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Study of transactivating effect of pre-S2 protein of hepatitis B virus and cloning of genes transactivated by pre-S2 protein with suppression subtractive hybridization

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Abstract

AIM: To investigate the transactivating effect of pre-S2 protein of hepatitis B virus (HBV) and construct a subtractive cDNA library of genes transactivated by pre-S2 protein with suppression subtractive hybridization (SSH) technique, and to pave the way for elucidating the pathogenesis of HBV infection.

METHODS: pcDNA3.1(-)-pre-S2 containing pre-S2 region of HBV genome was constructed by routine molecular methods. HepG2 cells were cotransfected with pcDNA3.1 (-)-pre-S2/pSV-lacZ and empty pcDNA3.1(-)/pSV-lacZ. After 48 h, cells were collected and detected for the expression of β-galactosidase (β-gal). SSH and bioinformatics techniques were used, the mRNA of HepG2 cells transfected with pcDNA3.1(-)-pre-S2 and pcDNA3.1(-) empty vector was isolated, respectively, cDNA was synthesized. After digestion with restriction enzyme *RsaI*, cDNA fragments were obtained. Tester cDNA was then divided into two groups and ligated to the specific adaptor 1 and adaptor 2, respectively. After tester cDNA was hybridized with driver cDNA twice and underwent two times of nested PCR, amplified cDNA fragments were subcloned into pGEM-Teasy vectors to set up the subtractive library. Amplification of the library was carried out with *E.coli* strain DH5α. The cDNA was sequenced and analyzed in GenBank with Blast search after PCR.

RESULTS: The pre-S2 mRNA could be detected in HepG2 cells transfected with pcDNA3.1(-)-pre-S2 plasmid. The activity of β-gal in HepG2 cells transfected with pcDNA3.1 (-)-pre-S2/pSV-lacZ was 7.0 times higher than that of control plasmid (P<0.01). The subtractive library of genes transactivated by HBV pre-S2 protein was constructed successfully. The amplified library contains 96 positive clones. Colony PCR showed that 86 clones contained 200-1 000 bp inserts. Sequence analysis was performed in 50 clones randomly, and the full length sequences were obtained with bioinformatics method and searched for homologous DNA sequence from GenBank, altogether 25 coding sequences were obtained, these cDNA sequences might be the target genes transactivated by pre-S2 protein.

CONCLUSION: The pre-S2 protein of HBV has transactivating effect on SV40 early promoter. The obtained sequences may be target genes transactivated by pre-S2 protein among which some genes coding proteins involved in cell cycle regulation, metabolism, immunity, signal transduction and cell apoptosis.This finding brings some new clues for studying the biological functions of pre-S2 protein and further understanding of HBV hepatocarcinogesis.

Key words: HBV; pre-S2 surface protein; Transactivation

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INTRODUCTION

Hepatitis B virus (HBV) causes acute and chronic infections of the liver. Acute infections may produce serious illness, and approximately 0.5% terminate with fatal, fulminant hepatitis. Chronic infections may also have serious consequences[1]. In addition to causing acute and chronic hepatitis, HBV is considered to be a major cliological factor in the development of human hepatocellular carcinoma (HCC), one of the most frequent fatal malignancies worldwide, and worldwide deaths from HCC caused by HBV infection probably exceed one million per year[2-4]. Epidemiological studies have demonstrated an approximately 10-fold increase in the relative risk of HCC among HBV carriers compared to noncarriers[5-8].

The precise role of HBV in the etiology of HCC is not well understood. Only occasionally, genes controlling the cell growth and differentiation are disturbed by HBV DNA sequences intergration, but since no common sites of viral DNA integration have been observed, the induction of cell

growth by HBV enhancer or promoter insertion cannot be regarded as a general mechanism of transformation in human HCC. An alternative mechanism of hepatocarcinogenesis could be the activation and/or repression, operated by HBV proteins, of the expression of genes crucial for cell growth regulation. Recent data have showed that some HBV proteins can exert a significant transactivational activity on both viral and cellular promoter[9]. Almost all HBV-associated HCCs studied so far harbor chromosomally integrated HBV DNA[10]. Integrated HBV DNA can encode two types of transcriptional transactivators: the already well studied HBx[11] and the pre-S2 transactivators: LHBs (large hepatitis B virus surface protein) and MHBs^t (C-terminally truncated middle surface protein)[12-14].

