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HCV NS5A and NS5B Enhance Expression of Human Ceramide Glucosyltransferase Gene*

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Abstract: Host genes involved in lipid metabolism are differentially affected during the early stages of hepatitis C virus (HCV) infection. Here we demonstrate that artificial up-regulation of fatty acid biosynthesis has a positive effect on the replication of the HCV full-length replicon when cells were treated with nystatin. Conversely, the HCV RNA replication was decreased when fatty acid biosynthesis was inhibited with 25-hydroxycholesterol and PDMP(D-*threo*-1-phenyl-2-decanoylamino-3- morpholino-1-propanol). In agreement with these results, the expression level of GlcT-1(ceramide glucosyltransferase), a host glucosyltransferase in the first step of GSL (glycosphingolipid) biosynthesis, was found to be closely associated with the expression and replication of HCV RNA. On the other hand, the viral RNA can also activate GlcT-1 in the early stage of viral RNA transfection *in vitro*. To identify viral factors that are responsible for GlcT-1 activation, we constructed ten stable Vero cell lines that express individual HCV proteins. Based on the analyses of these cell lines and transient transfection assay of the GlcT-1 promoter regions, we conclude that HCV proteins, especially NS5A and NS5B, have positive effects on the expression of GlcT-1. It is possible that NS5A and NS5B stimulate transcription factor(s) to activate the expression of GlcT-1 by increasing its transcription level.

Key words: Hepatitis C virus; Fatty acid biosynthesis; Ceramide glucosyltransferase; Stable cell lines

Hepatitis C virus (HCV) infection is a major health problem world-wide. HCV infection causes chronic hepatitis in up to 60-80% of infected adults and is associated with steatosis, cirrhosis and hepatocellular carcinoma[8, 10]. HCV is an enveloped, positive-sense RNA (~9.6kb) virus of the family *Flaviviridae*. It has a central ORF flanked by the 5' and 3' noncoding regions. The ORF encodes one large polyprotein that can produce different proteins: the core protein, the envelope proteins (E1, E2, p7), and the nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B)[2, 6]. Our understanding of the biology of HCV RNA replication has been facilitated by the

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development of full-length HCV replicons that produce active HCV virions.

Chronic HCV infection is characterized by several histological features of the liver such as bile duct damage, lymphoid follicles, low cholesterol levels and steatosis[18]. During the early stages of HCV infection, host genes involved in lipid metabolism are differentially affected. Recently, genomic analysis of the livers of HCV-infected chimpanzees revealed that the transcriptional levels of the genes involved in the lipid metabolism and homeostasis are altered *in vivo* and *in vitro*[3, 25]. Francis V. Chisari *et al* also demonstrated that several cellular genes involved in lipid metabolism, such as ATP citrate lyase, clathrin light polypeptide, lipase A and GlcT-1, are differentially up-regulated during the early acute infection of HCV in chimpanzees[25]. Furthermore, it has been demonstrated that HCV genomic RNAs induce the expression of the elevated levels of ATP citrate lyase which is involved in cholesterol biosynthesis[15]. On the other hand, Ceramide glucosyltransferase (GlcT-1), plays an important role in the infection of some RNA viruses such as HIV-1[11]. GlcT-1, which catalyzes the first glycosylation step in glycosphingolipid biosynthesis, the transfer of glucose to ceramide, is a pivotal enzyme in the GSL synthesis. Therefore, it is important to investigate the relationship between HCV and host lipid metabolism by exploring the activity change of GlcT-1 caused by HCV proteins.

In this study, using the HCV full-length replicon system, we validated a positive correlation between HCV replication and the expression of genes involved in the fatty acid synthesis[9]. Our replicon system was constructed by the transfection of pHCV-WHU-1 to the Vero cells. Furthermore, we constructed ten stable Vero cell lines to carry individual HCV proteins. Study of these cell lines revealed that HCV NS5A and NS5B may regulate the transcription of GlcT-1.

MATERIALS AND METHODS

Cell culture

Vero cells and Huh7 cells were maintained at 37°C in 5%CO_2 in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum (Gibco).

