Table 2
Relative luciferase activity under p35 promoter in various cell lines[a]

Cell line	Relative luciferase activity (fold increase)	
	pGL3-basic	pGL.hr5.p35prom
Sf9	1 ± 0.02	108.8 ± 14.1
Huh-7	1 ± 0.2	14.6 ± 0.9
B16	1 ± 0.5	6.0 ± 0.6
Raw264.7	1 ± 0.1	1.7 ± 0.1
YAC-1	1 ± 0.02	0.61 ± 0.29

[a] The relative luciferase activity as described in the legend Fig. 4C.

with the AcMNPV *p35* gene [32,33]. The AcMNPV *p35* gene also induces replication of the viral genome and inhibits its host protein synthesis in insect cells [34,35]. Moreover, Takramah et al. [36] reported that baculovirus p35 interacts with RNA polymerase II and enhances β-actin promoter activity in human cells. Additionally, Hershberger et al. [37] reported that the *p35* gene product is found in wild-type AcMNPV viral particles. Taken together, our results indicate that AcMNPV p35 inhibits apoptosis and enhances foreign gene expression in permissive mammalian cells, due to p35 inhibiting an anti-viral cellular factor, such as interferon-α or 2′–5′ oligoadenylate synthetase, and/or enhancing the CMV-IE promoter-containing recombinant baculovirus.

The purpose of the present study was to examine in various mammalian cell lines the infectivity and transduction of AcMNPV in vitro. Our data demonstrate that AcMNPV infected permissive and non-permissive mammalian cells, but transduction only occurred in permissive cell lines. YAC-1 cells inhibited the nuclear transport of the viral genome. In Huh-7 and B16 cells, AcMNPV *p35* mRNA was expressed and inhibited apoptosis and protein synthesis, and enhanced promoter activity. These results suggest that AcMNPV is useful for the development of more efficient baculovirus vectors for gene therapy.

Acknowledgments

This work was supported in part by a Grant-in-Aid for High Technology Research, (No. 09309011) from the Ministry of Education, Science, Sports and Culture, Japan, and by a Grant-in-Aid for AIDS research from the Ministry of Health, Labor, and Welfare, Japan (H17-AIDS-002). This manuscript is dedicated to Professor Wojciech J. Stec on the occasion of his 65th birthday.

References

[1] M.D. Ayres, S.C. Howard, J. Kuzio, M. Lopez-Ferber, R.D. Possee, The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus, Virology 202 (1994) 586–605.
[2] L.S. England, S.B. Holmes, J.T. Trevors, Persistence of viruses and DNA in soil, World J. Microb. Biotech. 14 (1998) 163–169.
[3] B.A. Keddie, G.W. Aponte, L.E. Volkman, The pathway of infection of *Autographa californica* nuclear polyhedrosis virus in an insect host, Science 243 (1989) 1728–1730.
[4] M. Kool, C.H. Ahrens, R.W. Goldbach, G.F. Rohrmann, J.M. Vlak, Identification of genes involved in DNA replication of the *Autographa californica* baculovirus, Proc. Natl. Acad. Sci. USA 91 (1994) 11212–11216.
[5] G. Croizier, L. Croizier, O. Argaud, D. Poudevigne, Extension of *Autographa californica* nuclear polyhedrosis virus host range by interspecific replacement of a short DNA sequence in the p143 helicase gene, Proc. Natl. Acad. Sci. USA 91 (1994) 48–52.
[6] E. Ito, D. Sahri, R. Knippers, E.B. Carstens, Baculovirus proteins IE-1, LEF-3, and P143 interact with DNA in vivo: a formaldehyde cross-linking study, Virology 329 (2004) 337–347.
[7] Y. Wang, Y. Qi, Y. Zhu, Z. Li, Y. Yang, Functional study on baculovirus anti-apoptosis genes, Mol. Cell. Biochem. 252 (2003) 103–107.
[8] R.J. Clem, L.K. Miller, Apoptosis reduces both the in vitro replication and the in vivo infectivity of a baculovirus, J. Virol. 67 (1993) 3730–3738.
[9] O. Argaud, L. Croizier, M. López-Ferber, G. Croizier, Two key mutations in the host-range specificity domain of the p143 gene of *Autographa californica* nucleopolyhedrovirus are required to kill *Bombyx mori* larvae, J. Gen. Virol. 79 (1998) 931–935.
[10] H. Tani, C.K. Limn, C.C. Yap, M. Onishi, M. Nozaki, Y. Nishimune, N. Okahashi, Y. Kitagawa, R. Watanabe, R. Mochizuki, K. Moriishi, Y. Matsuura, In vitro and in vivo gene delivery by recombinant baculoviruses, J. Virol. 77 (2003) 9799–9808.
[11] S. Ylä-Herttuala, K. Alitalo, Gene transfer as a tool to induce therapeutic vascular growth, Nat. Med. 9 (2003) 694–701.
[12] W.E. Delaney, R. Edwards, D. Colledge, T. Shaw, J. Torresi, T.G. Miller, H.C. Isom, C.T. Bock, M.P. Manns, C. Trautwein, S. Locarnini, Cross-resistance testing of antihepadnaviral compounds using novel recombinant baculoviruses which encode drug-resistant strains of hepatitis B virus, Antimicrob. Agents Chemother. 45 (2001) 1705–1713.
[13] C.J. McCormick, D.J. Rowlands, M. Harris, Efficient delivery and regulable expression of hepatitis C virus full-length and minigenome constructs in hepatocyte-derived cell lines using baculovirus vectors, J. Gen. Virol. 83 (2002) 383–394.
[14] C. Fipaldini, B. Bellei, N. La Monica, Expression of hepatitis C virus cDNA in human hepatoma cell line mediated by a hybrid baculovirus-HCV vector, Virology 255 (1999) 302–311.
[15] J. Barsoum, R. Brown, M. McKee, F.M. Boyce, Efficient transduction of mammalian cells by a recombinant baculovirus having the vesicular stomatitis virus G glycoprotein, Hum. Gene Ther. 8 (1997) 2011–2018.
[16] C.A. Schauber, M.J. Tuerk, C.D. Pacheco, P.A. Escarpe, G. Veres, Lentiviral vectors pseudotyped with baculovirus gp64 efficiently transduce mouse cells in vivo and show tropism restriction against hematopoietic cell types in vitro, Gene Ther. 11 (2004) 266–275.
[17] H. Tani, M. Nishijima, H. Ushijima, T. Miyamura, Y. Matsuura, Characterization of cell-surface determinants important for baculovirus infection, Virology 279 (2001) 343–353.
[18] F.M. Boyce, N.L. Bucher, Baculovirus-mediated gene transfer into mammalian cells, Proc. Natl. Acad. Sci. USA 93 (1999) 2348–2352.
[19] J.P. Condreay, S.M. Witherspoon, W.C. Clay, T.A. Kost, Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector, Proc. Natl. Acad. Sci. USA 96 (1999) 127–132.
[20] C.Y. Liang, H.Z. Wang, T.X. Li, Z.H. Hu, X.W. Chen, High efficiency gene transfer into mammalian kidney cells using baculovirus vectors, Arch. Virol. 149 (2004) 51–60.
[21] I. Nusinzon, C.M. Horvath, Interferon-stimulated transcription and innate antiviral immunity require deacetylase activity and histone deacetylase 1, Proc. Natl. Acad. Sci. USA 100 (2003) 14742–14747.
[22] K.C. Wang, J.C. Wu, Y.C. Chung, Y.C. Ho, M.D. Chang, Y.C. Hu, Baculovirus as a highly efficient gene delivery vector for the expression of hepatitis delta virus antigens in mammalian cells, Biotechnol. Bioeng. 89 (2005) 464–473.

[23] R.V. Merrihew, W.C. Clay, J.P. Condreay, S.M. Witherspoon, W.S. Dallas, T.A. Kost, Chromosomal integration of transduced recombinant baculovirus DNA in mammalian cells, J. Virol. 75 (2001) 903–909.

[24] N.D. van Loo, E. Fortunati, E. Ehlert, M. Rabelink, F. Grosveld, B.J. Scholte, Baculovirus infection of nondividing mammalian cells: mechanisms of entry and nuclear transport of capsids, J. Virol. 75 (2001) 961–970.

[25] P. Ghazal, J.A. Nelson, Enhancement of RNA polymerase II initiation complexes by a novel DNA control domain downstream from the cap site of the cytomegalovirus major immediate-early promoter, J. Virol. 65 (1991) 2299–2307.

[26] M. Salminen, K.J. Airenne, R. Rinnankoski, J. Reimari, O. Välilehto, J. Rinne, S. Suikkanen, S. Kukkonen, S. Ylä-Herttuala, M.S. Kulomaa, M. Vihinen-Ranta, Improvement in nuclear entry and transgene expression of baculoviruses by disintegration of microtubules in human hepatocytes, J. Virol. 79 (2005) 2720–2728.

[27] S.G. Kamita, K. Majima, S. Maeda, Identification and characterization of the p35 gene of *Bombyx mori* nuclear polyhedrosis virus that prevents virus-induced apoptosis, J. Virol. 67 (1993) 455–463.

[28] A. Sugimoto, P.D. Friesen, J.H. Rothman, Baculovirus p35 prevents developmentally programmed cell death and rescues a ced-9 mutant in the nematode *Caenorhabditis elegans*, EMBO J. 13 (1994) 2023–2028.

[29] B.A. Hay, T. Wolff, G.M. Rubin, Expression of baculovirus P35 prevents cell death in drosophila, Development 120 (1994) 2121–2129.

[30] D.R. Beidler, M. Tewari, P.D. Friesen, G. Poirier, V.M. Dixit, The baculovirus p35 protein inhibits Fas- and tumor necrosis factor-induced apoptosis, J. Biol. Chem. 270 (1995) 16526–16528.

