

• VIRAL HEPATITIS •

Transactivating effect of complete S protein of hepatitis B virus and cloning of genes transactivated by complete S protein using suppression subtractive hybridization technique

Gui-Qin Bai, Yan Liu, Jun Cheng, Shu-Lin Zhang, Ya-Fei Yue, Yan-Ping Huang, Li-Ying Zhang

Gui-Qin Bai, Shu-Lin Zhang, Ya-Fei Yue, Yan-Ping Huang, The First Hospital of Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China

Yan Liu, Jun Cheng, Li-Ying Zhang, Gene Therapy Research Center, Institute of Infectious Diseases, 302 Hospital of PLA, 100 Xisihuanzhong Road, Beijing 100039, China

Supported by the National Natural Science Foundation of China, No. C03011402, No. C30070690; the Science and Technique Foundation of PLA during the 9th Five-year Plan period, No. 98D063; the Launching Foundation for Students Studying Abroad of PLA, No. 98H038; the Youth Science and Technique Foundation of PLA during the 10th Five-year plan period, No. 01Q138; and the Science and Technique Foundation of PLA during the 10th Five-year Plan period, No. 01MB135

Correspondence to: Dr. Gui-Qin Bai, Department of Obstetrics and Gynecology of First Hospital, Xi'an Jiaotong University, Jiankang Road 1, Xi'an 710061, Shaanxi Province, China
Telephone: +86-29-85213194 Fax: +86-29-85252812

Received: 2004-11-08 Accepted: 2004-12-26

Abstract

AIM: To investigate the transactivating effect of complete S protein of hepatitis B virus (HBV) and to construct a subtractive cDNA library of genes transactivated by complete S protein of HBV by suppression subtractive hybridization (SSH) technique and to clone genes associated with its transactivation activity, and to pave the way for elucidating the pathogenesis of hepatitis B virus infection.

METHODS: pcDNA3.1(-)-complete S containing full-length HBV S gene was constructed by insertion of HBV complete S gene into *BamH I/Kpn I* sites. HepG2 cells were cotransfected with pcDNA3.1(-)-complete S and pSV-lacZ. After 48 h, cells were collected and detected for the expression of β -galactosidase (β -gal). Suppression subtractive hybridization and bioinformatics techniques were used. The mRNA of HepG2 cells transfected with pcDNA3.1(-)-complete S and pcDNA3.1(-) empty vector was isolated, and detected for the expression of complete S protein by reverse transcription polymerase chain reaction (RT-PCR) method, and 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 adaptors 1 and 2, respectively. After tester cDNA had been hybridized with driver cDNA twice and underwent nested PCR twice, amplified cDNA fragments were subcloned into pGEM-Teasy vectors to set up the subtractive library. Amplification of the library was carried out within *E. coli* strain DH5 α . The cDNA was sequenced

and analyzed in GenBank with BLAST search after polymerase chain reaction (PCR) amplification.

RESULTS: The complete S mRNA could be detected by RT-PCR in HepG2 cells transfected with the pcDNA3.1(-)-complete S. The activity of β -gal in HepG2 cells transfected with the pcDNA3.1(-)-complete S was 6.9 times higher than that of control plasmid. The subtractive library of genes transactivated by HBV complete S protein was constructed successfully. The amplified library contains 86 positive clones. Colony PCR showed that 86 clones contained DNA inserts of 200-1 000 bp, respectively. Sequence analysis was performed in 35 clones randomly, and the full length sequences were obtained with bioinformatics method and searched for homologous DNA sequence from GenBank, altogether 33 coding sequences were obtained. These cDNA sequences might be target genes transactivated by complete S protein of HBV. Moreover, two unknown genes were discovered, full length coding sequences were obtained by bioinformatics techniques, one of them was named complete S transactivated protein 1 (CSTP1) and registered in GenBank (AY553877).

CONCLUSION: The complete S gene of HBV has a transactivating effect on SV40 early promoter. A subtractive cDNA library of genes transactivated by HBV complete S protein using SSH technique has been constructed successfully. The obtained sequences may be target genes transactivated by HBV complete S protein among which some genes coding proteins are involved in cell cycle regulation, metabolism, immunity, signal transduction, cell apoptosis and formation mechanism of hepatic carcinoma.

