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World J Gastroenterol 2004;10(12):1735-1739
World Journal of Gastroenterology
 ISSN 1007-9327

• VIRAL HEPATITIS •

Cloning and identification of *NS5ATP2* gene and its spliced variant transactivated by hepatitis C virus non-structural protein 5A

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Received: 2003-11-21 **Accepted:** 2003-12-29

Abstract

AIM: To clone, identify and study new *NS5ATP2* gene and its spliced variant transactivated by hepatitis C virus non-structural protein 5A.

METHODS: On the basis of subtractive cDNA library of genes transactivated by NS5A protein of hepatitis C virus, the coding sequence of new gene and its spliced variant were obtained by bioinformatics method. Polymerase chain reaction (PCR) was conducted to amplify *NS5ATP2* gene.

RESULTS: The coding sequence of a new gene and its spliced variant were cloned and identified successfully.

CONCLUSION: A new gene has been recognized as the new target transactivated by HCV NS5A protein. These results brought some new clues for studying the biological functions of new genes and pathogenesis of the viral proteins.

Yang Q, Cheng J, Liu Y, Hong Y, Wang JJ, Zhang SL. Cloning and identification of *NS5ATP2* gene and its spliced variant transactivated by hepatitis C virus non-structural protein 5A. *World J Gastroenterol* 2004; 10(12): 1735-1739
http://www.wjgnet.com/1007-9327/10/1735.asp

INTRODUCTION

Hepatitis C virus (HCV) is the major causative agent of non-A, non-B hepatitis worldwide, which often leads to cirrhosis and an increased risk of hepatocellular carcinoma. The single-stranded RNA genome of HCV is a 9.6 kb-long positive-sense molecule, belonging to the *Flaviviridae* family. The viral genome encodes a single polyprotein precursor of approximately 3 010 amino acids, which is cleaved by both host and viral proteases to generate putative structural proteins (core, E1, and E2/p7) and the nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B)[1-3]. The nonstructural protein 5A (NS5A) is a phosphoprotein consisting of 447 amino acid residues. NS5A exists in two forms of polypeptide p56 and p58, which are phosphorylated mainly at serine residues both *in vitro* and *in vivo*[4,5].

It was previously shown that NS5A could function as a transcriptional trans-activator. Although these reports implicate a functional role of NS5A in transcription, the exact nature of its role or the mechanism (s) involved in regulating the cellular transcription has not been investigated. NS5A is localized to the endocytoplasmic reticulum (ER), whereas transcriptional trans-activation traditionally requires the protein to be in the nucleus. The NS5A protein must participate in signal transduction pathways that are initiated in the cytoplasm where it resides[6,7]. However, some studies show NS5A protein possesses a nuclear localization-like signal sequence and is present in the nuclear periplasmic membrane fraction related to transcription or translation[8]. The present study shows NS5A protein has transactivating effect on SV40 early promoter.

MATERIALS AND METHODS

Plasmid construction

The HCV-NS5A sequences were generated by PCR amplification of HCV plasmid (HCV strain 1b). The plasmid contains coding sequences for all of the nonstructural proteins. The PCR conditions were as follows: 94 ℃ for 40 s. 10 ng of PCR product was cloned with pEGM-T vector (Promega). The primary structure of insert was confirmed by direct sequencing. To create pcDNA3.1 (-)-NS5A, the fragments of encoding NS5A were released from the pEGM-T-NS5A by digestion with *Eco*R I and *Kpn* I, and ligated to pcDNA3.1 (-)[9].

Cell culture and transfection

The hepatoblastoma cell line HepG2 was propagated in DMEM supplemented with 10% FBS, 200 μmol/L L-glutamine, penicillin, and streptomycin. The HepG2 cells were plated at a density of 1×10^6 /well in 35-mm dishes. About 60-70% confluent HepG2 cells were cotransfected with plasmids pcDNA3. 1(-)-NS5A and pCAT3-promoter, transfected with pcDNA3.1 (-)-NS5A, pcDNA3.1 (-) with FuGENE 6(Roche).

Confirmation of protein expression of HCV-NS5A

Expression plasmid pcDNA3.1 (-)-NS5A was transfected using FuGENE 6 into HepG2 cells. The proteins expressed in these cells were analyzed on an immunoblot using the NS5A-specific antibody. The proteins were resolved by electrophoresis on a sodium dodecyl sulfate 125 g/L polyacrylamide gel. The lysate of cells transfected with expression vector pcDNA3. 1(-) served as negative control[10].