[31] M. Zhuo, F.R. Yu, D.H. Xu, L.Y. Sun, X.Y. Liu, Baculovirus p35 gene greatly enhances PC12 cell's resistance against oxidative stress, J. Neurol. Sci. 216 (2003) 135–141.

[32] S. Gomi, K. Majima, S. Maeda, Sequence analysis of the genome of *Bombyx mori* nucleopolyhedrovirus, J. Gen. Virol. 80 (1999) 1323–1337.

[33] N. Morishima, K. Okano, T. Shibata, S. Maeda, Homologous p35 proteins of baculoviruses show distinctive anti-apoptotic activities which correlate with the apoptosis-inducing activity of each virus, FEBS Lett. 427 (1998) 144–148.

[34] P.A. Hershberger, J.A. Dickson, P.D. Friesen, Site-specific mutagenesis of the 35-kilodalton protein gene encoded by *Autographa californica* nuclear polyhedrosis virus: cell line-specific effects on virus replication, J. Virol. 66 (1995) 5525–5533.

[35] X. Du, S.M. Thiem, Responses of insect cells to baculovirus infection: protein synthesis shutdown and apoptosis, J. Virol. 71 (1997) 7866–7872.

[36] D. Takramah, B.M. Seiffert, S. Schaller, M. Vigneron, G. Häcker, Baculovirus P35 interacts with a subunit of human RNA polymerase II and can enhance promoter activity in human cells, J. Gen. Virol. 84 (2002) 3011–3019.

[37] P.A. Hershberger, D.J. LaCount, P.D. Friesen, The apoptotic suppressor P35 is required early during baculovirus replication and is targeted to the cytosol of infected cells, J. Virol. 68 (1994) 3467–3477.

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Biochemical and Biophysical Research Communications 343 (2006) 988–994
BBRC
www.elsevier.com/locate/ybbrc

Inhibition of hepatitis C virus RNA replication by short hairpin RNA synthesized by T7 RNA polymerase in hepatitis C virus subgenomic replicons

Hiroyuki Hamazaki [a], Saneyuki Ujino [a], Naoko Miyano-Kurosaki [a,b], Kunitada Shimotohno [c], Hiroshi Takaku [a,b,*]

[a] *Department of Life and Environmental Sciences, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino, Chiba 275-0016, Japan*
[b] *High Technology Research Center, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino, Chiba 275-0016, Japan*
[c] *Department of Viral Oncology, Institute for Virus Research, Kyoto University, Kyoto 606-0507, Japan*

Received 6 March 2006

Abstract

RNA interference (RNAi) is a cellular process that induces gene silencing by which small duplexes of RNA specifically target a homologous sequence for cleavage by cellular ribonucleases. Here, to test the RNAi method for blocking hepatitis C virus (HCV) RNA replication, we created four short hairpin RNAs (shRNAs) targeting the HCV internal ribosome entry site/Core gene transcript using T7 RNA polymerase. shRNA suppressed the replication of HCV RNA in the HCV replicon. On the other hand, short interfering RNAs synthesized using the T7 RNA polymerase system trigger a potent induction of interferon-α and -β in a variety of cells. We examined whether the shRNAs synthesized using the T7 RNA polymerase system activated double-stranded RNA-dependent protein kinase, 2′-5′ oligoadenylate synthetase, or interferon-regulatory factor-3. Our results demonstrated that the T7-transcribed shRNA did not activate these proteins in Huh-7 cells and the HCV replicon. These shRNAs are a promising new strategy for anti-HCV gene therapeutics.

Keywords: Hepatitis C virus; RNAi; Synthesized shRNA; T7 RNA polymerase; IFN; PKR; 2′-5′OAS; Hepatitis C virus subgenomic replicons

RNA interference (RNAi) occurs in a variety of organisms, including *Caenorhabditis elegans* [1], *Trypanosoma brucei* [2], plants [3], *Drosophila* [4], planaria [5], zebra fish [6], and mouse embryos [7]. In most of these organisms, the injection of a double-stranded RNA (dsRNA) longer than 500 bp specifically suppresses the expression of the gene with the corresponding DNA sequence, but has no effect on genes with unrelated sequences.

RNAi is initiated by the RNase III-like nuclease Dicer, which promotes progressive cleavage of long dsRNAs into 21 to 27 nucleotide (nt) short interfering RNAs (siRNAs) with two nt 3′-overhangs. Subsequently, the siRNAs are incorporated into an RNA-induced silencing complex (RISC), identified in *Drosophila*, and the protein-RNA effector nuclease complex recognizes and destroys the target mRNAs [8–10].

Hepatitis C virus (HCV) is one of the main causes of liver-related morbidity and mortality [11]. The virus establishes a persistent infection in the liver, leading to the development of chronic hepatitis, liver cirrhosis, and hepatocellular carcinomas [11]. HCV replication occurs in the cytoplasm and is associated with membranes that appear to be derived from the endoplasmic reticulum. Genomic HCV RNA is translated to produce a 3000-amino acid polypeptide that is processed into at least 10 proteins. The nonstructural proteins 3, 4A, 4B, 5A, and 5B

* Corresponding author. Fax: +81 47 471 8764.
E-mail address: hiroshi.takaku@it-chiba.ac.jp (H. Takaku).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.03.053

form a replicase complex that promotes transcription of a genomic (−) strand intermediate. This serves as a template for the production of (+) strands that are either translated or packaged into virions as genomic RNAs [12,13]. A satisfactory treatment for HCV infection has yet to be developed, however, because studies of HCV have been hampered by the lack of a stable cell-culture system and a small-animal model. One recently reported HCV replicon is a selectable subgenomic HCV RNA, which replicates efficiently and continuously in human hepatoma Huh-7 cells [14,15]. HCV RNA replication is also sensitive to RNAi [16–18].

On the other hand, it was previously reported that dsRNA triggers the production of type I interferon (IFN), and activates dsRNA-dependent protein kinase (PKR) [19] and 2′-5′oligoadenylate synthetase (2′-5′OAS). Furthermore, two recent studies demonstrated that the mechanism of the IFN response might include recognition of the siRNAs by Toll-like receptor-3 (TLR-3) [20]. One simple method for limiting the risk of inducing an IFN response is to use the lowest effective dose of short hairpin RNA (shRNA) vector, as advocated by Bridge et al. [21]. Recently, Kim et al. reported that siRNAs synthesized using the T7 RNA polymerase system can trigger the potent induction of IFN-α and -β in a variety of cells [22].

In the present study, we synthesized four shRNAs targeting the HCV internal ribosome entry site (IRES)/Core gene transcript using T7 RNA polymerase. The greatest inhibitory effects occurred with both HCV 330-349-shRNA and HCV 340-359-shRNA, as the target of the HCV RNA. We also examined whether the shRNAs synthesized using the T7 RNA polymerase system activated PKR, 2′-5′OAS, or IFN-regulatory factor-3 (IRF-3). shRNA synthesized using T7 RNA polymerase did not, however, activate these proteins in Huh-7 cells and HCV replicons.

Materials and methods

shRNA synthesis by T7 RNA polymerase. Desalted DNA oligonucleotides were obtained from Sigma Proligo (Boulder, CO): the T7 promoter 5′-TAATACGACTCACTATAG-3′; EGFP 418–437nt as 5′-CTGGGGC ACAAGCTGGAGTA-3′; HCV 120–139nt as 5′-CCCCCCCTCCCGGG AGAGCC-3′, 260–279nt as 5′-AGTGTTGGGTCGCGAAAGGC-3′, 330–349nt as 5′-AGA CCGTGCACCATGAGCAC-3′, and 340–359nt as 5′-CCATGAGCAC GAATCCTAAA-3′. Loop used CCACACC [23] and overhang used CUU. The oligonucleotide-directed production of small RNA transcripts with T7 RNA polymerase was described previously [24]. For each transcription reaction, the oligonucleotide was annealed in sH_2O by heating at 95 °C; after 5 min, the heating block was allowed to cool down slowly to obtain the dsDNA. Transcription was performed using AmpliScribe™ T7 High Yield Transcription Kits (EPICENTRE Biotechnologies, Madison, WI) according to the manufacturer's recommended protocol. After incubation at 37 °C for 2 h, 1U RNase free-DNase was added at 37 °C for 15 min. Single-stranded 51nt RNAs were annealed by heating at 95 °C for 5 min followed by 1 h at 37 °C to obtain shRNAs.

Cell culture. Human hepatoma-derived Huh-7 cells and human uterus cancer-derived HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HCV subgenomic replicons were cultured in DMEM-supplemented with 10% FBS, 0.1 mM MEM non-essential amino-acid solution, 2 mM L-glutamine, and penicillin–streptomycin. The growth medium contained the active ingredient G418 (300 μg/ml) [25].

Quantification of HCV RNA by real-time reverse transcription-polymerase chain reaction. HCV replicons were seeded at 1×10^5 cells on 12-well plates for 24 h and transfected with the shRNAs (6, 30 or 60 nM) using Lipofectamine 2000 transfection reagent, according to the manufacturer's recommendation (Invitrogen, Carlsbad, CA). After 48 h, total RNA was isolated from cell cultures using TRIZOL (Invitrogen). HCV RNA was quantified by real-time reverse transcription-polymerase chain reaction (RT-PCR) using an ABI 7700 sequence detector (Perkin-Elmer Applied Biosystems, Foster City, CA). Real-time RT-PCR was performed using the following primers and the TaqMan probe located in the five untranslated region (UTR): forward primer (nt 130–146), 5′-CGGGAGA GCCATAGTGG-3′; reverse primer (nt 272–290), 5′-AGTACCACAAG GCCTTTCG-3′; TaqMan probe (nt 148–168), 5′-CTGCGGAACCGG TGAGTACAC-3′. These reagents were purchased from Applied Biosystems. The reporter dye, FAM, was attached to the five end and the quencher dye, TAMRA, was joined to the three end of the probe sequence [26].

