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Characterization of baculovirus *Autographa californica* multiple nuclear polyhedrosis virus infection in mammalian cells

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Abstract

The baculovirus *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) is used as a vector in many gene therapy studies. Wild-type AcMNPV infects many mammalian cell types in vitro, but does not replicate. We investigated the dynamics of AcMNPV genomic DNA in infected mammalian cells and used flow cytometric analysis to demonstrate that recombinant baculovirus containing a cytomegalovirus immediate early promoter/enhancer with green fluorescent protein (GFP) expressed high levels of GFP in Huh-7 cells, but not B16, Raw264.7, or YAC-1 cells. The addition of butyrate, a deacetylase inhibitor, markedly enhanced the percentage of GFP-expressing Huh-7 and B16 cells, but not Raw264.7 and YAC-1 cells. The addition of 5-aza-2′-deoxycytidine, a DNA methylation inhibitor, had no enhancing effect. Polymerase chain reaction analysis using AcMNPV-*gp64*-specific primers indicated that AcMNPV infected not only Huh-7 and B16 cells, but also Raw264.7 and YAC-1 cells in vitro. The genomic DNA was detected in Huh-7 and B16 cells 96 h after infection. Genomic AcMNPV DNA in YAC-1 cells was not transported to the nucleus. Luciferase assay indicated that AcMNPV *p35* gene mRNA and p35 promoter activity were clearly expressed only in Huh-7 and B16 cells. These results suggest that viral genomic DNA expression is restricted by different host cell factors, such as degradation, deacetylation, and inhibition of nuclear transport, depending on the mammalian cell type.
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Keywords: Baculovirus; Vector; *gp64*; *p35*; Mammalian cells

The ability of baculovirus to infect insects has many applications. In particular, *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) is used in many protein expression systems as a viral vector and plant insecticide.

AcMNPV has a double-stranded circular DNA genome of approximately 130 kbp containing more than 150 open-reading frames [1]. AcMNPV produced from infected cells has two viral phenotypes, occlusion-derived virus (ODV) and budded virus (BV) [2]. ODV enters midgut cells, and BV buds from infected midgut cells establish systemic infection by infecting hemocytes and other tissues in the larval lepidopteron host [3]. The viral genomic DNA of both phenotypes transports to the nucleus of the infected cells and replicates using nine essential viral genes, *ie-1*, *ie-2*, *p143*, *dnapol*, *lef-1*, *lef-2*, *lef-3*, *pe38*, and *p35* [4]. Moreover, the host range of AcMNPV in insect cells is detected by measuring the expression of viral helicase *p143* and pan-caspase inhibitor *p35* [5–8]. Argaud et al. [9] reported that recombinant AcMNPV *p143* replaced with *Bombyx mori* nuclear polyhedrosis virus (BmNPV) could replicate in non-permissive Bm5 cells. Clem and Miller [8], using a *p35* mutant virus, demonstrated protection of viral-induced apoptosis by expression of the AcMNPV *p35* gene within the host range.

AcMNPV infects a variety of mammalian cell types in vitro, but not certain hematopoietic cell lines, by unknown

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mechanisms. The inability of baculoviruses to replicate in mammalian cells makes them attractive candidate vectors for in vitro gene therapy studies [10,11]. These recombinant vectors contain compatible promoters and are highly effective in infecting primary hepatocyte and hepatoma cell lines, making them very useful tools for studies of hepatitis B and hepatitis C viruses [12–14].

To enhance gene delivery of the baculovirus vector into mammalian cells, a vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped baculovirus vector was generated to improve foreign gene expression in mammalian cells compared to wild-type baculovirus vector [15]. The VSV-G pseudotyped virus is thought to facilitate particle entry and escape from endolysosomes. Schauber et al. [16], however, reported that transduction of the baculovirus *gp64* pseudotyped lentiviral vector is similar to that of the VSV-G pseudotyped lentiviral vector, except for in hemapoietic cells. Furthermore, Tani et al. [17] suggested that baculovirus *gp64* vectors directly interact with general phospholipids in the surface membrane of mammalian cells, and the VSV-G pseudotyped virus interacts with general phosphatidylserine. Therefore, we hypothesized that baculovirus *gp64* mediates transduction in the presence of intracellular or viral factors.

In the present study, we investigated baculovirus infection and transduction in permissive and non-permissive mammalian cells in vitro. We report that AcMNPV penetrated not only Huh-7 cells, but also the hematopoietic cell lines, Raw264.7 and YAC-1 cells, while Raw264.7 and YAC-1 cells inhibited GFP gene expression. Nuclear transport of the AcMNPV genome was inhibited in YAC-1 cells. Our data suggest that AcMNPV might be useful for the development of more efficient baculovirus vectors for gene therapy.

