Transactivating effect of hepatitis C virus core protein: A suppression subtractive hybridization study

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INTRODUCTION
Hepatitis C virus (HCV) is a major causative agent of chronic liver diseases including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide[1-4]. The majority of individuals infected with HCV cannot resolve their infection and suffer from persistent chronic hepatitis. The molecular mechanism of HCV persistence and pathogenesis is not well understood. HCV contains a single-stranded positive-sense RNA genome which encodes a precursor polypeptide of approximately 3,000 amino acids. After translation, a capsid protein (core), envelope glycoproteins (E1 and E2), and nonstructural proteins (NS2, NS3a, NS3b, NS4A, NS4b, NS5a, and NS5b) are processed from the polypeptide by cellular and viral proteases[5-10]. HCV core gene contains the most conserved sequences in the coding region of most HCV genotypes, which implies an important biological function. Since suitable viral culture systems are not generally available[10-13], analysis of HCV genome organization and viral-product function is important to understand the viral life cycle and the pathogenesis of HCV infection. In order to understand the pathogenesis of HCV infection, we investigated the transactivating effect of HCV core protein.

MATERIALS AND METHODS
Construction and identification of expression vectors
pcDNA3.1(-)-core containing full-length HCV core gene was constructed by insertion of HCV core gene into EcoRI/BamHI site, which could directly express core protein. pcDNA3.1(-) was obtained from Invitrogen. The gene was identified by PCR and digested by EcoRI, BamHI, and Hind III (Takara). PCR primers were: up primer, 5'-GGA TCC AGG CTG AAG CCG GCA CA-3'(Shanghai BioAsia Biotechnology Co., Ltd); down primer, 5'-GGA TCC AGG CTG AAG CCG GCA CA-3'(Shanghai BioAsia Biotechnology Co., Ltd). pcDNA3.1(-)-core was cotransfected with pSV-lacZ (0.1-1.8 µg) (Promega). Expression of β-gal activity was detected by using β-gal assay kit (Promega). Expression of pcDNA3.1(-)-core and pSV-lacZ were cotransfected. At the same time, HepG2 cells were cotransfected with empty pcDNA3.1(-) and pSV-lacZ as control. After 48 h, cells were collected and total β-gal was detected.

Cotransfection with reporter vectors pSV-lacZ
HepG2 cells were transfected by various concentrations of pSV-lacZ (0.1-1.8 µg) (Promega). Expression of β-gal was detected by using β-gal assay kit (Promega). The best concentration of pSV-lacZ was selected, HepG2 cells with pcDNA3.1(-)-core and pSV-lacZ were cotransfected. At the same time, HepG2 cells were cotransfected with empty pcDNA3.1(-) and pSV-lacZ as control. After 48 h, cells were collected and the expression of β-gal was detected.

Expression of pcDNA3.1(-)-core in HepG2 cells
HepG2 cells were transiently transfected with pcDNA3.1(-)-core using Lipofectamine. At the same time, empty vectors were also transfected into cells as control. HepG2 cells were

RESULTS:
The core mRNA and protein could be detected in HepG2 cell lysate which was transfected by the pcDNA3.1(-)-core. The activity of β-galactosidase in HepG2 cells transfected by pcDNA3.1(-)-core was 5.4 times higher than that of HepG2 cells transfected by control plasmid. The subtractive library of genes transactivated by HCV core protein was constructed successfully. The amplified library contained 233 positive clones. Colony PCR showed that 213 clones contained 100-1,000 bp inserts. Sequence analysis was performed in 63 clones. Six of the sequences were unknown genes. The full length sequences were obtained by using bioinformatics method, accepted by GenBank. It was suggested that six novel cDNA sequences might be target genes transactivated by HCV core protein.

CONCLUSION:
The core protein of HCV has transactivating effects on SV40 early promoter/enhancer. A total of 63 clones from cDNA library were randomly chosen and sequenced. Using the BLAST program at the National Center for Biotechnology Information, six of the sequences were unknown genes. The other 57 sequences were highly similar to known genes.

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plated at a density of 1x10^6 on a 35 mm plate in RPMI1640 containing 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 100 mL/L heat-inactivated FBS. After 24 h of growth to 40-50% confluence, the cells were transfected with plasmids by using Lipofectamine according to the manufacturer’s protocol (Gibco Co.).

**mRNA and cDNA isolation**

Total cellular RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s instructions. cDNAs were reversely-transcribed from total RNA. The result was identified by PCR. Cells were collected and mRNA was isolated by using a micro mRNA purification kit (Amersham Biosciences).