Although it is true the transactivator functions are preformed by the LHBs or MHBs^t, on closer inspection, one can easily see that this role is mostly performed by the pre-S region of the envelope protein. This observation naturally leads to the question, whether pre-S protein fragments alone might also have the transactivating role in the absence of the S region. Actually, *in vitro* studies with protease-treated HBV particles showed specific cleavage of pre-S fragments from LHBs, which indicate the presence of protease-sensitive sites within the junction of the pre-S and S proteins[15,16]. So, while the proteolytic cleavage and generation of the free pre-S fragments are quite evident, their biological meanings remain obscure[17-19]. Recently, we have studied the transactivator function of pre-S proteins. In the present study, we showed that pre-S2 protein functions as a transcriptional transactivator, and constructed the subtractive library of genes transactivated by HBV pre-S2 protein, successfully.

MATERIALS AND METHODS

Plasmid construction

The pre-S2 gene was amplified by PCR from the plasmid G376-7 (GenBank number: AF384371)[20-22] using sense (5'- GAT ATC ATG CAG TGG AAC TCC ACC -3') and antisene (5'- GGA TCC TTA GTT CGG TGC AGG GTC -3') primers (Shanghai BioAsia Biotechnology Co., Ltd). As these primers contain *Eco*RV and *Bam*HI (Takara) recognition sites on their respective 5'- ends, the amplified 180 bp PCR fragment was subcloned into the *Eco*RV/*Bam*HI sites of pcDNA3.1(-) vector (Invitrogen), the expression vector, pcDNA3.1(-)-pre-S2 which could direct expression of pre-S2 fusion protein was obtained, then identified by PCR and digested of *Eco*RV/*Bam*HI.

Cell culture and DNA transfection

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 100 IU of penicillin and 100 μg of streptomycin per mL, supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), at 37 ℃ in a 50 mL/L CO_2 atmosphere. 1.5×10^6 cells were seeded in 35-mm plates 12 h prior to transfection and reached 50% confluence at the time of transfection. Cells were transfected with FuGENE6 Transfection Reagent (Roche), the transfection was carried out with 2.0 μg of pcDNA3.1 (-) empty and pcDNA3.1(-)-pre-S2 plasmid DNA according to the manufacture's protocol, respectively.

mRNA and cDNA isolation

The mRNA from HepG2 cells transfected with pcDNA3.1 (-)-pre-S2 and pcDNA3.1(-) empty vector was isolated by using a micro mRNA purification kit (Amersham Biosciences), respectively, cDNAs were reverse-transcribed from mRNA. Identification was done by PCR with pre-S2-specific primers.

Effect of pre-S2 protein on SV40 early promoter

HepG2 cells were transfected with various concentrations of pSV-lacZ (0.1-1.8 μg) (promega) plasmid DNA, expression of β-gal was detected by using a β-gal assay kit (promega). In pSV-LacZ plasmid, the LacZ gene is under the control of the SV40 early promoter element. The best concentration of pSV-lacZ was selected, HepG2 cells with pSV-lacZ (1.0 μg) and pcDNA3.1(-)-pre-S2 (2.0 μg) were cotransfected as a test group. At the same time, cotransfected with empty pcDNA3.1 (-) (2.0 μg) and pSV-lacZ (1.0 μg) was used as control as control group The cells were collected after 48 h, and the expression of β-gal was detected. Seven separate transfections were present in test and control groups, respectively, and Stata 7.0 program was used to analyze the experimental data.

