total of 43 previously untreated patients who were chronically infected with HCV-4a (34 patients), HCV-4m (3 patients), HCV-4n (3 patients), or HCV-4o (3 patients) were consecutively evaluated for antiviral treatment at Cairo University Hospital, Cairo, Egypt, between January 2008 and September 2010. The HCV subtype was determined according to the method of Okamoto et al. (31). The patients were treated with PEG-IFN α-2a (180 μg/week, subcutaneously) and RBV (1,000 to 1,200 mg daily, *per os*) for 48 weeks. The quantification of serum HCV RNA titers was performed as previously reported (14). To minimize the therapeutic burdens, including the high cost and possible side effects, therapy was discontinued if HCV RNA titers at week 12 did not drop by 2 log compared with baseline values or if HCV RNA was still detectable at week 24. These were considered a null response (see Results).

Sequence analysis of the NS5A and core regions of the HCV genome. Blood samples were collected using Vacutainer tubes. The sera were separated within 2 h of blood collection, transferred to sterile cryovials, and kept frozen at −80°C until use. HCV RNA was extracted from 140 μl of serum using a commercially available kit (QIAmp viral RNA kit; Qiagen, Tokyo, Japan). The extracted RNA was reverse transcribed and amplified for the HCV genome encoding a carboxy terminus of NS5A (amino acids [aa] 2193 to 2417) and the core protein (aa 1 to 191) using SuperScript III one-step RT-PCR Platinum *Taq* HiFi (Invitrogen, Tokyo, Japan). The resultant reverse transcription (RT)-PCR product was subjected to a second-round PCR by using Platinum *Taq* DNA polymerase high fidelity III (Invitrogen). Primers used for amplification of the 3′ half of the NS5A region of HCV-4 were as follows: NS5A-4/F1 (5′-CTCAAYTCGTTCGTRGTGGGATC-3′; sense) and NS5A-4/R1 (5′-CGAAGGTCACCTTCTTCTGCCG-3′; antisense) for one-step RT-PCR; and NS5A-4/F2 (5′-ATGCGAGCCYGAGCCGGACGT-3′; sense) and NS5A-4/R2 (5′-GCTCAGGGGGYTRATTGGCAGCT-3′; antisense) for the second-round PCR. Primers for amplification of the core region of HCV-4 were 249-F (5′-GCTAGCCGAGTAGTGTTG-3′; sense) and 984-R (5′-GATGTGRTGRTCGGCCTC-3′; antisense) (40) for one-step RT-PCR; and 319-F (5′-GGAGGTCTCGTAGACCGTGC-3′; sense) (40) and primer-186 (5′-ATGTACCCCATGAGGTCGGC-3′; antisense) (2) for the second-round PCR. RT was performed at 45°C for 30 min and terminated at 94°C for 2 min, followed by the first-round PCR over 35 cycles, with each cycle consisting of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 68°C for 90 s. The second-round PCR was performed under the same conditions. The sequences of the amplified fragments were determined by direct sequencing without subcloning. The amino acid sequences were deduced and aligned using Genetyx Win software version 7.0 (Genetyx Corp., Tokyo, Japan). The numbering of amino acid residues for HCV-4 isolates is according to the polyprotein of ED43 isolate (accession no. Y11604) (10). Consensus sequences of the carboxy terminus of NS5A of a given HCV-4 subtype were inferred by alignment of all sequences obtained in this study as well as all available NS5A sequences of HCV-4a (accession no. Y11604, DQ418782 to DQ418789, DQ516084, and DQ988073 to DQ988079), HCV-4m (FJ462433), HCV-4n (FJ462441), and HCV-4o (FJ462440) from the databases.

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

Nucleotide sequence accession numbers. The sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB725987 through AB726066.

TABLE 1 Virological responses of HCV-4-infected patients treated with PEG-IFN/RBV

Virological response	Proportion (%) of patients with indicated response (no. of patients/total no.)				
	HCV-4[a]	HCV-4a	HCV-4 m	HCV-4n	HCV-4o
SVR	58 (25/43)	56 (19/34)	100 (3/3)	33 (1/3)	67 (2/3)
Non-SVR	42 (18/43)	44 (15/34)	0 (0/3)	67 (2/3)	33 (1/3)
Null response	30 (13/43)	32 (11/34)	0 (0/3)	67 (2/3)	0 (0/3)
Relapse	12 (5/43)	12 (4/34)	0 (0/3)	0 (0/3)	33 (1/3)

[a] Includes all 43 cases with HCV-4 infection (34 cases with HCV-4a and 3 cases each with HCV-4m, -4n, and -4o).