© 2005 The WJG Press and Elsevier Inc. All rights reserved.

Key words: Complete S protein; Transactivated genes; Hepatitis virus B

Bai GQ, Liu Y, Cheng J, Zhang SL, Yue YF, Huang YP, Zhang LY. Transactivating effect of complete S protein of hepatitis B virus and cloning of genes transactivated by complete S protein using suppression subtractive hybridization technique. *World J Gastroenterol* 2005; 11(25): 3893-3898
<http://www.wjgnet.com/1007-9327/11/3893.asp>

INTRODUCTION

Hepatitis B virus (HBV) genome is defined as four open

read frames (ORFs), which are named as the regions of S, C, P, X, respectively. The region of S is divided into the sub-regions of pre-S1, pre-S2 and S according to different initial code ATG in frame. Dong *et al.*^[1] have shown that there is ORF before pre-S1 region in the genome of HBV from serum of patients with long and accurate polymerase chain reaction (LA-PCR). This region is 135 bp, which is named temporarily as pre-pre-S and its promoter activities are confirmed in 277 bp upstream nucleotide sequences before pre-S1 gene^[2]. Pre-pre-S, pre-S1, pre-S2 and S genes are translated in frame according to the same ORF. It is well-known that HBV causes acute and chronic infections of the liver, especially chronic infections may result in remarkable consequences^[3]. HBV is considered to be a major etiological factor in the development of human hepatocellular carcinoma (HCC)^[4-9]. Although the precise role of HBV in the etiology of HCC is not well understood, data have shown that some HBV proteins can exert a significant transactivating activity on both viral and cellular promoter^[10]. This mechanism may have a close relation with the formation of HCC.

Suppression subtractive hybridization (SSH) is a widely used new technique in the cloning of genes transactivated by viral proteins^[11]. Complete S of HBV includes pre-pre-S, pre-S1, pre-S2 and S regions, complete S protein functions as a transcriptional transactivator. In the present study, we have successfully constructed the subtractive library of genes transactivated by HBV complete S protein.

MATERIALS AND METHODS

Construction and identification of expression vector

The complete S gene was prepared by PCR amplification using plasmid G376 A7 (GenBank number: AF384371) as template^[1,12,13], sense (5'-GGA TCC ATG CAG TTA ATC ATT ACT TCC-3') and antisense (5'-GGT ACC AAT GTA TAC CCA AAG ACA AAA G-3') primers (Shanghai BioAsia Biotech Co., Ltd, China). As these primers contain *Bam*HI and *Kpn*I (Takara) recognition sites on their respective 5'-ends, the amplified 1 338 bp PCR fragment was subcloned into the *Bam*HI and *Kpn*I sites of pcDNA3.1(-) vector (Invitrogen Co., USA). The expression vector, pcDNA3.1 (-)-complete S which could directly express complete S fusion protein was obtained, then identified by PCR and digested by *Bam*HI/*Kpn*I.

Expression of pcDNA3.1 (-)-complete S in HepG2 cells

HepG2 cells were transiently transfected with pcDNA3.1 (-)-complete S. At the same time, empty vectors were also transfected into cells as controls. HepG2 cells were plated at a density of 1.5×10^6 in a 35 mm plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 mL/L heat-inactivated fetal bovine serum (FBS). After 24 h of growth to 40-50% confluence, the cells were transfected with plasmids using FuGENE6 transfection reagent following the manufacturer's protocol (Roche C, USA).

mRNA and cDNA isolation

The mRNA from HepG2 cells transfected with pcDNA3.1

(-)-complete S and pcDNA3.1(-) empty vector was isolated using micro mRNA purification kit (Amersham Biosciences, Co., USA). cDNAs were reverse-transcribed from total RNA. Identification was done by PCR with complete S sequence-specific primers.

Cotransfection with reporter vector pSV-lacZ

HepG2 cells were transfected with various concentrations of plasmid pSV-lacZ (0.1-1.8 μ g) (Promega Co., USA). Expression of β -gal was detected by a β -gal assay kit (Promega, Co., USA). In pSV-LacZ the LacZ gene was under the control of the SV40 early promoter element. The optimal concentration of pSV-lacZ plasmid DNA was selected, HepG2 cells was cotransfected with pSV-lacZ and pcDNA3.1 (-)-complete S (2.0 μ g). At the same time, cotransfected HepG2 cells transfected with empty pcDNA3.1(-) (2.0 μ g) and pSV-lacZ were used as controls. After 48 h, cells were collected and detected for the expression of β -gal.