RT-PCR. Total cellular RNA was prepared using TRIZOL (Invitrogen). Toll-like receptor (TLR)-3 and IFN-β mRNAs were detected by a RT-PCR High-Plus kit (Toyobo, Kyoto, Japan) with primers specific for these proteins. Primers specific for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were used as a loading control in a separate reaction. After 2 min at 94 °C, the reaction parameters were set for 1 min at 94 °C, followed by 1.5 min at 60 °C, for 40 cycles. The sequences of the primers were as follows: TLR-3 sense, 5′-AGCCACCTGAAGTTGACTCAGG-3′; TLR-3 antisense, 5′-CAGTCAAATTCGTGCAGAAGGC-3′ [27]; IFN-β sense, 5′-ACCAACAAGTGTCTCCTCCA-3′; and IFN-β antisense, 5′-GAGGTAACCTGTAAGTCTGT-3′ [28].

Huh-7 cells and HCV replicon transfection and reporter gene assay. Huh-7 cells and HCV replicons were seeded at 5×10^4 cells on 24-well plates for 24 h and transfected with the plasmid DNA pIRF-3/Luc (0.5 μg) using Lipofectamine 2000 transfection reagent according to the manufacturer's recommendation (Invitrogen). After 4 h, shRNA (60 or 200 nM) or polyinosinic acid:polycytidylic acid (polyI:C) (0.2 μg/ml) were transfected using Lipofectamine 2000 transfection reagent. Untreated cells were used as a control. After 20 h, luciferase activity was measured in the cell lysates using a luminometer (Berthold, Bad Wildbad, Germany).

Western blot analysis. Transfections of the shRNAs or polyI:C were performed in Huh-7 cells and HCV replicons with Lipofectamine 2000 reagent in accordance with the manufacturer's recommendation (Invitrogen). After 24 h, the cell extracts were prepared with lysis buffer. A sample (80 μg) of the total cell lysate was separated using 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. The membrane was incubated with monoclonal anti-PKR (Thr446) (Cell Signaling Tech., Beverly, MA) and detection was performed via a chemiluminescence reaction.

Ribosomal RNA-cleavage assay. Huh-7 cells and HCV replicons were transfected with the shRNAs or polyI:C using Lipofectamine 2000 in accordance with the manufacturer's recommendation (Invitrogen). After 24 h, the cells were harvested, washed in phosphate-buffered saline (PBS), and stored at −80 °C. The cell pellets were lysed in 1.5 pellet volumes of NP-40 lysis buffer containing 10 mM Hepes (pH 7.5), 90 mM KCl, 1.0 mM magnesium acetate, 0.5% (v/v) Nonidet P-40, 2.0 mM fresh 2-mercaptoethanol, and 100 μg/ml fresh leupeptin. The buffer was added to the frozen pellet and left on ice until it had thawed. The cell pellets were then dispersed in the buffer and left on ice for at least 5 min. The crude lysate was centrifuged at 10,000*g* and 4 °C for 10 min before the supernatant was transferred to a clean tube. The cell lysate containing 200 μg protein was then combined with 2 μl of 10 cleavage buffer [comprising 100 mM Hepes (pH 7.5), 1 M KCl, 50 mM magnesium acetate, 10 mM ATP, and 0.14 M 2-mercaptoethanol], plus 2 μl of a 10 concentration of the desired 2–5A activator and sH_2O to a final reaction volume of 20 μl. Immediately after the addition of the 2–5A activator, incubation was initiated at 30 °C [29]. The positive control 2–5A sample was a gift from

Professor Sawai of the Department of Chemistry, Faculty of Engineering, Gunma University, Japan.

Results

Inhibition of HCV RNA replication of synthesized shRNAs using T7 RNA polymerase in the HCV replicon

We synthesized four shRNAs targeting the HCV IRES/Core gene transcript using T7 RNA polymerase (Fig. 1A) and verified the sequences using 18% polyacrylamide gel electrophoresis (data not shown). The sequences are shown in Fig. 1B. To assess the inhibitory effects of the shRNAs on the intracellular replication of HCV, we used HCV replicons. Transfection of the shRNA into the HCV replicons (Fig. 1A), which stably express the HCV subgenome, indicated that the 330-349-shRNA and 340-359-shRNA both inhibited HCV RNA replication in a dose-dependent manner (Fig. 2). On the other hand, the control, EGFP-shRNA, did not induce efficient inhibition (Fig. 2).

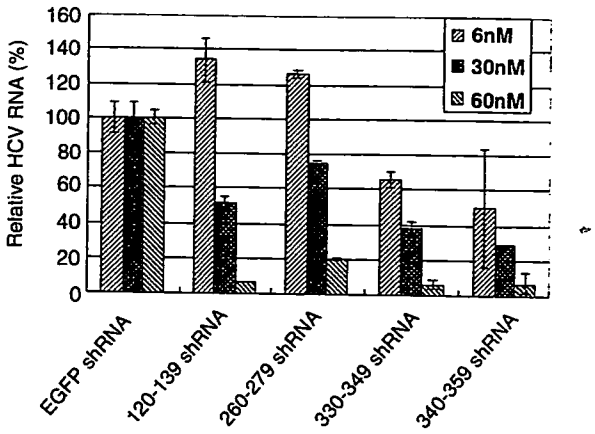

Fig. 2. Inhibition of HCV replication by shRNAs synthesized using the T7 RNA polymerase system in the HCV replicons. The effect of transfection with shRNAs or control EGFP HCV replication was measured by RT-PCR of HCV RNA 48 h after transfection. All values are shown as the percentages of the EGFP negative control.

shRNA stimulates TLR-3 in Huh-7 cells but not in the HCV replicons

To date, in mammals, 11 TLRs have been identified that recognize pathogen-associated molecular patterns, such as bacterial cell wall materials, bacterial or viral genomic DNA and RNA, and small molecules. At least four TLRs (TLR-3, 7, 8, and 9) recognize and respond to mono-, oligo-, and polynucleotides of natural and/or synthetic origin. TLRs 3, 7, and 8 recognize viral and synthetic single-stranded and dsRNAs, such as polyI:C and siRNA [30].

Toll-like receptor-3 recognizes dsRNA, which is commonly produced during viral replication, and is required for the full induction of IFN-α/β and pro-inflammatory

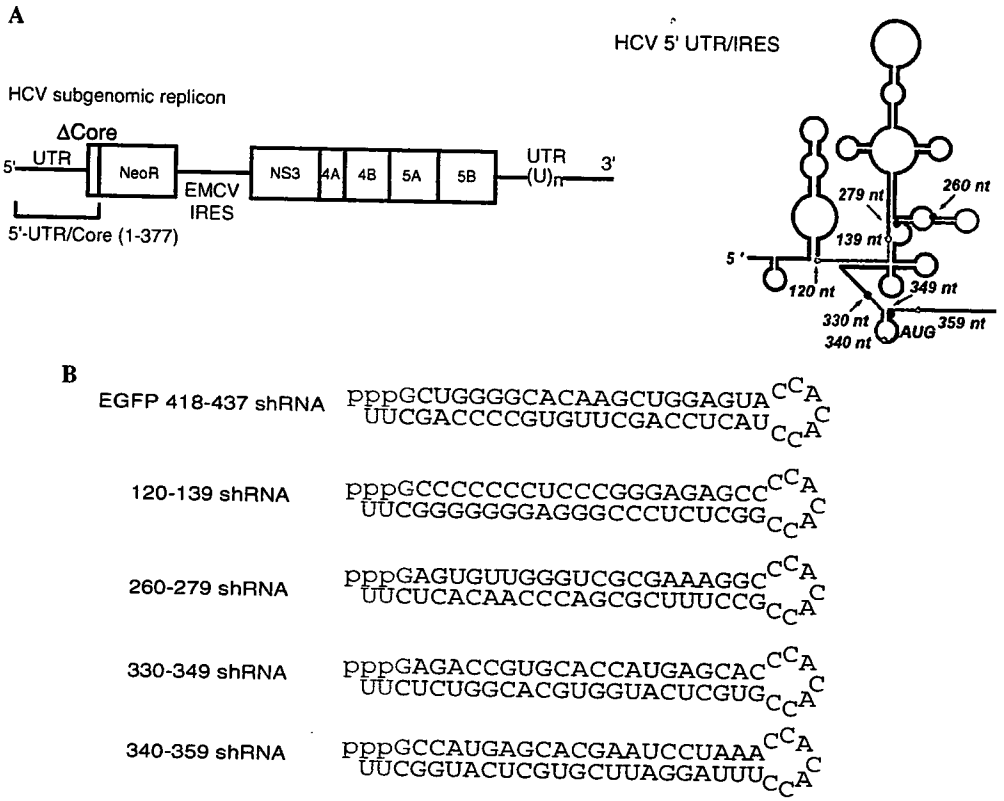

Fig. 1. shRNA-targeted regions of the HCV subgenomic replicons and shRNA sequences. (A) Schematic representation of HCV subgenomic replicons and shRNA targeted regions on HCV 5′-UTR/IRES secondary structure. (B) Sequences of shRNAs synthesized by phage polymerase. The control shRNA:EGFP-shRNA.

cytokines in response to exogenous stimulation by synthetic dsRNA or virus-derived dsRNA. Similar to TLR-4, TLR-3 activation can induce IFN-α/β expression via a MyD88-independent, TRIF-, NAP1-, and TBK1- dependent signaling pathway [31].