RESULTS

Patients' responses to PEG-IFN/RBV combination therapy. Among 43 patients enrolled in this study, 30 (70%) patients completed the entire course of PEG-IFN/RBV treatment for 48 weeks and follow-up for 24 weeks. On the other hand, the treatment was discontinued for 13 (30%) patients due to poor virological responses at 12 or 24 weeks after initiation of the therapy. Overall, 25 (58%) patients achieved SVR while 18 (42%) patients had non-SVR (Table 1). When analyzed on the basis of the subtype classification, SVR was achieved by 56% (19/34), 100% (3/3), 33% (1/3), and 67% (2/3) of patients infected with HCV-4a, -4m, -4n, and -4o, respectively.

Non-SVR patients are classified into two groups: (i) patients with null response, who did not achieve >2-log reduction of the initial viral load at week 12 or who had detectable viremia at week 24 of the treatment period; and (ii) patients with relapse, who were negative for HCV-RNA at the end of the treatment period (week 48) followed by a rebound viremia at a certain time point during the follow-up period of 24 weeks. Patients with null response represented 30% (13/43) of all the HCV-4-infected subjects analyzed, while those with relapse represented 12% (5/43). A similar tendency was observed for subtype HCV-4a.

Among various patients' demographic characteristics, SVR patients had a significantly lower average age than that of non-SVR patients (Table 2). Furthermore, a tendency for SVR patients to have a lower average titer of initial viral load than that of non-SVR was noted, although the difference was not statistically significant, due possibly to the small number of patients analyzed ($P = 0.07$).

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TABLE 2 Demographic characteristics of HCV-4-infected patients with SVR and non-SVR[a]

Factor	SVR	Non-SVR	P value
Age	38.47 ± 9.51	45.80 ± 5.65	0.014
Sex (male/female)	18/7	15/3	0.48
BMI	27.36 ± 3.65	27.67 ± 5.28	0.85
Platelets (× 10^3/μl)	204.4 ± 40.63	216.7 ± 87.25	0.59
Hemoglobin (g/dl)	14.54 ± 1.38	15.08 ± 1.39	0.25
WBC count	7,041 ± 1,876	7,078 ± 2,977	0.96
Albumin (g/dl)	4.12 ± 0.36	4.328 ± 0.41	0.11
ALT (IU/liter)	78.72 ± 59.68	82.39 ± 41.80	0.83
AST (IU/liter)	64.94 ± 27.63	58.17 ± 23.98	0.44
HCV-RNA (IU/ml)	84,290 ± 186,300	501,800 ± 816,700	0.07

[a] Values are means ± standard deviations. SVR, sustained virological response; BMI, body mass index; WBC, white blood cell; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Correlation between NS5A sequence heterogeneity and SVR in HCV-4 infection. We and other researchers reported significant correlation between sequence polymorphisms within the C-terminal half of NS5A, including that in ISDR and IRRDR, and PEG-IFN/RBV treatment outcome in HCV-1 and HCV-2 infections (13, 15, 18, 30). However, this information is quite limited in HCV-4 infection. To clarify this issue, part of the HCV-4 genome encoding a carboxy terminus (aa 2193 to 2417) of NS5A in pretreatment sera was amplified and sequenced, and amino acid sequences were deduced. The sequences obtained as well as all available NS5A sequences of HCV-4a, -4m, -4n, and -4o from the databases were aligned, and the consensus sequences for a desired HCV-4 subtype were inferred (see Materials and Methods). Next, to identify an NS5A region(s) that would be significantly correlated with treatment outcome, we carried out a sliding window analysis with a window size of 30 residues over the C-terminal half (aa 2193 to 2417) of NS5A sequences obtained from all SVR (n = 25) and non-SVR (n = 18) patients along with corresponding consensus sequences of each HCV-4 subtype as described previously (30). This analysis revealed that the difference in the overall number of amino acid mutations between SVR and non-SVR isolates exceeded the significant threshold only in a region corresponding to IRRDR of HCV-1b (13), ranging from aa 2331 to 2383, thus being referred to as IRRDR[HCV-4] (Fig. 1). Indeed, the average number of amino acid mutations in IRRDR[HCV-4] was significantly larger in SVR than in non-SVR (P = 0.0005) isolates (Fig. 2A). Sequences of IRRDR of HCV-4a, -4m, -4n, and -4o obtained from SVR and non-SVR patients along with the number of IRRDR mutations of each isolate are shown in Fig. 2B.

