

# Viral replication modulated by synthetic peptide derived from hepatitis B virus X protein

Chang-Zheng Song, Qing-Wei Wang, Chang-Cheng Song, Zeng-Liang Bai

**Chang-Zheng Song, Zeng-Liang Bai**, Laboratory of Immunobiology, College of Life Sciences, Shandong University, Jinan 250100, Shandong Province, China

**Chang-Zheng Song**, Shandong Research Center for Medical Biotechnology, Shandong Academy of Medical Sciences, Jinan 250062, Shandong Province, China

**Qing-Wei Wang**, Cancer Research Center, Qilu Hospital of Shandong University, Jinan 250012, Shandong Province, China

**Chang-Cheng Song**, Basic Research Laboratory, National Cancer Institute at Frederick, MD 21702, USA

**Correspondence to:** Dr. Chang-Zheng Song, Project of Viral Vaccine, Shandong Research Center for Medical Biotechnology, Shandong Academy of Medical Sciences, Jinan 250062, Shandong Province, China. songcz@life.sdu.edu.cn

**Telephone:** +86-531-2919607

**Received:** 2003-08-06 **Accepted:** 2003-09-24

## Abstract

**AIM:** A strategy for viral vaccine design is the use of conserved peptides to overcome the problem of sequence diversity. At present it is still unclear whether conserved peptide is safe as a candidate vaccine. We reported it here for the first time not only to highlight the biohazard issue and safety importance for viral peptide vaccine, but also to explore the effect of a fully conserved peptide on HBV replication within the carboxyl terminus of HBx.

**METHODS:** We synthesized the fully conserved peptide (CP) with nine residues, FVLGGCRHK. HBV-producing 2.2.15 cells were treated with or without 3.5  $\mu$ M CP for 36 hours. Quantitative detection of viral DNA was performed by real-time PCR. HBV antigens were determined by enzyme-linked immunosorbent assay (ELISA). Quantitative analyses of p53 and Bax proteins were based on immunofluorescence. Flow cytometry was performed to detect cell cycle and apoptosis.

**RESULTS:** Both extracellular and intracellular copies of HBV DNA per ml were significantly increased after incubation with 3.5  $\mu$ M of CP. HBsAg and HBeAg in the cultured medium of CP-treatment cells were as abundant as untreated control cells. CP influenced negatively the extracellular viral gene products, and 3.5  $\mu$ M CP could significantly inhibit intracellular HBsAg expression. In response to CP, intracellular HBeAg displayed an opposite pattern to that of HBsAg, and 3.5  $\mu$ M CP could efficiently increase the level of intracellular HBeAg. Flow cytometric analyses exhibited no significant changes on cell cycle, apoptosis, p53 and Bax proteins in 2.2.15 cells with or without CP.

**CONCLUSION:** Together with the results generated from the synthetic peptide, we address that the conserved region, a domain of HBx, may be responsible for modulating HBV replication. As conserved peptides from infectious microbes are used as immunogens to elicit immune responses, their latent biological hazard for human beings should be evaluated.

Song CZ, Wang QW, Song CC, Bai ZL. Viral replication modulated by synthetic peptide derived from hepatitis B virus X protein. *World J Gastroenterol* 2004; 10(3):389-392

<http://www.wjgnet.com/1007-9327/10/389.asp>

## INTRODUCTION

Human hepatitis B virus (HBV) is known as an important cause of acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma<sup>[1]</sup>. Although the incidence of new infections has decreased after the introduction of vaccination programmes, HBV infection remains an important global health problem, with the number of chronic HBV carriers exceeding 350 million worldwide<sup>[2]</sup>. HBV is a small DNA virus belonging to the hepadnavirus family that includes hepatitis viruses of woodchuck, ground squirrel, Peking duck, *etc.* These viral species all contain a highly conserved small open reading frame (ORF) encoding HBV x protein (HBx). HBx is a multifunctional viral regulator that modulates transcription, signaling pathways, protein degradation, and cell responses to genotoxic stress. These modulations affect viral replication and viral proliferation, directly or indirectly. HBx also affects cell cycle checkpoints, cell death, and carcinogenesis<sup>[3]</sup>.

