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Suppression of hepatitis C virus replication by baculovirus vector-mediated short-hairpin RNA expression

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Abstract **Short-hairpin RNAs (shRNAs) inhibit gene expression by RNA interference. Here, we report on the inhibition, by baculovirus-based vector-derived shRNAs, of core-protein expression in full-length hepatitis C virus (HCV) replicon cells. shRNAs were designed to target the highly conserved core region of the HCV genome. In particular, the core-shRNA452 containing nucleotides 452–472, as the target in the HCV core gene, dramatically inhibited the expression of the HCV core protein in replicon cells. Furthermore, HCV core-protein expression was inhibited more strongly by the vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped baculovirus vector than by the wild-type baculovirus vector.**

Keywords: Baculovirus vector; Hepatitis C virus; RNA interference; Short-hairpin RNA; Vesicular stomatitis virus glycoprotein

1. Introduction

The hepatitis C virus (HCV) is a major cause of liver-related morbidity and mortality [1]. The virus establishes a persistent liver infection, leading to the development of chronic hepatitis, liver cirrhosis, and hepatocellular carcinomas [1,2]. However, a highly effective anti-HCV drug has yet to be developed, partly due to the lack of detailed information about the life cycle of the virus. In an effort to develop an alternative to combined interferon–ribavirin treatment [3,4], we used RNA interference (RNAi) based on short-hairpin RNA (shRNA), which is a powerful tool for suppressing gene function [5]. RNAi is triggered by small-interfering RNAs (siRNAs) that are processed from long double-stranded or hairpin precursors, and become part of the ribonucleoprotein complex, the RNA-induced silencing complex (RISC) [6,7]. siRNAs expressed from DNA templates through the action of the Dicer enzyme silence gene expression as effectively as exogenously introduced synthetic siRNAs [5,8,9]. The use of RNAi has recently been extended to differentiated cultured mammalian cells [10]. It has also been used to inhibit viral replication in the HCV subgenomic replicon [11–13].

The baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) has long been used as a biopesticide, and as a tool for efficient recombinant protein production in insect cells [14]. Its host specificity was originally thought to be restricted to cells derived from arthropods; however, with an appropriate eukaryotic promoter, it can express foreign genes in several types of mammalian cells [15–17] and animal models [18,19]. Its advantages for use in gene-therapy applications are its inherent inability to replicate, its lack of cytopathic effect (CPE) in mammalian cells even at a high multiplicity of infection (MOI), and the absence of preexisting antibodies against baculoviruses in animals.

Here we designed baculovirus vector-mediated shRNAs against the highly conserved core-protein region of the HCV [20,21]. The shRNA452 construct mediated more effective inhibition of HCV replication than the other core-shRNAs (Ac-shRNA479 and Ac-shRNA523). We also found that HCV core-protein expression was more significantly inhibited by the vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped baculovirus vector than by the wild-type baculovirus vector.

2. Materials and methods

2.1. Baculovirus transfer vector constructs

We designed baculovirus transfer vectors expressing shRNAs against the following regions of the HCV core-protein sequence: nucleotides 452–472, containing the nuclear localization signal site (pU6-core-shRNA452); nucleotides 479–499 (pU6-core-shRNA479); and nucleotides 523–543 (pU6-core-shRNA523) [22]. Sense and antisense strands of shRNA oligonucleotides were synthesized, annealed at 95 °C for 3 min, and then slowly cooled in phosphate-buffered saline (PBS; pH 7.4, containing 50 mM NaCl). The oligonucleotides contained the loop CCACACC sequence, and Kpn I and BamHI ends, which were inserted into a pU6 vector, based on pSV2-neo. A Pol III-type U6 promoter allowed the constant expression of shRNAs. The following three sites in the core region of the common sequences of the HCV strain M1LE (GenBank accession number AB080299) were chosen as the targets for shRNAs: 5′-GCCGCGCAGGGGCCCCAGGUU-3′ (shRNA452); 5′-GCGCGCGACUAGGAAGACUUC-3′ (shRNA479); and 5′-GCGACAACCUAUCCCCAAGGC-3′ (shRNA523). Fragments of U6-core-shRNAs, ranging from the EcoRI site upstream of the U6 promoter to the BamHI site downstream of the terminating sequences, were sequenced and then inserted into the cloning site of the baculovirus transfer vector, pVL1393 (BD Biosciences, San Jose, CA, USA) in an opposite orientation to the PH promoter (Fig. 1C), in order to create pVL1393-core-shRNA452, shRNA479, shRNA523, and pVL1393-U6-terminator. A spacer was inserted between the inverted sequences to form a hairpin structure,

