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Hepatitis B Virus e Antigen Downregulates Cytokine Production in Human Hepatoma Cell Lines

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Abstract

Disease activities of hepatitis B are affected by the status of hepatitis B e antigen (HBeAg). The function of the hepatitis B virus (HBV) precore or HBeAg is unknown. We assumed that HBeAg blocks aberrant immune responses, although HBeAg is not required for viral assembly, infection, or replication. We examined the interaction of HBeAg and the immune system, including cytokine production. The inflammatory cytokine TNF, IL-6, IL-8, IL-12A, IFN-α1, and IFN-β mRNA were downregulated in HBeAg-positive HepG2, which stably expresses HBeAg, compared to HBeAg-negative HepG2 cells. The results of real-time RT-PCR-based cytokine-related gene arrays showed the downregulation of cytokine and IFN production. We also observed inhibition of the activation of NF-κB- and IFN-β-promoter in HBeAg-positive HepG2, as well as inhibition of IFN and IL-6 production in HBeAg-positive HepG2 cell culture fluids. HBeAg might modify disease progression by inhibiting inflammatory cytokine and IFN gene expression, while simultaneously suppressing NF-κB-signaling- and IFN-β-promoter activation.

Introduction

More than 2 billion people have been exposed to hepatitis B virus (HBV), and 350 million remain chronically infected worldwide. HBV is a noncytopathic DNA virus with a partially double-stranded 3.2-kb genome. HBV causes acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (2,6,21,25,37). Viral clearance and its pathogenesis during acute HBV infection require the induction of a vigorous $CD8^+$ T-cell response, and the induction of hepatic immunopathology, including cytokine responses.

The HBV genome consists of four open reading frames coding for the surface, core, polymerase, and X proteins. Viral DNA, upon entry into cells during productive infection, undergoes a repair process and forms covalently closed circular DNA. Transcription of this DNA produces longer (precore) and shorter (pregenomic) 3.5-kb RNAs. The pregenomic RNA is packaged into nucleocapsides along with the viral polymerase, and serves as the template for viral genome replication. Precore and pregenomic RNAs encode core, polymerase (by pregenomic RNA), and hepatitis B e antigen (HBeAg) (by precore RNA) (47).

Disease severity of hepatitis B is affected by the status of HBeAg. The presence of HBeAg in serum is also known to be a marker of a high degree of viral infectivity. Although there are diverse opinions, fulminant hepatitis may occur in persons who are negative for HBeAg in highly endemic areas (29). Infants born to HBeAg-positive mothers tend to be HBsAg-positive more than those born to HBeAg-negative mothers (44). HBeAg-positive asymptomatic carriers (ASCs) have higher viral load, but most do not display any liver dysfunction (10). These clinical cases can be assumed to have immune tolerance for HBeAg.

The core gene of 183 codons (at least for genotypes B and C) is preceded by an in-frame pre-ATG codon that extends the protein by 29 hydrophobic amino acids (Fig. 1A). Proteins like this are translated from a 3.5-kb precore RNA and converted to HBeAg by two proteolytic cleavage events in the secretory pathway (12,26,38). First, the N-terminal 19 residues encoded by the precore region serve as the signal peptide for translocation of the precore/core protein into the endoplasmic reticulum lumen, where the peptide is clipped away by a signal peptidase. Next, 30 residues are removed from the C terminus in a post-endoplasmic reticulum compartment to generate mature HBeAg of ~17 kDa (12). A single point mutation has been reported to produce a stop codon in the precore region of HBV DNA and prevent the formation of the precore protein required to make HBeAg (7). HBeAg is thought to involve immune tolerance via an unknown mechanism, although it is not required for viral

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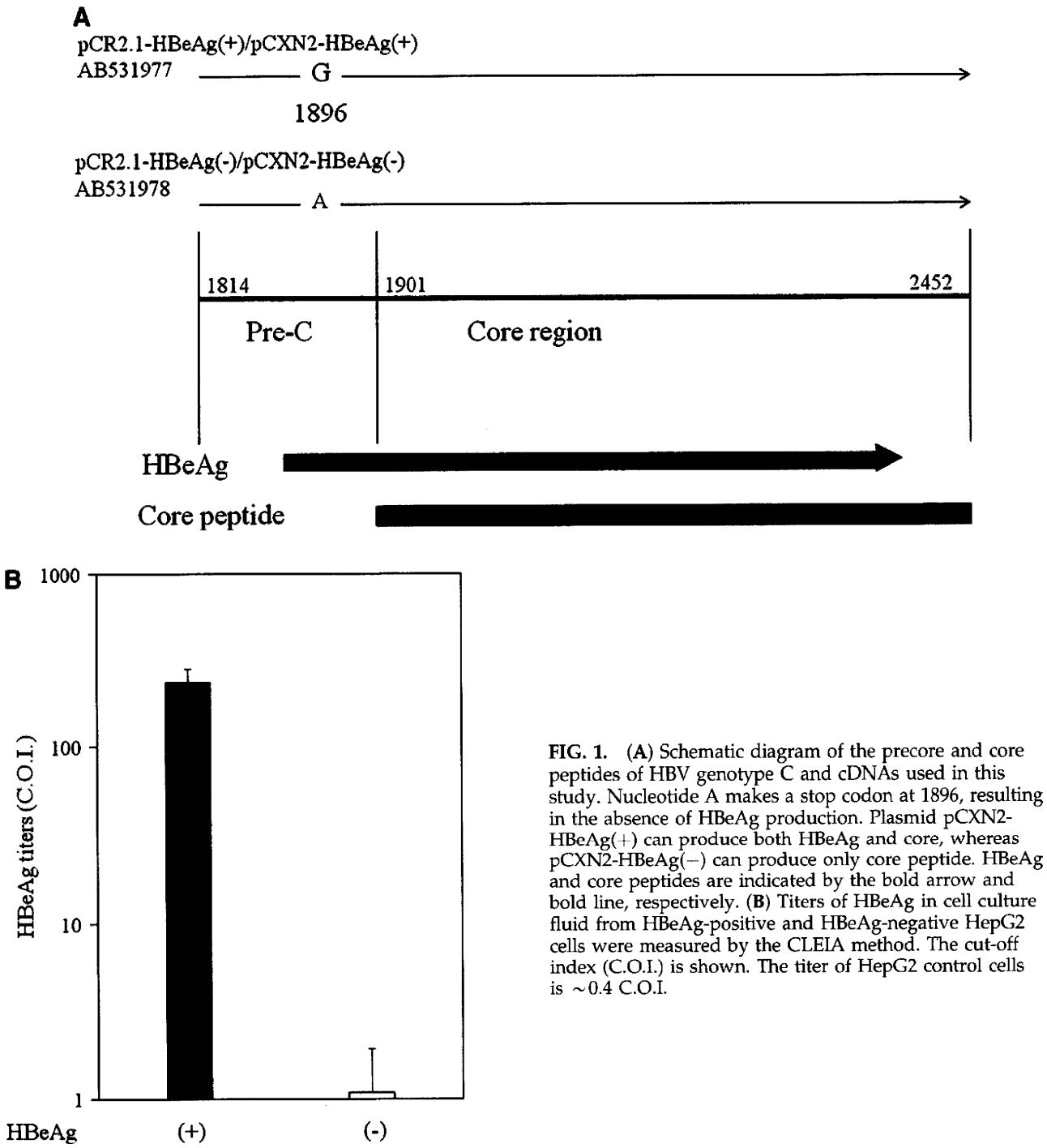

FIG. 1. (**A**) Schematic diagram of the precore and core peptides of HBV genotype C and cDNAs used in this study. Nucleotide A makes a stop codon at 1896, resulting in the absence of HBeAg production. Plasmid pCXN2-HBeAg(+) can produce both HBeAg and core, whereas pCXN2-HBeAg(−) can produce only core peptide. HBeAg and core peptides are indicated by the bold arrow and bold line, respectively. (**B**) Titers of HBeAg in cell culture fluid from HBeAg-positive and HBeAg-negative HepG2 cells were measured by the CLEIA method. The cut-off index (C.O.I.) is shown. The titer of HepG2 control cells is ~0.4 C.O.I.

assembly, infection, or replication (3,42). Visvanathan *et al.* (43) reported that the expression of TLR2 on hepatocytes, Kupffer cells, and peripheral monocytes, was significantly reduced in HBeAg-positive chronic hepatitis B patients. Although the precise function of HBV precore or HBeAg is unknown, it is possible that HBeAg suppresses the TLR pathways, thereby allowing HBV to establish persistent infection in the host (43).

Toll-like receptors (TLRs) play important roles in the innate immune response and are thought to have therapeutic potential for infectious diseases and cancers (18). Some of them are expressed on many different cells, including hepatocytes (32,36). Preiss *et al.* (32) demonstrated mRNA transcription for most TLRs, with the exception of TLR8. TLR5 mRNA was not detectable in HepG2 cells. Hepatocytes may themselves play an active role in innate immune responses to viruses such as HBV (32). Once these pattern recognition receptors (PRRs) have identified the pathogen-associated molecular patterns (PAMPs), the effector cells function and respond immediately. Ligand recognition by TLRs leads to the recruitment of various TIR domain-containing adaptors, such as myeloid differentiation primary

response gene (88) (MyD88), toll-interleukin 1 receptor domain containing adaptor protein (TIRAP), TIR domain-containing adapter inducing interferon-β (TRIF), and TRIF-related adapter molecule (TRAM), which in turn triggers the cascade of the signaling pathway, and ultimately the activation of transcription factors such as nuclear factor-κB (NF-κB) and interferon regulatory factors (IRFs), leading to the expression of various cytokines (e.g., tumor necrosis factor [TNF], interleukin-6 [IL-6], IL-8, interferon-α1 [IFN-α1], and IFN-β). Hepatic cytokines also play an important role in the progression of hepatitis B-associated liver diseases. A number of viruses have been shown to encode proteins that have the potential to inhibit antiviral activity of the innate and adaptive immune responses. Inflammatory cytokines contributing to viral clearance in HBV infection may have therapeutic value (20). In the present study, we assumed that HBeAg blocks aberrant immune responses, and we examined the role of HBeAg protein in cytokine production to test the interaction between HBeAg and the immune system in human hepatocytes. Our results demonstrated that cytokine production is inhibited by HBeAg, and that it also enhances IFN-sensitive hepatitis C virus (HCV) replication.

Materials and Methods

Plasmids

pNF-κB-luc, which expresses luciferase upon promoter activation by NF-κB, was purchased from Stratagene (La Jolla, CA). This vector has five repeats of the binding site for NF-κB (TGGGGACTTTCCGC). pIFN-β-luc, which expresses luciferase under the control of an IFN-β-dependent promoter, was kindly provided by Dr. N. Kato (Institute of Medical Science, University of Tokyo, Japan). To construct plasmids including HBV precore and core regions, HBV DNA was used from the serum of a genotype C HBeAg-positive asymptomatic carrier (ASC) patient as previously described (10). The DNA sequence information from this study will appear at GenBank (accession numbers AB531977 and AB531978). To make pCR2.1-HBeAg(+), the PCR product was cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). Using the Quickchange II site-directed mutagenesis kit (Stratagene), precore stop codon mutant G1896A was induced into pCR2.1-HBeAg(+) to pCR2.1-HBeAg(−) according to the manufacturer's instructions. To obtain the mammalian cell expression vectors, we performed subcloning using the EcoRI site of pCXN2 (kindly provided by Prof. J. Miyazaki, Osaka University, Osaka, Japan), a mammalian expression vector with a β-actin-based CAG promoter and SV40 origin (28). The constructs pCXN2-HBeAg(+) and pCXN2-HBeAg(−) were generated by this method (Fig. 1A). All sequences of these plasmids were confirmed using Big Dye Terminator on a 3730 DNA sequencer (Applied Biosystems, Foster City, CA).

