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• VIRAL HEPATITIS •

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

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Abstract

AIM: To investigate the transactivating effect of hepatitis C virus (HCV) core protein and to screen genes transactivated by HCV core protein.

METHODS: pcDNA3.1(-)-core containing full-length HCV core gene was constructed by insertion of HCV core gene into *Eco*RI/*Bam*HI site. HepG2 cells were cotransfected with pcDNA3.1(-)-core and pSV-lacZ. After 48 h, cells were collected and detected for the expression of β-gal by an enzyme-linked immunosorbent assay (ELISA) kit. HepG2 cells were transiently transfected with pcDNA3.1(-)-core using Lipofectamine reagent. Cells were collected and total mRNA was isolated. A subtracted cDNA library was generated and constructed into a pGEM-Teasy vector. The library was amplified with *E. coli* strain JM109. The cDNAs were sequenced and analyzed in GenBank with BLAST search after polymerase chain reaction (PCR).

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 the 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 with 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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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 polyprotein 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 *Eco*R I, *Bam*H I, and Hind III (Takara). PCR primers were: up primer, 5' -GAA TTC AAT GAG CAC GAA TCC TAA-3' ;down primer, 5' -GGA TCC AGG CTG AAG CGG GCA CA-3' (Shanghai BioAsia Biotechnology Co., Ltd).

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

plated at a density of 1×10^6 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% confluentce, 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 *RsaI*. 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 tester molecules with different ends. After the ends were filled with DNA polymerase, the entire population of molecules was subjected to nested PCR with two adapter-specific primer pairs.

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 ℃ 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/cm^2), and isopropyl-β-D-thiogalactoside (IPTG; 12.1 μg/cm^2) and incubated overnight at 37 ℃. 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 *Eco*RI/*Bam*H I yielded two bands: 4 900 bp empty pcDNA3.1 (-) and 573 bp HCV core. Cleavage with *Hind* III 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.

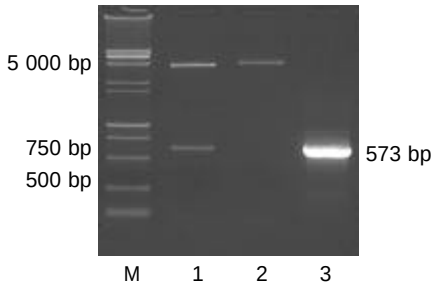

Figure 1 Products of pcDNA3.1(-)-core PCR and restriction enzyme cleavage were electrophoresed in 1% agarose gel. Lane 1: *Eco*RI / *Bam*HI cleaved; lane 2: *Hind*III cleaved; lane 3: products of plasmid PCR; M: DNA marker, (15 000 bp+ 2 000 bp).

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 (-) co-tranfected HepG2 cells were negative.

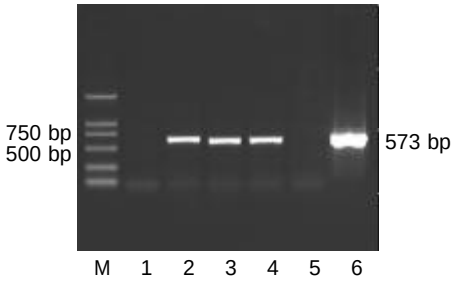

Figure 2 RT-PCR products were electrophoresed in 1% agarose gel. Lane 1: negative control; lanes 2-4: total RNA was isolated from pcDNA3.1(-)-core and RT-PCR was performed by three different Oligo dT; lane 5: blank control; lane 6: positive control; M: DNA marker (2 000 bp).

Table 1 Transient expression of pcDNA3.1(-)-core in HepG2 cells

Group	Coat Ag	*P*	P / N (n=0.05)	Results (+ / -)
pcDNA3.1(-)-core	pcDNA3.1(-)-core lysate	0.219	4.38	+
Blank plasmid	pcDNA3.1(-) lysate	0.034	0.68	-
Positive control	HCV core Ag	1.299	24.60	+
Negative control	PBS	0.012	0.24	-

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.

