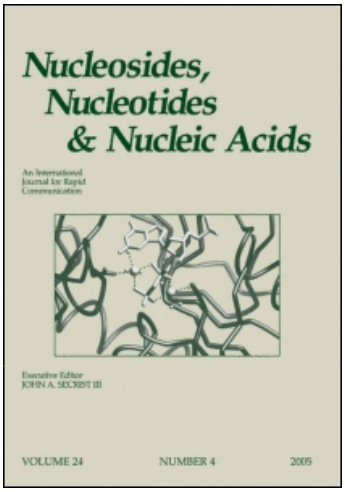

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INHIBITION OF HCV REPLICATION IN HCV REPLICON BY shRNAs

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□ *We show that the vector-derived long dsRNA specifically inhibits the replication of HCV RNA in HCV replicon. We designed a long dsRNA targeted to the full-length HCV IRES/core elements (1-to 377-nt). Our results revealed that the replication of HCV RNA was reduced to near background levels in a sequence-specific manner by the long dsRNAs in the HCV replicon. We also designed four shRNAs against several regions (120- to 139-nt, 260- to 279-nt, 330- to 349-nt, and 340- to 359-nt) of the HCV IRES/Core elements. The two HCV IRES/core-specific shRNAs, 330- to 349-nt and 340- to 359-nt, containing the AUG initiation codon sequence showed stronger HCV inhibitory effects than the other two shRNAs, 120- to 139-nt and 260- to 279-nt.*

Keywords RNAi; Long dsRNA; shRNA; HCV IRES/Core; HCV replicon; Anti-HCV

INTRODUCTION

Hepatitis C virus (HCV) is one of the main causes of liver-related morbidity and mortality. The virus establishes a persistent infection in the liver, leading to the development of chronic hepatitis, liver cirrhosis, and hepatocellular carcinomas. A highly effective anti-HCV drug, however, has yet to be developed, in part due to the lack of detailed information about the life cycle of this virus. The genomic HCV RNA is translated to produce a

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3000 amino acid polypeptide that is processed into at least 10 proteins. The nonstructural (NS) proteins 3, 4A, 4B, 5A, and 5B form a replicase complex that promotes transcription of a genomic (−) strand intermediated. This serves as a template for production of (+) strands that are either translated or packaged into virions as genomic RNAs. A recent report indicated that a synthetic HCV subgenomic RNA including the neomycin-resistance gene, instead of the structural protein-encoding region, replicated efficiently in Huh-7 cells.[1,2] The RNA interference (RNAi) mechanism is the process by which sequences complementary to cognate genes exhibit sequence-specific post-transcriptional gene silencing initiated by double-stranded RNA (dsRNA). The silencing is performed by the RNase III-like nuclease (Dicer), which promotes the cleavage of long dsRNAs into 21- to 23-nt short interfering RNAs (siRNAs) with 2-nt 3′ overhangs. Subsequently, the siRNAs are incorporated into an RNA-induced silencing complex (RISC), as identified in mammalian cells, and the protein–RNA effector nuclease complex recognizes and destroys the target mRNAs.[2–5]

Here, we constructed long dsRNA expression vectors and synthesized shRNAs targeted to the HCV RNA IRES/core region to evaluate their effects on viral replication using an HCV replicon system.

MATERIALS AND METHODS

In order to test for a dsRNA-mediated silencing effect, we designed a long hairpin dsRNA containing the internal ribosomal entry site (IRES) in the 5′ untranslated region (UTR) and the upstream part of core region (1–377) of the hepatitis C virus (HCV) genome. We then cloned the PCR-amplified fragment into the EcoR I and XbaI cloning site in the pCSII-CMV-MCS vector, downstream of the CMV promoter, in order to generate HCV IRES/core-targeting sense (pCMV-IRES-sense), antisense (pCMV-IRES-antisense), and long hairpin dsRNA (plh-dsRNA, nucleotides 1–377) vectors and to evaluate the efficacy of the long dsRNA-silencing effect on viral replication. For a control, we designed and constructed a second dsRNA, which included the HIV-1 env gene (env-dsRNA: 1032–1562, 531 bp). To determine the efficiency of gene expression with this construct, we transiently transfected it into Huh-7 cells (3×10^5), using the Lipofectamine 2000 transfection reagent according to the manufacturer's protocol. Since the expression of the longer dsRNA (HCV IRES) is an important determinant of its efficacy, we measured the intracellular expression of the plh-dsRNA target. Subsequently, the expression of the long hairpin dsRNA was also examined by RT-PCR analyses.

We also designed four shRNAs against several regions (120- to 139-nt, 260- to 279-nt, 330- to 349-nt, and 340- to 359-nt) of the HCV IRES/Core elements. For a control, we designed the EGFP shRNAs. For *in vitro*

transcription, DNA template oligonucleotides with a T7 promoter sequence (TAATACGACTCACTATAG) were designed to produce 51-nt single-stranded RNA (ssRNA). The ssRNAs were annealed by mixing both crude transcription reactions, heating at 95°C for 5 min, and cooling for 90 min at 4°C to obtain short hairpin dsRNAs. The hairpin includes the CCACACC sequence.

To determine the efficacy of gene silencing with these constructs, we transiently transfected them into HCV replicon (1×10^5), using the Lipofectamine 2000 transfection reagent according to the manufacturer's protocol. The RNA content was assessed by real-time RT-PCR at two days post-transfection and was related to the amount produced in the absence of plh-dsRNA and synthesized shRNAs.