Generation of subtractive cDNA library

Gene expression comparisons by suppression subtractive hybridization according to the manufacturer's instructions of PCR-selectTM cDNA subtraction kit (Clontech Co., USA). In brief, 2 μ g of mRNA from the tester and the driver was subjected to cDNA synthesis. Tester and driver cDNAs were digested with *Rsa*I. 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 cDNA was added to each sample of tester. The samples were heat denatured and allowed to anneal. 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, two primary hybridization samples were mixed without denaturation. Only the remaining equalized and subtracted single-stranded tester cDNAs could re-associate 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.

PCR analysis of subtraction efficiency

PCR was performed on un-subtracted and subtracted secondary PCR products with the G3PDH 5'- and 3'-primers, respectively. From each reaction, 5 μ L sample was removed, and the rest of the reaction was put back into the thermal cyclor for five more cycles. The above step was repeated thrice, and then 5 μ L sample (i.e., the aliquots were removed from each reaction after 18, 23, 28, and 33 cycles) was examined on 2.0% agarose gel.

Cloning subtractive library into pGEM-teasy vector

The amplified products containing a subtractive cDNA library (6 μ L) were cloned into a pGEM-teasy vector (Promega Co., USA). Subsequently, Plasmid DNA was transformed into *E. coli* strain DH5 α . Bacteria were taken up in 800 μ L of LB medium and incubated for 45 min at 37 $^{\circ}$ C. Bacteria were plated onto agar plates containing

ampicillin (100 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. Positive (white) colonies were picked out and identified by PCR. Primers were T7/SP6 primer of pGEM-teasy vector. After the positive colonies were sequenced (Shanghai BioAsia Biotechnology Co., Ltd, China), nucleic acid sequence 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(-)-complete S plasmid cleaved with *Bam*HI and *Kpn*I yielded two bands: 5 396 bp empty pcDNA3.1(-) and 1 338 bp HBV complete S, and only one 6 734 bp band (5 396 bp+1 338 bp), it was cleaved with *Kpn*I. The plasmid by PCR amplification with complete S-specific primers got a clear band with the expected size (1 338 bp). The sequence of the PCR product was correct (Figure 1).

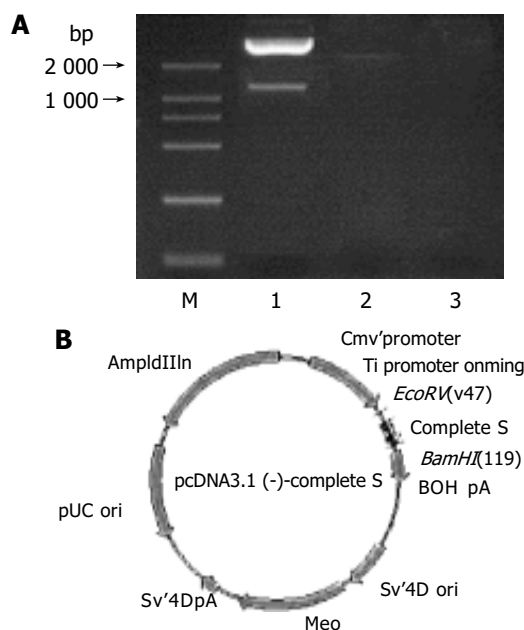


Figure 1 Electrophoresis of PCR products of pcDNA3-complete S and cleaved restriction enzyme (A). Structure of expression vector pcDNA3.1(-)-complete S plasmid(B).

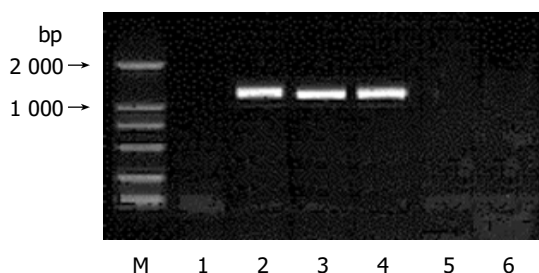


Figure 2 Electrophoresis of RT-PCR products in 0.9% agarose gel. Lane 1: negative control; lanes 2-4: mRNA isolated from pcDNA3.1(-)-complete S; lane 5: blank control; M: DNA marker (2 000 bp).