HBx is well conserved among the mammalian hepadnaviruses<sup>[4,5]</sup>. Regarding the viral life cycle, in related woodchuck hepatitis viruses, ablation of their X protein start codon or creation of C-terminal truncations has been found to decrease viral replication *in vitro* and to inhibit the establishment of productive infection *in vivo*<sup>[6,7]</sup>. It is not known which pathways influenced by HBx are needed or sufficient to establish an environment required for viral replication. HBx could not only form intracellular aggregates by itself<sup>[8-11]</sup>, but also involve clumping and organelle aggregation leading to an abnormal mitochondrial distribution<sup>[12,13]</sup>. The precise role of HBx in the HBV life cycle remains uncertain.

Several novel HBV therapeutic peptides containing immunodominant T helper (Th) and cytotoxic T lymphocyte responses (CTL) epitopes of HBV have been screened out. Epitope-based HBV vaccines were designed and synthesized<sup>[14]</sup>. Epitope peptides derived from HBx have been used in peptide vaccines to induce specific cellular immunity<sup>[15]</sup>. In order to overcome the problem of sequence diversity, a strategy for vaccine design was the use of conserved peptides<sup>[16]</sup>. The biological importance of HBx in HBV replication remains largely undefined. In transient transfection assays the full-length X-gene encoding a product in cells appeared to form intracellular aggregates and to accumulate in large granules, with a tendency of apoptosis<sup>[9]</sup>. There was a fully conserved nine amino acid sequence within the carboxyl terminus of all HBx. Deletion of this segment resulted in a drastic loss of transactivation activity of HBx<sup>[17]</sup>. To obtain a different approach to investigate the possible functions of the X gene in HBV replication, we recently performed experiments with synthetic peptide derived from HBx. The peptide could avoid forming aggregates and be employed to investigate the effect of HBx on HBV replication. We report it here for the first

time to highlight the biohazard issue and safety importance regarding the synthetic peptide used as a candidate vaccine.

## MATERIALS AND METHODS

### *In vitro synthesis of conserved peptide*

HBx sequences of hepadnaviruses used for analysis were taken from GenBank. Accession numbers were as follows: arctic ground squirrel hepatitis B virus, U29144; woodchuck hepatitis virus, M19183; woolly monkey hepatitis B Virus, NC\_001896; orangutan hepadnavirus, NC\_002168; hepatitis B virus subtype adr, D12980; and hepatitis B virus subtype adw, M54923. Sequence alignment was performed with the software provided by Vector NTI (USA). The fully conserved peptide of nine residues from HBx, FVLGGCRHK (Figure 1), was synthesized by the solid-phase method (GL Biochem Ltd.). Purity (>97%) was assessed by high-pressure liquid chromatography, amino acid analysis, and molecular weight determination by mass spectrometry.

|  |     |                            |
|--|-----|----------------------------|
| Arctic ground squirrel hepatitis B virus | 123 | SRLPL <u>FVLGGCRHK</u> YKM |
| Woodchuck hepatitis virus                | 123 | PRLSIF <u>FVLGGCRHK</u> CM |
| Woollymonkey hepatitis B virus           | 125 | PRLKV <u>FVLGGCRHK</u> LV  |
| Orangutan hepadnavirus                   | 127 | IRLKV <u>FVLGGCRHK</u> LV  |
| Hepatitis B virus subtype adr            | 127 | IRLKV <u>FVLGGCRHK</u> LV  |
| Hepatitis B virus subtype adw            | 127 | IRLKV <u>FVLGGCRHK</u> LV  |

**Figure 1** Alignment of amino acid sequences of HBx from six mammalian hepadnaviruses.

The numbers refer to the first amino acid of each peptide. Underlined region is a fully conserved sequence.

### *Cell culture treated with peptide*

2.2.15 cell line containing HBV ayw strain genome derived from a human hepatoblastoma HepG2 cells<sup>[18]</sup>, was grown and cultured in Dulbecco's modified Eagle's medium (Gibco Life Technologies) supplemented with 10% heat-inactivated fetal calf serum. 2.2.15 cells were seeded into 24-well tissue culture plates. Once cell culture dishes were subconfluent ( $\sim 5 \times 10^6$  cells), the culture medium was replaced by the same amount of fresh media harboring CP. Cells were incubated with 3.5  $\mu$ M CP for 36 hours, the culture medium was collected for the analysis of extracellular HBV DNA and HBV antigens. The cells were washed and detached from the dishes. The collected cells were lysed. After centrifugation, the supernatant was stored for the analysis of intracellular HBV genomic forms and HBV antigens. The protein concentration was estimated by Bradford method.