Suppression subtractive hybridization (SSH)

Genome comparisons was done by suppression subtraction hybridization according to the manufacturer's instructions of PCR-select™ cDNA subtraction kit (Clontech). In brief, 2 μg of mRNA from the tester and the driver was subjected to cDNA synthesis. Tester and driver cDNAs were digested with *RsaI*. The tester cDNA was split into two groups, and each was ligated with a different cDNA adapter. In a first hybridization reaction, an excess of driver was added to each sample of tester. The samples were heat denaturated and allowed to anneal. Because of the second-order kinetics of hybridization, the concentration of high- and low-abundance sequences is equalized among the single-stranded tester molecules. At the same time, single-stranded tester molecules were significantly enriched for differentially expressed sequences. During a second hybridization, the two primary hybridization samples were mixed together without denaturation. Only the remaining equalized and subtracted single-stranded tester cDNAs can reassociate forming double-stranded tester molecules with different ends. After filling in the ends with DNA polymerase, the entire population of molecules was subjected to nested PCR with two adapter-specific primer pairs. After that, secondary PCR products were used as templates for PCR amplification of G3PDH (a housekeeping gene) at 18, 23, 28, 33 cycles to assure subtracted efficiency.

Cloning of subtracted cDNA libraries

Products of these amplified overhangs containing a subtracted cDNA library (6 μL) were ligated into a pGEM-Teasy vector (Promega). Subsequently, the plasmid was transformed into *E. coli* strain DH5α. Bacteria were taken up in 800 μL of LB medium and allowed to incubate for 45 min at 37 ℃ and 225 r/m. after incubation, Bacteria were plated onto agar plates containing ampicillin (100 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 selected and identified by PCR. Primers were T7/SP6 primer of pGEM-Teasy vector. After sequencing the plasmids DNA of positive colonies (Shanghai BioAsia Biotechnology Co., Ltd), 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

Restriction enzyme analysis of pcDNA3.1(-)-pre-S2 plasmid with *Eco*RV/*Bam*HI yielded two bands: 5 396 bp empty pcDNA3.1(-) and 172 bp HBV pre-S2, cleaved with *Pst*I got only one 5 568 bp band (5 396 bp+172 bp). The plasmid by PCR amplification with pre-S2-sepcific primers got a clear band of the expected size (180 bp, Figure 1). Sequencing the PCR products was correct.

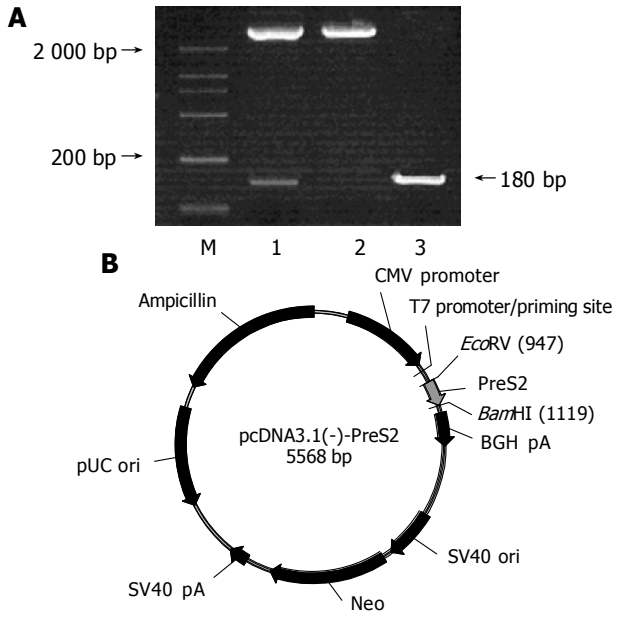

Figure 1 **A**: Products of pcDNA3.1(-)-pre-S2 PCR and restriction enzyme cleaved were eletrophoresed in 2.0% agarose gel. Lane 1: *Eco*RV/*Bam*HI cleaved, lane 2: *Pst*I cleaved, lane 3: production of plasmid PCR, M: DNA marker (2 000 bp). **B**: Structure of expression plasmid pcDNA3.1(-)-pre-S2.