Cell culture

Human hepatoma cells, HepG2 and Huh7 cells, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. Approximately 1×10^5 HepG2 cells were placed on 35-mm tissue culture dishes (Iwaki Glass, Tokyo, Japan) 24 h prior to transfection (13). The cells were transfected with pCXN2-HBeAg(+) or pCXN2-HBeAg(−) in Effectene transfection reagent (Qiagen, Hilden, Germany). After 48 h, G418 was added at 1000 μg/mL for the selection of stable cell lines, and HBeAg-positive and HBeAg-negative HepG2 cells were designated. After 3 wk, to avoid monoclonal selection, all cells were collected for further analysis.

RNA extraction, cDNA synthesis, and real-time PCR

The cells were seeded into 6-well plates, and total cellular RNA was extracted 48 h later using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA samples were then stored at −80°C until use. RNA quality was examined using the A_{280}/A_{260} ratio (Pharmacia Biotech, Bedford, MA). cDNA synthesis was performed using a random hexamer. For RNA quantitation, real-time PCR was conducted using SyBr Green I (ABI PRISM 7300; Applied Biosystems). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization, and data were analyzed by the comparative threshold cycle (C_T) method (16). The primers used are shown in Table 1.

Real-time PCR arrays

Gene expression profiling for TLR target genes was performed using RT^2 profiler PCR arrays (SuperArray, Frederick, MD) according to the manufacturer's instructions. In brief, 1 μg RNA was reverse-transcribed with the RT^2 profiler PCR array first-strand synthesis assay (SuperArray), followed by

TABLE 1. PRIMERS USED FOR QUANTITATIVE REAL-TIME PCR

Gene name	*Sequences (forward/reverse)*
GAPDH	5′-ACCCACTCCTCCACCTTTG-3′/ 5′-CTCTTGTGCTCTTGCTGGG-3′
TLR7	5′-GGAGGTATTCCCACGAACACC-3′/ 5′-GACCCCAGTGGAATAGGTACAC-3′
TNF	5′-CCAGACCAAGGTCAACCTC-3′/ 5′-CCAGATAGATGGGCTCATACC-3′
IL-6	5′-AAAAGTCCTGATCCAGTTC-3′/ 5′-GAGATGAGTTGTCATGTCC-3′
IL-8	5′-ACATACTCCAAACCTTTCCAC-3′/ 5′-CCAGACAGAGCTCTCTTCC-3′
IL-12A	5′-CCCTTGCACTTCTGAAGAG-3′/ 5′-AGGCAACTCTCATTCTTGG-3′
IFN-α1	5′-GGGATGAGGACCTCCTAGAC-3′/ 5′-GGAGTCCGCATTCATCAGG-3′
IFN-β	5′-GATTCATCTAGCACTGGCTGG-3′/ 5′-CTTCAGGTAATGCAGAATCC-3′
LY96 (MD-2)	5′-ATTTGCCGAGGATCTGATG-3′/ 5′-GGTGTAGGATGACAAACTCC-3′
RIPK2	5′-AGACACTACTGACATCCAAG-3′/ 5′-CACAAGTATTTCCGGGTAAG-3′
NF-κB1	5′-GAAGAAAATGGTGGAGTCTG-3′/ 5′-GGTTCACTAGTTTCCAAGTC-3′
MAP3K1	5′-CCACTGCATGTCAATTTGGG-3′/ 5′-CGTGGCTGTAGAAATCATGAG-3′
HCV	5′-TCTGCGGAACCGGTGAGTA-3′/ 5′-TCAGGCAGTACCACAAGGC-3′

real-time PCR with RT^2 real-time PCR master mix SyBr green (SuperArray). Gene expression was normalized to two internal controls (GAPDH and β-actin), to determine the fold change in gene expression between the test sample (HBeAg-positive HepG2) and the control sample (HBeAg-negative HepG2) by the 2^{-ddCT} (comparative cycle threshold) method (17). Data were analyzed with RT^2 Profiler™ PCR Array Data Analysis software (http://www.superarray.com/pcrarraydataanalysis.php). Genes with more than twofold change were also confirmed by real-time RT-PCR in at least triplicate. For this we used GAPDH for normalization.

Transfection and reporter assay

Approximately 1×10^5 cells were placed on 6-well plates (Iwaki Glass) 24 h prior to transfection. Cells were transfected with 0.4 μg of plasmid pIFN-β-luc or pNF-κB-luc in Effectene (Qiagen). For luciferase assay of NF-κB activation, cells were treated for 4 h with 0.5 or 5 ng/mL TNF-α, 10 or 50 μg/mL TLR4 ligand:lipopolysaccharide (LPS), or none at 44 h post-transfection (22,31,34,35,40). For IFN-β promoter assay, 50 μg/mL TLR3 ligand:poly(I-C), or none was added to cell culture fluid at 32 h post-transfection (16). At 48 h post-transfection, the cells were lysed with reporter lysis buffer (Promega, Madison, WI), and luciferase activity was determined by luminometer (Luminescencer-JNR II AB-2300; ATTO Bio Instruments, Tokyo, Japan) as previously described (16). Relative luciferase activity was measured at 48 h post-transfection and compared with that of an untreated control. Relative luciferase activity of HBeAg-negative cells was set as 1.

Chemiluminescent enzyme immunoassay

The supernatants of these cell lines were used for measuring the levels of HBeAg by the chemiluminescent enzyme immunoassay (CLEIA) system (Fujirebio Inc., Tokyo, Japan).

ELISA

Cell culture fluid was analyzed for IL-6 by enzyme-linked immunosorbent assay (ELISA; KOMA Biotech Inc., Seoul, Korea) following the manufacturer's protocol. Briefly, cell culture fluid samples were incubated in plates at 4°C overnight, followed by incubation with biotinylated monoclonal antibodies. Avidin-conjugated peroxidase was added to the plates, and enzyme activity was detected with an ELISA plate reader.

MTS assay

MTS assays were performed with the CellTiter 96 AQ One Solution Cell Proliferation Assay (Promega) (15). Twenty microliters/well of the MTS reagent was added to 100 μL of media containing cells in each well of 96-well plates, and left for 4 h at 37°C in a humidified 5% CO_2 atmosphere. For analysis, absorbance at 490 nm was measured using a Bio-Rad iMark microplate reader (Bio-Rad, Hercules, CA).

Antiviral assay using HCV subgenomic replicon

Huh7 cells harboring HCV genotype 1b subgenomic replicon, termed C13-3 cells, were used for antiviral bioassay (14). Intracellular HCV subgenomic RNA was measured by real-time RT-PCR. C13-3 cells were incubated in cell culture supernatant from HBeAg-positive, HBeAg-negative HepG2, or control HepG2 cells for 24–48 h. Post-incubation, RNA was extracted and stored at −80°C until analysis.

Statistical analysis

Results were expressed as mean ± SD. Student's *t*-test was used to determine statistical significance.

Results

Detection of stable expression of HBeAg by CLEIA

First, we examined the HBeAg production in cell culture fluid in HepG2 stably expressing HBV precore and core regions. HBeAg was detected in cell culture supernatants of HBV precore and core region-expressing cells (HBeAg-positive HepG2, 241 ± 47.9 C.O.I.) by CLEIA (cut-off index [C.O.I.]). On the other hand, expression of the core region without precore did not produce HBeAg in cell culture fluid (HBeAg-negative HepG2, 1.1 ± 0.84 C.O.I.) (Fig. 1B). Next, we performed an MTS assay to examine whether HBeAg affected cell proliferation or cell viability in our system. Cell proliferation/viability of HBeAg-positive cells (100 ± 0.87% at 24 h [n = 4]; 98.5 ± 0.7% at 48 h [n = 4]) was not statistically different from that of HBeAg-negative HepG2 (100 ± 0.4% at 24 h [n = 4]; 100 ± 1.21% at 48 h [n = 4]).

HepG2 cells respond to TLR3 ligand, TLR4 ligand, and tumor necrosis factor

Next we examined whether human hepatoma cell lines HepG2 and Huh7 respond to TLR3 ligand, TLR4 ligand, and tumor necrosis factor (TNF). Here we examined the NF-κB- and IFN-signaling pathways in HepG2 and Huh7 cells. To examine whether HepG2 possesses a functional TLR4 pathway, we initially characterized LPS-induced activation of NF-κB in HepG2 and Huh7 by luciferase reporter assay.

TABLE 2. NUCLEAR FACTOR (NF)-κB ACTIVATION FOLLOWING EXPOSURE TO LIPOPOLYSACCHARIDE (LPS), AND FOLLOWING EXPOSURE TO TUMOR NECROSIS FACTOR (TNF)-α, AND INTERFERON (IFN)-β-PROMOTER ACTIVATION FOLLOWING EXPOSURE TO POLY(I-C) BY LUCIFERASE ASSAYS

Ligand	*HepG2 (fold)*	*Huh7 (fold)*
NF-κB activation		
LPS (10 μg/mL)	23.3 ± 3.11**	1.82 ± 0.17*
LPS (50 μg/mL)	56.0 ± 13.6*	3.01 ± 0.69*
TNF-α (0.5 ng/mL)	9.47 ± 1.37**	1.45 ± 0.27
TNF-α (5 ng/mL)	14.4 ± 0.82***	8.59 ± 1.18**
IFN-β-promoter activation		
Poly (I-C) (50 μg/mL)	1.69 ± 0.14*	0.93 ± 0.10

Cells were transfected with 0.4 μg of plasmid pIFN-β-luc or pNF-κB-luc in Effectene (Qiagen). For the luciferase assay of NF-κB activation, cells were treated for 4 h with 0.5 or 5 ng/mL TNF-α, 10 or 50 μg/mL LPS, or none, at 44 h post-transfection (22,31,34,35,40). For the IFN-β promoter assay, 50 μg/mL poly(I-C) or none was added to cell culture fluid at 32 h post-transfection (16). Relative luciferase activity was measured at 48 h post-transfection and compared with that of an untreated control. Results are expressed as mean ± SD.

*$p < 0.01$, **$p < 0.001$, and ***$p < 0.0001$ in HepG2 or Huh7 induced by each ligand compared with untreated controls.

