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Specific expression of human interferon-gamma controls hepatitis B virus replication *in vitro* in secreting hepatitis B surface antigen hepatocytes

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Interferon-gamma (IFN-γ) has been reported to have antiviral activity against Hepatitis B virus (HBV) and to suppress HBV replication noncytolytically *in vivo*. Since systemic administration of IFN-γ may cause severe adverse effects, studies of the effects of liver-specific IFN-γ expression from adenoviral vectors *in vivo* have been investigated. In this study, a novel strategy has been described that drives specific expression of human IFN-γ in HBsAg-secreting hepatocytes. A bicistronic expression vector has been developed, pcDNA3.1-HBV antisense S gene-HCV core protein gene-HCV internal ribosome entry sites (IRES)-IFN-γ (pcDNA-SCIγ), by inserting four DNA fragments into pcDNA3.1. Tight modulation of HCV IRES-dependent translation by the HCV core protein was achieved using an antisense RNA technique with a bicistronic expression vector. HepG2 cells and HepG2.2.15 cells stably expressing HBV were transduced with pcDNA-SCIγ to test the responsiveness of IFN-γ to HBsAg expression. Gene transfer resulted in a low background and a 30-fold induction of IFN-γ expression from pcDNA-SCIγ in a cell-specific fashion. Hepatocyte-specific IFN-γ expression controlled effectively HBV replication in HBsAg-secreting HepG2.2.15 cells without cell toxicity.

1. Introduction

Interferon gamma (IFN-γ) plays a crucial role in the control of mammalian hepatitis B virus (HBV). Data from HBV-infected animal models *in vitro* and *in vivo* indicate that IFN-γ has direct antiviral actions against HBV (Schultz and Chisari, 1999; Dumortier et al., 2005; Parvez et al., 2006). Studies in HBV-transgenic mice show that a noncytopathic mechanism contributes to the direct inhibition of HBV replication by IFN-γ (Guidotti and Chisari, 2001). In addition, IFN-γ-mediated control of HBV infection has been demonstrated in acutely infected chimpanzees and in woodchucks infected by woodchuck hepatitis virus (Guidotti et al., 1999; Hodgson and Michalak, 2001).

Clinical application of cytokines such as IFN-α, IFN-γ, tumor necrosis factor alpha and interleukin-12 for controlling HBV replication is hindered by potential toxicity following systemic administration. Therefore, regulated, local gene expression in the liver is an attractive alternative. Previous studies have demonstrated that the combination of a liver-specific promoter and a tetracycline-regulated (Tet) gene expression system results in regulated intrahepatic gene expression for a variety of viral vectors (Dumortier et al., 2005; Aurisicchio et al., 2001, 2005). Despite many successful applications, the Tet regulatory system has limitations, such as a requirement for relatively high levels of doxycycline to achieve full activation. In addition, although novel versions of these viral vectors have been developed for gene transfer, none have fully overcome fundamental limitations of undesired expression of the potentially toxic wild type adenovirus genes and high production costs.

This study investigated whether the pcDNA3.1(+) vector is able to mediate conditional and specific gene expression in HBsAg-secreting hepatocytes. A recombinant eukaryotic bicistronic expression vector has been developed, pcDNA3.1-HBV antisense S gene-HCV core protein gene-HCV IRES-IFN-γ (pcDNA-SCIγ), by inserting four DNA fragments into pcDNA3.1(+) (Fig. 1a). HBsAg secreted by hepatocytes infected with HBV acts as the endogenous inducer for this gene transfer system. In this novel transfer system, the HBV antisense S gene, acting as a regulating element, causes IFN-γ expression specifically in HBsAg-secreting hepatocytes using an antisense RNA technique. HCV core protein acts as the response element in the system with its expression modulated by the regulating element. The self-modulating quality of the HCV genome was used for inhibiting HCV IRES-dependent translation by the HCV core protein (Li et al., 2003; Zhang et al., 2002; Shimoike et al., 2006). Thus, the expression of HCV core protein inhibits HCV IRES-dependent translation. When HCV core protein is not expressed,