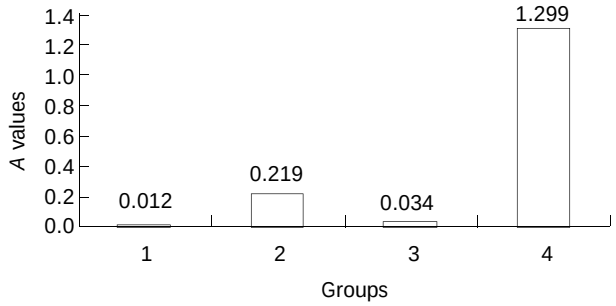

Figure 3 Comparison of *A* values among transfection groups. 1. Negative control (PBS); 2. pcDNA3.1(-)-core; 3. Blank plasmid; 4. Positive control.

Analysis of subtraction library

Using suppression subtractive hybridization technique (SSH), we obtained a total of 233 positive clones. These clones were prescreened by using PCR amplification to make sure that only clones with different inserts were subjected to sequencing (Figure 4). Two hundred and thirteen clones contained 100-1 000 bp inserts. 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 full length sequences accepted by GenBank were obtained with bioinformatics method. The other 57 sequences had a high similarity to known genes. Twenty-one of the sequences were 100% identical in nucleotide sequence to previously described sequences. Summary of the data is presented in Table 2.

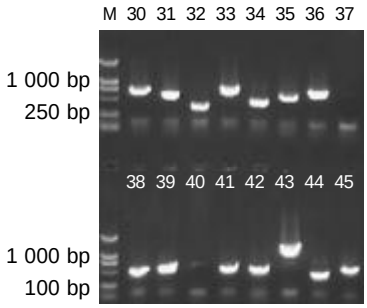

Figure 4 Agarose gel electrophoresis of PCR products of some clones (30-45). M: DNA marker (2 000 bp).

Table 2 Comparison between positive clones and similar sequences in GenBank

High similarity to known genes	Number of similar clones
Ribosomal protein	21
Eukaryotic translation elongation factor	6
Eukaryotic translation initiation factor	5
Heat shock factor binding protein (HSBP)	5
Apolipoprotein	5
NADH dehydrogenase	3
Heterogeneous nuclear ribonucleoprotein	3
D-like (HNRPDL), transcript variant	
Alpha-2-macroglobulin (A2M)	2
Bile acid-binding protein (BABP)	2
Cathepsin C (CTSC)	1
Fibronectin (FN precursor)	1
Magnesium-dependent protein phosphatase	1
Thioredoxin-related transmembrane protein	1
WEE1 gene for protein kinase	1

DISCUSSION

The diverse functional activities of HCV putative core protein have already been noted by a number of investigators. These include nucleocytoplasmic localization[14], regulation of cellular and unrelated viral promoters in *in vitro* studies[15-18], inhibition of apoptosis under certain conditions[19], physical association with apolipoprotein II[20] and cytoplasmic tail of the lymphotoxin β-receptor[21,22], promotion of normal cells to a transformed phenotype[23], and transactivation of suppression of cell growth[12,24].

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[23].

The nucleocapsid core protein of HCV has been shown to trans-act on several viral or cellular promoters[15,16,25,26]. 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 (BABP).

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[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[28]. Barba *et al*.[20] by double immunofluorescence and confocal analysis of the core and apo revealed colocalization of apoAII and the HCV core protein in the globular structures. Sabile *et al*.[29] investigated the binding of HCV core protein to cellular proteins expressed in HepG2 cells by combining 2 yeast hybrid, confocal and surface plasmon resonance assays. The results showed the direct binding of the viral protein to apolipoprotein AII (apoAII) and mapped the interaction domain to the C-terminal of HCV core protein. Taking advantage of the well-established increase in apoAII expression caused by fibers in HepG2 cells, they identified that apoAII was one of the cellular targets for HCV core protein and the intervention of fenofibric acid in cellular lipid metabolism directly affected the expression pattern of HCV core protein.

It has been discovered that bile acids are the natural ligand for a nuclear receptor termed farnesoid X receptor (FXR; NR1H4)[30-32]. 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[33]. Bile acids have been shown to modulate a variety of other cellular functions, such as secretion of lipoproteins

from hepatocytes[34,35] and translocation of bile acid transporters to the hepatocyte canalicular membrane[36]. Our results indicate that HCV core protein interacts with bile acid-binding proteins. Maybe it is a way that HCV core protein affects cellular lipid metabolism.

Our results revealed a number of novel and known genes that responded to HCV core. The 6 unknown nucleotide sequences of SSH fragments are deposited in GenBank under accession numbers of AY038359, AY038361, AY039041, AY039042, AY039043, and AY039044.

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