### *Real-time PCR assay*

Fluorescence quantitation of HBV DNA was performed with real-time PCR reagent kit (Shenzhen PG Biotech, China). The primers and probe were selected in the S gene of HBV genome and generated a product of 70 bp. HBV DNA was extracted from 100  $\mu$ l of the sample. Real time PCR was done using 2  $\mu$ l of HBV DNA, 0.06  $\mu$ l of UNG (1 u/ $\mu$ l), 0.4  $\mu$ l Taq (5 u/ $\mu$ l) and 37.6  $\mu$ l PCR mix. After incubation for 5 min at 37 °C, the DNA polymerase was activated at 94 °C for 1 min. The PCR cycling program consisted of 42 two-step cycles of 5 s at 95 °C and 30 s at 60 °C. Viral DNA was extracted and purified from 100  $\mu$ l of the sample. Amplification and detection were performed with a Line-gene real-time PCR analysis system (Japan). Three independent experiments were performed.

### *HBsAg and HBeAg assay*

Hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were determined using licensed ELISA kits (Wantai

Biotech Inc., Beijing) following the manufacturer's package insert procedure.

### *Flow cytometric analysis*

2.2.15 cells treated with or without 3.5  $\mu$ M CP for 36 hours were released by trypsinization and resuspended in phosphate-buffered saline at a density of  $2 \times 10^6$  cells/ml. Quantitative analyses of p53 and Bax proteins were based on immunofluorescence of cells stained with fluorescein isothiocyanate-conjugated monoclonal antibodies (PharMingen, USA) respectively. Cell cycle and apoptosis were monitored using propidium iodide staining of nuclei. Flow cytometry was performed and analyzed on a Becton Dickinson flow cytometer using CellQuest software.

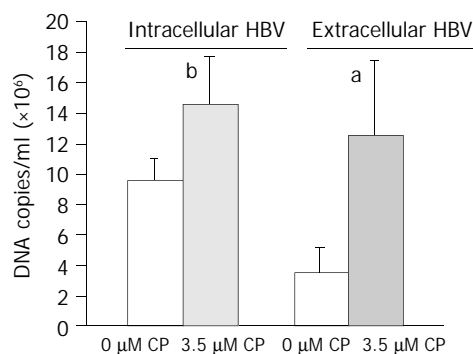
### *Statistical analysis*

Statistical analysis was performed by Student's *t* test. The data were analyzed with SigmaPlot 2000 software (SPSS Inc., USA). Differences were considered statistically significant when *P* value was less than 0.05.

## RESULTS

### *Enhancement of HBV DNA replication after CP treatment of 2.2.15 cells*

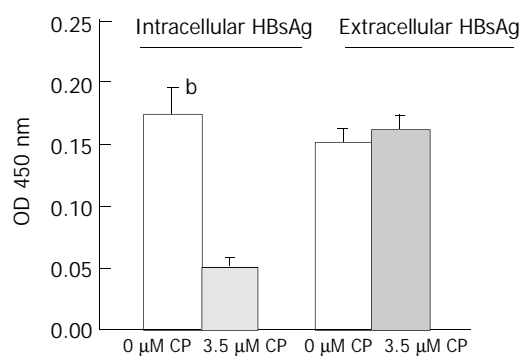
Parallel cultures, with or without CP, were maintained at 37 °C for 36 hours, and then investigated for the presence of viral DNA in cell lysates. HBV DNA was analyzed in a quantitative manner for the overall levels of HBV DNA (both extracellular and intracellular DNA) and the relative rate of HBV replication (intracellular DNA). HBV DNA was analyzed by real-time PCR. As shown in Figure 2, both extracellular and intracellular copies of HBV DNA per ml were significantly increased after incubation with 3.5  $\mu$ M of CP. We observed that HBV DNA replication of treated 2.2.15 cells was substantially enhanced by the conserved peptide.



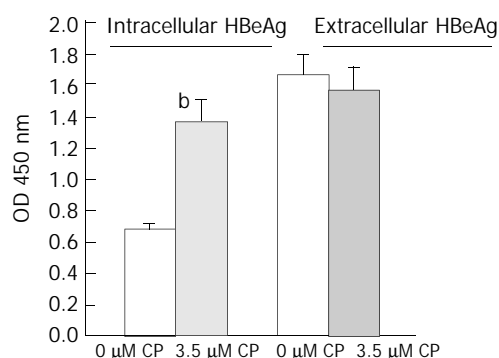
**Figure 2** Quantitative detection of HBV DNA by real-time PCR. HBV DNA levels were significantly increased by 3.5  $\mu$ M of CP (<sup>a</sup>*P*=0.005, <sup>b</sup>*P*=0.038).

