Functional Characterization of Interferon Regulation Element of Hepatitis B virus Genome In Vivo

Feng-Jun Liu · En-Qiang Chen · Qiao-Ling Zhou ·
Tao-You Zhou · Cong Liu · Li Liu ·
Xing Cheng · Hong Tang

Received: 25 March 2012 / Accepted: 6 July 2012 / Published online: 6 September 2012
© Indian Virological Society 2012

Abstract The roles of interferon regulatory element (IRE) in Hepatitis B virus (HBV) genome on inhibitory effect of interferon against HBV are controversial in vitro. This study aimed to determine the functional characterization of HBV-IRE sequence in vivo. Wild-type or IRE-mutant HBV replication-competent mice were firstly established, and mice were subsequently treated with polyinosinic-polytidylin acid (polyI.C) or phosphate-buffered saline via intraperitoneal. Results showed that PolyI.C inhibited viral replication, and increased the level of 2',5'-oligoadenylate synthase mRNA transcripts, a marker of $\text{INF-}\alpha/\beta$ induction. Between wild-type and IRE-mutant HBV replication-competent mice, the levels of HBV-RNA and HBV-DNA replication intermediates were similar. After PolyI.C treatment, the decreasing of HBV-RNA was similar between two groups, but HBV-DNA replication intermediates decreased significantly less in IRE-mutant than wild-type HBV replication-competent mice. These findings suggested that IRE mutant reduced the

inhibitory effect of interferon on HBV replication, which played a role in antiviral effect of interferon against HBV.

Keywords Interferon regulatory element · Hepatitis B virus · Transcription · Replication · Polyinosinic-polytidylin acid · Mice model

Introduction

Hepatitis B virus (HBV) infection is a worldwide health problem [17, 19]. Although a highly effective preventive vaccine is available [5], it does not help the estimated 350 million people who have already been infected chronically and are at higher risk of developing hepatic cirrhosis and hepatocellular carcinoma, the predominant causes of mortality due to HBV infection [3]. In recent years, several antiviral drugs have been developed for the treatment of chronic hepatitis B patients. Interferon, one of the approved antiviral agents against chronic HBV infection, can suppress HBV replication and improve the clinic outcome in patients with chronic HBV infection [8, 16, 33]. However, the detailed mechanisms responsible for these actions are not clearly understood.

HBV belongs to the family of hepadnaviruses, a group of small enveloped viruses with liver tropism. The HBV genome is a relaxed, circular, partially double-stranded 3.2-kb DNA molecule. HBV replicate by reverse transcription mechanism. The DNA genome of the virus is transcribed in the nucleus of the hepatocyte to generate the 3.5, 2.4, 2.1 and 0.7 kb HBV RNA which encode the HBV core(HBcAg and HBsAg) and polymerase polypeptide, the large surface antigen polypeptide, the middle and major surface antigen polypeptides(HBsAg), and the HBx polypeptide. The expression levels of those transcripts are regulated by the

Feng-Jun Liu, En-Qiang Chen contributed equally to this paper.

F.-J. Liu · E.-Q. Chen · Q.-L. Zhou · T.-Y. Zhou · C. Liu ·
L. Liu · X. Cheng · H. Tang (✉)
Center of Infectious Diseases, West China Hospital
of Sichuan University, No.37 Guo Xue Xiang, Chengdu 610041,
People's Republic of China
e-mail: htang6198@hotmail.com

F.-J. Liu · E.-Q. Chen · Q.-L. Zhou · T.-Y. Zhou · C. Liu ·
L. Liu · X. Cheng · H. Tang
Division of Infectious Diseases, State Key Laboratory
of Biotherapy, Sichuan University, Chengdu 610041,
People's Republic of China

F.-J. Liu
Department of Infectious Diseases, North Sichuan Medical
College, Nanchong 637000, People's Republic of China

enhancer II/core promoter, the large surface antigen promoter, the major surface antigen promoter and the enhancer I/X gene promoter, respectively. In addition to encoding the core polypeptide and the HBV DNA polymerase, the greater-than-genome 3.5-kb pregenomic RNA serves as the reverse transcription template to synthesize HBV DNA. Consequently, pregenomic 3.5-kb RNA is the precursor of viral DNA synthesis, and regulation of its transcription can influence directly HBV replication and synthesis of the virus.