Materials and methods

Animals and cell lines. Female C57BL/6 mice were purchased from Nippon SLC (Hamamatsu, Japan) and used at 6–8 weeks of age. A human hepatoma cell line (Huh-7), a mouse melanoma cell line (B16), a mouse lymphoma cell line (YAC-1), and a mouse macrophage cell line (Raw267.4) were obtained from the Riken Cell Bank (Wako, Japan). Huh-7, B16, and Raw264.7 cells were cultured in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St Louis, MO) and YAC-1 in RPMI 1640 (Sigma Chemical Co.), both supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Where indicated, culture medium was supplemented with 5-aza-2′-deoxycytidine (Aza-C; Sigma Chemical Co.) or sodium butyrate (Wako Chemical Co., Osaka, Japan) after virus treatment.

Generation of recombinant baculovirus. Wild-type baculovirus AcMNPV was purchased from BD Bioscience (San Diego, CA). The GFP was moved from pLEGFP-C1 (BD Bioscience) to pcDNA3.1(+) (Invitrogen Corp., Carlsbad, CA) as a *Nhe*I-*Xho*I fragment to construct pcDNA.GFP. Viruses were constructed using shuttle vectors derived from pVL1393 (BD Bioscience). The shuttle plasmid DNA was digested using a *Sma*I-*Eco*RI fragment. A 3.1 kbp BamHI-MunI fragment from pcDNA.GFP, which contains the CMV-IE promoter/enhancer with a GFP gene and polyadenylation signal expression cassette, was inserted into the pVL1393 backbone (pVL1393/CMV-GFP). Recombinant baculovirus AcCMV-GFP was generated according to the manufacturer's instructions (BD Bioscience). Purification of the baculovirus was performed as described previously [18].

Transduction of mammalian cells by baculovirus. Cells were seeded in 35 mm culture dishes at 3×10^5 cells per dish. Culture medium was removed, replaced with virus treatment, and incubated for 1 h at 37 °C. After removal of the virus, fresh medium was added and cultures were incubated at 37 °C. Cultures were harvested, washed, and resuspended in phosphate-buffered saline. The GFP-expressing populations were analyzed by FACSCalibur (Becton Dickinson, Mountain View, CA).

Polymerase chain reaction. Total DNAs were extracted from cells using the GenEluteTM Mammalian Genomic DNA Miniprep Kit (Sigma Chemical Co.) according to the manufacturer's instructions. Locations of the baculovirus genome were determined as described previously [15]. One-step polymerase chain reaction (PCR) was performed on 50 ng DNA samples using KOD-Plus—(Toyobo, Osaka, Japan). The specific primer pairs are presented in Table 1.

Construction of plasmids and luciferase assay. The enhancer *hr5* and p35 promoter were cloned from the AcMNPV genome to a pCR2.1-TOPO vector (Invitrogen), and the sequence homology was verified. The sequences of the specific primers were as follows: hr5: 5′-GAGCTCTTGC ACAATGTAACTAGTGCACTC-3′ (sense) and 5′-GCTAGCCGTCCG TTTGATTTAACG-3′ (anti-sense); p35 promoter: 5′-CTCGAGGTCGG TCACCATGTACAAAAG-3′ (sense) and 5′-AGATCTGCTCAAATGC TCACTTAATACAAG-3′ (anti-sense). The pGL.hr5.p35prom vector was constructed by a 705-bp SacI-NheI fragment containing hr5 and a 503-bp XhoI-BglII fragment containing the p35 promoter in a firefly luciferase reporter pGL3-basic vector (Promega Corp., Madison, WI). Transfections were performed by using Fugene 6 (Roche Applied Science), according to the manufacturer's instructions. Cells were plated at $0.8–3.0\times 10^5$ cells per well in 24-well plates 24 h before transfection. Reporter plasmid (400 ng) was mixed with 2 μl Fugene 6 in 50 μl serum-free medium, left for 15 min at room temperature, and then added to the cells. The pRL-TK vector (Promega) containing the herpes simplex virus thymidine kinase (HSV-TK) promoter driving the expression of a renilla luciferase reporter was used as an internal control for transfection efficiency (20 ng per transfection). Cells were harvested and lysed 24 h post-transfection. The firefly and renilla luciferase activities were measured using the Promega Dual luciferase assay system with 20 μl of cell extract according to the manufacturer's instructions with a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA).

Results and discussion

Recombinant baculovirus-mediated GFP gene expression in mammalian cells in vitro

Infectivity and intensity of expression of AcCMV, which uses the CMV-IE promoter to express the GFP gene, was

Table 1
Primers used for PCR[a]

Gene	Sense	Anti-sense	Annealing temp (°C)
gp64	CTACTAGTAAATCAGTCACACC	CCAAGTTTTTAATCTTGTACGG	50
p35	GGTAGAAATCGACGTGTCCCAGA	CGTGAGCAAACGGCACAATAAC	56
p143	TAATGTATCCAGGGTCGGTGCTCT	CGCATCATCATGTCCAAAGTGGAC	56
G3PDH	TCCACCACCCTGTTGCTGTA	ACCACAGTCCATGCCATCAC	60

[a] All sequences are presented in the 5′–3′ direction.