RESULTS AND DISCUSSION

Since the expression and accessibility of the plh-dsRNA to the target cognate gene in Huh-7 cells are critical determinant factors for effective silencing, we confirmed the presence of the long dsRNA in the cells by RT-PCR (Figure 1). For the purpose of testing for effective silencing mediated by dsRNA, we designed a long hairpin dsRNA containing the HCV IRES/core region together with a control long dsRNA, which encoded the HIV-1 env gene (531 bp). These vectors were transfected into HCV replicon with the aid of the Lipofectamine 2000 transfection reagent. The HCV subgenomic RNA replication in the HCV replicon was quantified by real- time RT-PCR at 2 days post transfection (Figure 2A). The observed down-regulation of the HCV RNA could be a consequence of direct sequence-specific degradation mediated by the expressed dsRNA in the HCV replicon. Although some mediated inhibition was also observed with

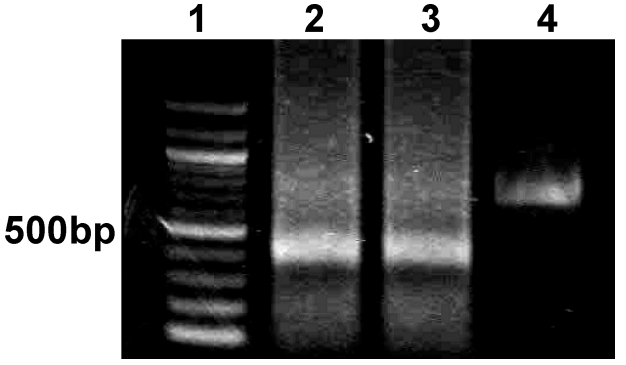

FIGURE 1 RT-PCR analysis of the sense, antisense, and dsRNA mRNA expression in Huh-7 cells. The RT-PCR amplification products, fractionated by electrophoresis on a 2.0% agarose gel with ethidium bromide staining. Lane 1: 100 bp DNA ladder marker; lane 2: pCMV-IRES-sense transfected Huh-7 cells; lane3: pCMV-IRES-antisense–transfected Huh-7 cells; lane 4: plh-dsRNA–transfected Huh-7 cells.

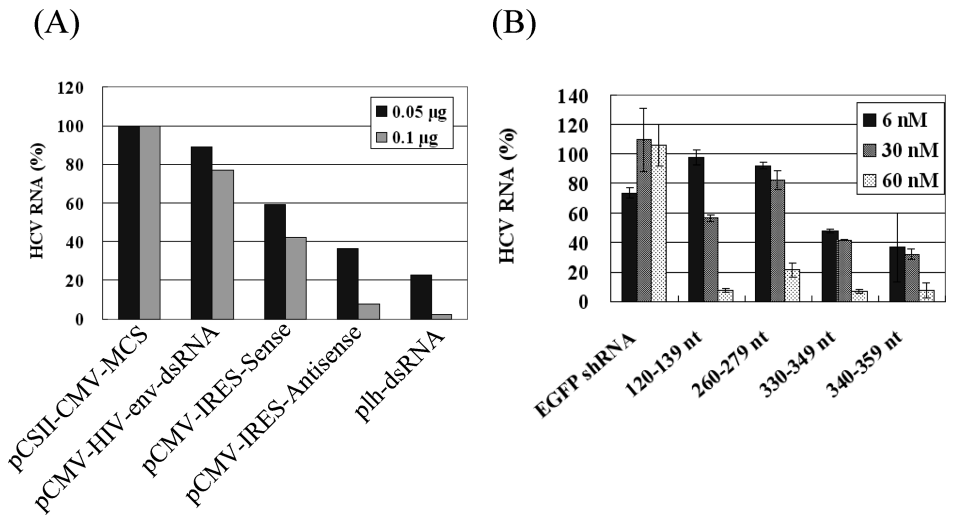

FIGURE 2 (A) Inhibition of HCV replication by sense, antisense, and dsRNA expression vectors. The dose-dependent inhibition of HCV replication by pCMV-IRES-sense, pCMV-IRES-antisense, and plh-dsRNA in HCV replicon. (B) Inhibition of HCV replication by synthesized shRNAs. The dose-dependent inhibition of HCV replication by the EGFP, 120- to 139-nt, 260- to 279-nt, 330- to 349-nt, 340- to 359-nt targeted shRNAs in HCV replicon.

the pCMV-IRES-sense, this may be due to the fact that the HCV minus strand RNA can act as an antisense molecule, which in turn binds to the 5′ end of the positive strand (IRES) to produce the observed inhibition. Speculatively, the sense strand may have the same structure as that of the IRES, and hence serve as a decoy to attract the ribosomes for interaction instead of the IRES.

Next, to test whether shRNAs can specifically inhibit HCV replication, we transfected the HCV replicon with the shRNAs corresponding to the HCV IRES/core. Especially, low levels of HCV RNA expression for both HCV IRES/core-specific shRNAs, 330- to 349-nt and 340- to 359-nt, containing the AUG initiation codon sequence were detected and no differences between these shRNAs were observed. Furthermore, the inhibition mediated by the shRNAs, 330- to 349-nt and 340- to 359-nt, containing the AUG initiation codon sequence, was higher than that seen with the shRNAs, 120- to 139-nt and 260- to 279-nt (Figure 2B).

The consensus results from this study support the potential use of long dsRNA and shRNAs as a gene therapy approach to inhibit HCV replication, which may prove to be a valuable means of treating hepatitis C virus infections.

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