**Generation of a subtracted cDNA library**

Genome comparisons were performed by suppression subtraction hybridization according to the manufacturer’s instructions of PCR-selectTM cDNA subtraction kit (Clontech). In brief, 2 µg aliquots each of poly(A)+ mRNA from the tester and the pooled driver were subjected to cDNA synthesis. Tester and driver cDNAs were digested with Rsal. The tester cDNA was subdivided into two portions, and each was ligated with a different cDNA adapter. In the first hybridization reaction, an excess of driver was added to each sample of tester. The samples were heat denatured and allowed to be annealed. Because of the second-order kinetics of hybridization, the concentration of high- and low-abundance sequences was equalized among the single-stranded tester molecules. At the same time single-stranded tester molecules were significantly enriched for differentially expressed sequences. During the second hybridization, the two primary hybridization samples were mixed without denaturation. Only the remaining equalized and subtracted single-stranded tester cDNAs could reassociate, forming double-stranded sequences. During the second hybridization, the two primary hybridization samples were mixed without denaturation.

**Cloning subtracted library into pGEM-Teasy vector**

Products of these amplified A overhangs containing a subtracted cDNA library (3 µL) were ligated into a pGEM-Teasy plasmid (Promega). Subsequently, the plasmid was introduced into Escherichia coli strain JM109. Bacteria were transferred into 800 µL of SOC medium and allowed to be incubated for 45 min at 37 °C and centrifuged at 225 rpm. Then they were plated onto agar plates containing ampicillin (50 µg/mL), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; 20 µg/cm²), and isopropyl-β-D-thiogalactoside (IPTG; 12.1 µg/cm²) and incubated overnight at 37 °C. White colonies were picked and identified by PCR. Primers were T7/SP6 primer of pGEM-Teasy plasmid. After the positive colonies (Shanghai BioAsia Biotechnology Co., Ltd) were sequenced, nucleic acid homology searches were performed using the BLAST (basic local alignment search tool) server at the National Center for Biotechnology Information.

**RESULTS**

**Identification of expression vector**

Figure 1 shows pcDNA3.1(-)-core of the PCR assay for plasmid and digestion of restriction enzyme analysis. Restriction enzyme analysis of pcDNA3.1(-)-core plasmid with EcoRI/BamHI I yielded two bands: 4 900 bp empty pcDNA3.1(-) and 573 bp HCV core. Cleavage with HindIII produced only one 5 500 bp band (4 900 bp+573 bp). The products of plasmid were amplified by PCR. Analysis of the PCR reaction products by agarose gel electrophoresis showed a clear band with the expected size (573 bp). Sequence of the PCR product was correct.

**Identification of HCV core transient expression**

The total mRNA was reversely-transcribed by three different Oligo dT, identification of cDNA by PCR yielded a common 573 bp band (Figure 2). Table 1 shows co-transfected pcDNA3.1(-)-core and pSV-lacZ into HepG2 cells, transient expression of HCV core was positive. On the contrary, empty pcDNA3.1(-)-core co-transfected HepG2 cells were negative.

**Result of pcDNA3.1(-)-core and pSV-lacZ cotransfection**

Selection of 0.3 µg pSV-lacZ served as the best concentration by analysis. After cotransfection of pcDNA3.1(-)-core and pSV-lacZ, the A value of expression of β-gal was 0.219. In contrast, the A value of expression of β-gal by cotransfected empty pcDNA3.1(-) and pSV-lacZ was 0.034. Expression of β-gal was 5.4-fold higher in cotransfected pcDNA3.1(-)-core and pSV-lacZ than in cotransfected empty pcDNA3.1(-) and pSV-lacZ (Figure 3). The significant increase of expression of β-gal was attributed to the transactivating effect of HCV core on early promoter of SV40, leading to the increased expression of downstream gene lacZ.
The diverse functional activities of HCV putative core protein have already been noted by a number of investigators. These include nucleocytoplasmic localization\cite{14}, regulation of cellular and unrelated viral promoters in *in vitro* studies\cite{15-18}, inhibition of apoptosis under certain conditions\cite{19}, physical association with apolipoprotein II\cite{20} and cytoplasmic tail of the lymphotixin β-receptor\cite{21,22}, promotion of normal cells to a transformed phenotype\cite{23}, and transactivation of suppression of cell growth\cite{24,25}.

We cotransfected HepG2 cells with pcDNA3.1(-)-core and pSV-lacZ and demonstrated that the HCV core was successfully expressed in transfected HepG2 cells. Expression of β-gal was 5.4-fold higher in cotransfected pcDNA3.1(-)-core and pSV-lacZ than in cotransfected empty pcDNA3.1(+) and pSV-lacZ. HCV core had significant transactivating effects on the early promoter of SV40, through increasing the expression of downstream gene lacZ. This result indicates that the HCV core protein expressed in HepG2 cells retains its biological activity in terms of transcriptional activation, which was inconsistent with the previous report\cite{26}.