HCV full-length replicon system

Construction of a highly efficient *in vitro* transfection system based on the Vero cell line was accomplished as previously described[9]. A HCV (1b) full-length cDNA clone (pHCV-WHU-1) was used that replicates and produces integrated and infectious virus particles in cultured Vero cells.

Effects of drugs that affect lipid metabolism on HCV replicons

2×10^5 Vero cells were infected with vTF7-3 (ATCC No. VR-2153™, 10^{10} $TCID_{50}$, 2000 fold dilution) for 2 h. Then vTF7-3 was removed and 1.6 μg pHCV-WHU-1 was transfected into Vero cells with Lipofectamine 2000 reagent (Invitrogen). Nystatin, 25-hydroxycholesterol and PDMP (inhibitor of GclT-1) (Alexis) at different indicated concentrations were added to the culture medium respectively. HCV RNA replication efficiency was evaluated by fluorescent quantitative PCR at 36h after transfection.

Relationship between HCV RNA and GlcT-1 mRNA

HCV RNA was obtained by *in vitro* transcription by T7 RNA polymerase (TaKaRa). 2×10^5 Vero cells or Huh7 cells were transfected with HCV RNA by Lipofectamine 2000 reagent (Invitrogen). HCV RNA

replication efficiency and GlcT-1 mRNA level were evaluated by fluorescent quantitative PCR at 2, 6, 12, 18, 24 h after transfection respectively.

Detection of HCV RNA

Fluorescent quantitative PCR was performed according to the manufacturer's protocol (Hepatitis C virus fluorescence polymerase chain reaction diagnostic kit was purchased from Daangene Co. Ltd, China). The RNA extract was treated with DNase I (37°C 30 min) before the quantification.

Detection of GlcT-1 mRNA

We estimated the expression level of the GlcT-1 mRNA by Fluorescent quantitative RT-PCR. Cells were scraped and washed with PBS once. Total RNA was extracted by Trizol reagent (Invitrogen). Oligonucleotides primers were designed based on GlcT-1 sequences available in GenBank (GeneID: 7357). Primers used were GlcT-1 FP (5'-CTTGGAGG GAATGGCCGTCTT-3') and GlcT-1 RP (5'-TCTTGT TGAGGTGTAATCGGGTGTA -3'). Probe used was (5'-FAM-TTCGTCCTCTTCTTGGTGCTGTGGCT-T AMRA-3').

Plasmids construction

Ten genes of HCV were amplified from pHCV-WHU-1[9] containing full-length HCV cDNA by using ten pair of primers (Table 1) respectively. Different size PCR products of different genes were cloned into the pcDNA3.1(+) vector (Invitrogen) by restriction enzymes (Table 1). All the plasmids were confirmed by DNA sequencing.

Construction of stable Vero cell lines

Construction of the stable Vero cell lines were carried out according to the manufacturer's instructionss (Invitrogen). Briefly, linearized pcDNA3.1(+)-Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B were transfected into the Vero cells using Lipofectamine 2000 reagent (Invitrogen) respectively, stable cell lines were selected by 600 μg/mL G418. Vero cells transfected with pcDNA3.1(+) was used as a system control. The selected stable Vero cell lines were maintained at 37°C in 5%CO_2 in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum and 300 μg/mL G418.

Detection of DNA, mRNA and foreign proteins in the stable Vero cell lines

Genomic DNA of HCV in the stable Vero cell lines was detected by PCR with the primers listed in Table 1. The mRNAs of HCV genes were detected by RT-PCR with the same primers. Expression of HCV E1, E2 and NS5B proteins in proper stable Vero cell lines were detected by Western blot analysis. The Western blot analysis was performed as described previously[23]. The primary polyclonal antibodies (rabbit anti-E1, E2 and NS5B proteins of HCV) were kindly provided by Pro. Ye Linbo (Wuhan University). The secondary antibody horse anti-rabbit IgG conjugated with alkaline phosphatase was purchased from Proteintech Group.Inc. GAPDH was used as a control.