Interferon binds to its receptor and activates the latent signal transducer and activator of transcription 1 and 2 (STAT1 and STAT2) and interferon regulatory factor (IRF)9/(ISGF3 γ), which form a heterotrimeric complex, the interferon-stimulated gene factor 3 (ISGF3). ISGF3 translocates into the nucleus where it binds to interferon-stimulated response element (ISRE) sequence in the promoter or enhancer of a variety of interferon-stimulated genes (ISG) and activates their expression. Interferon regulatory factors (IRFs) are some of target genes of ISGs. After being activating, IRFs could bind to interferon regulatory element (IRE) and regulate its function [15, 35, 36]. Therefore, INF- α/β can activate transcription of genes possessing ISRE/IRE sequence in their promoters or enhancers [6, 18, 27]. Previous analysis suggested that an ISRE/IRE sequence was present in the enhancer I/X gene promoter region of HBV genomic DNA [9, 25]. The presence of HBV-IRE suggested that interferon might directly regulate HBV transcription and, consequently, HBV replication [24]. Studies using reporter gene constructs have demonstrated that the HBV-IRE in enhancer I/X gene promoter was a functional regulatory site capable of mediating regulatory effect of IFN α , ISGF3, IRF1 and IRF7 on gene expression [1]. However, the roles of this HBV-IRE sequence in the inhibitory effect of interferon on HBV are somewhat controversial [26]. Therefore, it is necessary to further define the role of HBV-IRE in antiviral effect of interferon on HBV.

It has been previously demonstrated that the inhibitory effect of interferon on HBV replication in vitro HBV replication system is not obvious [1, 28], and therefore, most studies about the mechanism by which interferon regulate HBV replication have been performed in vivo by using HBV transgenic mice at present. However, the acquisition of transgenic mice is very time-consuming and laborious. We and others have reported previously that a mouse model with HBV replication was developed by using hydrodynamic-based procedure [21, 34] and this model was rapid, convenient and economical, in particular, allow the application of mutagenesis to the functional study of the HBV genomic region.

Based on these facts, we attempted in the current study to define the role of the HBV-IRE sequence in antiviral effect of interferon against HBV in vivo using the hydrodynamic-based mouse model and the IRE-mutated HBV DNA construct.

Materials and Methods

Plasmids

Plasmid pHBV4.1 containing 1.3 copies of HBV genome has been described previously [21, 30]. The plasmid HBV4.1-IREmut was derived by introducing clustered point mutations into the IRE of the enhancer I/X gene promoter in the pHBV4.1. The mutation converted the 10 nt IRE sequence located between nucleotide coordinates 1,091 to 1,100 from GAAAGTGAAA to GAAGGTAAAG, but did not alter the HBV polypeptide sequence [1]. The plasmids were prepared by using a plasmid purifying Kit (QIAGEN TIP-500) according to the manufacturer's instructions.

Injection of Naked Plasmid DNA and Treatment of PolyIC

Male BALB/C mice at SPF, weighing 18–20 g, were provided by the Huaxi Laboratory Animal Center of Sichuan University. Mice were injected via tail vein with 10 μ g of pHBV4.1 or pHBV4.1-IREmut in 2.0 ml of saline within 5–8 s (hydrodynamics-based in vivo transfection) [20].

Polyinosinic acid-polycytidylic acid (polyIC), as a synthetic analog of double-stranded RNA, was originally synthesized as a potent inducer of type I IFN. For polyIC treatment, at twenty-four hours after transfection, the wild-type or IRE-mutated HBV replication-competent mice were matched by body weight and serum HBeAg and treated as following: Both the wild type and the IRE mutant HBV replication-competent mice were matched and divided into two groups and treated with polyinosinic-polycytidylic acid (polyI.C) (sigma, P-0913) or phosphate-buffered saline (PBS), respectively. PolyIC was injected intraperitoneally at a dose of 200 μ g in 200 μ l PBS for three times at intervals of twenty-four hours, and PBS was injected as control. The mice were sacrificed 4 to 6 h after the final injection. The serum was stored at -20°C . Their liver tissue was frozen in liquid nitrogen and stored at -70°C prior to analysis for HBV DNA replication intermediates and RNA.