CAT assay

Cells were then harvested after 48 h for CAT assay. Lysates of transfected cells were analyzed for CAT density using a commercial enzyme-linked immunosorbent assay (Roche Molecular Biochemicals). The absorbance of the samples was measured at 405 nm[11].

RNA extraction and SSH

mRNAs from HepG2 cells transfected with plasmids pcDNA3.1

(-)-NS5A and pcDNA3.1(-) were extracted by using QuickPrep micro mRNA Purification Kit (Amersham Pharmacia). The amount of mRNA from two samples was 3-4 μg.

SSH was performed with the cDNA Substraction Kit (Clontech) according to the manufacturer' s protocol. cDNA was synthesized from 2 μg of poly A+RNA from two samples being compared. The cDNA from pcDNA3.1 (-)-NS5A acted as the tester, the cDNA from pcDNA3.1 (-) as the driver. The tester and driver cDNAs were digested with *Rsa* I, which yielded blunt ends. Two different PCR adaptors that could join only 5' ends DNA were ligated to different aliquots of tester DNA. These ligated DNAs were denatured, mixed with an excess of driver DNA (that had no adaptors), and allowed to anneal. The two DNA pools were then mixed together, and more denatured driver DNAs were added to further bind tester that was also present in the driver. Remaining complementary single strands of tester DNA were allowed to anneal, and the adaptor sequences were copied into their 3' ends. PCR was then performed to obtain exponential amplification of tester DNAs with different adaptors at each end. PCR amplifications products were directly purified by using Wizard PCR Preps DNA Purification System (Promega), and subcloned into pEGM-T easy vectors (Promega) to set up the subtractive library[12,13].

New gene cloned

On the basis of subtractive cDNA library of genes transactivated by NS5A protein of hepatitis C virus, the coding sequence of a new gene, named NS5ATP2, was obtained by bioinformatics methods. The standard PCR cloning technique was used to amplify NS5ATP2 gene. Cytoplasmic RNA was isolated from HepG2 cells. RNA was used for RT-PCR as described previously, primers were: sense 5' -GGA TTC ATG GCT TCG GTC TCC TCT GC-3' , antisense 5' -GGT ACC TCA GGA GTG TGG CTC ACT GG -3' (HepG2 cDNA). The PCR condition was as follows: at 94 ℃ for 60 s, at 60 ℃ for 60 s, at 72 ℃ for 60 s, for 30 cycles. The PCR product was cloned with pGEM-T vector (Promega). The primary structure of insert was confirmed by direct sequencing.

RESULTS

NS5A protein expressed in HepG2 cells

NS5A protein expressed in cells was analyzed by Western blot. The lysates of cells transfected with plasmid pcDNA3.1 (-)-NS5A were specifically detected by NS5A specific antibody (Figure 1).

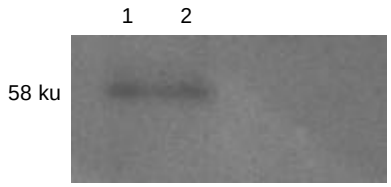

Figure 1 Western blotting of NS5A protein expression in HepG2 cells. Lane 1: Expression plasmid pcDNA3.1 (-)-NS5A; Lane 2: Plasmid pcDNA3.1 (-)-vector.

Transactivating effect of NS5A on SV40 early promoter

To determine whether NS5A protein has transactivating effect, we constructed plasmid pcDNA3.1 (-)-NS5A, and HCV NS5A protein expressed in Hep G2 cells was detected by reverse transcription PCR (RT-PCR) and Western blotting. HepG2 cells were transiently cotransfected with pcDNA3.1 (-)-NS5A/pCAT3-promoter, pcDNA3.1(-)/pCAT3-promoter. Chloramphenicol acetyltransferase (CAT) activity in cells that were cotransfected with pcDNA3.1 (-)-NS5A/pCAT3-promoter is shown in Figure 2.