Identification of HBV complete S transient expression

Reverse-transcription by three different Oligo dT, identification of cDNA by PCR yielded a common 1.338 bp band (Figure 2).

The stable expression of pcDNA3.1(-)-complete S in HepG2 cells was also confirmed by Western blotting hybridization at a high level.

Result of cotransfection of pSV-lacZ and pcDNA3.1(-)-complete S

The best concentration was at 1.0 µg of pSV-lacZ. When cotransfected with pcDNA3.1(-)-complete S and pSV-lacZ, the β -gal data about the expression of β -gal were 0.228. In contrast, the expression of β -gal cotransfected with empty pcDNA3.1(-) and pSV-lacZ was 0.033. Expression of β -gal was 6.9-fold higher when cotransfected with pcDNA3.1(-)-complete S and pSV-lacZ than when cotransfected with empty pcDNA3.1(-) and pSV-lacZ. The significant increase of expression of β -gal was attributed to the transactivating effect of HBV complete S protein on early promoter of SV40, thus increasing the expression of downstream gene lacZ (Figure 3).

Result of PCR analysis of subtraction efficiency

The G3PDH (a housekeeping gene) primers were used to confirm the reduced relative abundance of G3PDH following the PCR selection procedure. The result displayed that the G3PDH abundance of subtracted secondary PCR products significantly decreased compared to the un-subtracted one, indicating that the subtractive library had a high subtraction efficiency (Figure 4).

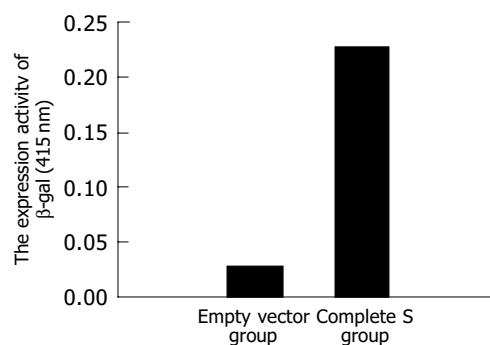


Figure 3 Result of β -galactosidase enzyme analysis.

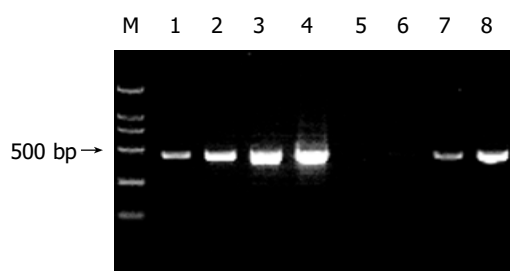


Figure 4 Reduction of G3PDH abundance by PCR-selection subtraction. Lanes 1 and 5: 18 cycles; lanes 2 and 6: 23 cycles; lanes 3 and 7: 28 cycles; lanes 4 and 8: 33 cycles. Lane M: marker (2 000 bp).

Analysis of subtractive library

Using suppression subtractive hybridization technique, we obtained a total of 86 positive clones. These clones were prescreened by PCR amplification to ensure that only clones with different inserts were subjected to sequencing analysis (Figure 5). Eighty-six clones contained 200-1 000 bp inserts. A total of 35 clones from cDNA library were randomly selected and sequenced, and 33 coding sequences were obtained. The data are presented in Table 1. The smear difference in subtractive library was displayed after electrophoresis of PCR products on 2% agarose gel (Figure 6).

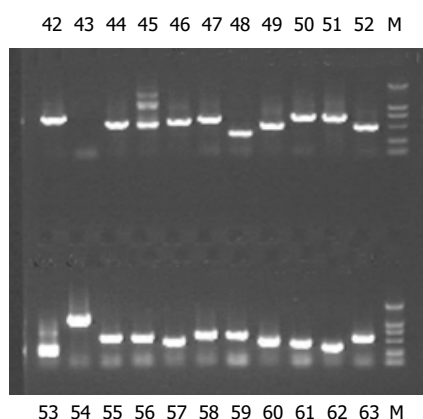


Figure 5 Electrophoresis of PCR products of part clones (42-63) on 0.9% agarose gel; M: marker (2 000 bp).