Analysis of HBV RNA and HBV DNA Replication Intermediates

Frozen liver tissue was mechanically pulverized in liquid nitrogen and used for preparation of total cellular RNA and HBV DNA replication intermediates. Total RNA were isolated by using TRIZOL reagent according to the manufacturer's instructions. HBV DNA replication intermediates were prepared as previously described [21]. One hundred and twenty microgram of liver tissue powder was lysed in 0.6 ml of 100 mM Tris hydrochloride (PH 8.0) and

0.2 % (vol/vol) NP40. The lysate was centrifuged for 1 min at 1,400 rpm in a microcentrifuge. The supernatant was adjusted to 6.75 mM magnesium acetate plus 200 µg of DNaseI/ml and incubated for 1 h at 37 °C. The supernatant was readjusted to 10 mM EDTA, 0.8 % (wt/vol) sodium lauryl sulfate, 100 mM NaCl and 1.6 mg of pronase/ml; it was incubated for additional 1 h at 37 °C. The supernatant was extracted twice with phenol/chloroform, precipitated with 0.7 volume of isopropanol and resuspended in 30 µl of 10 mM Tris hydrochloride (pH 8.0) and 1 mM EDTA.

RNA (Northern) and DNA (Southern) filter hybridization analysis was performed with 30 µg of total RNA and 30 µl of viral replication intermediates as described previously [31] with some modification. Filters were probed with digoxigenin-labeled full-length HBV DNA to detect HBV sequence, and for RNA analysis, the mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA was used as an internal control. Filter hybridization was detected by using DIG Luminescent Detection Kit (Roche Applied Science) and X ray. The relative abundances of specific RNA and DNA molecules were quantitated with Quantity One system.

Serological Analysis for HBeAg

Hepatitis B virus e-antigen (HBeAg) analysis was performed with 50 µl of mouse serum by using enzyme-linked immunosorbent assay (ELISA). The HBeAg Detection Kit (Shanghai Shiyehua company, China) was used.

RT-PCR Analysis for 2OAS mRNA in Mouse Liver

Intrahepatic mouse 2',5'-oligoadenylate synthase (2OAS) mRNA transcripts were analyzed by RT-PCR. Isolated total RNA from livers of mice received polyIC or PBS treatment was used as a template for reverse transcriptase (TAKARA, China) first-strand reaction with an Oligo dT primer. Multiplex PCR was performed with 2 primer pairs. (1) β -actin-specific primers (sense: CGTTGACATCCGTAAAGACC; anti-sense: AACAGTCCGCCTAGAAGCAC); (2) mouse 2OAS-specific primers as previously CAACAATGGCGC ACGGACTCAGGAG; anti-sense: AAAGAGCTC TTA-CAGCA GGATACATGTCC). Reaction products were analyzed by gel electrophoresis on a 1 % agarose gel. The expected bands were 281 bp for β -actin and 1,100 bp for 2OAS. The relative abundances of 2OAS mRNA were quantitated with Quantity One system.

Statistical Analysis

Data were analyzed using the SPSS version 13.0 software package (SPSS, Inc., Chicago, IL, USA). The difference between groups of quantitative data was analyzed by *t* test;

and test of significance was two-tailed, with significance being indicated by a *P* value <0.05.

Results

Effect of PolyIC Injection on HBV Transcription and Replication in HBV-Transfected Mice

To investigate whether the effect of interferon on HBV transcription and replication could be achieved in the HBV transfected mouse model, we initially investigated the effect of polyIC injection on the level of viral transcripts and replication intermediates in mice transfected with wild-type HBV DNA construct (Fig. 1). Multiple rather than single polyIC injections were employed in an attempt to sustain the alpha/beta interferon induction and to confirm the effect of interferon on HBV transcription. As shown in Fig. 1a, compared with the control mice that received PBS injection, the levels of the 3.5-, 2.4- and 2.1-kb transcripts in the livers of the mice that received polyIC treatment slightly decreased. The 3.5-kb HBV RNA was reduced by less than one fold based on this RNA filter hybridization analysis (Fig. 1d). The effect of multiple polyIC injections on HBV transcript in these mice supports the suggestion that induction of alpha/beta interferon does decrease viral RNA levels, although less than previously reported.