The nucleocapsid core protein of HCV has been shown to trans-act on several viral or cellular promoters\cite{15,16,20,25-29}. To get insight into the trans-action mechanism of HCV core protein, an SSH was used for identification of relatively transactivated target genes of HCV core protein. HCV core protein relatively transactivated gene subtractive library was set up successfully. Sequence analysis was performed in 63 clones. Five of the sequences matched strongly (>97% nucleotide identity) with the apolipoprotein (Apo) sequence. Two of those sequences matched strongly (>97% nucleotide identity) with the bile acid-binding protein (BABB).

Several lines of evidence suggest that HCV core protein may modulate cellular transduction signals and alter lipid metabolism. A characteristic of HCV infection is the presence of liver steatosis. It is plausible that this steatosis could arise, at least in part, from direct effects of HCV proteins on lipid metabolism. The levels of apoAI and high density lipoprotein are independent predictive factors response to treatment\cite{27}. Apo, a protein that bind to lipids and renders them water soluble in the form of lipoproteins, could be a good candidate for an interaction with the core protein. HCV has been described as a lipid-containing virus and in plasma of patients shows a heterogeneous density distribution partially due to its binding to low density lipoprotein, very low density lipoprotein, IgG, and to a minor degree of IgM and high density lipoprotein. It has been discovered that bile acids are the natural ligand for a nuclear receptor termed farnesoid X receptor (FXR; NR1H4)\cite{30,31}. Therefore, bile acids may be important regulators of gene expression in the liver and intestines. To date, the genes that have been shown to be responsive to regulation by FXR encoded proteins are involved in the biosynthesis and transport of bile acids\cite{32}. Bile acids have been shown to modulate a variety of other cellular functions, such as secretion of lipoproteins.
Our results revealed a number of novel and known genes that responded to HCV core. The 6 unknown nucleotide
that HCV core protein interacts with bile acid-binding proteins.

REFERENCES

1. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 1989; 244: 359-362

2. Di Bisceglie AM. Hepatitis C and hepatocellular carcinoma. Hepatology 1997; 26(3 Suppl 1): 345-385

3. Hasan F, Jeffers LJ, De Medina M, Reddy KR, Parker T, Schiff ER, Houghton M, Choo QL, Kuo G. Hepatitis C-associated hepatocellular carcinoma. Hepatology 1990; 12(3 Pt 1): 599-591

4. Ito T, Mukaigawa J, Zuo J, Hirabayashi Y, Mitauma K, Yasek K. Cultivation of hepatitis C virus in primary hepatocyte culture from patients with chronic hepatitis C results in release of high titre infectious virus. J Gen Virol 1996; 77(Pt 5): 1043-1054

5. Clarke B. Molecular virology of hepatitis C virus. J Gen Virol 1997; 78(Pt 10): 2397-2410

6. Bukh J, Purcell RH, Miller RH. Sequence analysis of the 5' noncoding region of hepatitis C virus. Proc Natl Acad Sci U S A 1992; 89: 4942-4946

7. Grakoui A, McCourt DW, Wychowski C, Feinestone SM, Rice CM. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. J Virol 1993; 67: 2832-2843

8. Takamizawa A, Mori C, Fuke J, Manabe S, Murakami S, Fujita J, Onishi E, Andoh T, Yoshida I, Okayama H. Structure and organization of the hepatitis C virus genome isolated from human carriers. J Virol 1991; 65: 1105-1113

9. Grakoui A, Wychowski C, Lin C, Feinestone SM, Rice CM. Expression and identification of hepatitis C virus polyprotein cleavage products. J Virol 1993; 67: 1385-1395

10. Hijikata M, Kato N, Ootsuyama Y, Nakagawa M, Shimotohko K. Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. Proc Natl Acad Sci U S A 1991; 88: 5547-5551

11. Kato N, Nakazawa T, Mizutani T, Shimotohko K. Susceptibility of human T-lymphotropic virus type I infected cell line M-T-2 to hepatitis C virus infection. Biochem Biophys Res Commun 1995; 206: 863-866

12. Lanford RE, Sureau C, Jacob JR, White R, Fuerst TR. Demonstration of in vitro infection of chimpanzee hepatocytes with hepatitis C virus using strand-specific RT/PCR. Virology 1994; 202: 606-614

13. Shimizu YK, Purcell RH, Yoshikura H. Correlation between the infectivity of hepatitis C virus in vivo and its infectivity in vitro. Proc Natl Acad Sci U S A 1993; 90: 6037-6041

14. Yasui K, Koike T, Tsukiyama-Kohara K, Funahashi SI, Ichikawa M, Kajita T, Moradpour D, Wands JR, Kohara M. The native form and maturation process of hepatitis C virus core protein. J Virol 1998; 72: 6048-6055

15. Ray RB, Lagging LM, Meyer K, Steele R, Ray R. Transcriptional regulation of cellular and viral promoters by the hepatitis C virus core protein. Virus Res 1995; 37: 209-220