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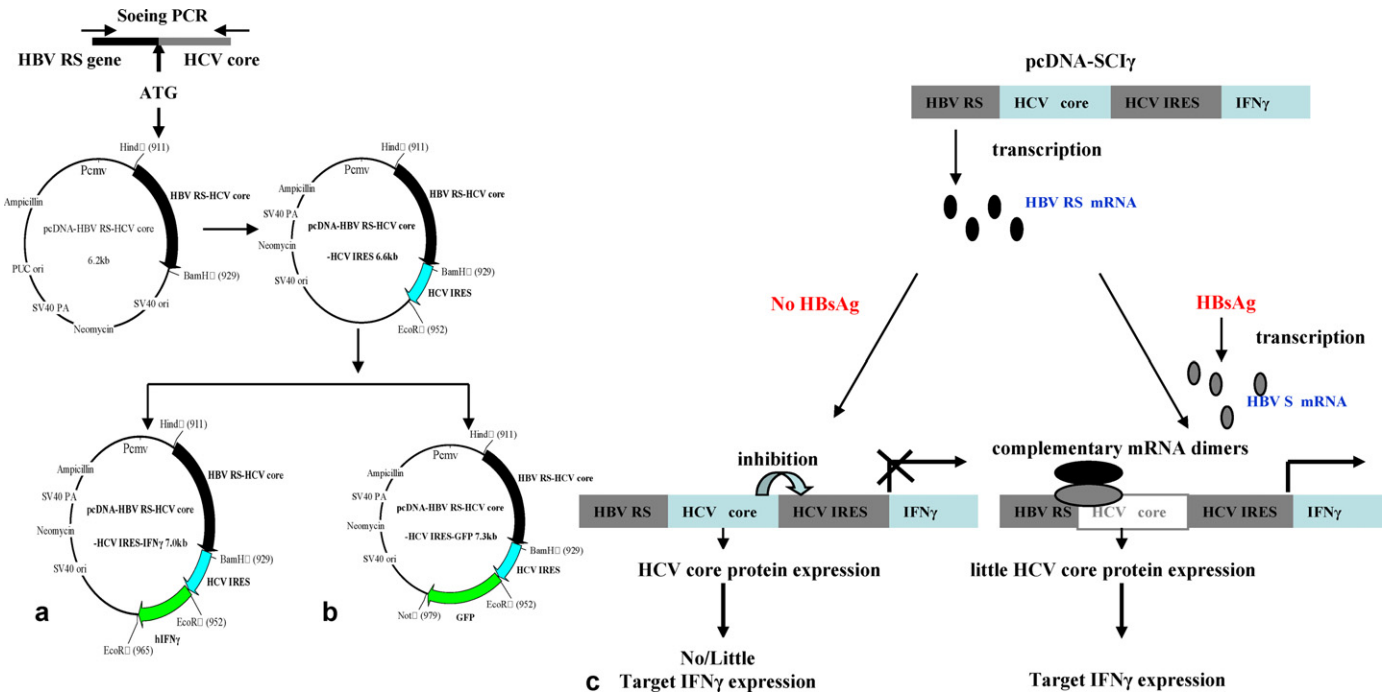

Fig. 1. Schematic diagrams of pcDNA3.1(+)-based recombinant eukaryotic bicistronic expression vector construction and function. Schematic illustration of (a) pcDNA-SCI; (b) control vector pcDNA-SCIGFP; (c) regulatory mode of pcDNA-SCIγ (HBV RS: HBV antisense S gene).

HCV IRES promotes the expression of the downstream target gene IFN-γ.