Next, we performed ROC curve analysis to estimate the optimal cutoff number of IRRDR[HCV-4] mutations for SVR prediction. This analysis estimated 4 mutations as the optimal number of IRRDR[HCV-4] mutations to predict SVR, since it achieved the highest sensitivity (84%; sensitivity refers to the proportion of SVR patients who were infected with HCV isolates of IRRDR[HCV-4] with 4 or more mutations) and specificity (72%; specificity refers to the proportion of non-SVR patients who were infected with HCV isolates of IRRDR[HCV-4] with 3 or fewer mutations) with an area under the curve (AUC) of 0.82 (Fig. 3). Accordingly, 21 (84%) of 25 patients with SVR, in contrast to only 5 (28%) of 18 patients with non-SVR, had IRRDR[HCV-4] with 4 or more mutations (referred to as IRRDR[HCV-4]≥4), with the difference between the two groups being statistically significant (P = 0.0004) (Table 3). It should be noted that 4 (31%) of 13 patients with null response and only 1 (20%) of 5 patients with relapse had HCV with IRRDR[HCV-4]≥4. These results collectively suggest that IRRDR[HCV-4]≥4 is significantly associated with SVR. In this connection, we also tested the impact of a higher (≥5) and a lower (≥3) degree of IRRDR mutations on treatment outcome. IRRDR[HCV-4]≥5 was significantly associated with SVR, though with a relatively lower sensitivity (64%) than that of IRRDR[HCV-4]≥4 (Table 3). On the other hand, there was no significant correlation between IRRDR[HCV-4]≥3 and SVR.

Correlation between core protein sequence heterogeneity and SVR in HCV-4 infection. A close correlation between core protein sequence patterns at positions 70 and 91 and treatment outcome has been proposed, especially in Japanese patients with HCV-1b infection (4–6). To examine this hypothesis in Egyptian patients infected with HCV-4, core sequences of the viral genome were amplified from the pretreated sera, and the amino acid sequences were deduced. Due to a high degree of sequence homology among core sequences of various HCV-4 subtypes, all sequences obtained were aligned with the prototype sequence, ED43 (10). The residues at positions 70 and 91 were both well conserved among the sequences analyzed, and therefore, no correlation with treatment outcome was observed for these residues (Fig. 4). All but two isolates had arginine at position 70 (Arg^{70}), the residue that has been associated with an IFN-sensitive phenotype as far as the core protein of HCV-1b is concerned (4–6). On the other hand, Pro at position 71 showed a tendency to be more frequent in SVR than in non-SVR patients; however, the frequency was not statistically different between the two groups.

Identification of independent predictive factors for SVR in HCV-4 infection. In order to identify significant independent

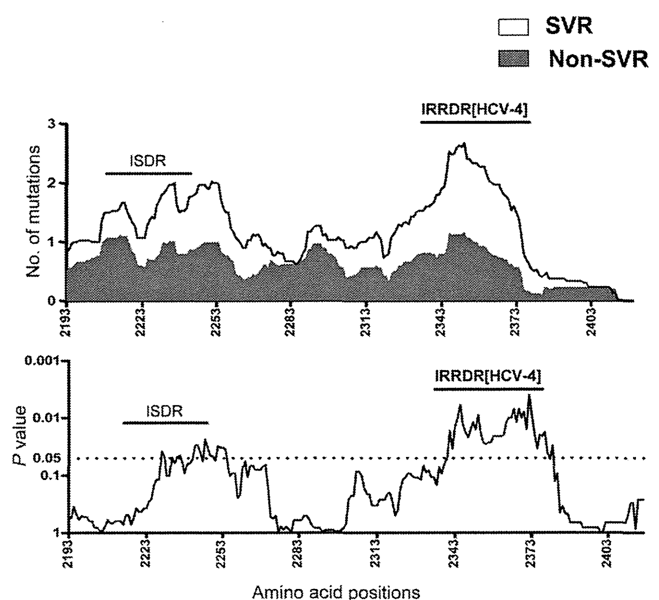

FIG 1 Sliding window analysis over the carboxy terminus (aa 2193 to 2417) of NS5A of HCV-4 obtained from SVR and non-SVR patients.

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