investigated by FACS analysis using the permissive cell lines Huh-7 and B16, and non-permissive cell lines Raw264.7 and YAC-1. These cells were infected with AcCMV-GFP (MOI 50) following the addition of a DNA methylation inhibitor, Aza-C, or a deacetylase inhibitor, butyrate, and the cells were harvested 24 h later. Condreay et al. and Liang et al. [19,20] reported that butyrate enhances the expression of recombinant baculovirus in mammalian cells, but the effects of DNA methylation inhibitior have not been reported. The percentage of GFP-expressing cells and the mean fluorescence intensity of AcCMV-GFP were detected by flow cytometric analysis. The percentage of GFP-expressing Huh-7 cells was 21.9%, higher than in the other cell lines (B16, Raw264.7, and YAC-1; Fig. 1A). Flow cytometric analysis did not detect GFP expression in Raw264.7 or YAC-1 cells. The mean fluorescence intensity of AcCMV-GFP-infected cell lines was higher in Huh-7 cells than in the other cell lines (Fig. 1B). Butyrate, but not Aza-C, increased the percentage of GFP-expressing cells approximately 2-fold in Huh-7 cells and 10-fold in B16 cells. Mean fluorescence intensity of GFP expression was enhanced approximately 3-fold in Huh-7 cells and 2-fold in B16 cells. Nusinzon and Horvat reported that a deacetylase inhibitor inhibits the anti-viral response of interferon-stimulated transcription [21]. Transduction of these cells was not markedly enhanced, however, when Aza-C was used as the DNA methylation inhibitor. Therefore, expression

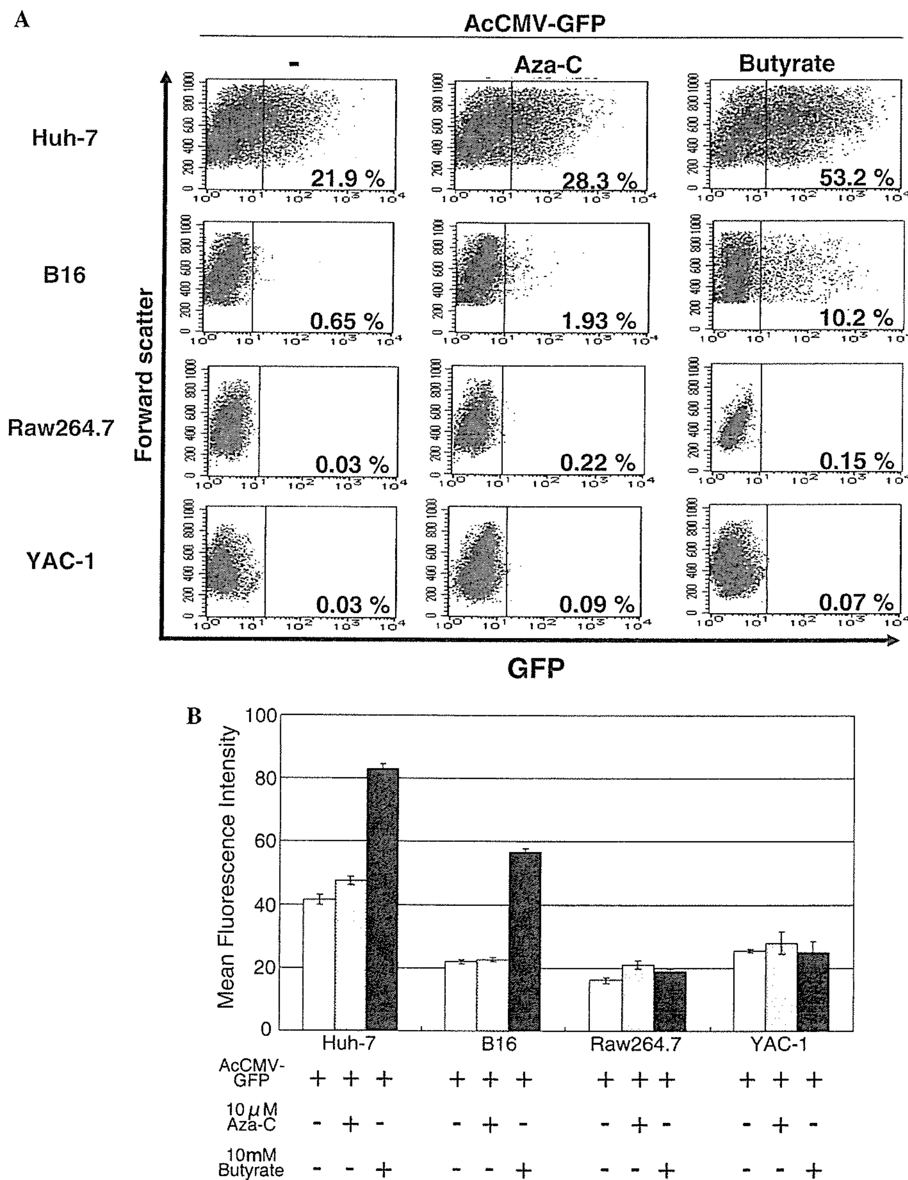

Fig. 1. In vitro recombinant baculovirus transduction in permissive or non-permissive cell lines. The cells were treated with recombinant baculovirus (AcCMV-GFP; MOI 50) or vehicle. After culturing in culture medium with or without 10 μM Aza-C or 10 mM butyrate for 24 h, harvested Huh-7, B16, Raw264.7, and YAC-1 cells were analyzed to determine the percentage of GFP-expressing cells (A) and mean fluorescence intensity of the GFP (B) using flow cytometry. The data shown are representative of duplicate experiments that gave similar results.

and transcription, but not infectivity, of recombinant baculovirus was enhanced in Huh-7 and B16 cells to which butyrate was added to induce hyperacetylation, but not in cells in which chromatin methylation was inhibited. Thus, we suggest that recombinant baculovirus can enter Huh-7 and B16 cells, although GFP expression of recombinant baculovirus was inhibited by acetylation in these cells due to cellular anti-viral function.