16. Ray RB, Steele R, Meyer K, Ray R. Transcriptional repression of p53 promoter by hepatitis C virus core protein. J Biol Chem 1997; 272: 10963-10966

17. Ray RB, Steele R, Meyer K, Ray R. Hepatitis C virus core protein represses p21WAF1/Cip1/S16 promoter activity. Gene 1998; 208: 331-336

18. Shih CM, Lo SJ, Miyamura T, Chen SY, Lee YH. Suppression of hepatitis B virus expression and replication by hepatitis C virus core protein in HuH-7 cells. J Virol 1993; 67: 5823-5832

19. Ray RB, Meyer K, Ray R. Suppression of apoptotic cell death by hepatitis C virus core protein. Virology 1996; 226: 176-182

20. Barba G, Harper F, Harada T, Kohara M, Goulisid E, Matsuya Y, Eder G, Schaff Z, Chapman M, Miyamura T, Brechot C. Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. Proc Natl Acad Sci U S A 1997; 94: 1200-1205

21. Chen CM, You LR, Wang LH, Lee YH. Direct interaction of hepatitis C virus core protein with the cellular lymphotixin-beta receptor modulates the signal pathway of the lymphotixin-beta receptor. J Virol 1997; 71: 9417-9426

22. Matsumoto M, Hiety Y, Zhu N, VanArsdale T, Hwang SB, Jeng KS, Gorbaleynia AE, Lo SY, Ou JH, Ware CF, Lai MM. Hepatitis C virus core protein interacts with the cytoplasmic tail of lymphotixin-beta receptor. J Virol 1997; 71: 1301-1309

23. Chang J, Yang SH, Cho YG, Hwang SB, Hahn YS, Sung YC. Hepatitis C virus core protein is required for transforming primary rat embryo fibroblasts in cooperation with the H-ras oncogene. J Virol 1998; 72: 3060-3065

24. Ray RB, Lagging LM, Meyer K, Ray R. Hepatitis C virus core protein cooperates with ras and transforms primary rat embryo fibroblasts to tumorigenic phenotype. J Virol 1996; 70: 4438-4443

25. Kim DW, Suzuki R, Harada T, Saito I, Miyamura T. Trans-suppression of gene expression by hepatitis C viral core protein. Jpn J Med Sci Biol 1994; 47: 211-220

26. Shih CM, Lo SJ, Miyamura T, Chen SY, Lee YH. Suppression of hepatitis B virus expression and replication by hepatitis C virus core protein in HuH-7 cells. J Virol 1993; 67: 5823-5832

27. Soardo G, Pirisi M, Fonda M, Fabris C, Falletti E, Toniutto P, Vitulli D, Cattin L, Gniano F, Bartoli E. Changes in bile lipid composition and response to interferon treatment in chronic hepatitis C. J Interferon Cytokine Res 1995; 15: 705-712

28. Tommassen R, Bonk S, Thiele A. Density heterogeneities of hepatitis C virus in human sera due to the binding of beta-lipoproteins and immunoglobulins. Mol Med (cibiol Immun 1993; 182: 329-334

29. Sabile A, Perlemuter G, Bonf F, Kohara K, Demaureg F, Kohara M, Matsuya Y, Miyamura T, Brechot C, Barba G. Hepatitis C virus core protein binds to apolipoprotein AI and its secretion is modulated by fibrates. Hepatology 1999; 30: 1064-1076

30. Makishima M, Okamoto AY, Repa J, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B. Identification of a nuclear receptor for bile acids. Science 1999; 284: 1362-1365

31. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consier TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD, Lehmann JM. Bile acids: natural ligands for an orphan nuclear receptor. Science 1999; 284: 1365-1368

32. Wang H, Chen J, Hollister K, Sowers LC, Forman BM. Endogenous bile acids are ligands for the nuclear receptor FXR/ BAR. Mol Cell 1999; 3: 543-553

33. Russell DW. Nuclear orphan receptors control cholesterol catabolism. Cell 1999; 97: 539-542

34. Lin Y, Havinga R, Choppin J, Verkade HJ, Vonk RJ, Kuipers F. Characterization of the inhibitory effects of bile acids on very-low-density lipoprotein secretion by rat hepatocytes in primary culture. Biochem J 1996; 316(2 Pt 2): 531-538

35. Lin Y, Havinga R, Verkade HJ, Moshage H, Slooff MJ, Vonk RJ, Kuipers F. Bile acids suppress the secretion of very-low-density lipoprotein by human hepatocytes in primary culture. Hepatology 1996; 23: 218-228

36. Misra S, Uijzay P, Gattainart Z, Varticovski L, Arias IM. The role of phosphoinositide 3-kinase in taurocholate-induced trafficking of ATP-dependent canalicular transporters in rat liver. J Biol Chem 1998; 273: 26638-26644

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