Therefore, we examined whether the shRNAs synthesized using T7 RNA polymerase induced type I IFN in Huh-7 cells and HCV replicons. First, we analyzed the expression of TLR-3 mRNA. Total cellular RNA was isolated from the cells and analyzed by RT-PCR using specific TLR-3-detection primers. TLR-3 mRNA was detected in all of the cells (Fig. 3A). Next, we examined whether 340-359-shRNA synthesized using T7 RNA polymerase activated IRF-3 in Huh-7 cells and HCV replicons. We assessed the trigger for IRF-3 phosphorylation by 340-359-shRNA in Huh-7 cells and HCV replicons. For this analysis, we constructed a luciferase reporter gene-expression vector (pIRF-3/Luc reporter) with an IRF-3 binding region (5′-GAAACCGAAACT-3′) in the pGL3-basic vector [32]. The pIRF-3/Luc and 340-359-shRNA were then co-transfected into Huh-7 cells and the HCV replicons using Lipofectamine 2000. IRF-3 activation was monitored using a luciferase assay (Fig. 3B). The internal control, polyI:C, simultaneously induced phosphorylation of IRF-3 and Luc gene expression in the Huh-7 cells. In contrast, the 340-359-shRNA mediated neither the phosphorylation of IRF-3 nor Luc gene expression in these cells. Both polyI:C and the 340-359-shRNA, however, failed to trigger the phosphorylation of IRF-3 and Luc gene expression in HCV replicons (Fig. 3B).

Huh-7 cells and HCV replicons that were transfected with the 340-359-shRNA were also used to evaluate the induction of IFN-β gene expression. The transcribed IFN-β mRNA was detected using RT-PCR analysis with an appropriate primer. IFN-β mRNA expression was detected in polyI:C-transfected Huh-7 cells (Fig. 4, lane 3), but not in cells transfected with the 340-359-shRNA (Fig. 4, lanes 4 and 5). In contrast, IFN-β mRNA expression was not observed when either the 340-359-shRNA (Fig. 4, lanes 4 and 5) or polyI:C (lane 3) was transfected into HCV replicons. These results suggested that the synthesized shRNAs using T7 RNA polymerase did not induce type I IFN production.

Detection of PKR phosphorylation induced by synthesized shRNAs using T7 RNA polymerase

Activation of PKR by viral dsRNA and synthesized dsRNA results in autophosphorylation and subsequent phosphorylation of the eukaryotic initiation factor 2α subunit, causing general inhibition of cellular protein synthesis. In addition to its role as a translational inhibitor, PKR is also a component of signal transduction pathways that regulate events such as cell growth and stress responses [33].

To investigate the association of these transductional pathways in relation to the inhibition of HCV replication, we analyzed the phosphorylated PKR activity induced by the 340-359-shRNA in Huh-7 cells and HCV replicons. The levels of phosphorylated PKR activity were detected with a Western blot assay. Phosphorylated PKR activity

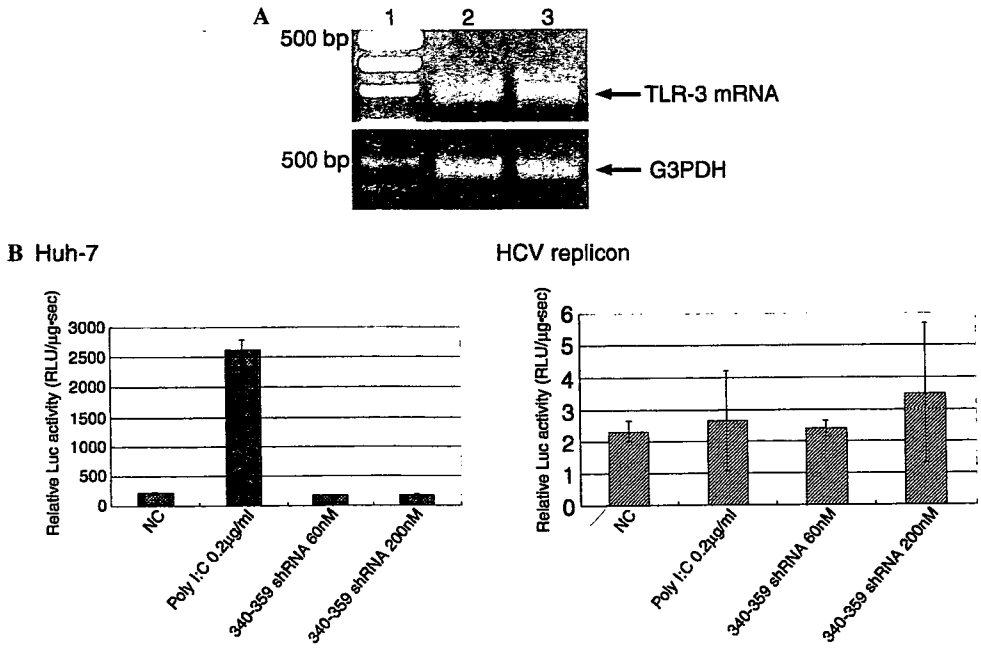

Fig. 3. Activation of IRF-3 after transfection of Huh-7 cells and HCV replicons with shRNAs synthesized using the T7 RNA polymerase system. (A) RT-PCR analysis of TLR-3 mRNA expression in Huh-7 cells and HCV replicons cells. The RT-PCR assay for TLR-3 mRNA was performed using TLR-3 mRNA-specific primers with concurrent amplification of G3PDH mRNA. The RT-PCR-amplified products were fractionated by electrophoresis on a 2.0% agarose gel and stained with ethidium bromide. Lane 1, DNA ladder; lane 2, Huh-7 cells; lane 3, HCV replicons. (B) Huh-7 cells and HCV replicons transfected with either polyI:C (0.2 μg) or the 330-349-shRNA (60 or 200 nM) were treated with 0.5 μg pIRF-3/Luc plasmid. After 24 h, the cell lysates were prepared and assayed for luciferase activity. NC, pIRF-3/Luc.

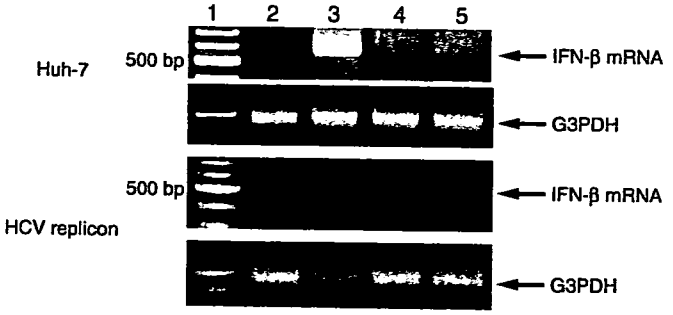

Fig. 4. RT-PCR analysis of IFN-β mRNA expression in Huh-7 cells and HCV replicons. The RT-PCR-amplified products were fractionated by electrophoresis on a 2.0% agarose gel and stained with ethidium bromide. Lane 1, DNA ladder; lane 2, negative control; lane 3, polyI:C (10 μg); lanes 4 and 5, 330-349-shRNA (60 and 200 nM, respectively).

was detected in polyI:C-transfected Huh-7 cells as an internal control (Fig. 5A, lane 3), whereas no such activity was detected in the cells transfected with the 340-359-shRNA (Fig. 5A, lanes 4 and 5). In addition, no phosphorylated PKR activity was detected in HCV replicons transfected with either polyI:C (Fig. 5A, lane 3) or the 340-359-shRNA (Fig. 5A, lanes 4 and 5).

Detection of activated RNase L following transfection with synthesized shRNAs using T7 RNA polymerase

Activation of 2′-5′OAS by viral dsRNA and synthesized dsRNA results in autophosphorylation and subsequent activation of RNase L, causing general inhibition of cellular protein synthesis. Thus, the RNase L induced by the synthesized shRNAs might have degraded the HCV RNA. Therefore, we examined whether synthesized shRNAs activated RNase L in Huh-7 cells and HCV replicons using a ribosomal RNA-cleavage assay to investigate the induction of RNase L activity in the target cells. The cells were transfected with either 340-359-shRNA or polyI:C using Lipofectamine 2000. The positive control contained the phosphorylated 2′-5′OAS. Total RNA was extracted with TRIzol and analyzed using a ribosomal RNA-cleavage assay with 1.0% agarose gel electrophoresis. RNase L activity was detected after the addition of the positive control (phosphorylated 2′-5′OAS) to both Huh-7 cells and HCV replicons (Fig. 5B, lane 2). In contrast, no RNase L activity was detected in cells that were transfected with the 340-359-shRNA (Fig. 5B, lanes 3 and 4). Notably, RNase L activity was not stimulated in Huh-7 cells and HCV replicons that were transfected with polyI:C as an internal control (Fig. 5B, lane 5). In addition, polyI:C induced negligible RNase L activity in HeLa cells (data not shown). These results demonstrated that the T7-transcribed shRNA did not induce RNase L activity in Huh-7 cells, HeLa cells, or HCV replicons.