The presence of the DNA, and the expression level of the mRNA and viral proteins were determined through different passages of the stable Vero cell lines.

GlcT-1 enzyme assay

GlcT-1 activity was determined according to the method of Shinichi[14]. C_6-NBD-Cer (6-{[(N-7-nitrobenz -2-oxa-1, 3-diazol- 4-yl)amino] caproyl} sphingosine, Cayman), a synthetic fluorescent substrate (50 μg), and lecithin (Sigma) (500 μg) were mixed in 100 μL of ethanol and the solvent was evaporated. 1 mL water was added and the mixture was

used to form liposomes. A standard reaction mixture (100 μL), composed of 20 mmol/L Tris·HCl (pH 7.5), 500 μmol/L UDP-Glc (UDP-Glucose, Sigma), 20 μL of liposomes, and 50 μg of cell protein was incubated at 32°C for 2h. After the incubation, the lipids were then redissolved in a small volume of $CHCl_3/CH_3OH$, 2:1 (vol/vol), and chromatographed on a precoated silica-gal TLC plate in $CHCl_3/CH_3OH/H_2O$, 65:25:4 (vol/vol). NBD lipids were visualized by a Variable Mode Typhoon 9200 Imager at 520 nm.

Construction of promoter reports

The 5'-gene regulation sequence of the GLcT-1 gene was obtained by genome PCR using LA Tag DNA polymerase (TaKaRa). The PCR fragments were amplified and cloned into the *Xho* I and *Hin*d III sites of the pGL3-Basic reporter (Promega) for determination of promoter activities. Clones were designated as pGL3(-529/+201), pGL3(-529/0), pGL3(-408/-156) and pGL3(-408/0), respectively. Site +1 indicates the transcription start site. Primer pairs are shown in Table 2.

Transient transfection assays

Activity of the promoter construct in the pGL3-Basic vector (Promega) was determined in the Huh7 cells by transient transfection. Plasmids were co-transfected into Huh7 cells using Lipofectamine 2000 reagent (Invitrogen) along with pRL-TK, a *Renilla* luciferase control reporter (Promega). After a 4 h transfection, cells were allowed to recover for 24 h in serum-containing medium and lysed for assays of promoter activity using the Dual-Glo™ Luciferase Assay System (Promega). The *Renilla*-driven luciferase activity was used to standardize the GlcT-1 promoter-driven luciferase activity for transfection efficiency.

RESULTS

Fatty acid biosynthesis affects HCV replication *in vitro*

In order to determine the relationship between the host genetic system of fatty acid biosynthesis and HCV RNA replication, we quantified the differences in HCV RNA expression after treating cells with different drugs that affect the fatty acid biosynthesis pathway. As shown in Fig. 1A, 25-hydroxycholesterol, an inhibitor blocking fatty acid synthesis, inhibited HCV replication in a dose-dependent manner. The inhibition did not result from general cytotoxicity of the drug, as judged by the MTT assay (data not shown). With the HCV full-length replicon system, viral replication was inhibited by nearly 70% by 4.96 μmol/L 25-hydroxycholesterol after 36 h treatment. In contrast, nystatin, which activates the SREBP (sterol-regulatory element-binding protein) pathway through cholesterol sequestration, caused a dose-dependent increase in viral replication (Fig. 1B). The HCV RNA level was increased by nearly 10 fold when cells were treated with 48.6 μmol/L nystatin. These results suggested possible stimulatory effects of cellular factors involved in fatty acid biosynthesis on HCV replication.