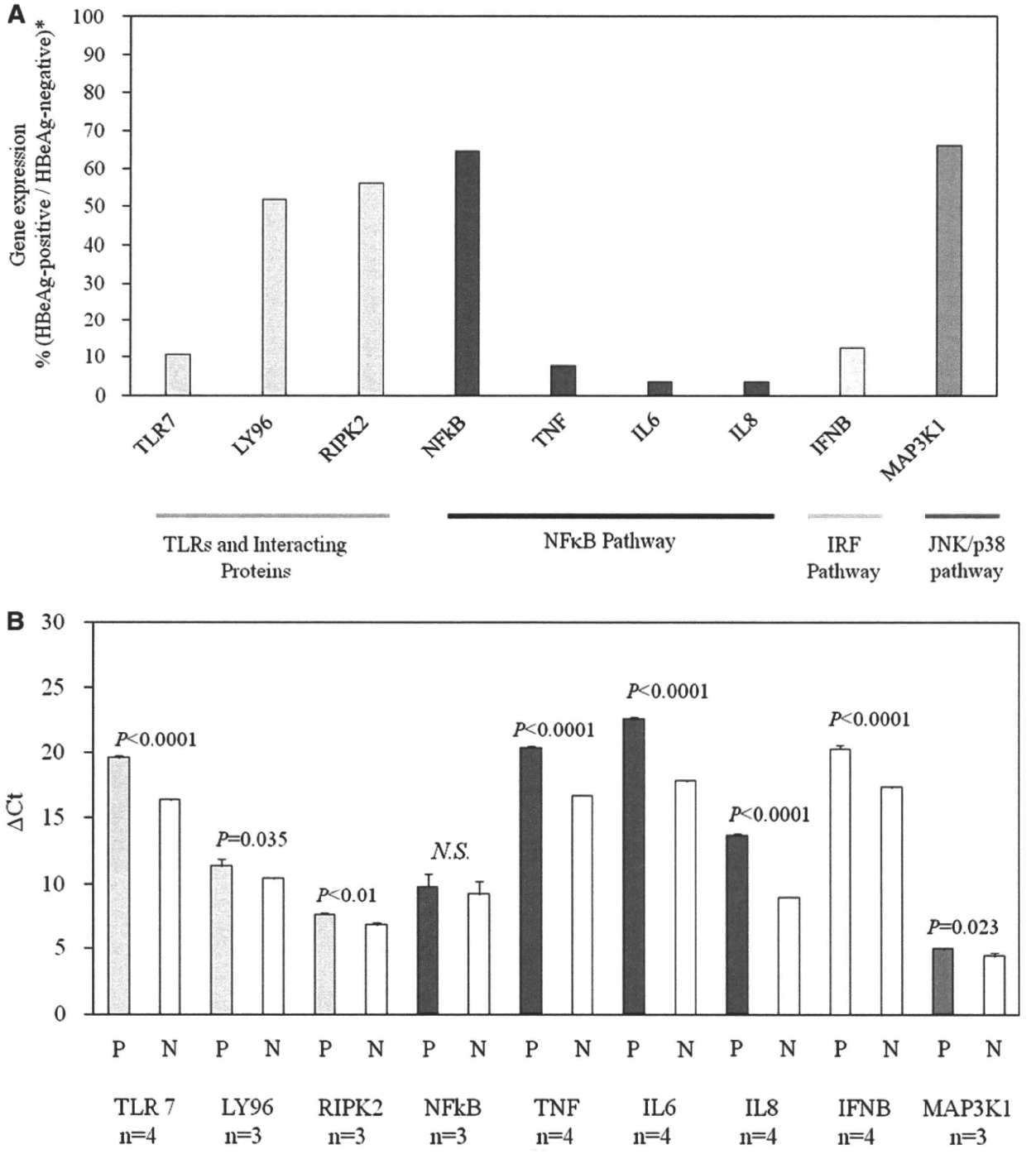

FIG. 2. Effects of HBeAg on toll-like receptor (TLR) signaling-related gene expression (comparison of genes expressed in HBeAg-positive HepG2 with those in HBeAg-negative HepG2). (**A**) TLR target gene expression examined by real-time RT-PCR in at least triplicate. GAPDH was used for normalization. These genes were screened by real-time PCR arrays, as described in the materials and methods section *(value of HBeAg-positive cells/value of HBeAg-negative cells)×100. (**B**) Statistical analysis of TLR signaling-related gene expression in HBeAg-positive and HBeAg-negative HepG2 cellular RNA by real-time RT-PCR by ΔCt. Results are expressed as mean ± SD (N.S., not statistically significant by Student's *t*-test; P, HBeAg-positive HepG2; N, HBeAg-negative HepG2; TLR7, toll-like receptor 7; LY96 [MD-2], lymphocyte antigen 96; RIPK2, receptor-interacting serine-threonine kinase 2; NFκB1, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 [p105]; IL-6, interleukin-6 [interferon-β2]; IL-8, interleukin-8; IFN-β, interferon-β1; MAP3K1, mitogen-activated protein kinase kinase kinase 1).

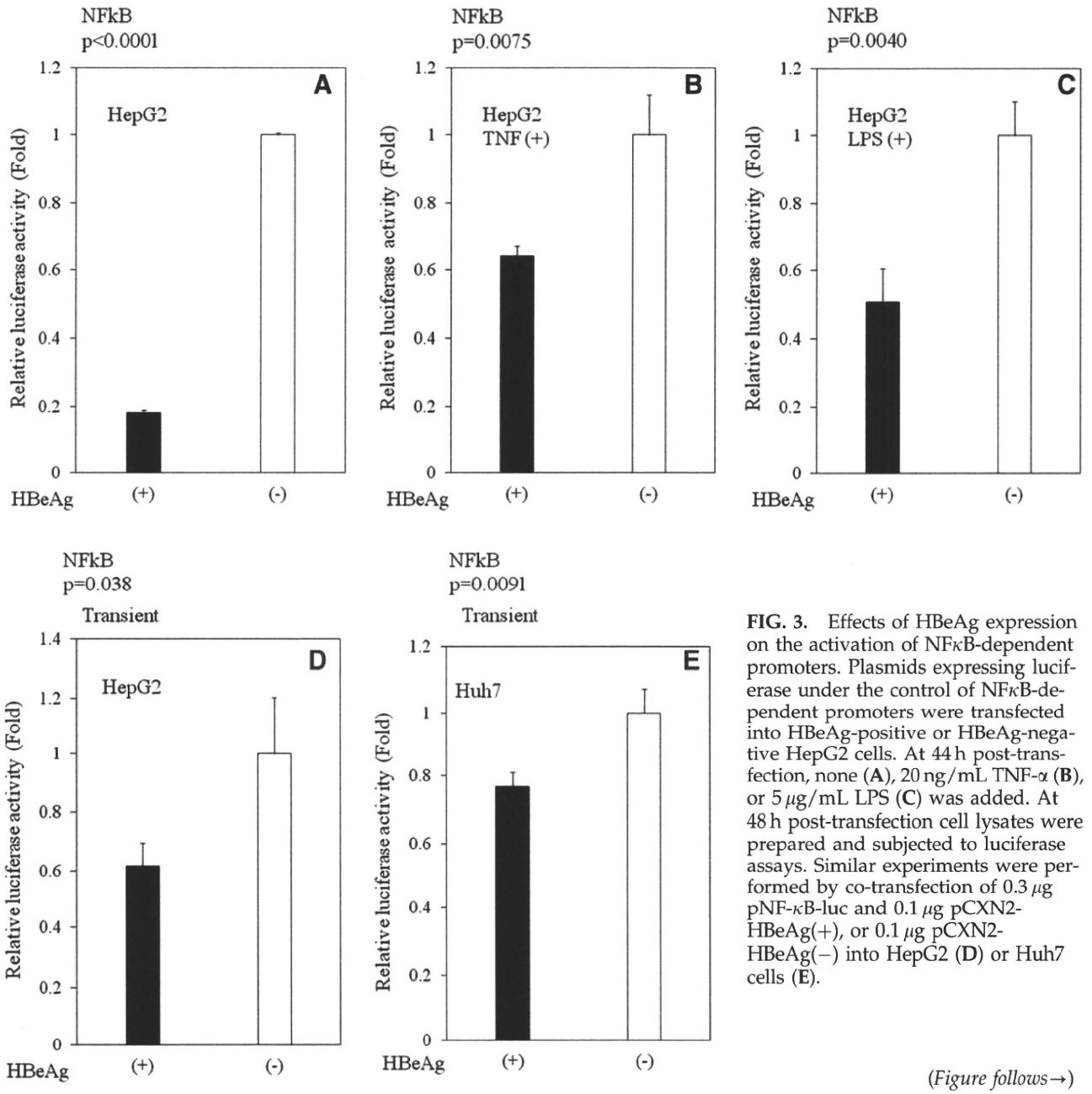

FIG. 3. Effects of HBeAg expression on the activation of NFκB-dependent promoters. Plasmids expressing luciferase under the control of NFκB-dependent promoters were transfected into HBeAg-positive or HBeAg-negative HepG2 cells. At 44 h post-transfection, none (**A**), 20 ng/mL TNF-α (**B**), or 5 μg/mL LPS (**C**) was added. At 48 h post-transfection cell lysates were prepared and subjected to luciferase assays. Similar experiments were performed by co-transfection of 0.3 μg pNF-κB-luc and 0.1 μg pCXN2-HBeAg(+), or 0.1 μg pCXN2-HBeAg(−) into HepG2 (**D**) or Huh7 cells (**E**).

(*Figure follows*→)

TLR4 plays an important role in the activation of NF-κB following exposure to extracellular LPS. When LPS was added to the cell culture medium of HepG2 and Huh7, approximately 23 ~ 56-fold and 1.8 ~ 3.0-fold activation, respectively, of NF-κB activity were observed (Table 2). Similarly, when TNF-α, another NF-κB activator, was added to the cell culture medium of HepG2 and Huh7, respectively, approximately 9 ~ 14-fold and 1.4 ~ 8.6-fold activation, respectively, of NF-κB activity were observed (Table 2). However, to examine for a functional TLR3 pathway by luciferase reporter assay, when poly(I-C) was added to the cell culture medium of HepG2 and Huh7, respectively, approximately 1.69-fold and 0.93-fold activation, respectively, of IFN-β-promoter activity were observed (Table 2), supporting the view that Huh7 cells are defective in the TLR3 and RIG-I pathway (16,39). Our results suggested that HepG2 possesses functional TLR3 and TLR4 pathways to some extent, but Huh7 does not possess a functional TLR3 pathway.

Downregulation of IFN and cytokine gene expression by HBeAg

Since HBeAg is associated with immune tolerance (3,42), we wanted to determine whether this might be related to HBeAg suppressing the host innate response, including the production of cytokines. To confirm the downregulation of IFN and cytokine genes, we performed real-time RT-PCR