Discussion

The present study examined whether HCV RNA replication was inhibited by an RNAi mechanism. We synthesized four shRNAs targeting the HCV IRES/Core gene transcript using T7 RNA polymerase (Figs. 1A and B). The 330-349-shRNA and the 340-359-shRNA both inhibited HCV RNA replication in a dose-dependent manner (Fig. 2). On the other hand, the control, EGFP-shRNA, did not induce efficient inhibition (Fig. 2). These findings

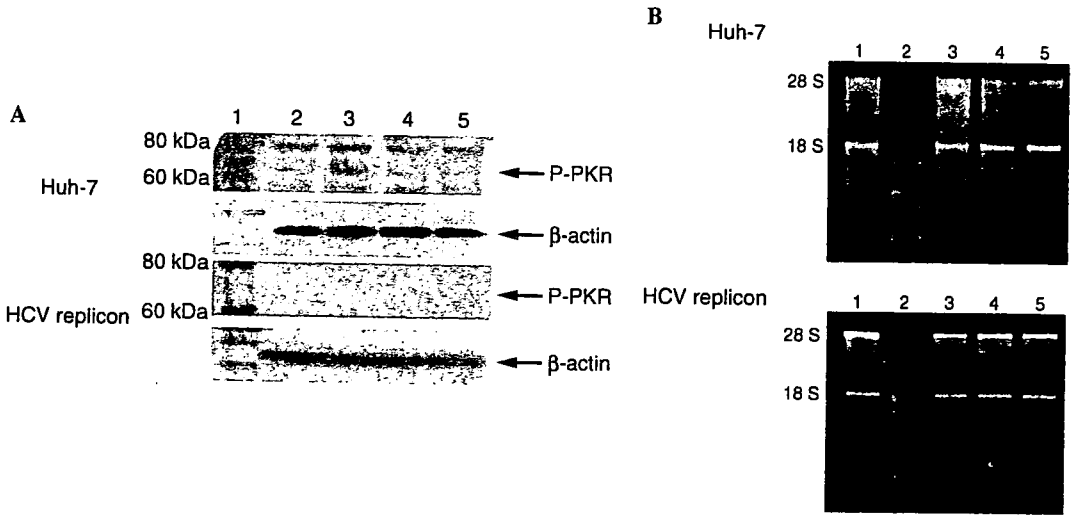

Fig. 5. Activation of PKR and RNase L with shRNAs synthesized using the T7 RNA polymerase system. (A) After 24 h, the Huh-7 cells and HCV replicons were lysed with lysis buffer. The lysates (80 μg protein/lane) were assayed by Western blot analysis with antibodies against phosphorylated PKR or β-actin as a control. The protein bands were visualized using the ECL method (Amersham). Lane 1, biotinylated protein marker; lane 2, negative-control cells; lane 3, cells transfected with 10 μg polyI:C; lanes 4 and 5, cells transfected with 330-349-shRNA (60 and 200 nM, respectively). (B) An RNase L assay was performed for Huh-7 cells and HCV replicons. Lane 1, negative control; lane 2, p5A2 p5 A2 p5 A (1 μM); lanes 3 and 4, 330-349-shRNA (60 and 200 nM, respectively); lane 5, Huh-7 cells and HCV replicons, polyI:C (10 μg).

suggest that targeting the region that includes the AUG of the HCV IRES/Core is effective.

It was previously reported that dsRNA triggered production of type I IFN, activation of PKR, and activation of 2′-5′OAS. Recently, Kim et al. demonstrated that siRNAs synthesized using the T7 RNA polymerase system can trigger the potent induction of IFN-α and -β in a variety of cells [22]. In the present study, we investigated whether the dsRNA stimulated various metabolic pathways in HCV replicons in addition to suppressing RNA replication.

First, we examined whether synthesized shRNAs induced IFN-β. It is expected that the induction of IFN-β occurs so that shRNA is recognized by TLR-3. We detected TLR-3 mRNA in Huh-7 cells and HCV replicons. Next, we examined the activation of IRF-3, which is one of the transcription factors. The results suggested that shRNAs did not induce activation of IRF-3 in Huh-7 cells and HCV replicons. Similarly, shRNAs did not induce IFN-β mRNA in Huh-7 cells and HCV replicons. Recent analyses of potential mediators of induction of the IFN response revealed that the initiating 5′-triphosphate is required for IFN induction in HEK 293 and HeLa cells [22]. Our synthesized shRNAs, however, did not induce IFN in Huh-7 cells and HCV replicons. Huh-7 cells might be less sensitive to dsRNA than HEK 293 and HeLa cells.

Next, we examined whether the synthesized shRNA activated PKR and 2′-5′OAS. PKR and 2′-5′OAS are activated by dsRNA and viral infection [34]. PKR activation stops translation. Activation of 2′-5′OAS activates RNase L and degrades RNA [35]. Our synthesized shRNAs, however, did not induce activation of PKR and 2′-5′OAS in Huh-7 cells and HCV replicons. Even polyI:C did not induce activation of these proteins in Huh-7 cells and HCV replicons. These proteins in Huh-7 cells might be insensitive to dsRNA. Therefore, we used HeLa cells, which were sensitive to dsRNA. The synthesized shRNAs also did not induce activation of PKR and 2′-5′OAS in HeLa cells. These results demonstrated an association between the sequence specific-inhibition via the RNAi mechanism without stimulating the TLR-3 signal pathway, PKR [36], or 2′-5′OAS by HCV proteins, because the TLR-3-adaptor protein TRIF is cleaved by HCV nonstructural 3/4A protease [37]. On the other hand, the nonstructural 5A protein might bind with PKR and block dimerization, which inhibits the activation of eukaryotic initiation factor 2α [38,39].

In conclusion, our results demonstrate that shRNAs targeting the HCV IRES/Core gene transcript using T7 RNA polymerase inhibited RNA replication in HCV replicons. In addition, our study revealed that the 330-349-shRNA and 340-359-shRNA inhibit the replication of HCV RNA via an RNAi mechanism without stimulating the TLR-3 signal pathway, PKR, or 2′-5′OAS. This suggests that RNAi might be an effective method for blocking HCV RNA replication in infected cells.

Acknowledgments

This work was supported by a Grant-in-Aid for High Technology Research (HTR) from the Ministry of Education, Science, Sports and Culture, Japan, and by a Grant-in-Aid for AIDS research from the Ministry of Health, Labor and Welfare, Japan (H17-AIDS-002).

References

[1] A. Fire, S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, C.C. Mello, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*, Nature 391 (1998) 806–811.

[2] Z. Wang, J.C. Morris, M.E. Drew, P.T. Englund, Inhibition of *Trypanosoma brucei* gene expression by RNA interference using an integratable vector with opposing T7 promoters, J. Biol. Chem. 275 (2000) 40174–40179.

[3] H. Vaucheret, C. Beclin, M. Fagard, Post-transcriptional gene silencing in plants, J. Cell Sci. 114 (2001) 3083–3091.

[4] H.H. Kavi, H.R. Fernandez, W. Xie, J.A. Birchler, RNA silencing in *Drosophila*, FEBS Lett. 579 (2005) 5940–5949.

[5] A.S. Alvarado, P.A. Newmark, Double-stranded RNA specifically disrupts gene expression during planarian regeneration, Proc. Natl. Acad. Sci. USA 96 (1999) 5049–5054.

[6] A. Dodd, S.P. Chambers, D.R. Love, Short interfering RNA-mediated gene targeting in the zebrafish, FEBS Lett. 561 (2004) 89–93.

[7] P. Svoboda, P. Stein, H. Hayashi, R.M. Schultz, Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference, Development 127 (2000) 4147–4156.

[8] L.J. Scherer, J.J. Rossi, Approaches for the sequence-specific knockdown of mRNA, Nat. Biotech. 21 (2003) 1457–1465.

[9] T. Sijen, J. Fleenor, F. Simmer, K.L. Thijssen, R.H.A. Plasterk, A. Fire, On the role of RNA amplification in dsRNA-triggered gene silencing, Cell 107 (2001) 465–476.

[10] D.S. Schwarz, G. Hutvagner, B. Haley, P.D. Zamore, Evidence that siRNAs function as guides, not primers, in the *Drosophila* and Human RNAi pathways, Mol. Cell 10 (2002) 537–548.

[11] J.G. McHutchison, K. Patel, Future therapy of hepatitis C, Hepatology 36 (2002) 245–252.

[12] R. Bartenschlager, V. Lohmann, Replication of hepatitis C virus, J. Gen. Virol. 81 (2000) 1631–1648.

[13] B. Clarke, Molecular virology of hepatitis C virus, J. Gen. Virol. 78 (1997) 2397–2410.

[14] K.J. Blight, A.A. Kolykhalov, C.M. Rice, Efficient initiation of HCV RNA replication in cell culture, Science 290 (2000) 1972–1974.

[15] V. Lohmann, F. Korner, J.-O. Koch, U. Herian, L. Theilmann, R. Bartenschlager, Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line, Science 285 (1999) 110–113.

[16] J.A. Wilson, S. Jayasena, A. Khvorova, S. Sabations, I.G. Rodrigue-Gervais, S. Arya, F. Sarangi, M. Harris-Brandts, S. Beaulieu, C.D. Richardson, RNA interference blocks gene expression and RNA synthesis from hepatitis C replicons propagated in human liver cells, Proc. Natl. Acad. Sci. USA 100 (2003) 2783–2788.

[17] G. Randall, A. Grakoui, C.M. Rice, Clearance of replicating hepatitis C virus replicon RNAs in cell culture by small interfering RNAs, Proc. Natl. Acad. Sci. USA 100 (2003) 235–240.

[18] T. Yokota, N. Sakamoto, N. Enomoto, Y. Tanabe, M. Miyagishi, S. Maekawa, L. Yi, M. Kurosaki, K. Taira, M. Watanabe, H. Mizusawa, Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs, EMBO Rep. 4 (2003) 602–608.

[19] C.A. Sledz, M. Holko, M.J. de Veer, R.H. Silverman, B.R.G. Williams, Activation of the interferon system by short-interfering RNAs, Nat. Cell Biol. 5 (2003) 834–839.