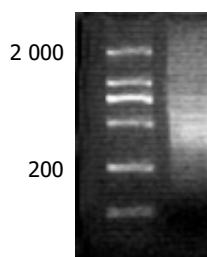


Figure 6 Smears of HBV complete S after PCR.

DISCUSSION

The open reading frame of HBV complete S gene consists of four coding regions: pre-pre-S, pre-S1, pre-S2 and S, each starting with an ATG codon in frame. Through in frame translational initiation at each of the four ATG codons, complete S (pre-pre-S + pre-S1 + pre-S2 + S), large (LHBs; pre-S1 + pre-S2 + S), middle (MHBs; pre-S2 + S) and small (SHBs; S) envelope glycoproteins can be synthesized^[14-18]. 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 Labs^[19,20]. Some studies indicate the biological significance of the pre-S2 transactivators^[21]. But we firstly discussed the transactivator function of HBV complete S protein in the present study.

We cotransfected HepG2 cells with pcDNA3.1(-)-complete S and pSV-lacZ and demonstrated that the HBV

Table 1 Comparison between positive clones and GenBank homology sequences

High similarity between proteins and known genes	Number of similar clones
<i>Homo sapiens</i> amino acid transporter system A2 (ATA2)	2
<i>Homo sapiens</i> heat shock 90 ku protein 1, (HSPCA)	4
<i>Homo sapiens</i> fibrinogen	1
<i>Homo sapiens</i> CDK4	5
<i>Homo sapiens</i> ribosomal protein	1
<i>Homo sapiens</i> translational initiation factor	1
<i>Homo sapiens</i> synaptophysin-like protein	1
Human mRNA for cytosolic malate dehydrogenase	1
<i>Homo sapiens</i> cytochrome c oxidase subunit I	2
<i>Homo sapiens</i> adenylate kinase 2 (AK2)	1
<i>Homo sapiens</i> NADH dehydrogenase	1
Human complement component C3 mRNA	1
<i>Homo sapiens</i> insulin-like growth factor binding protein 1	1
<i>Homo sapiens</i> SMT3 suppressor of mif two 3 homolog 2	1
<i>Homo sapiens</i> succinate dehydrogenase complex	1
<i>Homo sapiens</i> apolipoprotein H (beta-2-glycoprotein I) (APOH)	1
<i>Homo sapiens</i> BRCA2 and Cip1/p21 interacting protein splice variant	1
<i>Homo sapiens</i> Sec23 homolog A	1
<i>Homo sapiens</i> glutamate dehydrogenase 1	1
<i>Homo sapiens</i> proteasome (prosome, macropain) subunit	2
<i>Homo sapiens</i> eukaryotic translation elongation factor 1	1
<i>Homo sapiens</i> polymerase (RNA) I polypeptide D	2

complete S protein was successfully expressed in transfected HepG2 cells. Expression of β -gal was 6.9-fold higher when cotransfected with pcDNA3.1(-)-complete S and pSV-lacZ than when cotransfected with empty pcDNA3.1(-) and pSV-lacZ. HBV complete S had significant transactivating effect on early promoter of SV40 virus, thus increasing the expression of downstream gene lacZ. This result indicates that the HBV complete S protein expressed in HepG2 cells retains its biological activity in terms of transcriptional activation.

To get insight into the transactivation mechanism of HBV complete S protein, SSH was used for identification of transactivating target genes of complete S protein, and subtractive library was set up successfully. Sequence analysis was performed for 35 clones, and 33 coding sequences were obtained. These genes can be divided into four groups:

(1) The genes involved in cell structure and cell cycle that possess the important ability to control cell growth, differentiation and adherence, such as ribosomal protein, mitochondrion, eukaryotic translation elongation factor.

(2) The genes related to cell energy or substance metabolism (i.e., NADH dehydrogenase 2, cytochrome C oxidase II, etc.).