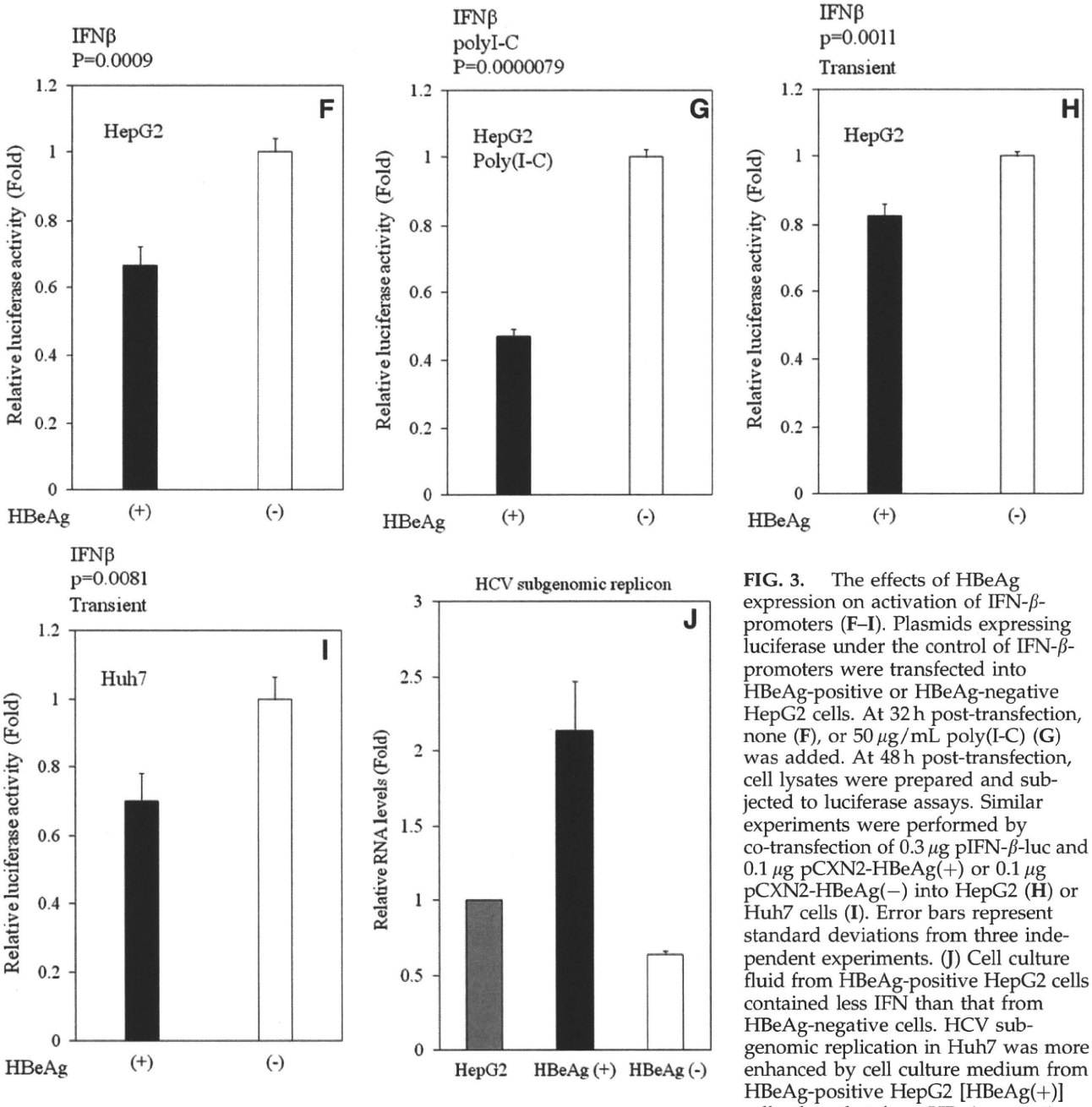

FIG. 3. The effects of HBeAg expression on activation of IFN-β-promoters (**F–I**). Plasmids expressing luciferase under the control of IFN-β-promoters were transfected into HBeAg-positive or HBeAg-negative HepG2 cells. At 32 h post-transfection, none (**F**), or 50 μg/mL poly(I-C) (**G**) was added. At 48 h post-transfection, cell lysates were prepared and subjected to luciferase assays. Similar experiments were performed by co-transfection of 0.3 μg pIFN-β-luc and 0.1 μg pCXN2-HBeAg(+) or 0.1 μg pCXN2-HBeAg(−) into HepG2 (**H**) or Huh7 cells (**I**). Error bars represent standard deviations from three independent experiments. (**J**) Cell culture fluid from HBeAg-positive HepG2 cells contained less IFN than that from HBeAg-negative cells. HCV subgenomic replication in Huh7 was more enhanced by cell culture medium from HBeAg-positive HepG2 [HBeAg(+)] cells, than that from HBeAg-negative HepG2 [HBeAg(−)] cells, and that from control HepG2 cells. Total cellular RNA was extracted at 24 h after adding cell culture medium. Intracellular gene expression levels of HCV and GAPDH were measured by real-time RT-PCR. The ratios of HCV/GAPDH are presented as n-fold relative to that in control HepG2 cells. The results are presented as means of data from three independent experiments.

assays. We compared six IFN and cytokine (IFN-α1, IFN-β, IL-6, IL-8, IL-12A, and TNF) gene expressions in HBeAg-positive HepG2 cells with those in HBeAg-negative HepG2 cells. The mRNAs of IFN-α1 and IL-12A were inhibited (6.7% and 11.6%, respectively, of those in HBeAg-negative HepG2), and ΔCt of HBeAg-positive HepG2/ΔCt of HBeAg-negative HepG2 in IFN-α1 mRNA and those in IL-12A mRNA were $14.36 \pm 0.11/10.47 \pm 0.02$ ($p < 0.001$, n = 3), and $17.74 \pm 0.11/14.65 \pm 0.17$ ($p < 0.001$, n = 3), respectively. As shown in Fig. 2, more inhibition of IFN-β, IL-6, IL-8, and TNF mRNA in HBeAg-positive HepG2 were also observed, compared with HBeAg-negative HepG2.

Effects of TLR-dependent target gene expression by HBeAg

To explore the upstream mechanism of IFN and cytokine production, we performed RT^2 profiler array assays to analyze important TLR-activated genes (84 target genes were included in the RT^2 profiler array), that could be modulated by

TLR-signaling, from HBeAg-positive HepG2. Expression profiling showed more than twofold inhibition of nine genes compared to that of HBeAg-negative HepG2 cells (TLR7, LY96, RIPK2, NFκB1, TNF, IL-6, IL-8, IFN-β, and MAP3K1) (Fig. 2A). To confirm these results, real-time PCR was performed. All of these genes except NF-κB1 were significantly downregulated in HBeAg-positive cells compared to HBeAg-negative cells. All of these genes have important roles in the immune response and activation of transcription (Fig. 2B).

Effects of HBeAg on NF-κB activation

Next, we assessed the mechanisms by which HBeAg affects cytokine and IFN production. HBV activates NF-κB, a major player in innate immune responses to viral infections (19). Therefore, we postulated that HBeAg inhibition of the activation of NF-κB might result in the inhibition of cytokine and IFN production, and the subsequent escape of an antiviral response. To test this assumption, we expressed luciferase reporter protein under the control of an NF-κB-dependent promoter in HBeAg-positive or HBeAg-negative HepG2 with or without TNF-α or LPS stimulation (Fig. 3A–E). As expected, HBeAg inhibited NF-κB promoter activity in HBeAg-positive HepG2 cells (Fig. 3A–C; $p < 0.001$ with no drug [n = 3]; $p = 0.004$ with LPS [n = 3]; $p = 0.0075$ with TNF [n = 3]). These results were also confirmed by the transient HBeAg-expression assay in HepG2 ($p = 0.038$, n = 3) and Huh7 cells ($p = 0.0091$, n = 3) (Fig. 3D and E). These findings suggest that HBeAg may affect cytokine production, at least in part, through NF-κB.

Effects of HBeAg on IFN-β activation

NF-κB stimulation leads to the expression of multiple cellular factors, including IFN-β, a central player in the innate immune response that is activated upon virus infection. In order to ascertain whether HBeAg inhibits IFN-β-promoters, we performed experiments using IFN-β-promoter luciferase reporter, essentially as described in the previous section. That is, we used the luciferase gene under the control of an IFN-β-stimulated promoter, and examined its expression in HBeAg-positive and HBeAg-negative HepG2 cells. HBeAg inhibited IFN-β-stimulated promoter activity in HBeAg-positive HepG2 cells with ($p < 0.001$, n = 3), or without poly(I-C) ($p < 0.001$, n = 3) (Fig. 3F and G). We also confirmed these results by transient transfection experiments with HepG2 ($p = 0.0011$, n = 3), and with Huh7 ($p = 0.0081$, n = 3) (Fig. 3H and I). These results demonstrated that HBeAg inhibits both NF-κB- and IFN-β-signaling pathways in hepatocytes.

Cell culture fluid from HBeAg-positive HepG2 enhanced HCV subgenomic RNA replication

To confirm the function of IFN production of these cell lines, we examined whether conditioned media from HBeAg-positive or HBeAg-negative HepG2 cells would cause any differences in HCV subgenomic RNA replication, which is IFN-sensitive replication (14), as it has been reported that there are no direct interactions between HBV and HCV replication in cell culture models and in a mouse study (1,9,11). Cell culture fluid from HBeAg-positive HepG2 cells enhanced HCV subgenomic RNA replication, more than that from HBeAg-negative HepG2 cells (Fig. 3J; $p = 0.0014$, n = 3), suggesting that HBeAg-expressing HepG2 cells contain less IFN than do HBeAg-negative cells, and that conditioned medium from HBeAg-positive HepG2 cells contains less IFN than that from HBeAg-negative cells. In this system, when we treated cells with 0, 1, 10, 100, and 1000 U/mL IFN-α, HCV subgenomic RNA levels were 100%, 57%, 39%, 28%, and 25%, respectively. We estimated that conditioned medium from HBeAg-negative HepG2 cells was equal to ~10 IU/mL IFN-α. Our results showed that HBeAg inhibits IFN production in cell culture medium.

Since the NF-κB target gene IL-6 has also been implicated in hepatitis B pathogenesis (30), the modulation of IL-6 involved in innate signaling by HBeAg was also verified at the protein level by ELISA. Our results demonstrated that IL-6 expression was downregulated in HBeAg-positive HepG2 cells (36.6 ± 30.1 pg/mL; 0 ± 0 pg/mL in conditioned medium from HepG2 control cells; 324.2 ± 15 pg/mL in conditioned medium from HBeAg-negative HepG2 cells). The concentration of IL-6 from HBeAg-positive HepG2 cells was significantly lower than that from HBeAg-negative HepG2 cells ($p = 0.00012$, n = 3).

Discussion

In this study, we investigated the regulation of HBeAg-induced suppression of IFN and cytokines in HepG2 stably expressing HBeAg protein as a model cell line. Our results demonstrated that HBeAg expression inhibits IFN and cytokine production. Transient expression of HBeAg also downregulated both NF-κB- and IFN-β-promoter activity in HepG2 or Huh7, although the mechanisms for this downregulation are unknown. In contrast to our findings, Yang *et al.* (46) observed that HBeAg activates NF-κB through IκBα degradation, and produces TNF-α and GM-CSF in the human hepatoma cell HA22T/VGH. These differences between their findings and ours may have been caused by the differences in the cell lines, and/or promoters (33). Extensive immunological studies by the Milich group (3,4,27) demonstrated that HBeAg appears more efficient at eliciting T-cell tolerance, including production of its specific cytokines IL-2 and IFN-γ, than HBV core antigen. Our observations support the immune-modulating role of HBeAg.

Locarnini *et al.* (23) used the Tet-off tetracycline gene expression system in Huh7, and revealed that core/precore expression affected gene expression, including cytokines. The system used in our present study, with HepG2 stably expressing HBeAg, supports these findings. Our results provide further direct evidence that hepatocytes exposed to HBeAg have enhanced HCV subgenomic RNA replication, and are significantly influenced in their ability to replicate. Several recent reports have also suggested that there was no evidence of direct interaction between HBV and HCV (1,9,11), although clinical studies showed interaction between HBV and HCV replication (24). It is possible that HBV might interfere with another virus by IFN or another cytokine. A cytokine response is critical for clearance of HCV, as failure to mount a potent and broad T-cell-repertoire response results in persistent HCV replication. This would explain how patients dual-infected with HBV and HCV exhibit a selective deficit of anti-HCV immunity, while demonstrating preservation of a normal immune response to unrelated antigens.

We used RT-PCR to observe the expression of TLRs 1, 3, 4, 5, 6, and 7 in HepG2 cells. We also confirmed in the present study that HepG2 has functional TLRs 3 and 4. Preiss *et al.*

(32) could not detect an NF-κB response to 1 ng/mL–1 μg/mL LPS in HepG2, whereas we could detect such a response to 10–50 μg/mL LPS (Table 2). Downregulation of TLR2 mRNA by genotype C HBV-derived HBeAg was not observed in our study, in contrast to the results of a previous study (43), in which genotype D HBV-derived clone (23) was used. Xu *et al.* (45) reported that TLR7 was suppressed in HBV infection, supporting our results. We do not know why LY96, an important molecule for TLR4, is downregulated (Fig. 2). Viruses encode proteins that target various intracellular signaling pathways, causing their constitutive or prolonged activation, resulting in increased cell proliferation and survival (41). It is well known that HBV activates the MAPK pathway (5). It is also known that RIPK2 activates the NF-κB- and IFN-β-dependent antiviral responses (8). These findings were in accordance with HBeAg's inhibition of the production of IFNs and cytokines (Fig. 2).