As shown in Fig. 1b, the levels of the HBV replication intermediates in mice received polyIC treatment were lower than that in the control mice received PBS treatment. Furthermore, the most significant reduction involved the more mature, high-molecular-weight DNA forms-relaxed circular DNA (RC DNA), which were almost undetectable in most polyIC-treated mice, while single-stranded DNA remained detectable, but at levels lower than those detected in control mice. Quantitative analysis showed that the injection of polyIC, by induction of interferon reduced the level of cytoplasmic HBV replication intermediates appropriately two fold in the mice transfected with wild HBV genome (Fig. 1e).

The expression 2OAS mRNA, a known marker of $\text{INF-}\alpha/\beta$ induction, was also detected by RT-PCR (Fig. 1c). It was observed that 2OAS mRNA was detectable in all HBV transfected-mice received PBS or polyIC injection, but the polyIC injection increased the level of the 2OAS gene expression to 1.8-fold (Fig. 1f). This indicated that injection of polyIC did induce the interferon response pathway, and its effects on HBV transcription and replication may be associated with interferon.

Effect of HBV IRE Mutation on Viral Transcription and Replication

To investigate whether the mutation of the enhancer1/X promoter IRE sequence in HBV genome will influence viral

Fig. 1 Modulation of HBV transcription and replication by interferon induced with polyIC in mice. Wild type HBV replication-competent mice are intraperitoneally injected with PBS or polyIC. **a** RNA (Northern) filter hybridization analysis of HBV transcripts. The glyceraldehydes-3-phosphatedehydrogease(GAPDH) RNA was used as an internal control for RNA loading per lane. **b** DNA (Southern) filter hybridization analysis of HBV replication intermediate. HBV RC DNA, HBV relaxed circular DNA; HBV SS DNA, HBV single-stranded DNA. **c** RT-PCR analysis for mouse OAS transcription.

d Quantitative analysis of the 3.5 kb HBV RNA. **e** Quantitative analysis of HBV DNA replication intermediates. The levels of 3.5 kb HBV RNA and HBV DNA intermediates (HBV DNA RI) are reported to the levels of the mice treated with PBS, which are set at 1.0. **f** Quantitative analysis of the OAS RNA. The RNA levels of OAS are reported to the levels of the mice treated with PBS, which are set at 1.0. The symbol “asterisk” represents the probability of a significant difference between the two groups

transcription and replication *in vivo* or not, we compared the levels of HBV transcription and replication from the mice with the wild or IRE mutant HBV DNA. The results of three independent experiments showed that the levels of the HBV 3.5- and 2.4-/2.1-kb mRNA derived from mice injected with wild or IRE mutant HBV DNA were not obviously different (Fig. 2a, c, $P > 0.05$). The levels of HBV DNA replication intermediates were also similar between the two groups of mice (Fig. 2b, d, $P > 0.05$). This suggested that mutations introduced in the HBV IRE region in this study did not greatly influence the transcription and replication of HBV *in vivo* in the HBV replication mouse model.

Influence of HBV IRE Mutation on the Inhibition Effect of Interferon on HBV Replication *In Vivo*

Having confirmed that effect of interferon on HBV transcription and replication can be achieved and HBV IRE

mutation does not influence HBV transcription and replication in the HBV replication mouse model *in vivo*, we used this model to investigate the role of HBV IRE in the antiviral effect induced by interferon. The difference between the modulation effect on transcription and replication from wild-type HBV and that from IRE-mutated HBV induced by polyIC was compared (Fig. 3). It was observed that the levels of the HBV 3.5- and 2.4-/2.1-kb transcripts in the livers of the HBV transfected mice with wild and IRE-mutated HBV DNA were both reduced by the induction of interferon response with polyIC injection (Fig. 3a, c). Quantitative analysis showed that interferon decreased the wild and mutant HBV transcripts to similar extent, and 3.5-kb HBV RNA were reduced similarly (Fig. 3c). These results suggested that the inhibitory effect of interferon on HBV transcription was not greatly influenced by IRE mutation.