GlcT-1 activity affects HCV replication *in vitro*

GlcT-1 is a pivotal enzyme in the GSL synthesis, which performs an important role in cell lipid metabolism. It catalyzes the first glycosylation step of GSL synthesis, the transfer of glucose from UDP-Glc to ceramide, and the production of GlcCer (glucosylcearmide or glucocerebroside) and UDP[12, 13]. In order to test the hypothesis that GlcT-1 might have a positive effect on HCV RNA replication, we added PDMP, a potent inhibitor of GlcT-1[17] into the HCV

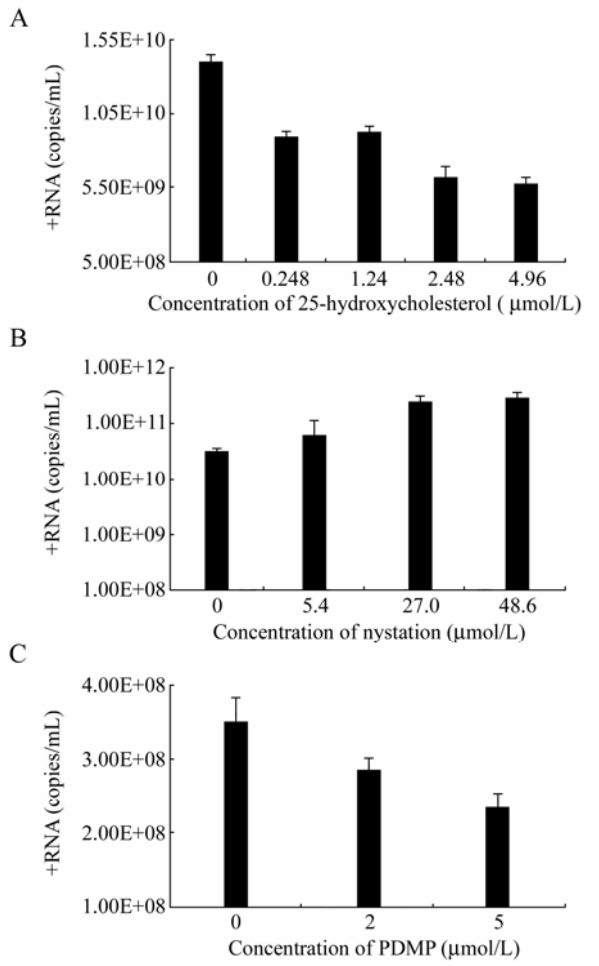

Fig. 1. Fatty acid biosynthesis affects HCV replication *in vitro*. A: Measurement of HCV RNA replication efficiency when treated with 25-hydroxycholesterol. B: Measurement of HCV RNA replication efficiency when treated with nystatin. C: Measurement of HCV RNA replication efficiency when treated with PDMP. The data of the column are the average of three detections.

full-length replicon system. As shown in Fig. 1C, viral replication was inhibited by nearly 40% after treatment for 36h with 5 μmol/L PDMP, in comparison with the untreated cells. These results suggest that an optimal GlcT-1 level is important for HCV replication.

Elevated levels of GlcT-1 transcription following HCV RNA transfection *in vitro*

Because HCV replication may require optimal

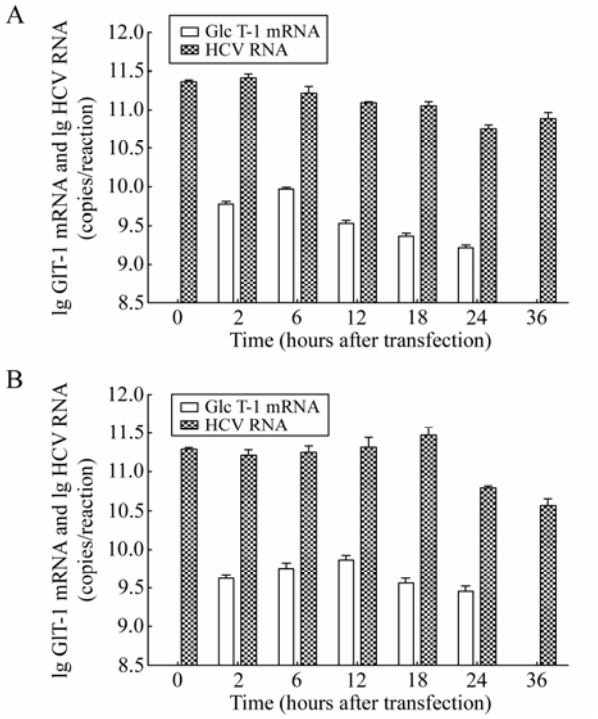

Fig. 2. GlcT-1 and HCV RNA are positively correlated *in vitro*. A: GlcT-1 mRNA and HCV RNA levels in Vero cells. B: GlcT-1 mRNA and HCV RNA levels inHuh7 cells.