What is the mechanism of the downregulation of cytokine production by HBeAg? From our results (Fig. 2), HBeAg appears to interact with the TLR signaling pathway upstream of NF-κB. In LPS stimulation, we observed downregulated TLR4 in HBeAg-positive HepG2 cells (data not shown). Although we are currently investigating this issue, TLR4 might be one of the more important molecules. Precore protein also may affect intracellular signal transduction pathways. Further studies will be needed to clear up these issues.

Many viruses have evolved strategies that block the effector mechanisms induced through IFN- and/or cytokine-signaling pathways (17). Although multiple mechanisms contribute to viral persistence, the ability of the virus to evade innate immune responses is likely to be particularly important. In this report, we have demonstrated that HBeAg suppresses IFN and cytokine mRNA expression. Exploration of the novel HBeAg-inhibiting signaling pathways could lead to the development of new therapeutic strategies for persistent HBV infection.

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Author Disclosure Statement

The authors have no conflicts with regard to financial interests. This material has not been previously reported and is not under consideration for publication elsewhere.

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SHORT REPORT Open Access

Inhibitory effects on HAV IRES-mediated translation and replication by a combination of amantadine and interferon-alpha

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Abstract

Hepatitis A virus (HAV) causes acute hepatitis and sometimes leads to fulminant hepatitis. Amantadine is a tricyclic symmetric amine that inhibits the replication of many DNA and RNA viruses. Amantadine was reported to suppress HAV replication, and the efficacy of amantadine was exhibited in its inhibition of the internal ribosomal entry site (IRES) activities of HAV. Interferon (IFN) also has an antiviral effect through the induction of IFN stimulated genes (ISG) and the degradation of viral RNA. To explore the mechanism of the suppression of HAV replication, we examined the effects of the combination of amantadine and IFN-alpha on HAV IRES-mediated translation, HAV replicon replication in human hepatoma cell lines, and HAV KRM003 genotype IIIB strain replication in African green monkey kidney cell GL37. IFN-alpha seems to have no additive effect on HAV IRES-mediated translation inhibition by amantadine. However, suppressions of HAV replicon and HAV replication were stronger with the combination than with amantadine alone. In conclusion, amantadine, in combination of IFN-alpha, might have a beneficial effect in some patients with acute hepatitis A.

Short report

Hepatitis A virus (HAV), a member of the family Picornaviridae, causes acute hepatitis and occasionally fulminant hepatitis, a life-threatening disease. As the broad epidemiological picture of hepatitis A changes, the public health importance of this disease is being increasingly recognized [1]. It is a significant cause of morbidity worldwide, although the mortality rate due to hepatitis A is low (improved intensive care and transplantation have contributed to a reduction in deaths). Improved sanitation and living standards mean that fewer countries remain highly endemic, but the risk of HAV infection is present in countries lacking HAV immunity or where the endemicity of hepatitis A is low or intermediate [1]. In such situations, these outbreaks can prove to be long and difficult to control. Vaccination and informing the general public about good hygienic measures are important for the prevention of HAV infection, but new therapeutic options are also desirable.

Amantadine, a tricyclic symmetric amine, inhibits HAV replication *in vitro* [2]. We previously reported that amantadine inhibits hepatitis A virus internal ribosomal entry site (IRES)-mediated translation in human hepatoma cells [2]. Interferons (IFNs) also exhibit antiviral effects against HAV infection [2,3]. In the present study, we examined the effects of amantadine with or without IFN-alpha, on HAV IRES activities, HAV subgenomic replicon replication and HAV replication *in vitro* as a proof of concept for the development of a more effective treatment to control HAV infection.

First, we evaluated the cytotoxicity of amantadine and IFN-alpha by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. Amantadine concentrations in a range of 1 - 125 μg/mL and those of 1 - 150 μg/mL for 12-h incubation were non-toxic for Huh7 cells and for HuhT7 cells, respectively (Figures 1A and 1B). Amantadine could be incubated for a short time, e.g., 12 h, with the cells, and then the dose of amantadine could be

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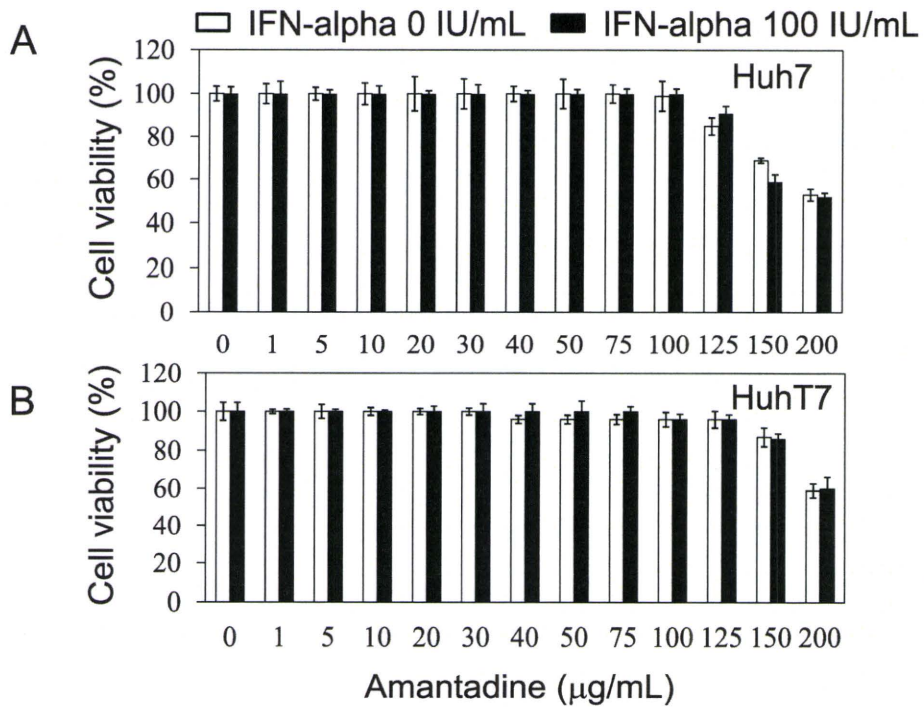

Figure 1 Effects of amantadine on cell growth and viability. MTS assays of cells 12 h after treatment with amantadine with or without 100 U/mL interferon (IFN)-alpha. (A) Huh7 cells. (B) HuhT7 cells. Data are expressed as mean ± SD.

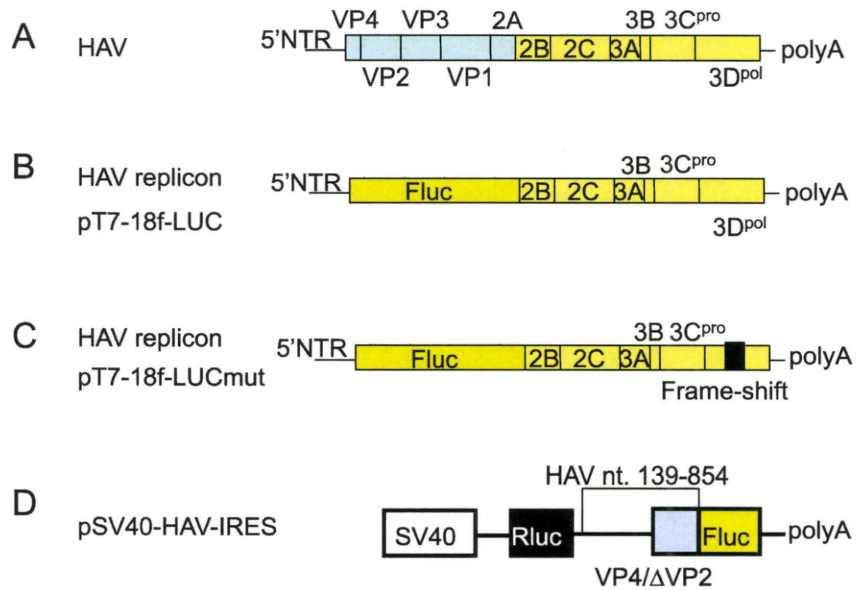

Figure 2 Structures of reporter constructs used in this study. (A) Structure of HAV genome. **(B)** Structure of the replication-competent HAV replicon (HAV replicon) pT7-18f-LUC, which contains an open-reading frame of firefly luciferase (Fluc) flanked by the first four amino acids of HAV polyprotein and by 12 C-terminal amino acids of VP1. This segment is followed by P2 and P3 domains of HAV polyprotein (HAV strain HM175 18f) [9,10]. **(C)** Structure of replication-incompetent HAV replicon (mut) (mut-HAV replicon) pT7-18f-LUCmut, which contains a frame-shift mutation in the polymerase 3 D [9,10]. **(D)** Bicistronic reporter constructs: pSV40-HAV IRES was described previously [2,4]. It encodes the Renilla luciferase genes (Rluc), the internal ribosomal entry site (IRES) HAV HM175, and the firefly luciferase gene (Fluc) under the control of the simian virus 40 promoter (SV40).

increased to higher than 100 μg/mL. With the combination of amantadine and 100 IU/mL IFN-alpha, we did not observe increased cytotoxicity compared with amantadine alone.

We previously reported that the introduction of siRNA targeted against the 5'NTR region of HAV HM175 inhibits HAV IRES-mediated translation and HAV replication [4]. Interestingly, amantadine and IFN also inhibited HAV IRES-mediated translation and HAV replication [2,3,5-8]. Accordingly, we planned to identify more effective strategies for suppressing HAV IRES-mediated translation and HAV replication. IRES is an attractive target for antivirals because HAV IRES is located in the 5'NTR region, the most conserved region among HAV strains. In the present study, we evaluated the HAV antiviral activity of amantadine and IFN-alpha. We initially examined the effects of this combination on HAV IRES-mediated translation using a luciferase reporter assay. Huh7 cells were transfected with pSV40-HAV IRES reporter vector, encoding SV40 promoter driven-*Renilla reniformis* and firefly luciferase, separated by HAV-IRES (Figure 2) [2], and treated with amantadine and/or IFN-alpha. Inhibition of luciferase activity at different levels was observed with amantadine with or without 100 IU/mL IFN-alpha (Figure 3A). Although the strongest suppression was noted with the combination of 10 μg/mL amantadine and 100 IU/mL IFN-alpha, IFN-alpha showed no additive effect on the translation inhibition by 50-100 μg/mL amantadine. This finding prompted us to examine whether IFN-alpha has additive suppression of HAV replicon replication by amantadine. We have reported that RNA replication of HAV can be analyzed in a DNA-based replicon system using HuhT7 cells that stably express T7-RNA polymerase in the cytoplasm (Figure 1) [9-11]. The luciferase activities determined after transfection of replicon DNA are a direct measure of RNA translation and replication. This is because replication in positive-stranded RNA viruses can be easily assessed with a viral replicon carrying the luciferase gene in place of viral structural genes. Moreover, luciferase activity due to translation or translation and replication can be evaluated when the transfection of a replication-competent replicon (HAV replicon) is compared with that of a replication-incompetent replicon (mut) (mut-HAV replicon) [8].