In contrast to the similar effects on HBV transcription, interferon reduced HBV replication intermediates from the

Fig. 2 The effect of IRE mutation on HBV transcription and replication in mice. The wild and IRE mutant HBV replication competent mouse model were established by using the wild and IRE-mutant HBV DNA, respectively. **a** RNA (Northern) filter hybridization analysis of HBV transcripts. The glyceraldehydes-3-phosphatedehydrogease (GAPDH) RNA was used as an internal control for RNA loading per lane. **b** DNA (Southern) filter hybridization analysis of HBV replication intermediate. HBV RC DNA, HBV relaxed circular DNA; HBV SS DNA, HBV single-stranded DNA. **c** Quantitative analysis of the 3.5 kb HBV RNA. **d** Quantitative analysis of HBV DNA RI. The levels of 3.5 kb HBV RNA and HBV DNA RI are reported relative to that of the wild HBV transfected mice, which are set at 1.0

wild and IRE mutant viral genome to different extent. The level of cytoplasmic HBV replication intermediates was reduced by appropriately twofold in the mice with wild type HBV DNA after polyIC injection (Fig. 3b, d). However, polyIC injection reduced HBV replication intermediates from the IRE mutant HBV DNA only onefold, to a somewhat lesser extent. And the difference in relative decreasing level of HBV replication intermediates induced by polyIC between two groups was statistically significant ($P < 0.05$). This indicated that mutation of HBV IRE reduced the inhibitory effect of interferon on HBV replication.

Discussion

Antiviral therapy is critical for treatment of patients with chronic HBV infection, and treatment with interferon is one of standard antiviral therapy at present [13]. Although the treatment with interferon can reduce virus load and ameliorate disease symptoms of chronically infected HBV patients, an effective reduction in virus load is only observed in less than half of the treated patients. The mechanism by which interferon inhibits HBV and the

reasons that the patient did not respond to interferon treatment remain unclear.

In this study, the role of HBV IRE in antiviral effect of interferon was investigated. It was observed that mutation of the enhancer1/X gene promoter IRE sequence in HBV genome partly reduced the inhibitory effect of interferon induced by polyIC on HBV replication. In addition, a clinical study in our department was performed to investigate the relationship between HBV IRE sequence mutation and antiviral effect of interferon in interferon treated patients. The results of that study suggested that, in some patients, the poor virological response to the interferon treatment is probably associated with a mutation in the HBV IRE region (unpublished data). Therefore, it appears that interferon inhibits viral replication from wild-type HBV genome partly through an IRE-dependent mechanism and mutation of HBV IRE might be one of the reasons that HBV does not respond to interferon treatment. These information may throw some light on clinical application, for example, antiviral effect of interferon may be predicted based on HBV IRE sequencing, and also, new antiviral agents targeting to this gene region can be designed in an attempt to improve antiviral effect.

Fig. 3 The influence of IRE mutation on the inhibition effect of interferon induced by polyIC injection on HBV transcription and replication. The wild and mutant HBV replication-competent mice are treated with polyIC or PBS, respectively. **a** RNA (Northern) filter hybridization analysis of HBV transcripts. The glyceraldehydes-3-phosphatedehydrogenase (GAPDH) RNA was used as an internal control for RNA loading per lane. **b** DNA (Southern) filter hybridization analysis of HBV replication intermediate. HBV RC

DNA, HBV relaxed circular DNA; HBV SS DNA, HBV single-stranded DNA. **c** Quantitative analysis of the 3.5 kb HBV RNA. **d** Quantitative analysis of HBV DNA RI. The levels of 3.5 kb HBV RNA and HBV DNA RI are reported relative to the levels of mice treated with PBS, which are set at 1.0, respectively. The symbol “asterisk” represents the probability of a significant difference between the two groups

2OAS, one of upregulated genes by interferon, is usually known as a marker of induction of interferon response [7, 32, 37]. In our current study, it was observed that the level of the 2OAS gene expression in polyIC-treated mice is higher than that of control mice treated with PBS. Furthermore, the levels of the HBV replication intermediates in mice received polyIC treatment were lower than that in the control mice received PBS treatment, and also, the levels of HBV RNA of the mice that received polyIC treatment decreased, although slightly. The observations suggest that polyIC induced interferon response in HBV-transfected mice, and interferon suppressed the replication of HBV. In addition, it was shown that polyIC injection inhibited viral replication from wild-type viral genome to a much greater degree than IRE-mutated HBV DNA. Therefore, all these results indicated that mutation of HBV IRE reduced the inhibitory effect of interferon on HBV replication, and the IRE play a role in inhibition effect of interferon on HBV.