(3) The genes involved in the mechanism underlying the development of hepatocellular carcinoma. Cyclin-dependent kinase 4 (CDK4) is a key regulator of cell cycle. It has been shown that a variety of cell cycle-related proteins play an important role in the process of hepatocarcinogenesis. CDK4 is related to the regulation of the cyclin G1 phase, and significantly elevates in HCC compared to surrounding cirrhotic tissues by Western blot and *in vitro* kinase assays. The enhanced cyclin D1-related kinase activity in HCC was accompanied with the up-regulation of CDK4 activity. In addition, the protein levels and kinase activities of CDK4 are higher in poorly differentiated and advanced HCC. In

conclusion, the increases of cyclin D1 and CDK4 play an important role in the development of HCC. CDK4 activation may be closely related to the histopathologic grade and progression of HCC^[22-24]. But CDK4 does not increase in children with hepatic carcinoma^[25]. Amino acid transporter system A2 (ATA2) is responsible for Na⁺-independent amino acid transporter system. When amino acid is starved, the expression of ATA2 increases, suggesting that this expression is directly related to the activity of amino acid transporter^[26]. Its mRNA increases in the biopsy of hepatocirrhosis and hepatic carcinoma^[27], especially in chorion after hepatectomy, suggesting that ATA2 is closely associated with hepatic regeneration^[26,28]. ATA2 is cloned in hepatic carcinoma HepG2, and increases in patients of hepatic carcinoma^[29].

(4) The genes controlling hepatic cell infection and apoptosis, such as adenylate kinase 2(AK2) protein. AK2 gene located at the right arm of the second chromosome may play a role in maintaining the levels of ADP/ATP by releasing two-molecule ADP from ATP to AMP^[30]. The release of two mitochondrial proteins, cytochrome C and apoptosis-inducing factor (AIF), into the soluble cytoplasm of cells undergoing apoptosis has been well established. Since only AK2 intermembrane proteins release from mitochondria during the early phase of the apoptotic process, AK2 is very important in cell apoptosis. AK2 and cytochrome C are translocated in cytoplasm gelatum in apoptosis model^[31]. Therefore AK2 plays a role in inducing cell apoptosis. Apolipoprotein H (APOH), is named as beta-2-glycoprotein I and anticoagulative serum protein. The polymorphous APOH gene is closely related to fat metabolism, coagulation and hypertension^[32]. It is the pre-S of HBsAg that results in HBsAg combination with APOH. Resecting phosphatide and oxygenated phosphatide could disturb this mutual action, suggesting that this action is involved in the ectoblastic phosphatide^[33]. Furthermore, it is restrained from rebuilding HBsAg, anti-HBsAg and APOH. APOH may be the vector of HBV and plays a role in HBV infection. APOH chiefly combines with complete Dane HBV particle with telescope, and the activity of APOH-HBsAg is the highest in patients with active duplication^[33]. This combination facilitates virus particle entrancing hepatic cells, and plays an important role in the beginning of HBV infection^[34].

We testified the transactivator ability of HBV complete S protein, and constructed the subtractive cDNA library of genes transactivated by complete S protein. These genes are closely correlated with carbohydrate metabolism, immunoregulation, occurrence and development of tumor. How these genes affect occurrence and development of chronic hepatitis B, hepatic fibrosis and hepatocarcinoma needs to be further studied.