To further determine the effects of the combination of amantadine and IFN-alpha on HAV replication, we transfected the HAV replicon or mut-HAV replicon into HuhT7 cells, and the drugs were added 24 h later. Reporter assays were performed 48 or 72 h after transfection. The transfection efficacy of HAV replicon was estimated as 20-30% in our systems. Luciferase activity was normalized with respect to the protein concentration of cell

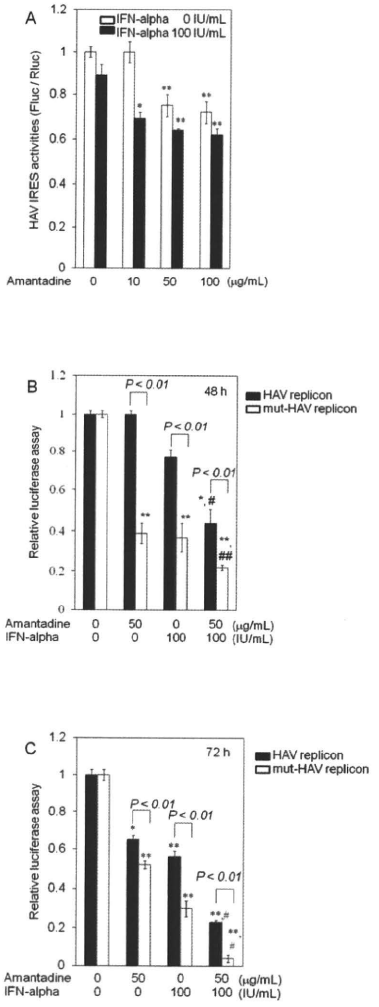

Figure 3 (A) Effects of amantadine with or without interferon on the hepatitis A virus (HAV) internal ribosomal entry site (IRES) activities in Huh7 cells. Approximately 2 × 10^5 cells were seeded on a 6-well tissue culture plate (Iwaki Glass, Tokyo, Japan) 24 h prior to transfection. pSV40-HAV-IRES (0.3 μg) was transfected into Huh7 cells using the Effectene transfection reagent (Qiagen, Tokyo, Japan). 24 h after transfection, amantadine and/or IFN in various concentrations was added to cells. 48 h after transfection, cell extracts were prepared, and luciferase assays were performed using the Dual Luciferase assay system (Toyo Ink, Tokyo, Japan) according to the manufacturer's instructions [2]. For controlling the variations in transcription, IRES activity was assessed by measuring the ratio of *Renilla* and firefly luciferases. All samples were run in triplicate. *Renilla* and firefly luciferase activities were measured as relative light units using a luminescencer (JNRII-AB-2300; ATTO, Tokyo, Japan). **(B, C) Effects of amantadine with or without interferon on the HAV subgenomic replicon replication in HuhT7 cells. (B)** 48 h after transfection and **(C)** 72 h after transfection. Black columns, replication-competent HAV replicon; white columns, replication-incompetent HAV replicon (mut). Relative luciferase activities without any treatments were set at 1. Data are expressed as mean (columns) ± SD (vertical lines). *$P < 0.05$ and **$P < 0.01$, compared with untreated control by Student's t test. #$P < 0.01$ and ## $P < 0.05$, compared with amantadine alone or IFN-alpha alone by Student's t test.

lysates. In this DNA-based system, 48 h after transfection, the replication rates of the HAV replicon were 100%, 77%, and 44% compared to those of control when treated with amantadine alone, IFN alone, and their combination, respectively (Figure 3B). On the other hand, since the mut-HAV replicon cannot replicate, the luciferase activity (39%, 37%, and 22% compared to those of control for the same test conditions, respectively) is due to translation of the viral RNA and not replication. Amantadine alone showed 52% at 72 h, higher than 37% at 48 h, supporting the notion that amantadine might suppress translation of the viral RNA. Suppression effects of these treatments were stronger in the mut-HAV replicon than in the HAV replicon. These findings support our observation of the suppression of HAV IRES-mediated translation by amantadine and IFN-alpha. Suppression effects at 48 h after transfection by the combination of amantadine and IFN-alpha against HAV replication were stronger than those by amantadine or IFN-alpha monotreatment. IFN-alpha was more effective than amantadine against the HAV replicon ($P = 0.0027$) (Figure 3B).

Seventy-two hours after transfection, the replication rates of the HAV replicon were 65%, 56%, and 23% compared to those of control when treated with amantadine alone, IFN-alpha alone, and their combination, respectively (Figure 3C). The replication rates of the mut-HAV replicon were 52%, 30%, and 4% of those of control, respectively. IFN-alpha was more effective than amantadine against the replication of HAV replicon or mut-HAV replicon ($P < 0.001$ or $P < 0.001$). Suppression effects of the combination of amantadine and IFN-alpha at 72 h post-transfection were stronger than those of amantadine or IFN-alpha monotreatment. Suppression effects of these treatments were stronger in the mut-HAV replicon than in the HAV replicon. Moreover, it is important to note that the effects of this combination were observed at earlier time points (Figure 3C).

Next, we performed an infectivity assay using the virus to investigate the effects of combination of amantadine and IFN-alpha on tissue culture-adapted HAV strain KRM003 (genotype IIIB, accession no. L20536) propagation in African green monkey kidney GL37 cells [12-14]. GL37 cell monolayers in 96-well culture plates were infected with HAV at a multiplicity of infection (MOI) of 5 or 50 for 1 h at 37°C in a CO_2 incubator. Without removing the inoculum, drug-containing media were added to appropriate wells. The final concentrations of amantadine, IFN-alpha, and their combination were 50 μg/ml, 100 IU/ml and 50 μg/ml of amantadine and 100 IU/ml of IFN-alpha, respectively. After incubation for 72 h, infected cells were evaluated with ELISA. Suppression of HAV replication by the combination of amantadine and IFN-alpha was stronger than those of amantadine alone, IFN-alpha alone, and untreated control (Figure 4).

IFNs are proteins induced by lymphocytes and other cells including hepatocytes in response to viruses such as HAV. In virus-infected cells, dsRNA activates antiviral interferon pathways and the production of IFN type I. The secreted IFN type I induces a positive feedback loop that results in the expression of interferon-stimulated genes (ISGs), including RNase L and protein kinase R (PKR) [15]. Our study supports the fact that the administration of IFN-alpha suppresses HAV replication through HAV IRES mediated-translation and other mechanisms and that, on the other hand, amantadine suppresses HAV replication mainly through HAV IRES mediated-translation.

There are several reports concerning HAV suppressing intracellular dsRNA-induced retinoic acid-inducible gene I (RIG-I)-mediated IFN regulatory factor 3 (IRF-3) activation to block induction of IFN [16,17]. Yang et al. reported that HAV proteins interact with mitochondrial antiviral signaling protein, an essential component of virus-activated signaling pathways that induce protective IFN responses [18]. However, in this study, the

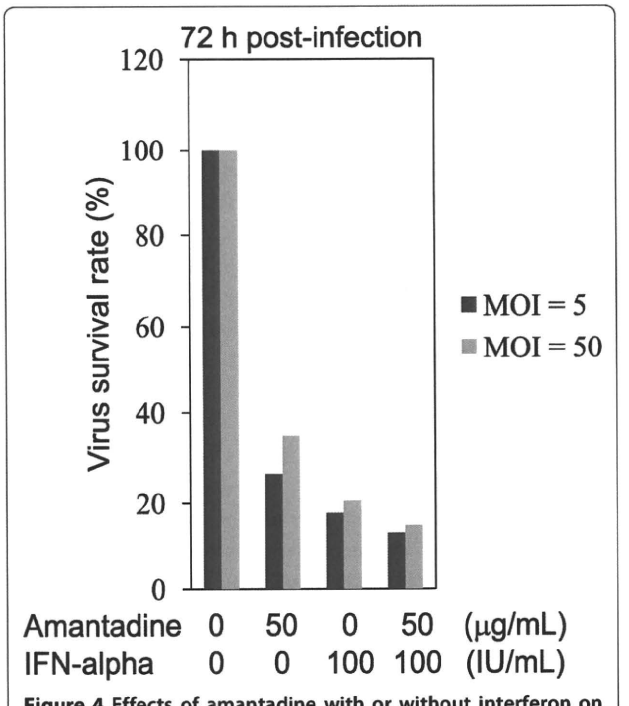

Figure 4 Effects of amantadine with or without interferon on HAV KRM003 genotype IIIB strain replication in African green monkey kidney cell GL37. GL37 cell monolayers in 96-well culture plates were infected with HAV [at a multiplicity of infection (MOI) of 5 or 40] for 1 h at 37°C in a CO_2 incubator. Amantadine and/or IFN was added to cells. After the incubation for 72 h, infected cells were evaluated with ELISA. The rate of virus survival was measured using this equation: Virus survival rate (%) = 100 × Absorbance with drug/Absorbance without drug.

administration of exogenous IFN-alpha could suppress HAV replication, although endogenous IFNs produced by cells also may play an important role in inhibiting viral replication. Further studies will be needed.

Amantadine inhibits the replication of many DNA and RNA viruses and is also used as a drug for the treatment of Parkinson's disease [2]. It is known that the M2 protein of influenza A virus is a target of amantadine [19]. Furthermore, it has been reported to inhibit HAV IRES-mediated translation and replication by our group and other researchers [2,3,5-8].

Therefore, we examined the possibilities of the combination of amantadine and IFN-alpha against HAV because these two drugs were previously reported to be effective against HAV [2,3,5-8]. To our knowledge, this is the first study demonstrating that a combination of amantadine and IFN-alpha can suppress HAV replication more effectively than amantadine or IFN-alpha alone.

Abbreviations

HAV: hepatitis A virus; **IRES**: internal ribosomal entry site; **IFN**: interferon; **MTS**: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.

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Authors' contributions

LY, Tatsuo Kanda, FI and OY conceived and designed the study. LY, Tomoko Kiyohara and Tatsuo Kanda performed the experiments. LY, Tomoko Kiyohara, Tatsuo Kanda and FI analyzed data and wrote the manuscript. Tomoko Kiyohara, KI and TW contributed to experiments using a whole HAV virus. Tomoko Kiyohara, Tatsuo Kanda and VG contributed to the interpretation of the interpretation of the results and took part to the critical revision of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Hepatitis A virus (HAV) proteinase 3C inhibits HAV IRES-dependent translation and cleaves the polypyrimidine tract-binding protein

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SUMMARY. Hepatitis A virus (HAV) infection is still an important issue worldwide. A distinct set of viruses encode proteins that enhance viral cap-independent translation initiation driven by an internal ribosome entry site (IRES) and suppress cap-dependent host translation. Unlike cytolytic picornaviruses, replication of HAV does not cause host cell shut off, and it has been questioned whether HAV proteins interfere with its own and/or host translation. HAV proteins were coexpressed in Huh-7 cells with reporter genes whose translation was initiated by either cap-dependent or cap-independent mechanisms. Among the proteins tested, HAV proteinase 3C suppressed viral IRES-dependent translation. Furthermore, 3C cleaved the polypyrimidine tract-binding protein (PTB) whose interaction with the HAV IRES had been demonstrated previously. The combined results suggest that 3C-mediated cleavage of PTB might be involved in down-regulation of viral translation to give way to subsequent viral genome replication.

Keywords: 3C protease, hepatitis A virus, IRES, PTB, translation.