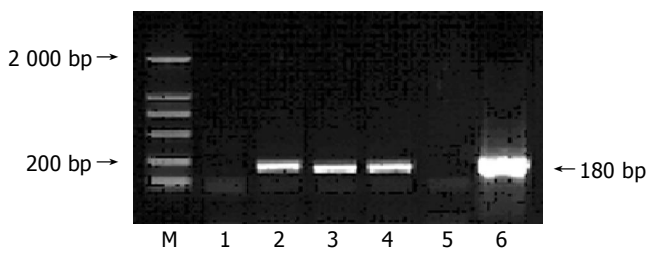

Figure 2 RT-PCR products were eletrophoresed in 0.9% agarose gel. Lane 1: negative control, lanes 2-4: mRNA was isolated from pcDNA3.1(-)-pre-S2 and RT-PCR was performed by three different Oligod T, lane 5: blank control, lane 6: positive control, M: DNA marker (2 000 bp).

Expression of HBV pre-S2 protein

Reverse-transcribed by three different Oligo dT, identification of cDNA by PCR yielded common 180 bp band (Figure 2).

Transactivation of HBV pre-S2 protein on early promoter of SV40

The *A* data about expression of β-gal of the test group (n = 7) was 0.238±0.007. In contrast, the *A* data about that of the control group (n = 7) was 0.034±0.009. Expression of β-gal were 7.0-fold higher when contransfected pcDNA3.1(-)-pre-S2 and pSV-lacZ than when contransfected empty pcDNA3.1(-) and pSV-lacZ. The significant increase of expression of β-gal (P<0.01) was attributed to the transactivating effect of HBV pre-S2 protein on early promoter of SV40, increasing the expression of downstream gene lacZ (Figure 3).

Analysis of the subtraction library

Subtraction efficiency analysis showed that the PCR products of G3PDH in unsubtracted library were visible after 18 cycles, however, 28 cycles were required in the subtracted one for G3PDH to be detected (Figure 4). The abundance of non-differentially expressed gene was effectively reduced, which indicated that the subtraction library had the high subtraction efficiency.

Using SSH technique, we obtained a total of 96 positive clones. These clones were prescreened by using PCR amplification to ensure that only clones with different inserts were subjected to sequencing (Figure 5). Among these clones,

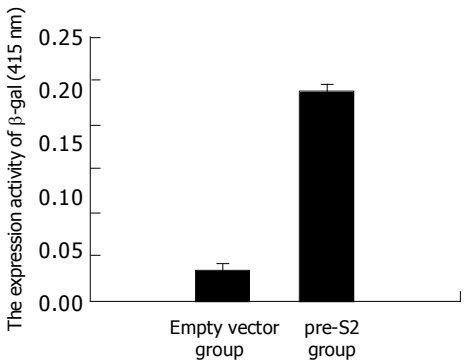

Figure 3 Result of β-galactosidase enzyme analysis. The numbers represent mean±SD of 7 separate transfections.

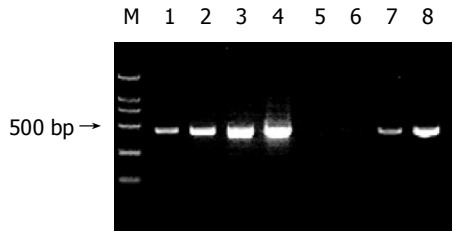

Figure 4 Reduction of G3PDH abundance by PCR-select subtraction. PCR was performed on unsubtracted (lanes1-4) or subtracted (lanes 5-8) secondary PCR products with the G3PDH 5' and 3' primers. Lanes 1, 5: 18 cycles; lanes 2, 6: 23 cycles; lanes 3, 7: 28 cycles; lanes 4, 8: 33 cycles. Lane M: DNA marker (2 000 bp).

eighty-six clones contained 200-1 000 bp inserts. A total of 50 clones from the cDNA library were randomly chosen and sequenced, altogether 25 coding sequences were obtained. Summary of the data is presented in Table 1.