### *Effects of CP on expression of HBV antigens*

Cell lysates and culture medium were analyzed for intracellular and extracellular viral gene products. ELISA results revealed that CP affected the expression of HBV antigens differently. As shown in Figure 3, there were no significant differences in extracellular HBsAg from 2.2.15 cells treated with or without CP, and 3.5  $\mu$ M CP could significantly inhibit intracellular HBsAg expression. Changes in HBeAg were evaluated as well. It was found that there was no statistically significant difference of extracellular HBeAg when the cells were treated with CP or without CP (Figure 4). In response to CP, the intracellular HBeAg displayed an opposite pattern to that of HBsAg. We observed that CP could efficiently increase the level of intracellular HBeAg (Figure 4).



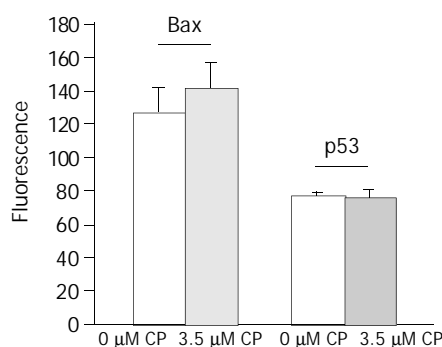
**Figure 3** ELISA detection of HBsAg. Three and a half μM CP could significantly inhibit intracellular HBsAg expression (<sup>b</sup> $P < 0.001$ ).



**Figure 4** ELISA detection of HBeAg. The level of intracellular HBeAg was significantly increased by 3.5 μM CP (<sup>b</sup> $P < 0.001$ ).

#### Flow cytometric analysis of virus-host cells treated with CP

When fluorescein isothiocyanate-conjugated monoclonal antibodies were used to assess p53 and Bax proteins, 2.2.15 cells treated with CP or without CP displayed no significant changes (Figure 5). As shown in Table 1, the results exhibited no significant changes of cell cycle and apoptosis in 3.5 μM CP treated cells compared with the cells not treated by CP.



**Figure 5** Flow cytometric analysis of Bax and p53 proteins.

**Table 1** Percentage of apoptosis and cell cycle phase

| HepG2 cells | Apoptosis | G0/G1      | G2-M       | S         |
|-------------|-----------|------------|------------|-----------|
| 0 μM CP     | 0.39±0.01 | 81.21±0.51 | 11.09±2.11 | 7.71±2.62 |
| 3.5 μM CP   | 0.62±0.27 | 79.87±1.22 | 10.24±0.99 | 9.89±0.30 |

## DISCUSSION

HBV is a 3.2 Kb DNA virus with only four genes having been identified namely C, S, P, and X<sup>[19]</sup>. The C gene codes for the core protein and the e antigen, the S gene codes for three related viral envelope proteins known as surface antigens, the P gene

codes for the viral DNA polymerase, and the X gene codes for a 16.5-kDa protein. HBV molecule is well organized. The X gene could overlap the viral polymerase gene and the precore gene<sup>[20,21]</sup>, thus increasing the utilization of the small genome. Uchida and colleagues analyzed nucleotide (nt) sequences of HBx from more than 130 clinical HBV isolates. They attempted to establish the correlation of nt substitutions with clinical pathological characteristics. They found the X gene (465nt) was crucial for the replication and expression of HBV because HBx could transactivate HBV DNA, and contained the core promoter, enhancer II, and two direct repeats. There were several mutational hotspots, some of which seemed to relate to the immunological epitopes of the X protein. One was an 8-nt deletion between nt 1 770 and 1 777, which could truncate 20 amino acids from the carboxyl terminus of HBx. This deletion could lead to the suppression of replication of HBV DNA<sup>[22]</sup>. There was a fully conserved nine amino acid sequence within the carboxyl terminus of all HBx. We synthesized the fully conserved peptide with nine residues, FVLGGCRHK, and queried for its physiological role in regulation of HBV replication.