INTRODUCTION

The messenger-sense RNA genome of hepatitis A virus (HAV) is about 7500 nucleotides in length and contains a single large open-reading frame (ORF) encoding a polyprotein with the capsid proteins representing the amino-terminal third and the remainder comprising a series of nonstructural proteins required for viral RNA replication: 2B, 2C, 3A, 3B, $3C^{pro}$ (cysteine proteinase responsible for most post-translational cleavage events within the polyprotein) and $3D^{pol}$ (RNA-dependent RNA polymerase, see Fig. 1a, top panel) [1]. In a regulated cascade, the viral polyprotein is cleaved by $3C^{pro}$ into intermediate and mature products that fulfill distinct functions in the viral life cycle. At both ends of the picornaviral genome, the ORF is flanked by highly structured nontranslated regions (5′NTR and 3′NTR). The down-stream part of the 5′NTR presents an internal ribosome entry site (IRES) that allows translation by a cap-independent mechanism [1–3]. Several IRES trans-acting factors (ITAF) have been identified as mediating IRES binding to the ribosome [4]. Whereas glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and La autoantigen suppress HAV IRES activity, the poly(C)-binding protein (PCBP) and the polypyrimidine tract-binding protein (PTB) were found to enhance HAV translation [3,5–9]. PTB, a 57-kDa protein, is a member of the heterogeneous nuclear ribonucleoprotein family that shuttles between the nucleus and cytoplasm [10]. While experimental data have demonstrated PTB binding to polypyrimidine tracts (UCUUU or UCUUC) in picornaviral IRES, the exact cellular functions of PTB are as yet incompletely defined [3,10,11].

Proteolytic cleavage of host proteins is a common mechanism executed by picornaviruses to shut off host cell protein synthesis and to regulate viral protein and RNA synthesis. These two synthetic processes are central in the viral life cycle and mutually exclusive on the same RNA template. As HAV does not shut off host protein synthesis, it seems that HAV cap-independent translation constantly

Abbreviations: GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; HAV, Hepatitis A virus; IRES, internal ribosome entry site; ITAF, IRES trans-acting factors; ORF, open-reading frame; PABP, poly(A)-binding protein; PCBP, poly(C)-binding protein; PTB, polypyrimidine tract-binding protein.

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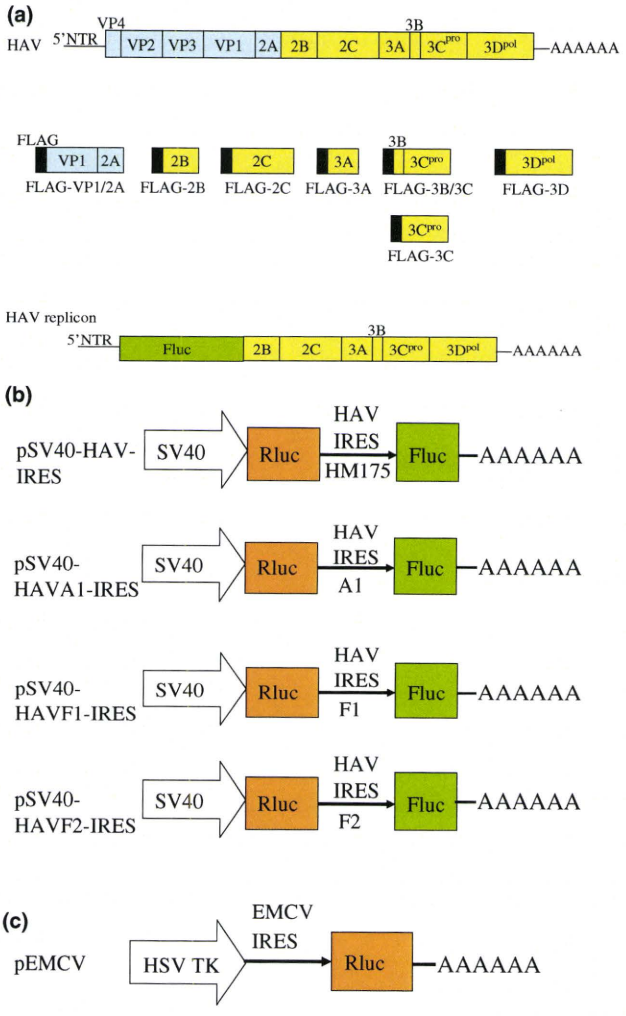

Fig. 1 Schematic representation of the hepatitis A virus (HAV) constructs used in this study. (a) Structures of the HAV genome (upper panel), FLAG-tagged HAV proteins (middle panel; ref. 17) and HAV replicon pT7-18f-luciferase (lower panel; ref. 13). AAAAAA, poly A tail. (b) Structure of the bicistronic plasmids used. pSV40-HAV-internal ribosome entry site (IRES) encodes the Renilla luciferase, the IRES of HAV strain HM175 and the firefly luciferase (Fluc) under the control of the simian virus 40 promoter (SV40) (ref. 16). pSV40-HAVA1-IRES, pSV40-HAVF1-IRES and pSV40-HAVF2-IRES encode IRES elements derived from an acute hepatitis and two fulminant hepatitis cases, respectively. (c) Structure of plasmid pEMCV.

competes with cap-dependent translation of host proteins [1,12]. In this study, we show that HAV $3C^{pro}$ cleaved PTB and suppressed cap-independent translation initiation. The data indicate that the viral proteinase might play an important role in the regulation of HAV IRES-mediated cap-independent translation by targeting noncanonical translation factors.

MATERIALS AND METHODS

Cell lines

Huh-7, a human hepatoma cell line, and its stably transformed derivative Huh-T7 that expresses the T7 RNA polymerase [3] were grown in Dulbecco's modified Eagle medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated foetal bovine serum with or without G418 sulfate (400 μg/mL; Promega, Madison, WI, USA), in addition to penicillin and streptomycin.

Plasmids

pT7-18f-luciferase (LUC), a replication-competent HAV replicon, containing an open-reading frame with the firefly luciferase (Fluc) flanked by the first four amino acids of the HAV polyprotein and by 12 C-terminal amino acids of VP1, followed by the P2 and P3 domains of the HAV polyprotein (HAV strain HM175 18f, GenBank Accession No. M59808), and pT7-18f-LUCmut, a replication-deficient replicon, were described previously [13] (Fig. 1a).

The constructs encoding the simian virus 40 (SV40) promoter-driven *Renilla reniformis* luciferase (Rluc), the IRES derived from the cell culture adapted HAV strain HM175 [14], and Fluc, named pSV40-HAV-IRES, was prepared as described previously [2,15] (Fig. 1b). To investigate the specific effect exerted by the HAV IRES sequences, bicistronic reporter constructs (pSV40-HAVA1-IRES, pSV40-HAVF1-IRES and pSV40-HAVF2-IRES; Kanda *et al.*, manuscript in preparation) were prepared, which included the IRES of clinical specimens. Construction of HAV protein expression plasmids was described previously [16]. Briefly, seven regions of the HAV genome were amplified by reverse transcription-polymerase chain reaction (PCR) with HAV region-specific primers [16]. These regions were HAV VP1-2A, 2B, 2C, 3A, 3BC, 3C and 3D. The amplified products were cloned into pCXN2 (kindly provided by Prof. J. Miyazaki, Osaka University, Japan), a mammalian expression vector with a beta-actin-based CAG promoter and a SV40 origin [17], to generate pCXN2-FLAG-VP1-2A, 2B, 2C, 3A, 3BC, 3C, 3D expressing FLAG-tagged proteins [16] (Fig. 1a). To control for the target specificity, pEMCV, which contains the encephalomyocarditis virus (EMCV) IRES upstream of Rluc, was generated (Fig. 1c). Transient expression of $3C^{pro}$ using vaccinia virus, pGEM-3C, and pEXT7-HAV3C was described before [12].

Transfection and protein analyses

Approximately 60% confluent Huh-7 cells, grown in 6-well culture plates, were transfected with 0.3 μg of the LUC reporter plasmid and 0.1 μg of each HAV protein-expressing plasmid using Effectene transfection reagent (Qiagen, Tokyo, Japan). Forty-eight hours after transfection, cell

extracts were prepared, and a LUC assay kit (Toyo Ink, Tokyo, Japan) was used according to the manufacturer's instructions. LUC activity was measured in relative light units with a luminometer (AB-2200-R; ATTO, Tokyo, Japan). The assays were adjusted to protein amount and were conducted, on average, in duplicate [18]. To determine cleavage of the host proteins PTB and poly(A)-binding protein (PABP), extracts of transfected cells were analysed for viral antigen and host proteins, as described previously [12]. Viral proteins were identified using anti-FLAG and anti-HAV 3C antibodies. PTB was recognized by the monoclonal antibody BB7 [19].

RESULTS

HAV proteinases 3BC and 3C suppress IRES-dependent translation

Translation of the HAV polyprotein is initiated cap-independently and is driven by an IRES. As a first approach to assess the role of HAV proteins, we examined cap-independent and cap-dependent translation using the bicistronic reporter constructs depicted in Fig. 1. pSV40-HAV-IRES, which contains the IRES of HAV strain HM175 (Fig. 1b), was transfected into Huh-7 together with various expression vectors encoding FLAG-tagged HAV protein (Fig. 1a). The expression of these proteins was confirmed by Western blotting with anti-FLAG antibodies (data not shown and ref. 16). Compared to the control (pCXN2) and to the other HAV proteins tested (VP1-2A, 2B, 2C, 3A and 3D), expression of HAV 3BC or 3C specifically inhibited cap-independent translation initiated by the HAV IRES as determined by the Fluc activity (Fig. 2).

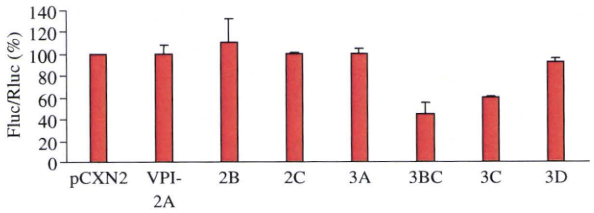

Fig. 2 Effects of hepatitis A virus (HAV) proteins on HAV internal ribosome entry site (IRES)-dependent translation. Relative luciferase activities are indicated (IRES/Cap; firefly luciferase/*Renilla reniformis* luciferase, %). Luciferase activities were determined in three independent experiments. Error bars represent standard errors of the mean.

To corroborate the observed suppression of HAV IRES-independent translation, we next examined the effect of $3C^{pro}$ on translation, which was dependent on HAV IRES elements derived from clinical isolates; IRES A1 was taken from an acute self-limited hepatitis (pSV40-HAVA1-IRES), and F1 and F2 were derived from fulminant HAV infections (pSV40-HAVF1-IRES and pSV40-HAVF2-IRES) (Fig. 3a–c). After coexpression of pSV40-HAVA1-IRES, pSV40-HAVF1-IRES and pSV40-HAVF2-IRES with 3BC or $3C^{pro}$, the Fluc activity was specifically suppressed when compared to the control (pCXN2, Fig. 3a–c). The results confirm our findings shown in Fig. 2 and demonstrate that HAV proteinases 3BC and $3C^{pro}$ suppress HAV IRES-dependent translation. For yet unknown reasons, the negative effect of 3BC was generally more pronounced than that exerted by $3C^{pro}$. However, as $3C^{pro}$ is the prevailing and stable form of the viral proteinase, only this form was used in the subsequent studies.