GlcT-1 expression, we explored possible enhancement of GlcT-1 expression by HCV genes. We quantified the syntheses of both GlcT-1 mRNA and HCV RNA following transfection of HCV RNA into Vero and Huh7 cells. The results showed that levels GlcT-1 mRNA increased with time, following a similar trend as HCV RNA synthesis (Fig. 2). The GlcT-1 mRNA increased during the early hours of transfection, and started to decrease at 12h in Vero cells (Fig. 2A), and at 18 h in Huh7 cells (Fig. 2B). These data suggest that HCV RNA can active GlcT-1 at the early hours of transfecion *in vitro*.

Construction of stable Vero cell lines carrying different HCV genes

It has been demonstrated that the expression of GlcT-1 and HCV RNA replication are positively related both *in vivo*[3] and *in vitro*. In order to identify

which HCV protein increases GlcT-1 activity, we constructed ten stable Vero cell lines carrying 10 different HCV genes. These cells were named as Vero-Core, Vero-E1, Vero-E2, Vero-p7, Vero-NS2, Vero-NS3, Vero-NS4A, Vero-NS4B, Vero-NS5A and Vero-NS5B. Each cell line expressed a single HCV protein. Vero-mock (transfected with pcDNA3.1(+)) was used as a control. All cell lines were selected and maintained in the presence of 300 μg/mL G418.

Stable maintenance of HCV genes in Vero cells

In order to confirm that the HCV genes transfected are stably maintained in the host cell lines, we analyzed the intracellular levels of the HCV DNAs and mRNAs, as well as the levels of E1, E2, NS5B proteins during each passage. As shown in Fig. 3A, we were able to detect the DNA and mRNA of HCV genes by PCR and RT-PCR respectively. The sizes of the fragments were consistent with the predicated sizes of the corresponding genes. We also confirmed the expression of E1, E2 and NS5B genes in Vero-E1, Vero-E2 and Vero-NS5B cell lines (Fig. 3B). Even with repeated freezing and thawing, we did not detect decreases in the expression levels of these genes in these cell lines, demonstrating that the HCV genes can be stably maintained.

Expression and activity of GlcT-1 in Vero cells

To determine which HCV proteins have a positive effect on the expression of GlcT-1, we quantified the expression level of GlcT-1 mRNA and its enzymatic activity in the stable Vero cell lines. As shown in Fig. 4A, we observed a linear correlation between the level of GlcT-1 mRNA and its enzymatic activity. The activity of GlcT-1 varied among different Vero cell lines expressing different HCV proteins. The GlcT-1 activity was significantly increased (from 2 to 6 folds compare with the control) in the Vero-NS2, Vero-NS5A and Vero-NS5B cell lines.