In this analysis, it was shown that the induction of interferon by polyIC also inhibited viral replication from IRE-mutated HBV DNA construct, although to a somewhat lesser extent than wild-type viral genome. This suggests that mutation of the IRE sequence in the HBV Ehn1/Xp region did only partially reduce but not completely block the interferon-mediated suppressive effect on viral

replication, which might be due to the fact that interferon inhibits HBV replication by more than one single mechanism. Firstly, interferon regulates HBV DNA replication by both immunomodulatory [4] and directly antiviral mechanism. Furthermore, as far as direct antiviral mechanism is concerned, except for HBV IRE-dependant pathway, interferon has the potential to trigger the activation of other multiple noncytolytic intracellular antiviral pathways that can target many steps in the viral life [2, 11, 12, 23]. In a word, multiple mechanisms might be involved in antiviral effect by interferon on HBV, and IRE-dependant mechanism investigated in our present study was one of them. Moreover, interferon regulates the function of IRE through activating a series of cytokines, so the level of these cytokines may influence its antiviral effect. These may explain the results that mutation of IRE sequence could only partly reduce the regulatory effect induced by interferon on HBV replication.

In this study, it was also observed that polyIC treatment reduced the level of cytoplasmic HBV replication intermediates to a greater extent than that of HBV RNA in the mice transfected with whether wild-type or IRE-mutated HBV genome. Additionally, mutation of HBV IRE reduced interferon-mediated inhibition of viral replication without obvious influence on transcription. These observations indicate that subtle alterations in viral transcription may be

associated with major alterations in levels of replication intermediates as observed in HBV transgenic mice. Evidence showed that a small variation of RNA transcription could probably lead to great changes of DNA levels. For example, analysis of the role of HNF1 α in modulating HBV replication in vivo indicated that the steady levels of HBV transcripts were not influenced by this transcription factor, but the levels of replication intermediates in the livers of the HNF1 α -null HBV transgenic mice were approximately two- and four-fold higher in male and female mice, respectively, compared with that observed in the HBV transgenic mice expressing functional HNF1 α . Similarly, peroxisome proliferators were shown to increase HBV replication in a PPAR α -dependent manner without significantly affecting the levels of the HBV transcripts. In addition, various stimuli including polyIC treatment result in dramatically reduced viral replication without readily detectable alteration in viral transcription [10]. Therefore, similarly to HBV transgenic mice, in HBV transfected mice viral replication can be regulated by activity of transcription factors without measurably affecting viral transcription.

Finally, we found that in our current study, the gene expression of 2OAS was detectable in all HBV transfected mice received PBS or polyIC treatment. This result indicates that transfection of viral DNA may induce interferon response. This is probably because that viral DNA introduced and nuclei and protein molecules produced in vivo triggered antiviral response, which was supported by previous observations [14, 22, 29, 34]. It had been previously observed that HBV transfected mouse established with immuno-competent mouse is a short-term HBV replication model, with viral gene expression lasting for only about 10–15 days. Furthermore, the disappearance of HBV antigens, viral transcripts and replication intermediates from the blood and liver coincided with the appearance of antiviral antibodies and evolution of the CTL response to HBV. In contrast, the virus persisted for at least 81 days after transfection of immunocompromised mice. All these observations indicated that transfection of HBV DNA triggered immune system to induce antiviral state. Because interferons stand in the frontline of defense against viral infection, it is not surprising that transfection of the viral genome induces interferon response.

It has been demonstrated that the levels of HBV replication presumably differ from mouse to mouse even in the same experiment conditions. Additionally, there were some variations in the response to interferon between different mice, as antiviral effect of interferon is relevant to host as well as virus factor, which is demonstrated in its clinical use for treatment of patients with chronic HBV infection. Therefore, in our study mice were matched by body weight and serum HBeAg before being treated with polyIC, more

than three mice were used in every experiment and all of these experiments were repeated for at least three times to ensure accuracy and reliability of the experiment results.

In conclusion, the function of IRE was investigated in vivo by using HBV replication-competent mouse model, and it was demonstrated that mutation of IRE sequence in the enhancer1/X gene promoter partly decreased the inhibitory effect of interferon on HBV replication, suggesting that interferon inhibits HBV replication partly by an IRE-dependent mechanism, means that HBV IRE plays a role in the antiviral effect on HBV induced by interferon.

Acknowledgments This work was supported by National Key Technologies Research and Development Program of China (2012ZX10002007 and 2008ZX10002-006), National Natural Science Foundation of China (No. 30972622), and National Basic Research Program of China (No.2007CB512902).