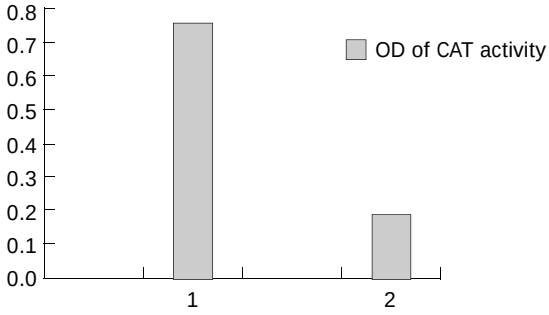

Figure 2 Transactivation on SV40 promoter by NS5A. 1: Plasmid pcDNA3.1 (-)-NS5A was cotransfected with pCAT3-promoter in HepG2 cells. 2: Plasmid pcDNA3.1 (-) was cotransfected with pCAT3-promoter in HepG2 cells.

Construction of subtractive cDNA library

Our studies showed NS5A protein had transactivation effect on SV40 promoter. In order to investigate influence of NS5A protein on cells gene expression, Suppression subtraction hybridization (SSH) was introduced to establish substractive cDNA library of HepG2 transfected with plasmid pcDNA3.1 (-)-NS5A. We performed the PCR experiment to analyse the ligation efficiency. The result showed that at least 25% of the cDNA had adaptors at both ends. The efficiency of subtraction was estimated by PCR experiment. The test was done by comparison of the abundance of G3PDH before and after subtraction. G3PDH primers were provided by the kit (Figure 3).

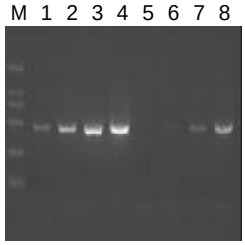

Figure 3 Reduction of G3PDH amount by PCR-selective subtraction. PCR was performed on unsubtracted (Lanes 1-4) and subtracted (Lanes 5-8) secondary PCR products with the G3PDH 5' and 3' primers. Lanes 1, 5: 18 cycles; Lanes 2,6: 23 cycles; Lanes 3,7: 28 cycles.

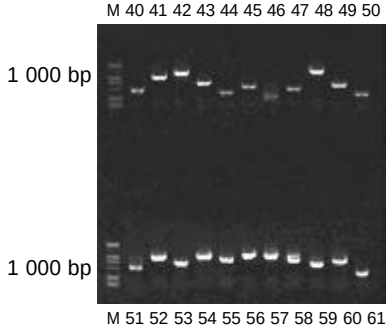

Figure 4 Map of colony PCR on 9 g/L agarose/EtBr gel.

After tester cDNA was hybridized with driver cDNA twice and underwent nested PCR twice, they were then subcloned into pGEM-T easy vectors to set up the subtractive library. Amplification of the library was carried out with *E. coli* strain JM109. The amplified library contained 121 positive clones.

Colony PCR showed that 115 clones contained 200-1 000 bp inserts (Figure 4). The nucleotide sequences of 90 clones from this cDNA library was analyzed, the full length sequences were obtained with Vector NTI 6 and BLAST database homology search (http://www. ncbi. nlm. nih. gov/). Altogether 44 kinds of coding sequences were obtained, consisting of 29 known and 15 unknown ones. Some genes code for proteins involved in cell cycle regulation, cell apoptosis, signal transduction pathway and tumour (Table 1).

Table 1 Sequence analysis of 46 clones isolated from subtractive cDNA library

Known genes	Number of clones	Homology (%)
Ribosomal protein	15	99
Eukaryotic translation initiation factor	4	99
HCV NS5A protein	4	98
Sentrin	4	99
Pro-oncosis receptor inducing membrane injury (Porimin)	3	100
Importin	3	98
Serine/threonine kinase	3	100
Cadherin-associated protein	2	100
Mitogen-activated protein kinase phosphatase	2	99
Adenylyl cyclase-associated protein	2	100
Serum response element	2	100
Rho GTPase activating protein	2	100
Fibronectin	3	99
Laminin	3	99
Lysophospholipase A2	2	100
Lysophospholipase B	2	100
Dual specificity phosphatase 6	1	99
Putative homeodomain transcription factor	2	92
Transcription factor B2 NF-E2-like basic leucine zipper	2	100
Transcriptional activator	2	98
Transcriptional elongation factor (TFIIS)	2	100
MHC-I binding protein	1	100
C response protein binding protein (CRPBP)	1	99
Integrin	2	99
Iron-regulated transporter (IREG)	1	99
Tumor associated protein L6	2	100
WW domain-containing protein 1 (WWP1)	1	100
Nascent polypeptide-associate complex α(NACA)	1	99
Thioredoxin reductase	1	99

Confirmation of new gene expression by RT-PCR

We found the spliced variant of NS5A-TP2 (Figures 5, 6). After EST database homology search (http://www. ncbi. nlm. nih. gov/), the locations of NS5A-TP2 and its spliced variant were detected on chromosome 6q22. 1-23. 3. The exons and introns of two new genes were compared (Figure 7). The direct sequencing showed we acquired the ORF of NS5A-TP2 (Figure 8).