Translation of the viral polyprotein is the first metabolic step in the viral life cycle and a prerequisite for viral RNA synthesis. It can be assumed that a negative effect on

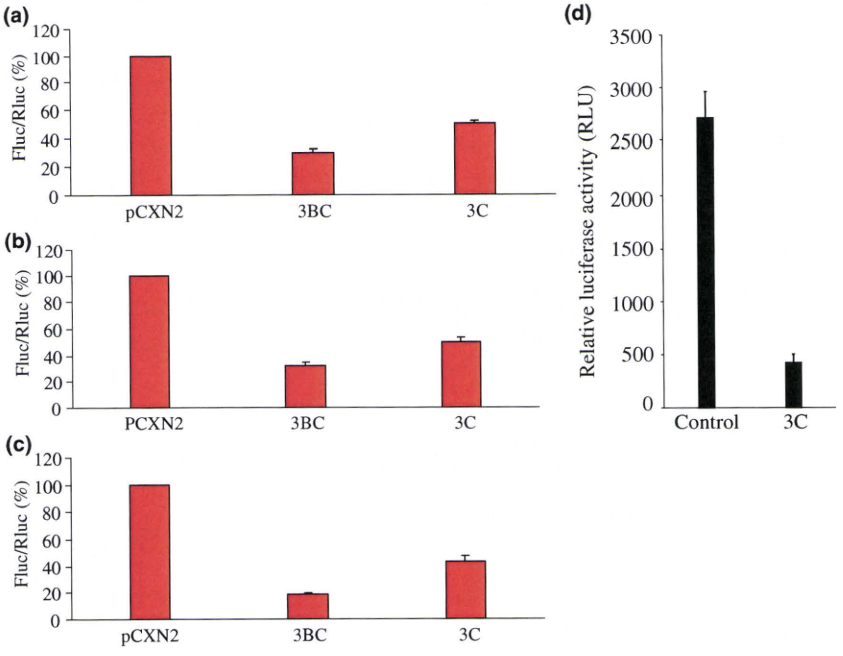

Fig. 3 Effects of hepatitis A virus (HAV) 3BC or $3C^{pro}$ on HAV internal ribosome entry site (IRES)-dependent translation (a–c) and on expression of the HAV replicon [pT7-18f-luciferase (LUC)] (d). HAV was derived from clinical isolates: (a) acute hepatitis; (b) and (c) two forms of fulminant hepatitis. Relative luciferase activities are indicated (IRES/Cap; firefly luciferase/*Renilla reniformis* luciferase, %) (a–c). LUC activities are presented as an average of three independent experiments. Error bars represent standard errors of the mean. RLU, relative light units.

translation might indirectly cause a reduction in viral genome production. HAV replication was efficiently studied using the viral replicon (see Fig. 1a, lower panel) with Fluc as reporter gene in place of the viral structural proteins [13]. The reporter gene activity is directly proportional to viral RNA synthesis. To investigate whether 3C-mediated suppression of translation affects genome replication, we cotransfected HAV replicon RNA with the 3C-expression or control vector into Huh-T7 cells (Fig. 3d). HAV replicon replication was monitored by reporter assay 72 h post-transfection. Compared to the replication-deficient replicon (pT7-18f-LUCmut), the reporter activity at this point was derived from newly synthesized viral genomes and therefore represents viral genome synthesis [13]. Compared to the control, HAV replication was significantly suppressed in the presence of excess 3C, indicating that 3C-mediated inhibition of translation restrained HAV genome replication in human hepatoma cells.

To assess the specificity of the inhibitory effect exerted by HAV proteinase $3C^{pro}$, translation initiated at the EMCV IRES was compared with the HAV IRES. For this, HAV $3C^{pro}$ was coexpressed with pEMCV (Fig. 1c), and the Rluc activity of the cell extracts collected 48 h post-transfection was determined. Compared to the HAV IRES tested in parallel experiments, the EMCV IRES activity was similar in the presence and absence of coexpressed HAV $3C^{pro}$ [118 ± 29 (%)]. Combined and in light of the results described in the following, these findings suggest that an essential ITAF was cleaved by HAV $3C^{pro}$. As shown in the following, HAV $3C^{pro}$ partially cleaved PTB, whose active role in picornaviral IRES-dependent translation has been demonstrated previously [6,8]. As EMCV IRES translation was unaffected by HAV $3C^{pro}$, PTB is not an essential ITAF for this IRES, confirming an earlier report [20]. Intriguingly, an excess of PTB even suppressed EMCV IRES-driven translation [21]. The abundance and distribution of PTB varies significantly among cell types [6,8]. Large amounts of PTB were found in the cytoplasmic fraction of Huh-7 cells that were used in our studies. Based on these observations, the results reported here suggest that HAV $3C^{pro}$ reduced the cytoplasmic levels of intact PTB to such a degree that only the activity of the HAV IRES was affected, but not that of the EMCV IRES.

$3C^{pro}$ cleaves PTB

It has been reported that the HAV IRES is associated with La autoantigen, GAPDH, PTB, PABP and PCBP [6,8,12–14]. The latter two proteins were cleaved by HAV $3C^{pro}$ [12,13]. Furthermore, it was shown that PTB is cleaved by polioviral $3C^{pro}$ and that PTB fragments inhibit polioviral IRES-dependent translation [22]. To assess whether the observed suppression of HAV IRES translation might be because of 3C-mediated cleavage of PTB, we tested the levels of endogenous PTB after transient expression of $3C^{pro}$ in Huh-7 cells. As GAPDH was found to suppress HAV IRES translation and to antagonize the enhancing effect of PTB [8], GAPDH levels were tested in parallel. As control for the proteolytic activity of $3C^{pro}$ *in vivo*, cleavage of the poly(A)-binding protein was also analysed. Recombinant $3C^{pro}$ was identified by immunoblot with anti-3C (Fig. 4, left panel) [12,13], and PABP was partially cleaved as demonstrated earlier (Fig. 4, middle panel). Whereas the levels of GAPDH were unchanged (data not shown), a PTB cleavage product of approximately 45 kDa and a slightly faster migrating polypeptide were clearly detectable when HAV $3C^{pro}$ was expressed (Fig. 4, right panel). The extent of host protein cleavage significantly depended on the amount of 3C expressed (compare lanes 1 and 3). Specific PTB cleavage was also observed when the extracts used in Fig. 2 were tested (not shown). Moreover, PTB of Huh-7 cells, the rabbit

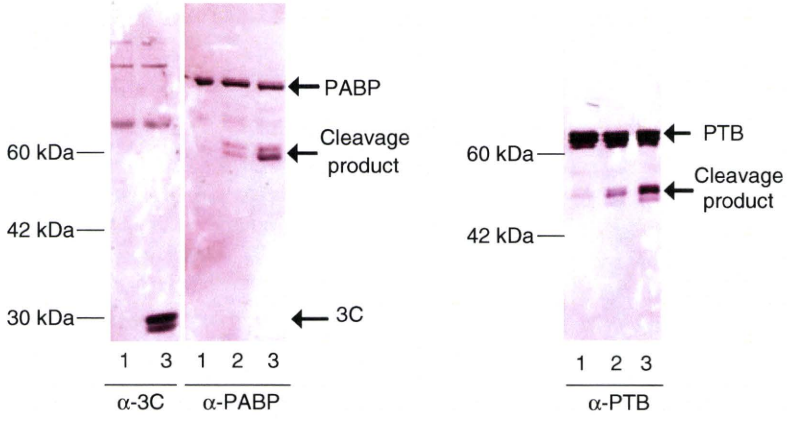

Fig. 4 Hepatitis A virus (HAV) $3C^{pro}$ cleaves the polypyrimidine tract-binding protein (PTB). Huh-7 cells were transfected with pGEM (lanes 1), pGEM-3C (lanes 2) and pEXT7-HAV3C (lanes 3) and infected with vaccinia virus T7. Cell lysates were collected 24 h post-transfection and subjected to immunoblot using anti-3C, anti-poly(A)-binding protein and anti- PTB. As less $3C^{pro}$ was produced by pGEM-3C in comparison with pEXT7-HAV3C, cleavage of host proteins was more pronounced in lanes 3 when compared to lanes2.

reticulocyte lysate, and recombinant PTB produced in *E. coli* was substrate to cleavage-mediated *in vitro* by purified recombinant HAV $3C^{pro}$ (data not shown). Combined with its well-documented translation enhancing effect and binding specificity to stem-loop IIIa of the HAV IRES [6–8], the results strongly suggest that the inhibitory effect of HAV $3C^{pro}$ on HAV IRES translation is because of proteolytic cleavage of PTB.

DISCUSSION

The expression level of the viral proteinase was found to substantially affect the detection of PTB cleavage products (see Fig. 4). Neither in HAV-infected cells nor in cells expressing the HAV replicon were PTB cleavage fragments detectable (not shown). A similar discrepancy was observed for PCBP, another ITAF that is essential for picornaviral translation and the molecular switching to RNA replication [9,23,24]. Whereas PCBP cleavage by recombinant HAV $3C^{pro}$ was clearly shown, PCBP-processing products were not apparent in extracts of HAV-infected cells [9]. Combined, our findings on HAV-3C-mediated cleavage of PCBP and PTB suggest that because of the protracted replication of HAV, very low quantities of $3C^{pro}$ are present in infected cells and cleavage of these host proteins is not discernible. This is in clear contrast to poliovirus whose highly efficient replication resulted in obvious cleavage of both PCBP and PTB [22,24].

The functional domains of PTB are four RNA recognition motifs that all bind short pyrimidine-rich sequences. By binding to different sites on the same RNA molecule, PTB can lead to distinctive RNA restructuring. Such conformational changes are thought to be critical in enabling the ribosomal recruitment in IRES-driven translation initiation. Our constructs do not include the 1–138 nt region of 5′NTR, in which a pyrimidine-rich-tract exists. PTB interacts with stem-loop IIIa of the HAV IRES that contains short polypyrimidine tracts [7]. These binding sites can be bridged by a single PTB molecule, which is an arrangement that favours a role for PTB as an RNA chaperone. It is likely that PTB stabilizes or alters the IRES structure to enable the recruitment of the ribosome and to position it correctly at the start codon.

For poliovirus, direct evidence was provided that PTB cleavage products inhibited IRES-dependent translation [22]. As outlined by the authors, it is possible that PTB fragments may interfere with the binding of intact PTB to poliovirus IRES or that cleaved PTB may no longer function as translational activator that facilitates the recruitment of translational machinery to the IRES element. Although not directly assessed here, it is assumed that suppression of HAV IRES translation is induced by similar mechanism(s). Moreover, in poliovirus-infected Hela cells, PTB cleavage fragments are redistributed to the cytoplasm [22]. As abundant quantities of PTB are present in the cytoplasm of Huh-7 cells used in our study [8], PTB redistribution might not be essential for the effect of PTB cleavage on HAV translation. Yet it is attractive to speculate that the PTB fragment(s) might have altered RNA-binding specificity. For poliovirus IRES translation, an attractive model was put forward for the participation of PTB and PCBP in the molecular switch from viral translation to RNA replication [22]. Supposedly, after viral 3C-mediated cleavage, PTB and PCBP lose their enhancing function. Once IRES translation is stalled, replication of the viral RNA consequently is turned on. Taken together with our earlier observations [9], HAV translation is inhibited indirectly by its own product, $3C^{pro}$, through the proteolytic cleavage of PCBP and PTB.

The HAV 3B and 3C proteins are 23 and 219 amino acids in length, respectively [25]. The 3B moiety was found to be essential for the 3AB interaction with 3CD [26]. It seems that 3BC was more suppressive than 3C in cap-independent translation. Further studies will reveal the 3B function in the interaction with PTB and 3BC. In conclusion, HAV proteinase 3C cleaved PTB and suppressed HAV IRES-dependent translation.

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STATEMENT OF PERSONAL INTERESTS

None of the authors have personal interests relevant to this research to declare.

DECLARATION OF FUNDING INTERESTS

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