REFERENCES

- 1 **Dong J**, Cheng J. Study on definition of pre-per-s region in hepatitis B virus genome. *Shijie Huaren Xiaohua Zazhi* 2003; **8**: 1091-1096
- 2 **Yang Q**, Dong J, Cheng J, Liu Y, Hong Y, Wang JJ, Zhuang SL. Definition of pre-pre-s promoter sequence from hepatitis B virus genome and identification of its transcription activity. *Jiefangjun Yixue Zazhi* 2003; **9**: 761-762
- 3 **Beasley RP**. Hepatitis B virus. The major etiology of hepatocellular carcinoma. *Cancer* 1988; **61**: 1942-1956
- 4 **Dong J**, Cheng J, Wang Q, Wang G, Shi S, Liu Y, Xia X, Li L, Zhang G, Si C. Quasispecies and variations of hepatitis B virus: core promoter region as an example. *Zhonghua Shiyang He Linchuang Bingduxue Zazhi* 2002; **16**: 264-266
- 5 **Dong J**, Cheng J, Wang Q, Shi S, Wang G, Si C. Cloning and analysis of the genomic DNA sequence of augmenter of liver regeneration from rat. *Chin Med Sci J* 2002; **17**: 63-67
- 6 **Deng H**, Dong J, Cheng J, Huangfu KJ, Shi SS, Hong Y, Ren XM, Li L. Quasispecies groups in the core promoter region of hepatitis B virus. *Hepatobiliary Pancreat Dis Int* 2002; **1**: 392-396
- 7 **Parkin DM**, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* 1999; **80**: 827-841
- 8 **Dong J**, Cheng J, Wang Q, Huangfu J, Shi S, Zhang G, Hong Y, Li L, Si C. The study on heterogeneity of hepatitis B virus DNA. *Zhonghua Yixue Zazhi* 2002; **82**: 81-85
- 9 **Xia X**, Cheng J, Yang J, Zhong Y, Wang G, Fang H, Liu Y, Li K, Dong J. Construction and expression of humanized anti-HBsAg scFv targeting interferon-alpha in *Escherichia coli*. *Zhonghua Ganzangbing Zazhi* 2002; **10**: 28-30
- 10 **Seeger C**, Mason WS. Hepatitis B virus biology. *Microbiol Mol Biol Rev* 2000; **64**: 51-68
- 11 **Kuang WW**, Thompson DA, Hoch RV, Weigel RJ. Differential screening and suppression subtractive hybridization identified genes differentially expressed in an estrogen receptor-positive breast carcinoma cell line. *Nucleic Acid Res* 1998; **26**: 1116-1123
- 12 **Liu Y**, Cheng J, Shao DZ, Wang L, Zhong YW, Dong J, Li K, Li L. Synergetic transactivating effect of HCV core and HBV X proteins on SV40 early promoter/enhancer. *Zhonghua Shiyang He Linchuang Bingduxue Zazhi* 2003; **17**: 70-72
- 13 **Huangfu J**, Dong J, Deng H, Cheng J, Shi S, Hong Y, Ren X, Li L. A preliminary study on the heterogeneity of preS2 region in hepatitis B virus. *Zhonghua Neike Zazhi* 2002; **41**: 233-236
- 14 **Borchani-Chabchoub I**, Mokdad-Gargouri R, Gargouri A. Glucose dependent [correction of dependant] negative translational control of the heterologous expression of the pre-S2 HBV antigen in yeast. *Gene* 2003; **311**: 165-170
- 15 **Soussan P**, Pol S, Garreau F, Brechot C, Kremsdorf D. Vaccination of chronic hepatitis B virus carriers with pre-S2/S envelope protein is not associated with the emergence of envelope escape mutants. *J Gen Virol* 2001; **82**: 367-371
- 16 **Park JH**, Lee MK, Kim HS, Kim KL, Cho EW. Targeted destruction of the polymerized human serum albumin binding site within the preS2 region of the HBV surface antigen while retaining full immunogenicity for this epitope. *J Viral Hepat* 2003; **10**: 70-79
- 17 **Borchani-Chabchoub I**, Gargouri A, Mokdad-Gargouri R. Genotyping of Tunisian hepatitis B virus isolates based on the sequencing of pre-S2 and S regions. *Microbes Infect* 2000; **2**: 607-612
- 18 **Tai PC**, Suk FM, Gerlich WH, Neurath AR, Shih C. Hypermodification and immune escape of an internally deleted middle-envelope (M) protein of frequent and predominant hepatitis B virus variants. *Virology* 2002; **292**: 44-58
- 19 **Hildt E**, Urban S, Hofschneider PH. Characterization of essential domains for the functionality of the MHBst transcriptional activator and identification of a minimal MHBst activator. *Oncogene* 1995; **11**: 2055-2066
- 20 **Bruss V**, Lu X, Thomssen R, Gerlich WH. Post-translational alterations in transmembrane topology of the hepatitis B virus large envelope protein. *EMBO J* 1994; **13**: 2273-2279
- 21 **Hildt E**, Munz B, Saher G, Reifenberg K, Hofschneider PH. The Pre-S2 activator MHBs (t) of hepatitis B virus activates c-raf-1/Erk2 signaling in transgenic mice. *EMBO J* 2002; **21**: 525-535
- 22 **Kita Y**, Masaki T, Funakoshi F, Yoshida S, Tanaka M, Kurokohchi K, Uchida N, Watanabe S, Matsumoto K,