AcMNPV infects permissive and non-permissive cell lines

Barsoum et al. [15] reported that the VSV-G pseudotype baculovirus as well as wild-type baculovirus could be modified for more efficient transduction, because baculovirus infection produces a low level of viral DNA. We then determined whether the cell lines were infected with AcMNPV. The cells were treated with one of several MOIs (1, 10, 50, 100, or 200) of AcMNPV and harvested 24 h later. AcMNPV-infected cells were detected from total DNA of infected cells by PCR amplification using AcMNPV-*gp64*-specific primers. Surprisingly, AcMNPV-*gp64*-specific bands from total DNA of infected cells were detected not only in Huh-7 and B16 cells, but also in Raw264.7 and YAC-1 cells (Fig. 2). Furthermore, specific bands of the viral genome were also detected in mouse primary splenocytes (data not shown). The AcMNPV-specific bands from cells infected with AcMNPV (MOI 1) were detected only in Huh-7 cells. These findings suggest that baculovirus AcMNPV can enter various cell lines, even when the recombinant baculovirus containing the expression cassette is not expressed.

Degradation and nuclear transport of viral DNA in the infected cells

We investigated whether the genomic DNA is degraded in the infected cells to produce cellular resistance to the virus. Wang et al. [22] demonstrated that baculovirus DNA is degraded in hepatoma cells. Condreay et al. and Merrihew et al. [19,23], however, reported that genomic DNA of recombinant baculovirus expressing neomycin phosphotransferase is integrated into CHO cells at a rate of approximately 1 in 50–100 cells. To determine if the cells contain viral DNA, the cells were treated with AcMNPV (MOI 50) and harvested at various time points. Cells invaded by AcMNPV were detected by PCR amplification using AcMNPV-*gp64* specific primers under dividing conditions. Viral DNA bands were confirmed in Huh-7 and B16 cells up to 96 h after infection (Fig. 3A). In the non-permissive cell lines Raw264.7 and YAC-1, however, AcMNPV (MOI 50) survived for up to only 48 h after infection. These results suggest that in Huh-7 and B16 cells, baculovirus viral DNA is taken up more easily or the viral DNA is slower to degrade.

Cells were infected with AcMNPV, total DNA was extracted 24 h later, and nuclear and cytoplasmic DNA was isolated as described in Materials and methods section. In adherent Huh-7, B16, and Raw264.7 cells, the AcMNPV genome was transported to the nucleus (Fig. 3B). In contrast, in suspension cells (YAC-1), the genomic DNA was not transported to the nucleus. van Loo et al. [24] suggested that the baculovirus genome is transported by actin filaments in mammalian cells, as in insect cells. The CMV-IE promoter strongly enhances binding with RNA polymerase II in the nucleus [25]. Recently, Salminen et al. [26] reported that human hepatocytes block nuclear transport of the baculovirus genome by microtubules, in contrast to insect cells. These results suggest that the recombinant

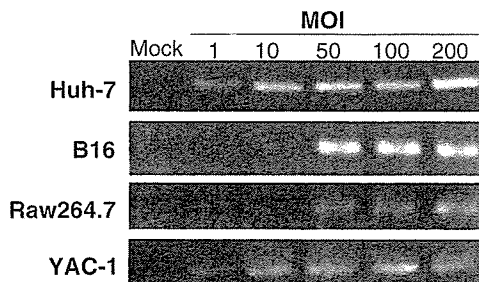

Fig. 2. AcMNPV MOI-dependent infection in various mammalian cells. The cells were treated with AcMNPV (MOIs 1, 10, 50, 100, and 200). The cells were harvested after 24 h. AcMNPV genomic DNA was detected in isolated total DNA of treated cells by PCR using AcMNPV-*gp64*-specific primers. The data shown are representative of duplicate experiments that gave similar results.