The approach was when HBsAg is secreted in cells infected with HBV, HBV S gene mRNA and transcripts from the HBV antisense S gene in the recombinant plasmid form complementary sense-antisense dimers. Since the ligation of the HBV antisense S gene with the HCV core gene was performed by Soeing PCR (Horton et al., 1989), the termination sequence of the HBV antisense S gene was contiguous with the upstream region of the initiation AUG codon of the HCV core protein gene, with no additional bases. As a result, the formation of mRNA dimers would cover the AUG of the HCV core protein mRNA, which leads to decreased expression of the HCV core protein. Decreased expression of the HCV core protein relieves the inhibition of HCV IRES-dependent translation by the HCV core protein, resulting in enhanced capacity of HCV IRES to promote IFN-γ expression. In contrast, very little expression of IFN-γ should occur in non-HBV-infected cells because of inhibition of HCV IRES-dependent translation by the HCV core protein, which is abundantly expressed in the absence of mRNA dimers of the HBV S gene at the HCV core protein gene initiation codon (Fig. 1c). This study represents the first application of the recombinant nonviral vector pcDNA3.1(+) to mediate cell-specific IFN-γ expression in HepG2.2.15 cells stably expressing HBV. The effects of the vector on viral replication and cell viability were also assessed in this study.

2. Materials and methods

2.1. Construction of pcDNA3.1(+) plasmid vectors

Three gene fragments encoding anti-HBV S, HCV core protein and the IRES element of HCV were generated by PCR amplification, as described previously (Kan et al., 2003). Human IFN-γ DNA was generated by PCR from plasmid pLY4-γ (Institute of Biochemistry and Cell Biology, Shanghai, China), using the following primers: 5′-CTGAATTCATGCAGGACCCGTACGTTAAAGAAGCT-3′ (sense), 5′-AGTGATATCCTATTACTGAGAAGCACGACGACCGCGGAACAGCAT-3′ (antisense) (Takara, Dalian, China).

The four fragments were inserted sequentially into pcDNA3.1(+) to generate pcDNA-SCIγ (Fig. 1a). Briefly, the ligation of the antisense fragment of the HBV-S gene with the HCV core protein gene was performed by Soeing PCR, adding a *Hind*III site 5′ and a *Bam*HI site 3′ as described (Kan et al., 2003). The PCR product was digested with *Hind*III and *Bam*HI and inserted into pcDNA3.1(+) digested with the same restriction enzymes thereby generating pcDNA-SC. The PCR products of HCV IRES as a *Bam*HI/*Eco*RI fragment were subcloned between the *Bam*HI and *Eco*RI sites of pcDNA-SC thereby generating pcDNA-SCI. Finally, the human IFN-γ gene was amplified by PCR to add *Eco*RI sites and subcloned into pcDNA-SCI, which generated the final construct pcDNA-SCIγ.

Insertion of the complete coding region of the green fluorescence protein (GFP) gene from pEGFP-N1 (Invitrogen) was by performed by *Eco*RI/*Not*I digestion and subcloned into the multiple cloning site of pcDNA-SCI to generate the control vector pcDNA-SCIGFP (Fig. 1b). Transfection efficiency of pcDNA-SCIγ vector in hepatocytes was tested with a vector pcDNA-GFP expressing GFP, obtained by inserting the complete coding region of GFP into *Eco*RI/*Not*I site of pcDNA3.1(+). All the recombinant vectors were verified by sequence analysis (Takara, Dalian, China). GFP expression was monitored by fluorescence microscopy (Nikon Eclipse, Omron, Japan) using a standard FITC-filter set with excitation by blue light (488 nm).

2.2. Cell culture and transfections

HepG2.2.15, which carries the double full HBV genome and can express stably a series of HBV antigens, is derived from HepG2. HepG2.2.15 cells and HepG2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2. For HepG2.2.15 cells, G418 (380 μg/ml) was included. Cells were seeded onto 6-well plate at a density of 8.0×10^5 per well and grown to 80% confluence for transfection. Cells were transfected with different doses of recombinant plasmid DNA (4, 8, and 16 μg) using Lipofectamine 2000 (10 μl, 20 μl