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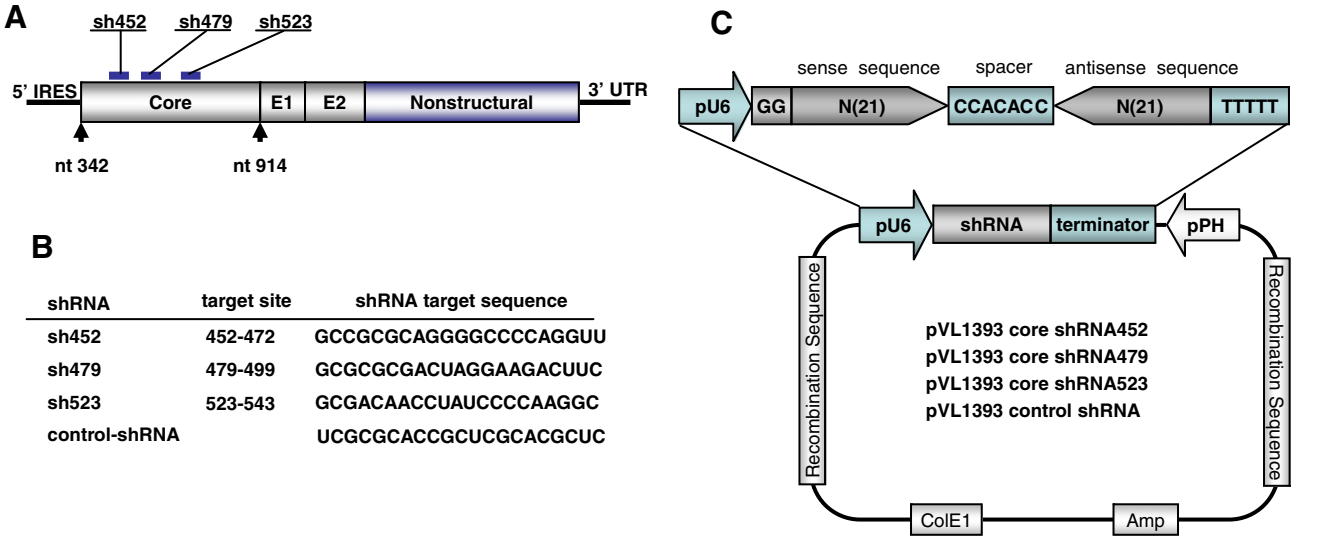

shRNA	target site	shRNA target sequence
sh452	452-472	GCCGCGCAGGGGCCCCAGGUU
sh479	479-499	GCGCGCGACUAGGAAGACUUC
sh523	523-543	GCGACAACCUAUCCCCAAGGC
control-shRNA		UCGCGCACCGCUCGCACGCUC

Fig. 1. (A) Genomic profile of HCV showing both coding and non-coding genes. (B) HCV core-region target sites and sequences used for the design of shRNAs. (C) Construction and schematic representation of baculovirus transfer vector expressing HCV core-shRNA.

and to enhance its stability (Fig. 1C). Scrambled shRNA (control-shRNA) cloned into the same vector was used as a negative control (pVL1393-control-shRNA) in all experiments. The VSV-G-pseudotyped baculovirus vector-transduced shRNA452 was constructed following previously published procedures [23].