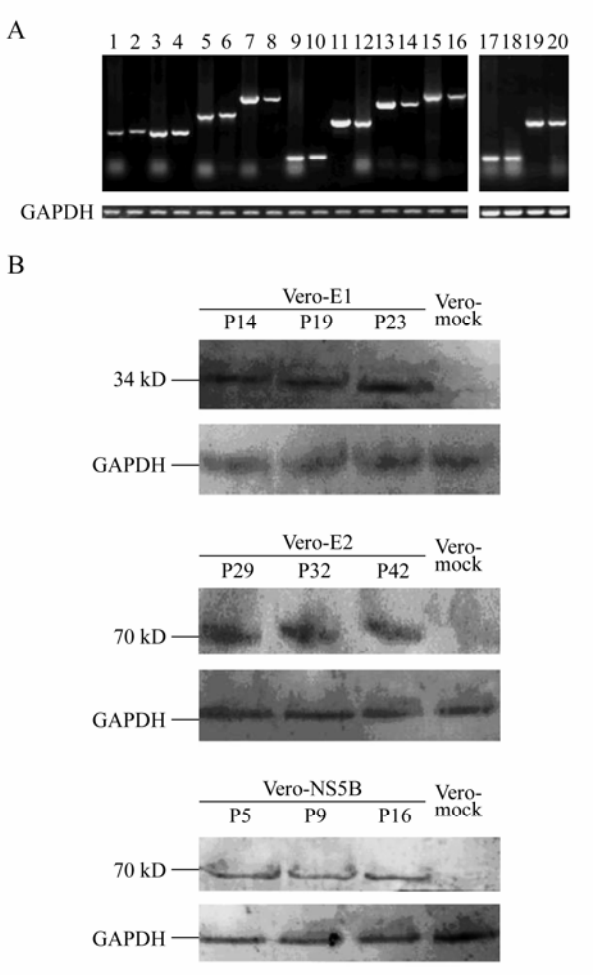

Fig. 3. Detection of HCV genes in the Vero stable cell lines. A: Measurement of the DNA and mRNA of HCV gene(s) in the stable cell lines by PCR and RT-PCR. 1and 2, Core, 573 bp (DNA/RNA); 3 and 4, E1, 576 bp (DNA/RNA); 5 and 6, E2, 1089 bp (DNA/RNA); 7 and 8, NS3, 1893 bp (DNA/RNA); 9 and 10, NS4A, 162 bp (DNA/RNA); 11 and 12, NS4B, 783 bp (DNA/RNA); 13 and 14, NS5A, 1341 bp (DNA/RNA); 15 and 16, NS5B, 1773 bp (DNA/RNA); 17 and 18, p7, 189 bp (DNA/RNA); 19 and 20, NS2, 651 bp (DNA/RNA). B: Measurement of the HCV protein(s): E1 (~35 kD), E2 (~70 kD), NS5B (~68 kD) expression in the stable cell lines: Vero-E1, Vero-E2, Vero-NS5B by Western blot. The passage number are shown at the top of the figure, the molecular weight at the left. Vero-mock was used as the system control and GAPDH acted as the experiment control.

NS5A and NS5B increase the transcription level of GlcT-1

To confirm that NS2, NS5A and NS5B enhance the transcription of the GlcT-1 gene, the promoter regions of GlcT-1 were cloned into the upstream of a luciferase reporter construct. The promoter activities of four of these constructs, pGL3(-529/+201), pGL3(-529/0), pGL3(-408/-156) and pGL3(-408/0), were examined by luciferase assays in Huh7 cells following transient transfection. Results are shown in Fig. 4B. The highest activity was obtained with the -529/+201 region. The 3'-deletion constructs showed decreased luciferase activities. Thus, in Huh7 cells, most of the regulatory elements required for the GlcT-1 promoter activity reside in the proximity of -156/+201. To determine that NS2, NS5A and NS5B proteins could also stimulate the GlcT-1 promoter, plasmids were co-transfected in Huh7 cells (Fig. 4C). Our results showed that NS5A and NS5B, but not NS2, can increase the activity of the promoter of pGL3 (-529/+201) when compared with the control. The enhancement was also dose-dependent as tested in NS5B