- Kuriyama S. Expression of G1 phase-related cell cycle molecules in naturally developing hepatocellular carcinoma of Long-Evans Cinnamon rats. *Int J Oncol* 2004; **24**: 1205-1211
- 23 **Edamoto Y**, Hara A, Biernat W, Terracciano L, Cathomas G, Riehle HM, Matsuda M, Fujii H, Scoazec JY, Ohgaki H. Alterations of RB1, p53 and Wnt pathways in hepatocellular carcinomas associated with hepatitis C, hepatitis B and alcoholic liver cirrhosis. *J Cancer* 2003; **106**: 334-341
- 24 **Masaki T**, Shiratori Y, Rengifo W, Igarashi K, Yamagata M, Kurokohchi K, Uchida N, Miyauchi Y, Yoshiji H, Watanabe S, Omata M, Kuriyama S. Cyclins and cyclin-dependent kinases: comparative study of hepatocellular carcinoma versus cirrhosis. *Hepatology* 2003; **37**: 534-543
- 25 **Kim H**, Lee MJ, Kim MR, Chung IP, Kim YM, Lee JY, Jang JJ. Expression of cyclin D1, cyclin E, cdk4 and loss of heterozygosity of 8p, 13q, 17p in hepatocellular carcinoma: comparison study of childhood and adult hepatocellular carcinoma. *Liver* 2000; **20**: 173-178
- 26 **Freeman TL**, Mailliard ME. Posttranscriptional regulation of ATA2 transport during liver regeneration. *Biochem Biophys Res Commun* 2000; **278**: 729-732
- 27 **Bode BP**, Fuchs BC, Hurley BP, Conroy JL, Suetterlin JE, Tanabe KK, Rhoads DB, Abcouwer SF, Souba WW. Molecular and functional analysis of glutamine uptake in human hepatoma and liver-derived cells. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G1062-1067
- 28 **Freeman TL**, Thiele GM, Tuma DJ, Machu TK, Mailliard ME. ATA2-mediated amino acid uptake following partial hepatectomy is regulated by redistribution to the plasma membrane. *Arch Biochem Biophys* 2002; **400**: 215-222
- 29 **Hatanaka T**, Huang W, Wang H, Sugawara M, Prasad PD, Leibach FH, Ganapathy V. Primary structure, functional characteristics and tissue expression pattern of human ATA2, a subtype of amino acid transport system A. *Biochim Biophys Acta* 2000; **1467**: 1-6
- 30 **Villa H**, Perez-Pertejo Y, Garcia-Estrada C, Reguera RM, Requena JM, Tekwani BL, Balana-Fouce R, Ordonez D. Molecular and functional characterization of adenylate kinase 2 gene from *Leishmania donovani*. *Eur J Biochem* 2003; **270**: 4339-4347
- 31 **Kohler C**, Gahm A, Noma T, Nakazawa A, Orrenius S, Zhivotovsky B. Release of adenylate kinase 2 from the mitochondrial intermembrane space during apoptosis. *FEBS Lett* 1999; **447**: 10-12
- 32 **Xia J**, Yang QD, Yang QM, Xu HW, Liu YH, Zhang L, Zhou YH, Wu ZG, Cao GF. Apolipoprotein H gene polymorphisms and risk of primary cerebral hemorrhage in a Chinese population. *Cerebrovasc Dis* 2004; **17**: 197-203
- 33 **Stefas I**, Rucheton M, D'Angeac AD, Morel-Baccard C, Seigneurin JM, Zarski JP, Martin M, Cerutti M, Bossy JP, Misse D, Graafland H, Veas F. Hepatitis B virus Dane particles bind to human plasma apolipoprotein H. *Hepatology* 2001; **33**: 207-217
- 34 **Gao P**, Guo Y, Qu L, Shi T, Zhang H, Dong C, Yang H. Relation between Beta-2-glycoprotein I and hepatitis B virus surface antigen *Zhonghua Ganzangbing Zazhi* 2002; **10**: 31-33