HBx is regarded as an important multifunctional protein for the viral life cycle and viral-host interactions. HBx has been shown to be essential for viral proliferation in the woodchuck<sup>[6,7]</sup>, but was not central to HBV replication and virion export<sup>[23]</sup>. In the absence of a convenient animal model system for studying HBV replication, the transgenic mouse could provide an alternative choice. The transgenic mice HBx was not absolutely essential for HBV replication and virion secretion, but replication was significantly decreased in the absence of X protein<sup>[24]</sup>. Utilizing real time quantitative PCR, we found that CP could increase both the level of intracellular HBV DNA and the production of HBV DNA secretion.

HBsAg and HBeAg were translated from preS/S mRNA and precore mRNA individually. In this report, we demonstrated that CP could induce intracellular HBeAg expression and downregulate intracellular HBsAg expression. The results agreed closely with a recent observation of Xu *et al.*, who found that the presence of HBx increased the level of C gene transcripts approximately three folds in mtTg04 mice. HBx had no apparent effect on the RNA level of the S gene in this mouse line<sup>[25]</sup>. Our result was also supported by the findings of Reifenberg's research group<sup>[26]</sup>. They found that the X gene provided in trans could stimulate the expression of C gene when they studied transgenic mice carrying subgenomic HBV DNA. Our data suggest that HBsAg and HBeAg in cultured medium of CP-treated cells were as abundant as untreated control cells. This might be due to the basal level released from 2.2.15 cells before incubated with CP. CP had a negative influence on extracellular viral gene products. Considering the possible influence of CP on the status of host cells might alter the biologic properties of HBV, we performed analysis of cell cycle and apoptosis. Our results showed no significant cellular changes by CP. Despite the association of multiple activities with HBx, none of them appeared to provide a uniform hypothesis regarding the true biological functions of HBx<sup>[27]</sup>. Taking advantage of the synthetic peptide, we are in an attempt to identify different roles of a domain in HBx in HBV replication.

Synthetic peptide derived HBx may be responsible for modulating HBV replication. HBx has been found to be a short-lived protein and proteolysed by a ubiquitin proteasome pathway<sup>[28,29]</sup>. We think that some short peptides converted from HBx through intracellular degradation process may still remain their basic functions for HBV replication.

## REFERENCES

- 1 Lee YH, Yun Y. HBx protein of hepatitis B virus activates Jak1-STAT signaling. *J Biol Chem* 1998; **273**: 25510-25515