References

- Alcantara FF, Tang H, McLachlan A. Functional characterization of the interferon regulatory element in the enhancer 1 region of the hepatitis B virus genome. *Nucleic Acids Res.* 2002;30: 2068–75.
- Barry G, Breakwell L, Fragkoudis R, Attarzadeh-Yazdi G, Rodriguez-Andres J, Kohl A, Fazakerley JK. PKR acts early in infection to suppress Semliki Forest virus production and strongly enhances the type I interferon response. *J Gen Virol.* 2009;90: 1382–91.
- Beasley RP, Hwang LY, Lin CC, Chien CS. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22,707 men in Taiwan. *Lancet.* 1981;2:1129–33.
- Bonjardim CA, Ferreira PC, Kroon EG. Interferons: signaling, antiviral and viral evasion. *Immunol Lett.* 2009;122:1–11.
- Chen DS. Hepatitis B. Vaccination: the key towards elimination and eradication of hepatitis B. *J Hepatol.* 2009;50:805–16.
- Darnell JE Jr, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science.* 1994;264:1415–21.
- Der SD, Zhou A, Williams BR, Silverman RH. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proc Natl Acad Sci USA.* 1998; 95:15623–8.
- Di Marco V, Craxi A. Chronic hepatitis B: who to treat and which choice of treatment? *Expert Rev Anti Infect Ther.* 2009;7: 281–91.
- Fujiyama A, Miyanohara A, Nozaki C, Yoneyama T, Ohtomo N, Matsubara K. Cloning and structural analyses of hepatitis B virus DNAs, subtype adr. *Nucleic Acids Res.* 1983;11:4601–10.
- Guidotti LG, Eggers CM, Raney AK, Chi SY, Peters JM, Gonzalez FJ, McLachlan A. In vivo regulation of hepatitis B virus replication by peroxisome proliferators. *J Virol.* 1999;73: 10377–86.
- Haller O, Kochs G, Weber F. Interferon, Mx, and viral countermeasures. *Cytokine Growth Factor Rev.* 2007;18:425–33.
- Hovanessian AG. On the discovery of interferon-inducible, double-stranded RNA activated enzymes: the 2'-5' oligoadenylate synthetases and the protein kinase PKR. *Cytokine Growth Factor Rev.* 2007;18:351–61.
- Jafri SM, Lok AS. Antiviral therapy for chronic hepatitis B. *Clin Liver Dis.* 2010;14:425–38.