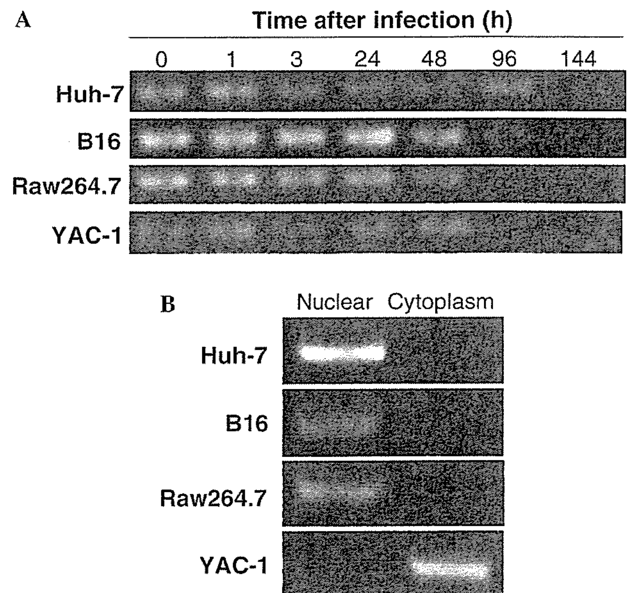

Fig. 3. Degradation and nuclear transport of the AcMNPV genome in various mammalian cells. (A) The cells were treated with AcMNPV (MOI 50). These cells were harvested at the indicated times, and total DNA was isolated. (B) The cells were treated with AcMNPV (MOI 50). The cells were harvested after 24 h, and the nuclear and cytoplasmic DNA was isolated as described in Materials and methods section. AcMNPV genomic DNA was detected in isolated DNA by PCR using AcMNPV-*gp64*-specific primers. The data shown are representative of duplicate experiments that gave similar results.

baculovirus did not activate the CMV-IE promoter in the infected macrophage cell line Raw264.7, and was inhibited by degradation of the viral genome and an unknown mechanism in infected YAC-1 due to inhibited nucleus transport of the genomic DNA into the nucleus.

AcMNPV expression determined using p35 gene detection in Huh-7 and B16 cells

The host range of AcMNPV in insect cells is involved in the expression of pan-caspase inhibitor p35 and viral helicase p143 [5–9]. We examined whether insect host range-detecting genes *p35* and *p143* were expressed in mammalian cells using reverse transcription RT-PCR analysis with *p35* or *p143*-specific primers. The cells were infected with AcMNPV and then harvested. Total RNA was extracted from infected cells 24 h later. The baculovirus *p35* gene was more clearly expressed in Huh-7 cells than in B16 cells, but was not expressed at all in the other cell lines (Fig. 4A). The AcMNPV *p143* gene, which is a host range-detecting gene in insect cells, was not expressed in mammalian cell lines.

To define the p35 promoter activity in mammalian cells, we constructed the luciferase reporter plasmid pGL.hr5.p35prom the inserted p35 promoter region and enhancer hr5 of the p35 promoter (Fig. 4B). For a transient reporter assay, cells were transfected with the reporter plasmid, and the reporter assay was examined 24 h later. Relative luciferase activity of baculovirus p35 promoter was clearly enhanced in Huh-7 and B16 cells. Based on the expression of *p35* mRNA, relative luciferase activity in Huh-7 cells was enhanced 2.4-fold compared to the activity in B16 cells. As expected, relative luciferase activity in host Sf9 cells was the highest in these cell lines (Table 2). These results suggest that the baculovirus p35 promoter was clearly activated in non-host mammalian cells, as in Huh-7 and B16 cells.

The AcMNPV *p35* gene has multiple functions. AcMNPV p35 inhibits apoptosis by inhibiting pan-caspase and mitigating eIF2α phosphorylation in various cell lines, C. elegans cells, drosophila cells, and mammalian cells [27–31]. Although the baculovirus BmNPV *p35* gene shares approximately 90% identity with the AcMNPV *p35* gene, BmNPV *p35* does not block apoptosis very well, compared