- 2 **Lee WM.** Hepatitis B virus infection. *N Engl J Med* 1997; **337**: 1733-1745
- 3 **Murakami S.** Hepatitis B virus X protein: a multifunctional viral regulator. *J Gastroenterol* 2001; **36**: 651-660
- 4 **Seeger C, Mason WS.** Hepatitis B virus biology. *Microbiol Mol Biol Rev* 2000; **64**: 51-68
- 5 **Murakami S.** Hepatitis B virus X protein: structure, function and biology. *Intervirology* 1999; **42**: 81-99
- 6 **Zoulim F, Saputelli J, Seeger C.** Woodchuck hepatitis virus X protein is required for viral infection *in vivo*. *J Virol* 1994; **68**: 2026-2030
- 7 **Chen HS, Kaneko S, Girones R, Anderson RW, Hornbuckle WE, Tennant BC, Cote PJ, Gerin JL, Purcell RH, Miller RH.** The woodchuck hepatitis X gene is important for establishment of virus infection in woodchucks. *J Virol* 1993; **67**: 1218-1226
- 8 **Henkler F, Hoare J, Waseem N, Goldin RD, McGarvey MJ, Koshy R, King IA.** Intracellular localization of the hepatitis B virus HBx protein. *J Gen Virol* 2001; **82**(Pt 4): 871-882
- 9 **Song CZ, Bai ZL, Song CC, Wang QW.** Aggregate formation of hepatitis B virus X protein affects cell cycle and apoptosis. *World J Gastroenterol* 2003; **9**: 1521-1524
- 10 **Pal J, Somogyi C, Szmolenszky AA, Szekeres G, Sipos J, Hegedus G, Martzinovits I, Molnar J, Nemeth P.** Immunohistochemical assessment and prognostic value of hepatitis B virus X protein in chronic hepatitis and primary hepatocellular carcinomas using anti-HBxAg monoclonal antibody. *Pathol Oncol Res* 2001; **7**: 178-184
- 11 **Urban S, Hildt E, Eckerskorn C, Sirma H, Kekule A, Hofschneider PH.** Isolation and molecular characterization of hepatitis B virus X-protein from a baculovirus expression system. *Hepatology* 1997; **26**: 1045-1053
- 12 **Rahmani Z, Huh KW, Lasher R, Siddiqui A.** Hepatitis B virus X protein colocalizes to mitochondria with a human voltage-dependent anion channel, HVDAC3, and alters its transmembrane potential. *J Virol* 2000; **74**: 2840-2846
- 13 **Takada S, Shirakata Y, Kaneniwa N, Koike K.** Association of hepatitis B virus X protein with mitochondria causes mitochondrial aggregation at the nuclear periphery, leading to cell death. *Oncogene* 1999; **18**: 6965-6973
- 14 **Guan XJ, Wu YZ, Jia ZC, Shi TD, Tang Y.** Construction and characterization of an experimental ISCOMS-based hepatitis B polypeptide vaccine. *World J Gastroenterol* 2002; **8**: 294-297
- 15 **Hwang YK, Kim NK, Park JM, Lee K, Han WK, Kim HI, Cheong HS.** HLA-A2.1 restricted peptides from the HBx antigen induce specific CTL responses *in vitro* and *in vivo*. *Vaccine* 2002; **20**: 3770-3777
- 16 **Nakamura Y, Kameoka M, Tobiume M, Kaya M, Ohki K, Yamada T, Ikuta K.** A chain section containing epitopes for cytotoxic T, B and helper T cells within a highly conserved region found in the human immunodeficiency virus type 1 Gag protein. *Vaccine* 1997; **15**: 489-496
- 17 **Kumar V, Jayasuryan N, Kumar R.** A truncated mutant (residues 58-140) of the hepatitis B virus X protein retains transactivation function. *Proc Natl Acad Sci U S A* 1996; **93**: 5647-5652
- 18 **Sells MA, Chen ML, Acs G.** Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proc Natl Acad Sci U S A* 1987; **84**: 1005-1009
- 19 **Lee JH, Ku JL, Park YJ, Lee KU, Kim WH, Park JG.** Establishment and characterization of four human hepatocellular carcinoma cell lines containing hepatitis B virus DNA. *World J Gastroenterol* 1999; **5**: 289-295
- 20 **Okamoto H, Tsuda F, Akahane Y, Sugai Y, Yoshida M, Moriyama K, Tanaka T, Miyakawa Y, Mayumi M.** Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. *J Virol* 1994; **68**: 8102-8110
- 21 **Yuh CH, Chang YL, Ting LP.** Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus. *J Virol* 1992; **66**: 4073-4084
- 22 **Uchida T, Saitoh T, Shinzawa H.** Mutations of the X region of hepatitis B virus and their clinical implications. *Pathol Int* 1997; **47**: 183-193
- 23 **Blum HE, Zhang ZS, Galun E, von Weizsacker F, Garner B, Liang TJ, Wands JR.** Hepatitis B virus X protein is not central to the viral life cycle *in vitro*. *J Virol* 1992; **66**: 1223-1227
- 24 **Reifenberg K, Nusser P, Lohler J, Spindler G, Kuhn C, von Weizsacker F, Kock J.** Virus replication and virion export in X-deficient hepatitis B virus transgenic mice. *J Gen Virol* 2002; **83**(Pt 5): 991-996
- 25 **Xu Z, Yen TS, Wu L, Madden CR, Tan W, Slagle BL, Ou JH.** Enhancement of hepatitis B virus replication by its X protein in transgenic mice. *J Virol* 2002; **76**: 2579-2584
- 26 **Reifenberg K, Wilts H, Lohler J, Nusser P, Hanano R, Guidotti LG, Chisari FV, Schlicht HJ.** The hepatitis B virus X protein transactivates viral core gene expression *in vivo*. *J Virol* 1999; **73**: 10399-10405
- 27 **Seeger C.** The hepatitis B virus X protein: the quest for a role in viral replication and pathogenesis. *Hepatology* 1997; **25**: 496-498
- 28 **Hu Z, Zhang Z, Doo E, Coux O, Goldberg AL, Liang TJ.** Hepatitis B virus X protein is both a substrate and a potential inhibitor of the proteasome complex. *J Virol* 1999; **73**: 7231-7240
- 29 **Kim JH, Kang S, Kim J, Ahn BY.** Hepatitis B virus core protein stimulates the proteasome-mediated degradation of viral X protein. *J Virol* 2003; **77**: 7166-7173

Edited by Wu XN and Wang XL