14. Ju Y, Hou N, Zhang XN, Zhao D, Liu Y, Wang JJ, Luan F, Shi W, Zhu FL, Sun WS, Zhang LN, Gao CJ, Gao LF, Liang XH, Ma CH. Blockade of Tim-3 pathway ameliorates interferon-gamma production from hepatic CD8+ T cells in a mouse model of hepatitis B virus infection. *Cell Mol Immunol*. 2009;6:35–43.
15. Kalvakolanu DV, Borden EC. An overview of the interferon system: signal transduction and mechanisms of action. *Cancer Invest*. 1996;14:25–53.
16. Keefe EB, Dieterich DT, Han SH, Jacobson IM, Martin P, Schiff ER, Tobias H. A treatment algorithm for the management of chronic hepatitis B virus infection in the United States: 2008 update. *Clinical gastroenterology and hepatology: the official clinical practice journal of the American Gastroenterological Association*. 2008;6:1315–41. quiz 286.
17. Kim SR, Kudo M, Hino O, Han KH, Chung YH, Lee HS. Epidemiology of hepatocellular carcinoma in Japan and Korea. A review. *Oncology*. 2008;75(Suppl 1):13–6.
18. Kimura T, Kadokawa Y, Harada H, Matsumoto M, Sato M, Kashiwazaki Y, Tarutani M, Tan RS, Takasugi T, Matsuyama T, Mak TW, Noguchi S, Taniguchi T. Essential and non-redundant roles of p48 (ISGF3 gamma) and IRF-1 in both type I and type II interferon responses, as revealed by gene targeting studies. *Genes Cells*. 1996;1:115–24.
19. Lavanchy D. Worldwide epidemiology of HBV infection, disease burden, and vaccine prevention. *J Clin Virol*. 2005;34(Suppl 1):S1–3.
20. Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther*. 1999;6:1258–66.
21. Liu FJ, Liu L, He F, Wang S, Zhou TY, Liu C, Deng LY, Tang H. Establishment and primary application of a mouse model with hepatitis B virus replication. *World J Gastroenterol*. 2007;13:5324–30.
22. Luo Y, Lo CM, Cheung CK, Lau GK, Wong J. Hepatitis B virus-specific CD4 T cell immunity after liver transplantation for chronic hepatitis B. *Liver Transpl*. 2009;15:292–9.
23. Mucha R, Bhide MR, Chakurkar EB, Novak M, Mikula I Sr. Toll-like receptors TLR1, TLR2 and TLR4 gene mutations and natural resistance to *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle. *Vet Immunol Immunopathol*. 2009;128:381–8.
24. Nakao K, Nakata K, Yamashita M, Tamada Y, Hamasaki K, Ishikawa H, Kato Y, Eguchi K, Ishii N. p48 (ISGF-3gamma) is involved in interferon-alpha-induced suppression of hepatitis B virus enhancer-1 activity. *J Biol Chem*. 1999;274:28075–8.
25. Okamoto H, Imai M, Shimozaki M, Hoshi Y, Iizuka H, Gotanda T, Tsuda F, Miyakawa Y, Mayumi M. Nucleotide sequence of a cloned hepatitis B virus genome, subtype ayr: comparison with genomes of the other three subtypes. *J Gen Virol*. 1986;67(Pt 11):2305–14.
26. Rang A, Heise T, Will H. Lack of a role of the interferon-stimulated response element-like region in interferon alpha -induced suppression of Hepatitis B virus in vitro. *J Biol Chem*. 2001;276:3531–5.
27. Reich N, Evans B, Levy D, Fahey D, Knight E Jr, Darnell JE Jr. Interferon-induced transcription of a gene encoding a 15-kDa protein depends on an upstream enhancer element. *Proc Natl Acad Sci USA*. 1987;84:6394–8.
28. Robek MD, Boyd BS, Wieland SF, Chisari FV. Signal transduction pathways that inhibit hepatitis B virus replication. *Proc Natl Acad Sci USA*. 2004;101:1743–7.
29. Suzuki T, Takehara T, Ohkawa K, Ishida H, Jinushi M, Miyagi T, Sasaki Y, Hayashi N. Intravenous injection of naked plasmid DNA encoding hepatitis B virus (HBV) produces HBV and induces humoral immune response in mice. *Biochem Biophys Res Commun*. 2003;300:784–8.
30. Tang H, McLachlan A. Transcriptional regulation of hepatitis B virus by nuclear hormone receptors is a critical determinant of viral tropism. *Proc Natl Acad Sci USA*. 2001;98:1841–6.
31. Tang H, Delgermaa L, Huang F, Oishi N, Liu L, He F, Zhao L, Murakami S. The transcriptional transactivation function of HBx protein is important for its augmentation role in hepatitis B virus replication. *J Virol*. 2005;79:5548–56.
32. Wang J, Jiang D, Zhang H, Lv S, Rao H, Fei R, Wei L. Proteome responses to stable hepatitis B virus transfection and following interferon alpha treatment in human liver cell line HepG2. *Proteomics*. 2009;9:1672–82.
33. Wiegand J, Hasenclever D, Tillmann HL. Should treatment of hepatitis B depend on hepatitis B virus genotypes? A hypothesis generated from an explorative analysis of published evidence. *Antivir Ther*. 2008;13:211–20.
34. Yang PL, Althage A, Chung J, Chisari FV. Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection. *Proc Natl Acad Sci USA*. 2002;99:13825–30.
35. Zhang Q, Wei L, Wang Y. ISGF3, a critical factor of the IFN-alpha pathway in the antiviral action of HBV. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi*. 2005;19:110–3.
36. Zhang Q, Wang Y, Wei L, Jiang D, Wang JH, Rao HY, Zhu L, Chen H, Fei R, Cong X. Role of ISGF3 in modulating the anti-hepatitis B virus activity of interferon-alpha in vitro. *J Gastroenterol Hepatol*. 2008;23:1747–61.
37. Zhou A, Hassel BA, Silverman RH. Expression cloning of 2–5A-dependent RNAase: a uniquely regulated mediator of interferon action. *Cell*. 1993;72:753–65.