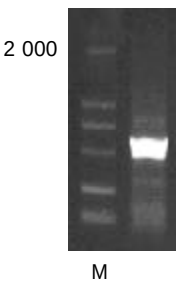

Figure 5 NS5A-TP2 fragment amplified by RT-PCR. M: Marker.

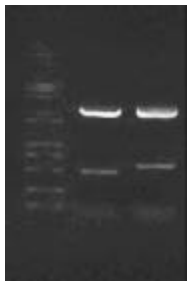

Figure 6 pEGM-T-NS5A-TP2 cut by EcoR I/Kpn I. M: Marker; Lane 1: A 512-bp fragment; Lane 2: A 615-bp fragment.

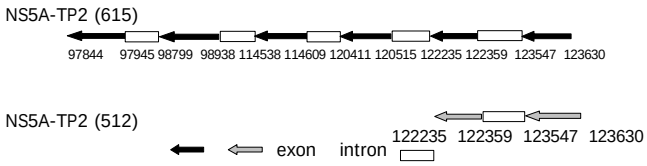

Figure 7 Comparison of exons and introns of NS5A-TP2 (615) and (512) gene.

615 **ATG** GCT TCG GTC TCC TCT GCG ACC TTC TCG
512 **ATG** GCT TCG GTC TCC TCT GCG ACC TTC TCG

615 GGC CAC GGG GCT CGG TCC CTA CTG CAG TTC
512 GGC CAC GGG GCT CGG TCC CTA CTG CAG TTC

615 CTG CGG CTG GTA GGG CAG CTC AAG AGA GTC
512 CTG CGG CTG GTA GGG CAG CTC AAG AGA GTC

615 CCA CGA ACT GGC TGG GTA TAC AGA AAT GTC
512 CCA CGA ACT GGC TGG GTA TAC AGA AAT GTC

615 CAG AGG CCG GAG AGC GTT TCA GAT CAC ATG
512 CAG AGG CCG GAG AGC GTT TCA GAT CAC ATG

615 TAC CGG ATG GCA GTT ATG GCT ATG GTG ATC
513 TAC CGG ATG GCA GTT ATG GCT ATG GTG ATC

615 AAA GAT GAC CGT CTT AAC AAA GAC CGA TGT
512 AAA GAT GAC CGT CTT AAC AAA GAC C-- ---

615 GTA CGC CTA GCC CTG GTT CAT GAT ATG GCA
512 --- --- --- --- --- --- --- --- --- ---

615 GAA TGC ATC GTT GGG GAC ATA GCA CCA GCA
512 --- --- --- --- --- --- --- --- --- ---

615 GAT AAC ATC CCC AAA GAA GAA AAA CAT AGG
512 --- --- --- --- --- --- --- --- --- ---

615 CGA GAA GAG GAA GCT ATG AAG CAG ATA ACC
512 --- --- --- G GAA GCT A**TG A**AG CAG ATA ACC

615 CAG CTC CTA CCA GAG GAC CTC AGA AAG GAG
512 CAG CTC CTA CCA GAG GAC CTC AGA AAG GAG

615 CTC TAT GAA CTT TGG GAA GAG TAC GAG ACC
512 CTC TAT GAA CTT TGG GAA GAG TAC GAG ACC

615 CAA TCT AGT GCA GAA GCC AAA TTT GTG AAG
512 CAA TCT AGT GCA GAA GCC AAA TTT GTG AAG

615 CAG CTA GAC CAA TGT GAA ATG ATT CTT CAA
512 CAG CTA GAC CAA TGT GAA ATG ATT CTT CAA

615 GCA TCT GAA TAT GAA GAC CTT GAA CAC AAA
512 GCA TCT GAA TAT GAA GAC CTT GAA CAC AAA

615 CCT GGG AGA CTG CAA GAC TTC TAT GAT TCC
512 CCT GGG AGA CTG CAA GAC TTC TAT GAT TCC
615 ACA GCA GGA AAA TTC AAT CAC CCT GAG ATA
512 ACA GCA GGA AAA TTC AAT CAC CCT GAG ATA

615 GTC CAG CTT GTT TCT GAA CTT GAG GCA GAA
512 GTC CAG CTT GTT TCT GAA CTT GAG GCA GAA

615 AGA AGC ACT AAC ATA GCT GCA GCT GCC AGT
512 AGA AGC ACT AAC ATA GCT GCA GCT GCC AGT

615 GAG CCA CAC TCC **TGA**
512 GAG CCA CAC TCC TGA

Figure 8 ORF comparison of NS5A-TP2 (615) and (512).