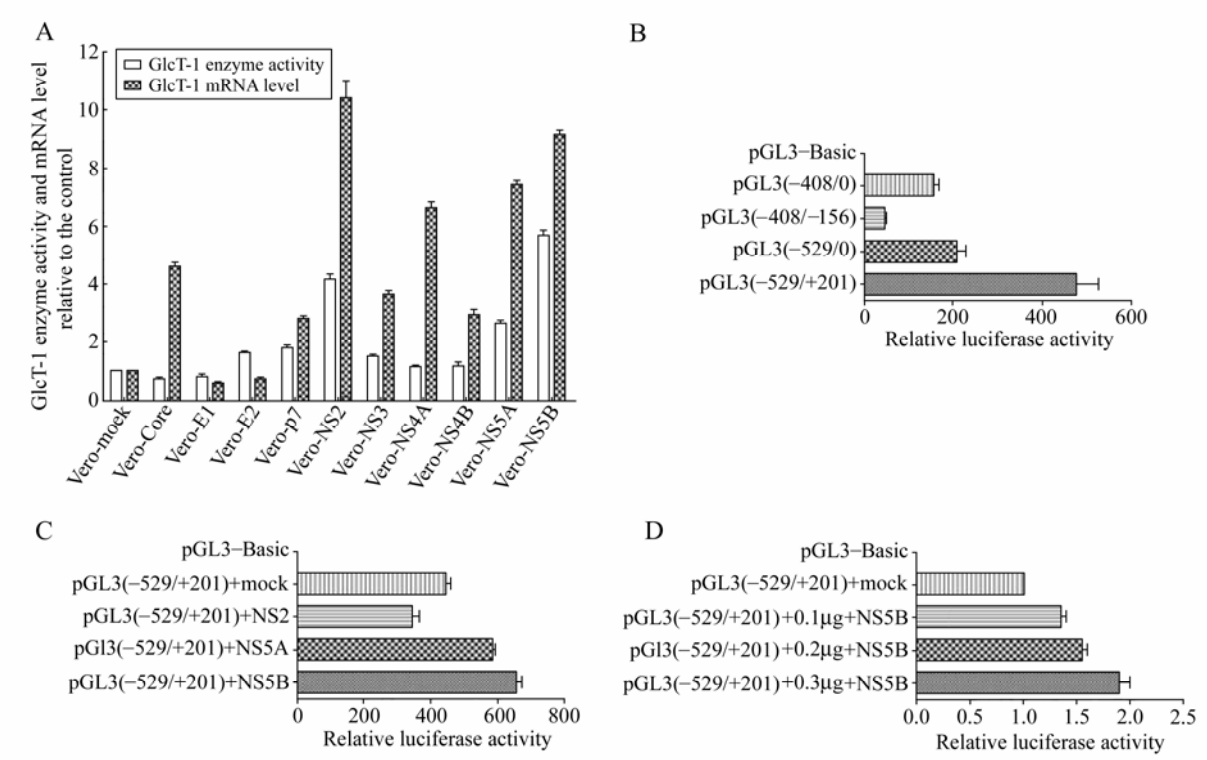

Fig. 4. Relationship between GlcT-1 and HCV functional proteins. A: Measurement of GlcT-1 in the stable Vero cell lines. GlcT-1 mRNA detection in the stable Vero cell lines was performed by Real-Time RT-PCR. GlcT-1 activity detection in the stable Vero cell lines was performed by TLC. Measurements were repeated twice and the error bars are shown at the top of each column. GlcT-1 activity of Vero-mock was taken as one. B: Transcription activity of the promoter reporter constructs of the GlcT-1 gene in Huh7 cells. Cells were transfected with 0.4 μg pGL3 promoter constructs of pGL3-Basic vector per transfection, together with 0.02 μg pRL-TK vectors for normalizing the transfection efficiency as described in the text. C: NS2, NS5A and NS5B affect the transcription activity of the GlcT-1 gene in Huh7 cells. Cells were transfected with 0.2 μg pGL3(-529/+201) and 0.2 μg pcDNA3.1-mock/ NS2/NS5A/NS5B per transfection, together with 0.02 μg pRL-TK vector. D: NS5B increases the transcription activity of the GlcT-1 gene in a dose-dependent manner. Cells were transfected with 0.2 μg pGL3(-529/+201) and 0.2 μg pcDNA3.1-mock or different doses of pcDNA3.1-NS5B per transfection, together with 0.02 μg pRL-TK vectors. The activity of pGL3(-529/+201)+mock was taken as one. The values represent at least three independent transfection experiments, each assayed in triplicate.

cotransfection (Fig. 4D). These results suggested that NS5A and NS5B enhance the mRNA level of GlcT-1 by simulating the GlcT-1 promoter regions.