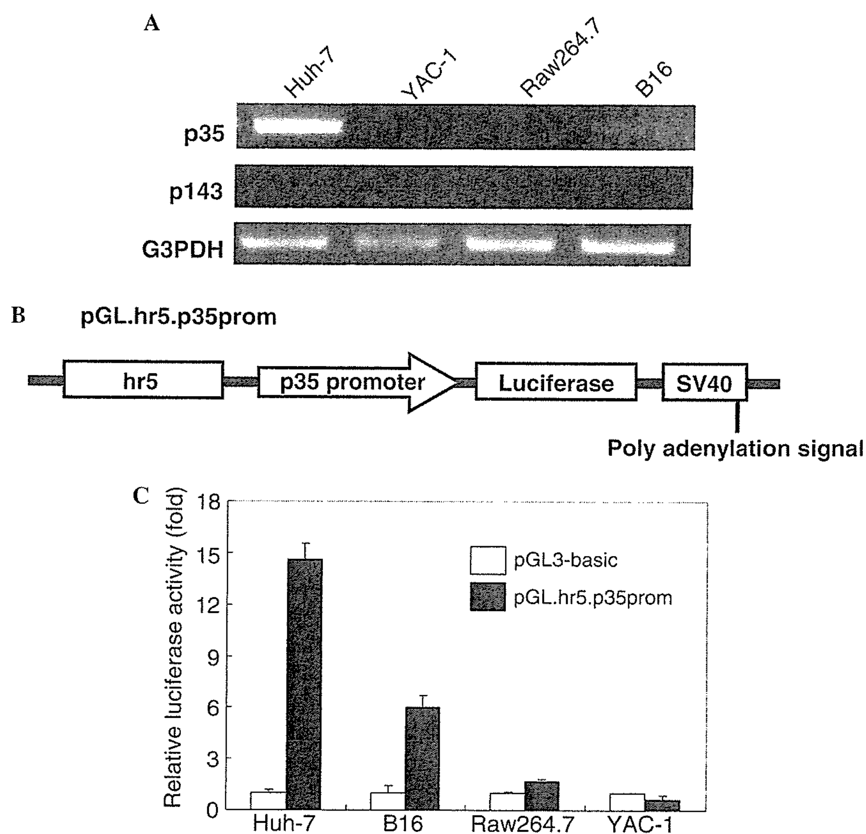

Fig. 4. Expression of AcMNPV p35 in various mammalian cells. (A) The cells were treated with AcMNPV (MOI 50). The cells were harvested after 24 h. The AcMNPV *p35* or *p143* gene was detected in isolated total RNA of treated cells by RT-PCR using specific primers. The data shown are representative of duplicate experiments that gave similar results. (B) Reporter plasmid containing the luciferase gene was placed under the control of the p35 promoter and enhancer hr5 was constructed. (C) The cells were co-transfected with pGL3-basic plasmid (no insert) or reporter plasmid (400 ng/well) and pRL-TK plasmid (20 ng/well) as an indicator for normalization of transfection efficiency. Cell extracts were prepared 24 h later and assayed for luciferase activity. Ratio of relative luciferase activity represents the firefly luciferase to renilla luciferase ratio with the pGL3-basic plasmid value defined as 1. The values reported represent averages of duplicate transfection.

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 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

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

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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.
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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

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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).

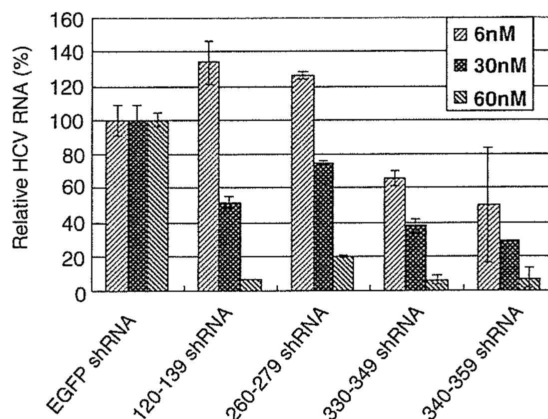

Fig. 2. Inhibition of HCV replication by shRNAs synthesized using the T7 RNA polymerase system in the HCV replicons. The effect of transfection with shRNAs or control EGFP HCV replication was measured by RT-PCR of HCV RNA 48 h after transfection. All values are shown as the percentages of the EGFP negative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

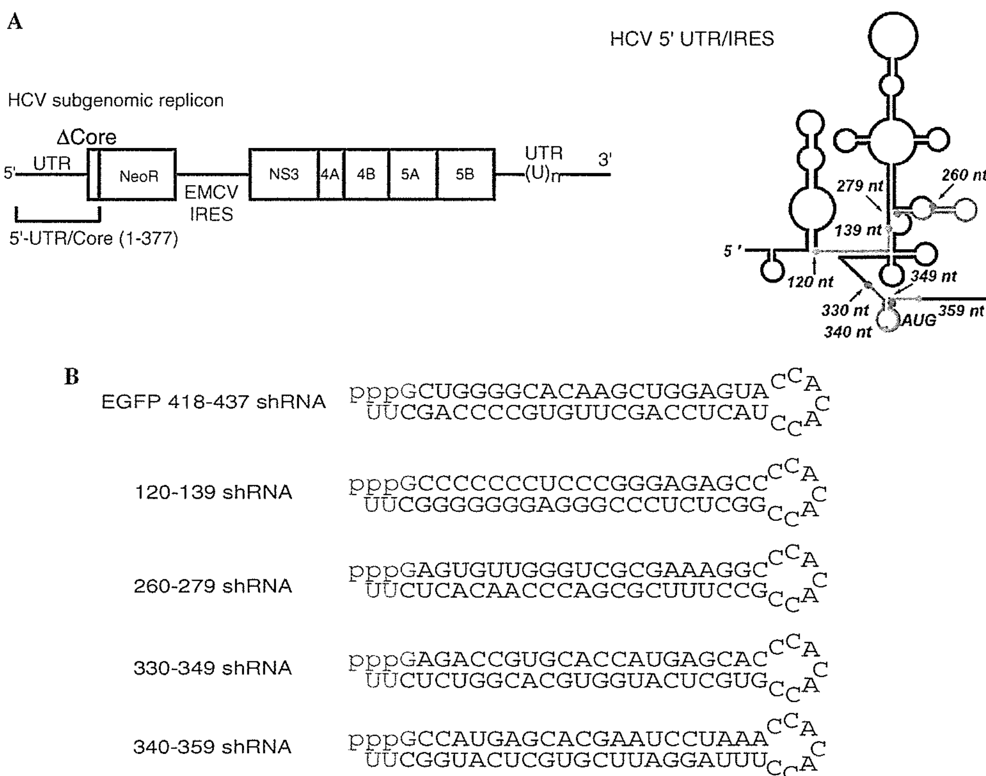

Fig. 1. shRNA-targeted regions of the HCV subgenomic replicons and shRNA sequences. (A) Schematic representation of HCV subgenomic replicons and shRNA targeted regions on HCV 5′-UTR/IRES secondary structure. (B) Sequences of shRNAs synthesized by phage polymerase. The control shRNA:EGFP-shRNA.