[20] K. Kariko, P. Bhuyan, J. Capodici, D. Weissman, Small Interfering RNAs mediate sequence-independent gene suppression and induce immune activation by signaling through Toll-like receptor 3, J. Immunol. 172 (2004) 6545–6549.
[21] A.J. Bridge, S. Pebernard, A. Ducraux, A.-L. Nicoulaz, R. Iggo, Induction of an interferon response by RNAi vectors in mammalian cells, Nat. Genet. 34 (2003) 263–264.
[22] D.H. Kim, M. Longo, Y. Han, P. Lundberg, E. Cantin, J.J. Rossi, Interferon induction by siRNAs and ssRNAs synthesized by phage polymerase, Nat. Biotech. 22 (2004) 321–325.
[23] J.M. Jacque, K. Triques, M. Stevenson, Modulation of HIV-1 replication by RNA interference, Nature 418 (2002) 435–438.
[24] O. Donze, D. Picard, RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase, Nucleic Acids Res. 30 (2002) e46.
[25] H. Kishine, K. Sugiyama, M. Hijikata, N. Kato, H. Takahashi, T. Noshi, Y. Nio, M. Hosoka, Y. Miyanari, K. Shimotohno, Subgenomic replicon derived from a cell line infected with the hepatitis C virus, Biochem. Biophys. Res. Commun. 290 (2002) 993–999.
[26] T. Takeuchi, A. Katsume, T. Tanaka, A. Abe, K. Inoue, K. Tsukiyama-Kohara, R. Kawaguchi, S. Tanaka, M. Kohara, Real-time detection system for quantification of hepatitis C virus genome, Gastroenterology 116 (1999) 636–642.
[27] S. Liu, D.J. Gallo, A.M. Green, D.L. Williams, X. Gong, R.A. Shapiro, A.A. Gambotto, E.L. Humphris, Y. Vodovotz, T.R. Billiar, Role of Toll-like receptors in changes in gene expression and NF-κB activation in mouse hepatocytes stimulated with lipopolysaccharide, Infect. Immun. 70 (2002) 3433–3442.
[28] K. Brack, I. Berk, T. Magulski, J. Lederer, A. Dotzauer, A. Vallbracht, Hepatitis A virus inhibits cellular antiviral defense mechanisms induced by double-stranded RNA, J. Virol. 76 (2002) 11920–11930.
[29] M.R. Player, E.M. Wondrak, S.F. Bayly, P.F. Torrence, Ribonuclease L, a 2-5A-dependent enzyme: purification to homogeneity and assays for 2-5A binding and catalytic activity, Methods 15 (1998) 243–253.
[30] L. Alexopoulou, A.C. Holt, R. Medzhltov, R.A. Flavell, Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3, Nature 413 (2001) 732–738.
[31] H. Oshiumi, M. Matsumoto, K. Funami, T. Akazawa, T. Seya, TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-α induction, Nat. Immunol. 4 (2003) 161–167.
[32] R. Lin, P. Genin, Y. Mamane, J. Hiscott, Selective DNA binding and association with the CREB binding protein coactivator contribute to differential activation of alpha/beta interferon genes by interferon regulatory factors 3 and 7, Mol. Cell Biol. 20 (2000) 6342–6353.
[33] H. Streitenfeld, A. Boyd, J.K. Fazakerley, A. Bridgen, R.M. Elliott, F. Weber, Activation of PKR by Bunyamwera Virus is independent of the viral interferon antagonist NSs, J. Virol. 77 (2003) 5507–5511.
[34] C.E. Samuel, Antiviral actions of interferons, Clin. Microbiol. Rev. 14 (2001) 778–809.
[35] R.H. Silverman, A. Zhou, M.B. Auerbach, D. Kish, A. Gorbachev, R.L. Fairchild, Skin allograft rejection is suppressed in mice lacking the antiviral enzyme, 2′,5′-oligoadenylate-dependent RNase L, Viral Immunol. 15 (2002) 77–83.
[36] L.-C. Hsu, J.M. Park, K. Zhang, J.-L. Luo, S. Maeda, R.J. Kaufman, L. Eckmann, D.G. Guiney, M. Karin, The protein kinase PKR is required for macrophage apoptosis after activation of Toll-like receptor 4, Nature 428 (2004) 341–345.
[37] E. Foy, K. Li, C. Wang, R. Sumpter Jr., M. Ikeda, S.M. Lemon, M. Gale Jr., Regulation of interferon regulatory factor-3 by the hepatitis C virus serin protease, Science 300 (2003) 1145–1148.
[38] T. Taguchi, M. Nagano-Fujii, M. Akutsu, H. Kadoya, S. Ohgimoto, S. Ishido, H. Hotta, Hepatitis C virus NS5A protein interacts with 2′,5′-oligoadenylate synthetase and inhibits antiviral activity of IFN in an IFN sensitivity-determining region-independent *manner*, J. Gen. Virol. 85 (2004) 959–969.
[39] J. Vyas, A. Elia, M.J. Clemens, Inhibition of the protein kinase PKR by the internal ribosome entry site of hepatitis C virus genomic RNA, RNA 9 (2003) 858–870.

Nucleosides, Nucleotides, and Nucleic Acids, 25:801–805, 2006

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770600726091

Taylor & Francis
Taylor & Francis Group

INHIBITION OF HCV REPLICATION IN HCV REPLICON BY shRNAs

Hiroyuki Hamazaki □ *Department of Life and Environmental Sciences, Chiba Institute of Technology, Chiba, Japan*

Hitoshi Takahashi and Kunitada Shimotohno □ *Department of Viral Oncology, Institute for Virus Research, Kyoto University, Kyoto, Japan*

Naoko Miyano-Kurosaki and Hiroshi Takaku □ *Department of Life and Environmental Sciences, and High Technology Research Center, Chiba Institute of Technology, Chiba, Japan*

□ *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

Received 4 November 2005; accepted 10 March 2006.
This work was supported in part by a Grant-in-Aid for High Technology Research, from the Ministry of Education, Science, Sports, and Culture, Japan.
Presented at the XVI International Roundtable, Minneapolis, MN, September 12–16, 2004.
Address correspondence to H. Takaku, Department of Life and Environmental Science and High Technology Research Center, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino-shi, Chiba 275-0016, Japan. E-mail: hiroshi.takaku@it-chiba.ac.jp

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

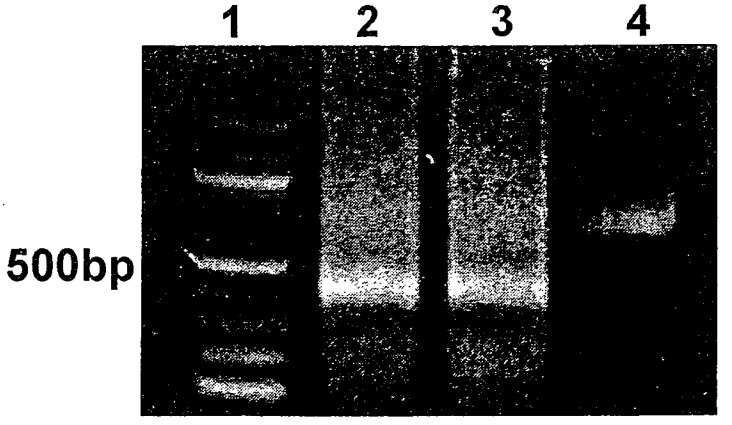

FIGURE 1 RT-PCR analysis of the sense, antisense, and dsRNA mRNA expression in Huh-7 cells. The RT-PCR amplification products, fractionated by electrophoresis on a 2.0% agarose gel with ethidium bromide staining. Lane 1: 100 bp DNA ladder marker; lane 2: pCMV-IRES-sense transfected Huh-7 cells; lane3: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

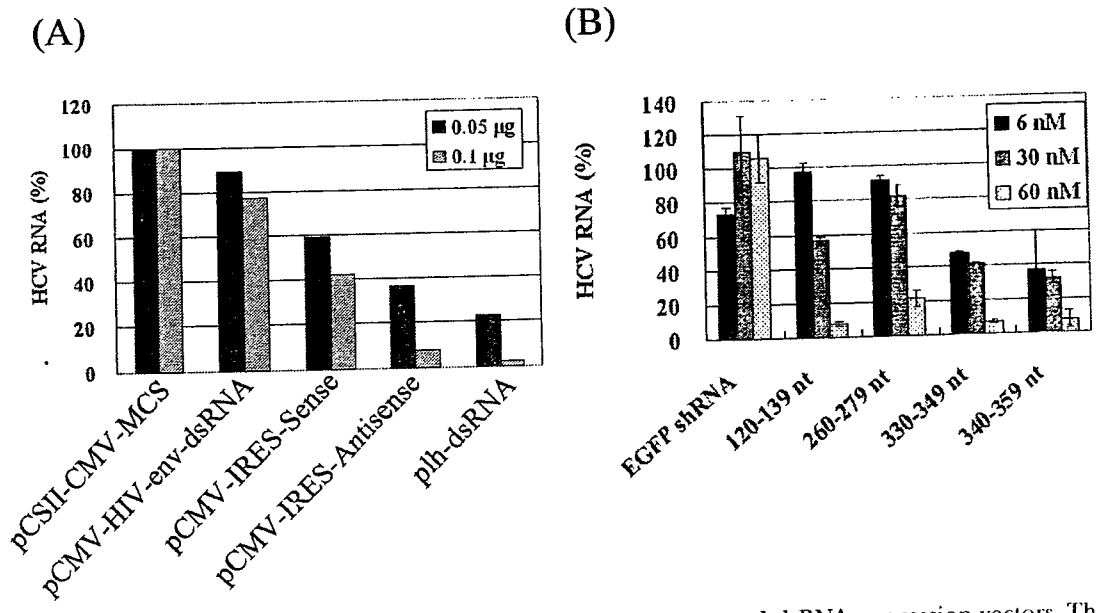

FIGURE 2 (A) Inhibition of HCV replication by sense, antisense, and dsRNA expression vectors. The dose-dependent inhibition of HCV replication by pCMV-IRES-sense, pCMV-IRES-antisense, and plh-dsRNA in HCV replicon. (B) Inhibition of HCV replication by synthesized shRNAs. The dose-dependent inhibition 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.

REFERENCES

1. Lohmann, V.; Korner, F.; Koch, J.O.; Herian, U.; Theilmann, L.; Bartenschlager, R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **1999**, 285, 110–113.