DISCUSSION

Hepatitis C virus often causes persistent infection with a significant risk of end-stage cirrhosis and hepatocellular carcinoma. HCV may benefit by regulation of celluar genes leading to the disruption of normal cell growth. Viral genes can override cellular control mechanisms, which in untransformed cells regulate cell cycle progression in response to various antiproliferative signals. In HCV persistently infected cells, the continued presence of viral gene products is likely to be detrimental for host cells. Many studies demonstrated NS5A protein of HCV transcriptionally modulates cellular genes and promotes murine fibroblast cell growth into a tumorigenic phenotype. It may be possible that the NS5A protein plays a role in hepatocarcinogenesis, since many other viral proteins that play a role in carcinogenesis often function as transcriptional activators[14-17]. However, the precise mechanism is still unknown.

In the present study, we investigated the possible mechanism by which NS5A protein transactivated gene expression and its role in hepatocarcinogenesis. NS5A protein in Hep G2 cells was detected by RT-PCR and Western blotting. HepG2 cells were transiently cotransfected with pcDNA3.1 (-)-NS5A/ pCAT3-promoter. CAT activity was evidently higher in the cotransfected cells than in control. It is suggested that NS5A protein has transactivating effect on SV40 early promoter. We predicted that NS5A protein transcriptionally regulated gene expression through regulating promoter activity, either directly or through signal transduction pathways.

On the basis of this study, we constructed subtractive cDNA library by SSH. After sequencing analysis, we obtained coding sequences of 46 genes, which consisted of 26 kinds of known and 15 kinds of unknown ones. Some genes code for proteins involved in cell cycle regulation, cell apoptosis, and tumor angiogenesis. Sentrin is a 101-amino acid ubiquitin-like protein that interacts with the death domains of Fas and TNFR1, with PML, a tumor suppressor implicated in the pathogenesis of promyelocytic leukemia, with Rad51 and Rad52, proteins that are involved in repairing double-stranded DNA breaks, and with RanGAP1, a GTPase-activating protein that is critically involved in nuclear protein transport[18-20]. Overexpression of sentrin in mammalian cells protects them against anti-Fas or tumor necrosis factor-induced cell death[21]. Porimin is a highly glycosylated protein that can be classified as a member of the cell membrane-associated mucin family[22]. Porimin is a membrane mucin that mediates cell death. Although mucins mainly affect cell adhesion and ligand binding, several membrane mucins have also been documented to trigger cell death or inhibit cell proliferation, such as CD43 (leukosialin, sialophorin), CD162 (PSGL-1), and CD164 (MGC-24v)[23]. Likewise, serine/threonine kinase, cadherin-associated protein, adenylyl cyclase-associated protein, mitogen-activated protein kinase phosphatase involving in cell cycle regulation, and cell growth may be correlated with hepatocarcinogenesis of NS5A protein[24-28].

Alternative pre-mRNA splicing is a fundamental mechanism for differential gene expression that has been reported to regulate the tissue distribution, the intracellular localization, and the activity of different protein kinases. In the process of our study on new genes, we accidentally acquired the spliced variant of NS5A-TP2 and confirmed the ORF of NS5A-TP2 (516) and its location on chromosome. Both of NS5A-TP2 (615) and its spliced variant- NS5A-TP2 (516) locate on 6q22.1-23.3, but they have different exons and introns[29-31].

The result of this study shows that the NS5A protein is a potent transcriptional activator and transactivates some genes involved in cell cycle regulation, cell apoptosis, and tumor angiogenesis. The study on new genes NS5A-TP2 (516), and NS5A-TP2 (615) brings some new clues to the biological functions of novel genes and pathogenesis of the viral proteins.

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Edited by Zhu LH and Chen WW **Proofread by** Xu FM