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

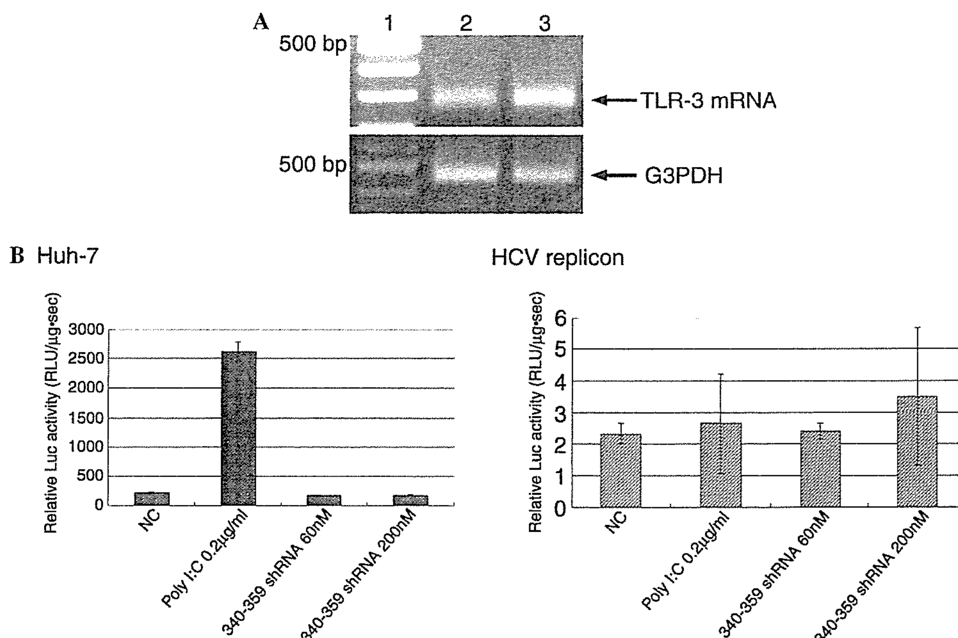

Fig. 3. Activation of IRF-3 after transfection of Huh-7 cells and HCV replicons with shRNAs synthesized using the T7 RNA polymerase system. (A) RT-PCR analysis of TLR-3 mRNA expression in Huh-7 cells and HCV replicons cells. The RT-PCR assay for TLR-3 mRNA was performed using TLR-3 mRNA-specific primers 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.

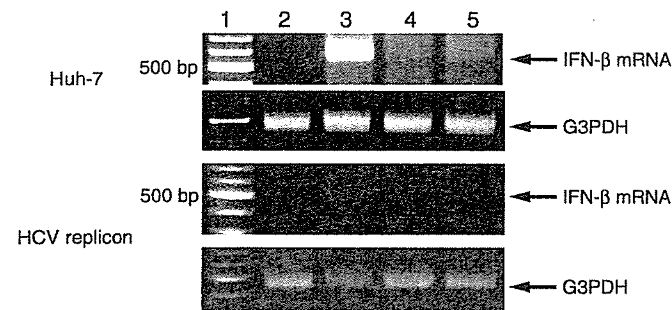

Fig. 4. RT-PCR analysis of IFN-β mRNA expression in Huh-7 cells and HCV replicons. The RT-PCR-amplified products were fractionated by electrophoresis on a 2.0% agarose gel and stained with ethidium bromide. Lane 1, DNA ladder; lane 2, negative control; lane 3, polyI:C (10 μg); lanes 4 and 5, 330-349-shRNA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