2. Kishine, H.; Sugiyama, K.; Hijikata, M.; Kato, N.; Takahashi, H.; Yasunori, N.; Hosaka, M.; Miyanari, Y.; Shimotohno, K. Subgenomic replicon derived from a cell line infected with the hepatitis C virus. *Biochemical and Biophysical Research Communications* **2002**, 293, 993–999.
3. Park, W.S.; Miyano-Kurosaki, N.; Hayafune, M.; Nakajima, E.; Matsuzaki, T.; Shimada, F.; Takaku, H. Prevention of HIV-1 infection in human peripheral blood mononuclear cells by specific RNA interference. *Nucleic Acids Research* **2002**, 30, 4830–4835.
4. Xia, H.; Mao, Q.; Paulson, H.L.; Davidson, B.L. siRNA-mediated gene silencing in vitro and in vivo. *Nature Biotechnology* **2002**, 20, 1006–1010.
5. Park, W.S.; Hayafune, M.; Miyano Kurosaki, N.; Takaku, H. Specific HIV-1 env gene silencing by small interfering RNAs in human peripheral blood mononuclear cells. *Gene Therapy* **2003**, 10, 2046–2050.

Nucleosides, Nucleotides, and Nucleic Acids, 25:795–799, 2006

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770600726083

Taylor & Francis
Taylor & Francis Group

SILENCING OF HIV-1 GENE EXPRESSION BY siRNAs IN TRANSDUCED CELLS

Masaaki Hayafune □ *Department of Life and Environmental Science, Chiba Institute of Technology, Narashino-shi, Chiba, Japan*

Naoko Miyano-Kurosaki and Hiroshi Takaku □ *Department of Life and Environmental Science and High Technology Research Center, Chiba Institute of Technology, Narashino-shi, Chiba, Japan*

Wee-Sung Park □ *Department of Life and Environmental Science, Chiba Institute of Technology, Narashino-shi, Chiba, Japan, and Human Genome Center, Laboratory of Molecular Medicine, Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo, Japan*

□ *The RNA interference (RNAi) phenomenon is a recently observed process in which the introduction of a double-stranded RNA (dsRNA) into cells causes the specific degradation of an mRNA containing the same sequence. To study dsRNA-mediated gene interference targeted to the* env *gene (NL4-3: 7490-7508) in HIV-1 infected cells, we constructed tandem-type and hairpin-type siRNA expression vectors, which were under the control of two U6 promoters. We also constructed lentiviral-based siRNA expression vectors for further assessment of their antiviral activity in transduced cells. At both the transient plasmid and lentiviral-mediated RNA expression levels, the siRNA encoding the* env *fragment exhibited sequence-specific suppression of target gene expression and strongly inhibited (≥ 90%) HIV-1 infection in the cells, as compared to the antisense RNA expression vector. Targeting the HIV-1* env *gene with siRNAs encoding the* env *gene fragment (7490–7508) might be an effective strategy for gene therapy applications in HIV-1/AIDS treatment and management.*

Keywords RNAi; shRNA; siRNA; HIV-1 *env*; Lentiviral vector

Received 4 November 2005; accepted 10 March 2006.

This work was supported in part by a Grant-in-Aid for High Technology Research, from the Ministry of Education, Science, and Culture, Japan, and a Research Grant from the Human Science Foundation (HIV-SA-14719).

Presented at the XVI International Roundtable Conference, Minneapolis, MN, September 12–16 2004.

Address correspondence to H. Takaku, Department of Life and Environmental Science and High Technology Research Center, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino-shi, Chiba 275-0016, Japan. E-mail: hiroshi.takaku@it-chiba.ac.jp

INTRODUCTION

RNA interference (RNAi) is a phenomenon in which double-stranded RNA (dsRNA) affects silencing of the expression of genes that are highly homologous to either of the RNA strands in the duplex. RNAi is initiated by the enzyme Dicer, a member of the RNase III family of double-stranded RNA-specific endonucleases, which promotes the processive cleavage of a long dsRNA into 21- to 23-nt long duplexes, each with a 2-nt, 3′ overhanging end.[1] These products, termed short interfering siRNAs, are then incorporated into an inactive protein complex. ATP-dependent unwinding of the double-stranded siRNA generates an active RNA-induced silencing complex (RISC), which then uses the antisense siRNA sequence to identify homologous mRNA through complementary base pairing. Degradation of the target mRNA is thought to proceed from the center of the region spanned by the guide siRNA.

We previously reported that four designed siRNA oligonucleotides containing HIV-1 *env* gene fragments (E7145, E7361, E7457, and E7490) mediated dsRNA gene interference in HIV-1 infected cells. The E7490 siRNA (NL4-3: 7490–7508) displayed ≥90% inhibitory efficacy in HIV-1 transfected cells.[2,3]

In this study, we analyzed the siRNA-mediated silencing of the *env* gene with the E7490 siRNA to establish its potential targets in the gene for HIV-1 gene therapy. For safe delivery of this gene fragment (E7490), which was chosen based on its efficient HIV-1 inhibitory potential identified in our previous study, we constructed a lentiviral-based siRNA (E7490) expression vector for further assessment of its antiviral activity in transduced MT-4 cells. Our results suggested that siRNA-mediated RNAi targeted to the HIV-1 *env* gene can be used in gene therapy for HIV/AIDS.

RESULTS AND DISCUSSION

To evaluate the gene interference mediated by siRNA (E7490) expression in HIV-1 infected cells, we constructed an siRNA (E7490) expression vector for the assessment of its antiviral activity. In order to express the siRNA targeted to HIV-1 (NL4-3: 7490–7508), we constructed a tandem-type siRNA expression vector, which was driven under the control of two U6 promoters, and an alternate hairpin-type siRNA expression vector, which was controlled by one U6 promoter (Figure 1B). For the control vector, a **G** was mutated to **C** in the sequence of the siRNA (Figure 1A). The sequence and orientation of the constructed vector inserts were confirmed by nucleotide sequence analysis.

In order to evaluate the anti-HIV-1 activity of the constructed vectors, they were cotransfected into COS cells with pNL4-3, using the FuGENE6 (Roche Applied Science, Mannheim, Germany), and cultured for three

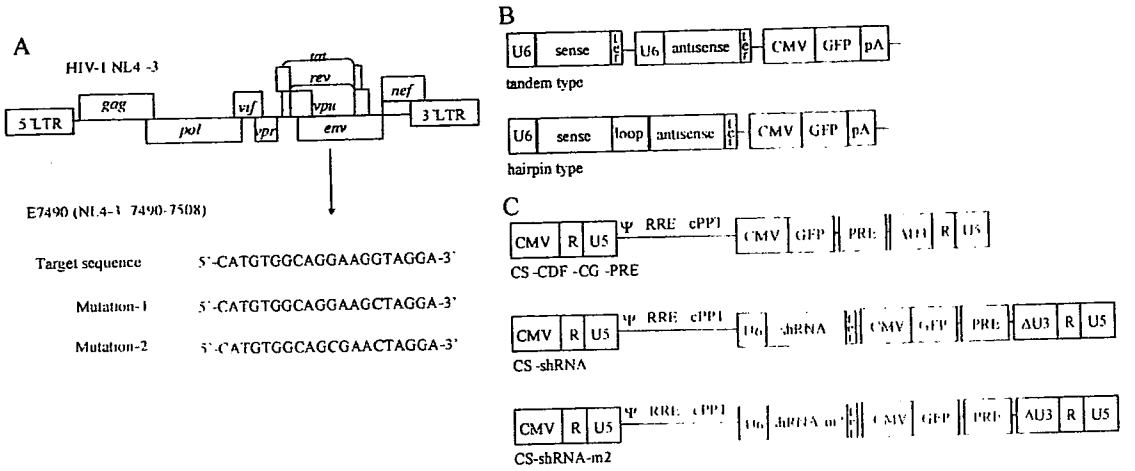

FIGURE 1 Construction of siRNA expression vectors and target sequences for siRNA design. (A) HIV-1 genomic sequence showing the target sequence in the *env* gene used for the siRNA design. (B) The sense and antisense oligos for both the tandem- and hairpin-type siRNAs were annealed at 95°C and gradually cooled down to 4°C. They were then cloned into the Kpn I site, downstream from the U6 promoter and the Xho I site behind the terminator sequence. (C) The generated U6 hairpin vectors were digested with EcoR I and Nhe I and then were cloned into the same site in the CS-CDF-CG-PRE (from Dr. Miyoshi, RIKEN, Japan).

days, and then the GFP expression was monitored by fluorescence microscopy. The GFP expression in these transfected cells was observed. The amount of p24 antigen was measured to check for HIV-1 inhibition by the CLEIA assay (Fujirebio, Tokyo, Japan). Both the tandem and hairpin-type siRNAs mediated about 90% inhibition, as compared to the empty vector (Figure 2). RT-PCR was performed for the downregulation of viral RNAs. The reduction of HIV-1 RNA was correlation with the p24 antigen products.