DISCUSSION

Alteration of lipid metabolism has been shown to occur in the presence of HCV gene expression. Multiple cellular genes, such as ATP citrate lyase, clathrin light polypeptide, lipase A and GlcT-1, as well as Apolipoprotein AII and acyl-CoA thioesterase I in the SREBP pathway, that are involved in lipid metabolism, are differentially affected in acute HCV infection in chimpanzees[3, 4, 15, 25]. Consistent with these previous findings, our research implies that the expression level of the GlcT-1 gene is closely related to HCV RNA replication and expression. Specifically, the existence of HCV RNA activates GlcT-1 in the early stage of HCV transfection *in vitro* (Fig. 2). Although the functional requirement for this activation is currently unknown, our result serves as an important step for future characterization of the molecular mechanisms involved.

HCV replication has been demonstrated to occur in an HCV induced ‘‘membranous web’’ consisting of modified lipid containing intracellular vesicles[6], it is possible that fatty acids may be needed for proper architecture of this structure that is critical for HCV RNA replication. Nevertheless, further experiments are needed to determine the exact role of fatty acids in HCV replication.

Activation of SREBP increases both fatty acid and cholesterol biosynthesis[19]. 25-hydroxycholesterol, which inhibits SREBP cleavage/translocation, causes a decrease in fatty acid biosynthesis. On the other hand, nystatin causes an increase in fatty acid biosynthesis/translocation of SREBP as a homeostatic response. HCV RNA level was affected by host fatty acid biosynthesis in HCV subgenomic replicon system[25]. Consistently, using these two inhibitors, our results demonstrate that up or down regulation of fatty acid biosynthesis has a positive or negative effect on the replication of HCV full-length replicons, respectively (Fig. 1A, 1B).

We also found that the drug, PDMP, inhibits HCV replication. It is possible that PDMP is also directly affecting P-glycoprotein activity and inhibits the enzyme GlcT-1[12]. Our results show that GlcT-1 has a positive effect on HCV replication in the HCV full-length replicon system (Fig. 1C). In order to find the contributing gene(s) which affect GlcT-1, we established ten stable cell lines expressing different HCV gene individually. This provide to be an effective tool for exploring the relationship between HCV gene(s) and host cell factor. Indeed, we found that some stable lines, especially Vero-NS5B, can greatly enhance the efficiency of HCV RNA replication (data not shown).

Many HCV nonstructural proteins are involved in the host lipid metabolism process. For example, NS2 contains several stretches of hydrophobic amino acids and is predicted to be a polytopic membrane protein[1]. NS2 also has an effect on liver and non-liver-specific promoters and enhancer elements that might be relevant for the pathogenesis of chronic HCV infection[22]. NS5A is a multifunctional phosphoprotein that is believed to have various functions in HCV replication and pathogenesis[6, 24]. It has recently been determined that NS5A is associated with apolipoproteins AI and AII[24], co-localizes with the HCV core protein on cytoplasmic lipid droplets, and interacts with apoB[5].

NS5A can also increase hepatic lipid accumulation[16], and activates a lipid kinase contributing to the integrity of the membranous viral replication complex[21]. Domitrovich *et al* discovered that NS5B is able to reduce the transcription and synthesis of MTP (microsomal triglyceride transfer protein), a major regulator of the assembly and secretion of triglyceride-rich lipoprotein particles[5]. These previous data strongly suggest that HCV NS2, NS5A and NS5B may affect some steps of the host lipid metabolism and are consistent with our results. As shown in this manuscript, the insertion of HCV genes changed the expression of GlcT-1 to various degree both at the level of mRNA and enzyme activity (Fig. 4A), suggesting that HCV proteins, especially NS5A and NS5B have an ability to up-regulate the activity of host GlcT-1. NS5A and NS5B are very likely to stimulate transcription factor(s) to activate the expression of GlcT-1 by increasing the transcription level of the GlcT-1 gene (Fig. 4C).

In conclusion, we found that the expression level of GlcT-1 gene is affected by the expression of HCV RNA. We also demonstrate that the expression of HCV functional proteins is capable of altering lipid metabolism in Vero cells by altering host genes, particularly those related to fatty acid biosynthesis. Our results may have implications in understanding the development of steatosis in chronically infected patients[7, 20, 26].

Acknowledgements

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