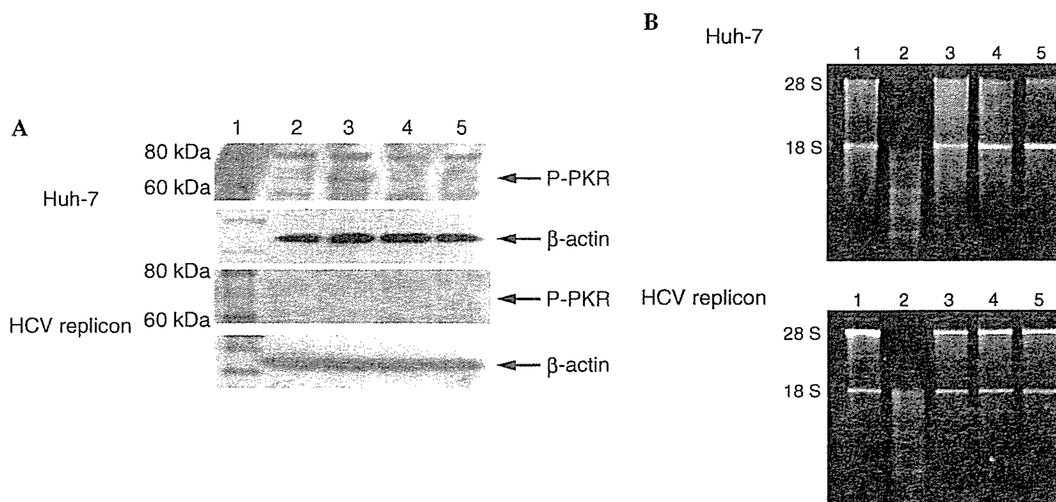

Fig. 5. Activation of PKR and RNase L with shRNAs synthesized using the T7 RNA polymerase system. (A) After 24 h, the Huh-7 cells and HCV replicons were lysed with lysis buffer. The lysates (80 μg protein/lane) were assayed by Western blot analysis with antibodies against phosphorylated PKR or β-actin 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

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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

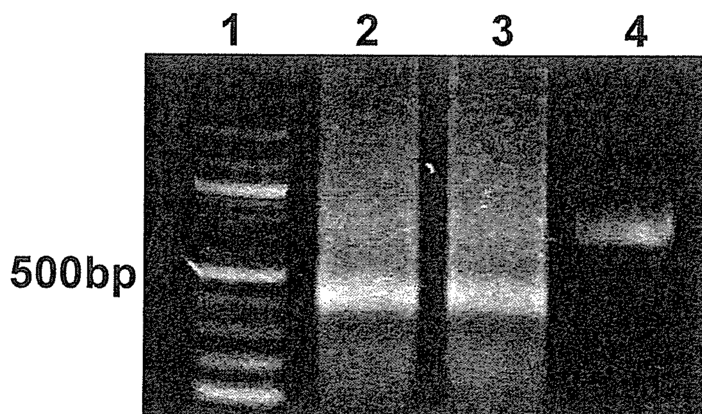

FIGURE 1 RT-PCR analysis of the sense, antisense, and dsRNA mRNA expression in Huh-7 cells. The RT-PCR amplification products, fractionated by electrophoresis on a 2.0% agarose gel with ethidium bromide staining. Lane 1: 100 bp DNA ladder marker; lane 2: pCMV-IRES-sense transfected Huh-7 cells; lane3: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

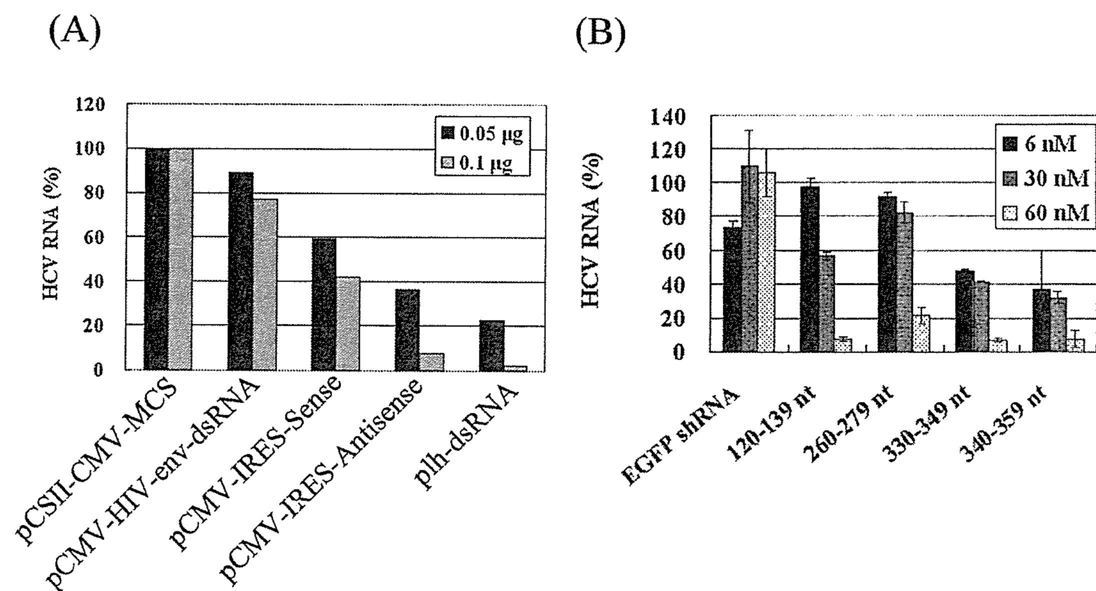

FIGURE 2 (A) Inhibition of HCV replication by sense, antisense, and dsRNA expression vectors. The dose-dependent inhibition of HCV replication by pCMV-IRES-sense, pCMV-IRES-antisense, and plh-dsRNA in HCV replicon. (B) Inhibition of HCV replication by synthesized shRNAs. The dose-dependent inhibition 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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SILENCING OF HIV-1 GENE EXPRESSION BY siRNAs IN TRANSDUCED CELLS

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□ *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

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