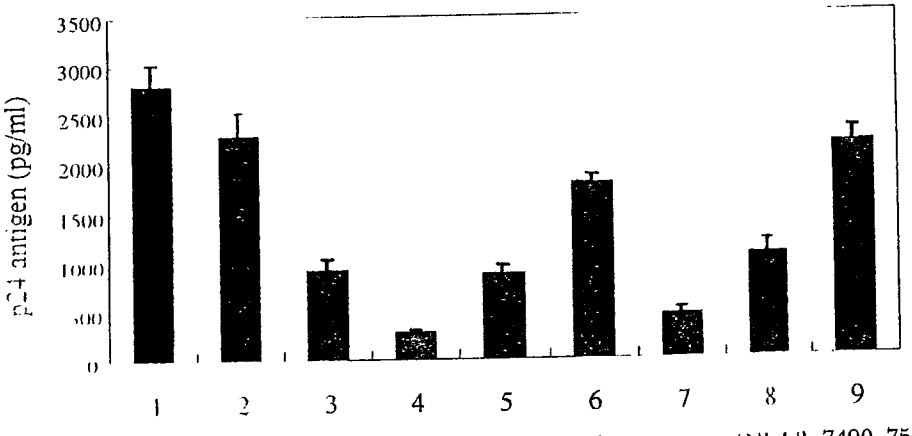

FIGURE 2 Effects of siRNA expression vector targeted against the *env* gene (NL4-3: 7490–7508). COS cells were cotransfected with the siRNA expression vector (1 μg) and pNL4-3 (0.1 μg). At 3 days post cotransfection, p24 antigen production was detected by the HIV-1 p24 CLEIA assay (each bar represents the average of 3 samples ± standard deviations). Lane 1: U6-C, empty vector (hairpin type); lane 2: U6-sen, only sense RNA expression; lane 3: U6-ant, only antisense RNA expression; lane 4: U6-sh, hairpin-type siRNA expression; Lane 5: U6-sh-m1, hairpin-type siRNA (mutation 1) expression; lane 6: U6-Sh-m2, hairpin- type siRNA (mutation 2) expression; lane 7: U6-SA; tandem type siRNA expression; lane 8: U6-SA-m1, tandem type siRNA (mutation-1) expression; lane 9: U6-SA m2, tandem- type siRNA (mutation-2) expression.

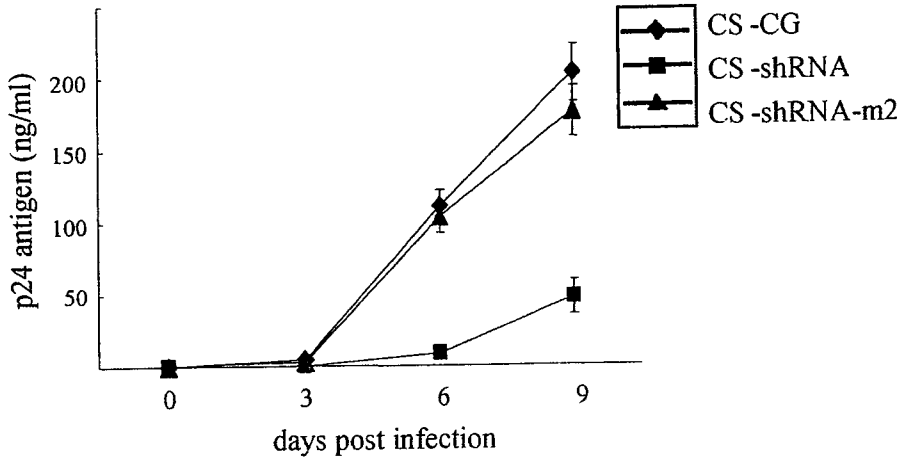

FIGURE 3 Evaluation of the inhibition efficacy of the transgene. The empty (CS-CG), shRNA, and shRNA-m2 lenti-vectors were used to infect MT-4 cells, which were challenged with 0.005 MOI of HIV-1_{NL4-3}. At 3, 6, and 9 days after infection, the amount of p24 was measured by the HIV-1 p24 CLEIA assay (each bar represents the average of 3 samples/replicate ± standard deviations).

Based on these results, siRNAs expressed by lentiviral vectors using the CS-CDF-CG-PRE (CS-CG) site were constructed for the purpose of transducing lymphoid cells and evaluating their anti-HIV-1 activity. The lentiviral vectors were pseudotyped with VSV-G and encoded green fluorescence protein (GFP) as a reporter gene, and the siRNA expression cassettes were inserted into the EcoR I site upstream of the CMV-GFP (Figure 1C).

The lentivirus was packaged in 293T cells by simultaneous transfection of the plasmid vectors. Then the produced virus was titrated for viral infectivity on MT-4 cells, using GFP expression as an indicator. Vector titer ranged from 8.7×10^6 to 1.5×10^7 for CS-CG, CS-shRNA, and CS-shRNA-m2, respectively. MT-4 cells were transduced by the CS-CG, CS-shRNA, and CS-shRNA-m2 at a multiplicity of infection (MOI) of 1. Twenty-four hours after transduction, MT-4 cells (2×10^6 cells/ml in 48-well plate) were challenged with HIV-1_{NL4-3} (MOI of 0.005) and cultured for three, six, and nine days, and the amount of p24 antigen was measured as an index for HIV-1 inhibition by the CLEIA assay. After six days, CS-shRNA showed a 93% inhibition effect, as compared to the empty vector (Figure 3).

Generally, at the plasmid and lentiviral RNA expression levels, the siRNA encoding the *env* fragment (NL4-3: 7490–7508) exhibited sequence-specific suppression of target gene expression and strongly inhibited HIV-1 infection in the cells as compared to the CS-CG and CS-shRNA-m. Hence, targeting the HIV-1 env gene with siRNAs encoding the *env* fragment will be an effective strategy for clinical application against HIV-1/AIDS.

REFERENCES

1. Elbashir, S.M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. Duplexes of 21-Nucleotide RNAs Mediate RNA Interference in Cultured Mammalian Cells. *Nature* **2001**, 411, 494–498.

2. Park, W.-S.; Miyano-Kurosaki, N.; Hayafune, M.; Nakajima, E.; Matsuzaki, T.; Shimada, F.; Takaku, H. Prevention of HIV-1 Infection in Human Peripheral Blood Mononuclear Cells by Specific RNA Interference. *Nucleic Acids Research* **2002**, 30, 4830–4835.
3. Park, W.-S.; Hayafune, M.; Miyano-Kurosaki, N.; Takaku, H. Specific HIV-1 Env Gene Silencing by Small Interfering RNAs in Human Peripheral Blood Mononuclear Cells. *Gene Therapy* **2003**, 10, 2046–2050.

Silencing of HIV-1 gene expression by two types of siRNA expression systems

Masaaki Hayafune[1], Naoko Miyano-Kurosaki[1,2], Wee-Sung Park[1], Yuka Moori[1] and Hiroshi Takaku[1,2]*

[1]Department of Life and Environmental Science, Chiba Institute of Technology, Narashino-shi, Chiba, Japan
[2]High Technology Research Centre, Chiba Institute of Technology, Narashino-shi, Chiba, Japan

*Corresponding author: Tel: +81 47 478 0407; Fax: +81 47 471 8764. E-mail: hiroshi.takaku@it-chiba.ac.jp

The RNA interference (RNAi) phenomenon is a recently discovered process in which the introduction of a double-stranded RNA (dsRNA) into cells causes the specific degradation of mRNA containing the same sequence. We designed mammalian expression vectors that direct the synthesis of small interfering RNA (siRNA)-like transcripts and examined them for their siRNA-mediated gene interference targeting the *env* gene (NL4-3:7490-7508, E7490). We constructed siRNA expression vectors for two different strands (sense and antisense; tandem promoter) and for siRNA expressed from the short hairpin RNA (shRNA). The inhibition efficacy on HIV-1 replication differed between these two vectors. Notably, the shRNA vector pU6-env-shRNA inhibited p24 production more effectively than the tandem promoter expression vector pU6-env-siRNA. Furthermore, we examined the ability of lentiviral vectors expressing shRNA to suppress HIV-1 expression in HIV-1-infected SupT1 cells. The env-shRNA (E 7490) almost completely suppressed HIV-1 expression in infected cells for up to 15 days.

Keywords: HIV/AIDS gene therapy, lentiviral vector, shRNA, siRNA, U6 promoter

Introduction

HIV-1 infection is a worldwide disease that requires alternative therapeutic strategies. Despite significant advances, current treatments are limited by toxicity, complexity, cost and resistance. The development of resistance is the most critical of these limitations, as partial viral suppression allows the evolution of resistant viruses and the creation of their reservoir cells, which are unaffected by therapeutic agents. The emergence of resistant viruses reduces drug activity and limits future treatment options (Montaner & Mellors, 1999).

HIV-1-specific small interfering RNAs (siRNAs) exert potent antiviral effects in a variety of cell culture systems (Jacque *et al.*, 2002; Novina *et al.*, 2002). siRNAs containing cognate sequences existing within different regions of the HIV-1 genome inhibit infection by specifically degrading genomic HIV-1 RNA, thereby preventing the formation of viral cDNA intermediates (Michienzi *et al.*, 2003). HIV-1-specific siRNAs can inhibit infection in permanent cell lines, primary $CD4^+$ T-cells and macrophages (Song *et al.*, 2003). An event in the viral life cycle is inhibited after fusion and before reverse transcription (RT) or during the transcription of viral RNA from the integrated provirus.

siRNA synthesized *in vitro* is now routinely transfected into a wide variety of cell lines using various lipid reagents. This approach is applicable to siRNA-mediated post-transcriptional gene silencing (PTGS) in cell lines only. The high cost of siRNA limits the number of targets that can be tested. To overcome these factors, a variety of different approaches have been employed. Each of these approaches involves driving siRNA expression under RNA polymerase III (*pol* III) promoters, such as human U6, mouse U6, human H1 and human 7SK promoters. The products normally generated by *pol* III promoters are small and highly structured and are found in a variety of subcellular compartments, so they are ideal for expressing siRNA. The most abundant RNA transcribed from a *pol* III promoter is the U6 small nuclear RNA (Brummelkamp *et al.*, 2002), which has a crucial role in the processing of premature RNA and H1 (Myslinski *et al.*, 2001), an RNA component of RNase P.

siRNA can be cloned as a hairpin (short hairpin RNA [shRNA]) (Paddison *et al.*, 2002; Paul *et al.*, 2002; Sui *et al.*, 2002), or the sense and antisense (tandem type) strands can be transcribed from two different *pol* III promoters in the same vector (Lee *et al.*, 2002). The main difference between the expression of siRNAs as two different strands (sense and antisense) and the expression of siRNAs from hairpin RNA is that shRNA depends on Dicer processing. It is difficult to determine which technology is a more efficient tool for inhibiting gene expression. We previously reported that synthetic siRNAs