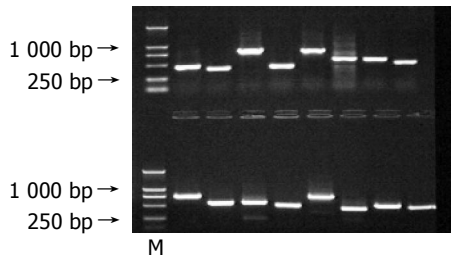

Figure 5 Results of PCR amplification of part clones (17-32). M: DNA marker (2 000 bp).

DISCUSSION

The sequence encoding the pre-S2 transactivators is localized in the HBV surface gene. The surface gene consists of a single open reading frame divided into three coding regions: pre-S1, pre-S2 and S, each starting with an in-frame ATG codon. Through alternate translational initiation at each of the three AUG codons, a large (LHBs, pre-S1+pre-S2+S), a middle (MHBs, pre-S2+S) and a small (SHBs, S) envelope glycoprotein can be synthesized[23-27]. The transactivator function of the surface protein requires the cytoplasmic orientation of the pre-S2 domain (the minimal functional unit) that occurs in the case of MHBs^t and in a fraction of LHBs[28,29]. In contrast, full-length MHBs displays no transcriptional activator function, here the pre-S2 domain directs into the lumen of the endoplasmic reticulum (ER). The generation of functional MHBs^t from MHBs requires deletion of the 3' end of the pre-S2/S gene encoding at least the last 70 amino acids corresponding to the hydrophobic region III of the S domain during the integration process[30]. Deletions of 3' sequences of the pre-S2/S gene generating functional MHBs^t transactivators were found in one-third of the integrates investigated so far[31,32]. This fact and the presence of complete pre-S/S genes in some other systematically studied integrates indicate the biological significance of the pre-S2 transactivators[33].

The transactivator function of the pre-S2 activators is based on the cytoplasmic orientation of the pre-S2 domain, and many reports have demonstrated that the pre-S2 region is sufficient for generation of the transcriptional transactivator function. The pre-S2 domain is PKC-dependent phosphorylated. Moreover, the pre-S2 domain binds of PKC α/β and triggers a PKC-dependent activation of the c-Raf-1/MAPKKK signal transduction cascade, resulting in an activation of transcription factors such as AP-1 and NF-κB. Furthermore, by activation of this signaling cascade, the pre-S2 activators cause an increased proliferation rate of hepatocytes. According to the two-step model of carcinogenesis (initiation/promotion), the pre-S2 activators could exert a tumor-promoter-like function by activation of the PKC/c-Raf-1/MAPKKK signaling cascade: cells harboring critical mutations (initiation) may be positively selected (promotion). Such a multistep process may account for the long latency period in HCC development, but it also leads to the hypothesis that each tumor reflects an individual case[34-37].

We cotransfected HepG2 cells with pcDNA3.1(-)-pre-

Table 1 Homolog searching of sequenced cDNA fragments from SSH library

High similarity proteins to known genes	GenBank No.	Number of similarity clones
Eukaryotic translation elongation factor1 alpha 1	BC018641	15
Ribosomal protein	BC004294	7
Fibronectin 1 (FN1)	BC016875	3
Mitochondrion	AY289054	2
Ferritin, heavy polypeptide 1	BC063514	2
Albumin	BC036003	2
Keratin 18	BC000698	1
Phospholipase A2	HUMPHPLA2	1
Pituitary tumor-transforming 1 interacting protein	BC034250	1
NADH dehydrogenase 2 (MTND2)	NM_173709	1
Cytochrome c oxidase II (MTCO2)	NM_173705	1
Laminin, beta 1	BC026018	1
Tumor rejection antigen (gp96)1 (TRA1)	NM_003299	1
Calmodulin 2 (phosphorylase kinase,delta)(CALM2)	NM_001743	1
M-phase phosphoprotein, mpp11	HSMPP11	1
Spermidine/spermine N1-acetyltransferase (SSAT)	HSU40369	1
Nucleophosmin	BC020467	1
Alpha-2-macroglobulin	HUMA2MGL	1
Tubulin	BC018948	1
Prostaglandin F synthase	AB032154S4	1
Methionine-tRNA synthetase (MARS)	NM_004990	1
Bile acid-binding protein	AB032151S1	1
Tyrosine 3-monooxygenase/tryptophan 5-monooxyge nase activation protein, zeta polypeptide	BC013265	1
Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1) (RAC1)	NM_198829	1
Aldo-keto reductase family 1,member C3(3-alpha hydroxysteroid dehydrogenase, type II)	BC001479	1