2.2. Preparation of baculoviruses

Recombinant baculovirus containing the shRNA genome (Ac-shRNA) was generated by homologous recombination of the transfer vector and linearized baculovirus DNAs (BD Biosciences) following previously published procedures [23].

2.3. Cell culture

NNC#2 (NN/1b/FL) cells [24] carrying a full genome replicon were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), non-essential amino-acids, L-glutamine, and 1 mg/ml G418 (Invitrogen, Carlsbad, CA, USA).

2.4. Measurement of HCV core protein

AcU6-HCV-core-shRNAs or AcU6-VSV-HCV-core-shRNAs were used to infect HCV replicon cells. After 48 h, intracellular HCV core-protein levels were measured using a fully automated HCV core-protein antigen chemiluminescent enzyme immunoassay (CLEIA) according to the manufacturer's instructions [25]. The relative chemiluminescence unit was measured, and was used to determine the concentration of HCV core antigen according to a standard curve generated using recombinant HCV core antigen. The concentration was expressed in units of femto-mol/L (fmol/L). Each CLEIA assay was performed in triplicate.

3. Results

3.1. Construction of baculovirus transfer vectors carrying shRNA-synthesizing cassettes

The core-protein forms the nucleocapsid, and modulates gene transcription, cell proliferation, and apoptosis [21]. HCV functions as a messenger RNA (mRNA) with a single-stranded RNA genome; thus, we hypothesized that cleavage of the core-protein mRNA would inhibit nuclear transport and virus duplication.

To characterize the efficiency of baculoviruses as vehicles for gene therapy, we selected the HCV core region as a target site (Fig. 1A and B), and constructed a recombinant baculovirus vector expressing the shRNA. Fig. 1C shows the baculovirus-transfer vectors used in this study. The baculovirus-transfer vector pVL1393-core-shRNA carries core-shRNA under the control of the PolIII, U6 promoter. Recombinant baculovirus containing the shRNA genome (Ac-shRNA) was generated by homologous recombination of the transfer vector and linearized baculovirus DNAs (BD Biosciences) in Sf9 cells. Viruses were produced at high titers, ranging from 1.5×10^8 to 1.2×10^9 pfu/ml. shRNA452 expression was confirmed by Northern blot analysis in Ac-shRNA452-infected Huh-7 cells (Supplementary Fig. S1 and Supplementary methods).

3.2. Inhibition of HCV RNA replication of baculovirus-mediated shRNA-expression vectors in the HCV replicon

We investigated whether intracellular expression of shRNA inhibited viral replication and affected HCV RNA levels in NNC#2 cells. The baculovirus-infection efficiency of NNC#2 cells ranged from 80 to 90% (Fig. 2A and Supplementary methods). The real-time reverse-transcription (RT) polymerase chain reaction (PCR) was used to examine the presence of HCV RNA, and the ability to induce RNA silencing in NNC#2 cells 48 h post-infection. When NNC#2 cells were infected with Ac-shRNAs at a MOI of 100, a significant reduction in HCV RNA levels was observed compared with a non-related shRNA control (Fig. 2B and Supplementary methods). Although inhibition of HCV RNA levels occurred with all three constructs, the greatest effect occurred with sh452 (68%), while sh479 decreased the levels by approximately 55% and sh523 by 25%. By contrast, the control baculovirus vectors (Ac-U6-terminator and Ac-control-shRNA [random sequence]) had no inhibitory effect on HCV replication (Fig. 2B). These results point to a sequence-specific inhibitory effect of shRNA on HCV replication.

3.3. Silencing of HCV core-protein expression through baculovirus-mediated shRNA in the HCV replicon

We confirmed the inhibitory effect of shRNAs using fluorescence microscopy to assess the localization and expression of the HCV core protein 48 h post-infection. The core proteins