S2 and pSV-lacZ and demonstrated that the HBV pre-S2 protein is successfully expressed in transfected HepG2 cells. Expression of β-gal were 7.0-fold higher when contransfected with pcDNA3.1(-)-pre-S2 and pSV-lacZ than when contransfected with empty pcDNA3.1(-) and pSV-lacZ. HBV pre-S2 protein has significant transactivating effect on early promoter of SV40, increasing the expression of downstream gene lacZ. This result indicates that the HBV pre-S2 protein expressed in HepG2 cells retains its biological activity in terms of transcriptional activation, which is consistent with the previous report[38,39].

To get insight into the transactivation mechanism of HBV pre-S2 protein, the SSH was used for identification of transactivating relative target genes of pre-S2 protein, and a subtractive library was set up successfully. Sequence analysis was performed in 50 clones, and 25 coding sequences were obtained. These genes can be divided into 4 groups: (1) The genes involving in cell structure and cell cycle. They possess the important ability to control cell growth, differentiation and adherence, such as ribosomal protein, mitochondrion, nucleophosmin, tubulin, keratin, M-phase phosphoprotein, eukaryotic translation elongation factor, and so on. (2) The genes relating to cell energy or substance metabolism, (i.e., ferritin, albumin, NADH dehydrogenase 2, cytochrome c oxidase II, spermidine/spermine N1-acetyltransferase, 3-alpha hydroxysteroid dehydrogenase, methionine-tRNA synthetase). Bile acids may be important regulators of gene expression in the liver and intestines. Bile acids have been shown to modulate a variety of cellular functions, such as secretion of lipoproteins from hepatocytes and translocation of bile acid transporters to the hepatocyte canalicular membrane[40-42]. Our results indicate that the HBV pre-S2 protein interact with the bile acid-binding protein, maybe it is a way that HBV affect cellular lipid metabolism. (3) The genes involving in the formation mechanism of hepatic fibrosis. alpha-2-macroglobulin (A2M) is a glycoprotein with high molecular weight, which can bind with different proteases, including interstitial collagenases and the other metalloproteases. A2M's proteinase inhibitor effect can inhibit the activity of collagenases, and participate the formation of hepatic fibrosis[43,44]. Laminin (LN) is a kind of serum fibrosis markers, during fibrogenesis LN continuously accumulate in liver tissues and form basement membrane resulting in sinusoidal capillaration[45], the stages of hepatic fibrosis are correlated with the serum levels of LN, so LN can be used in detecting the degree of hepatic fibrosis. The serum levels of LN were elevated from S1 to S4, because of the increase of hepatic fibrosis. Our data suggest that HBV pre-S2 protein may play a role in the progress of liver cirrhosis[46-48]. (4) The genes controlling cell signal transduction and apoptosis. Such as tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, calmodulin 2, ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1) (RAC1), Phospholipase A2 (PLA2). PLA2 is a family of enzymes thought to play a key role in inflammation by releasing arachidonic acid for the synthesis of eicosanoids and lysophospholipid for the synthesis of platelet-activating factor and take part in different singaling pathways, including apoptosis and MAPKKK signaling cascade. CPLA2 (cytosolic phospholipase A2) is activated by MAP kinase due to a phosphorylation of serine residue 505[49].

Altogether, we testified the transactivator ability of HBV pre-S2 protein again, and obtained the subtractive cDNA library of genes transactivated by pre-S2 protein with SSH technique, successfully. Furthermore, we brought some new clues for studying the biological functions of HBV pre-S2 protein and and pave the way for elucidating the pathogenesis of HBV infection.

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