INHIBITION OF EXPRESSION OF RNA POLYMERASE WITH SMALL INTERFERING RNAs TARGETING A CONSERVED MOTIF IN THE RESPECTIVE VIRAL GENES IN VIRUSES OF THE FAMILY *FLAVIVIRIDAE*

Z. XU[#], X. LI[#], R. LIU, Y. SI, M. SUN, M. JIN, H. CHEN, P. QIAN[*]

Laboratory of Animal Virology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, Hubei 430070, P.R. China

Received March 19, 2007; accepted August 22, 2007

Summary. – The RNA-dependent RNA polymerase (RdRp) in viruses of the family *Flaviviridae* plays an important role in the viral replication process and in the forming of a replicase complex. We used small interfering RNAs (siRNAs) corresponding to the highly conservative Motif V of RdRp gene of different viruses to examine their role in modulating the expression of RdRp. Evaluation of the expression of RdRps was performed by the fluorescence, flow cytometry, Western blotting, and real-time PCR. We found that Classical swine fever virus (CSFV) siRNA could completely block the transcription and expression of RdRp. Additionally, Hepatitis C virus (HCV) siRNA could cause effective inhibition of RdRp, whereas Japanese encephalitis virus siRNA did not show significant repression of corresponding RdRp. These results demonstrated that siRNAs inhibited the expression of tested RdRps at the transcription level or at the posttranscriptional processing to a different extent.

Key words: Classical swine fever virus; Japanese encephalitis virus; Hepatitis C virus; small interfering RNAs; RNA-dependent RNA polymerase

Introduction

The members of the family *Flaviviridae* are single-stranded, positive-sense RNA viruses. CSFV, one of the most important animal pathogen belonging to the genus *Pestivirus* causes a highly contagious disease of domestic pigs and wild boars. It has still broken out worldwide and led to the huge economical loss (Li *et al*., 2006). HCV, a major human pathogen belonging to the genus *Hepacivirus,* can evoke acute and chronic hepatitis that may lead to a cirrhosis and liver cancer with 170 million infected people worldwide (Ferron *et al*., 2005). Japanese encephalitis virus (JEV) belonging to the genus *Flavivirus* is an emerging pathogen causing acute Japanese encephalitis in humans and many kinds of animals exhibiting mental disturbances for 40%–70% of survivors (Konishi *et al*., 2000). The members of the genus *Pestivirus* show a broader similarity in the genome structure and translation strategy to the genus *Hepacivirus* than to the genus *Flavivirus* (Lindenbach and Rice, 2001).

The RdRp of *Flaviviridae* plays a crucial role in the viral replication cycle and in the forming of a replicase complex (Al-Mutairy *et al*., 2005). The primer-dependent or primer-independent mechanism is used in the RNA replication process of positive-sense single-stranded RNA viruses at or near the 3'-end of the RNA template (Paul *et al*., 1998). In *Flaviviridae,*

[#]Contributed equally to this work.

[*]Corresponding author. E mail: qianp@mail.hzau.edu.cn; fax: +8627-87281795.

Abbreviations: eGFP = enhanced green fluorescent protein; BVDV = Bovine viral diarrhea virus; CSFV = Classical swine fever virus; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HCV = Hepatitis C virus; JEV = Japanese encephalitis virus; RISC = RNA induced silencing complex; siRNA = small interfering RNA; RdRp = RNA-dependent RNA polymerase

de novo initiation is the likely mechanism occurring during virus replication in the infected cells (Selisko *et al*., 2006). For example, both of the RdRps of Bovine viral diarrhea virus (BVDV) and HCV can use the *de novo* mechanism to initiate RNA replication without a primer (Choi *et al*., 2004). Thus, the virus propagation may be inhibited, when the normal function and protein expression of RNA polymerase is repressed.

RNA interference has become an essential tool in the study of specific viral gene function and represented a promising approach for the development of an effective antiviral drug. The RNA-induced silencing complex (RISC) uses small interfering RNAs (siRNAs) to select its targets in a sequence-dependent manner (Bengert *et al*., 2005). The RISC can cleave one strand of siRNAs, leaving the remaining unpaired guide strand to search for complementary mRNA target. Once recognized, the corresponding mRNA is cut by an argonaute endonuclease in the RISC, leading to specific gene silencing. (Dykxhoorn and Lieberman, 2006).

In this paper, we investigated the inhibitory effect of the siRNAs on truncated RNA polymerases of CSFV, HCV and JEV, as members of the family *Flaviviridae*. To aim directly at each virus species, the three siRNAs were invented as complementary to the conserved Motif V of corresponding RdRps of these viruses. HCV and CSFV siRNAs were identified as strong repressors of mRNA accumulation and protein expression.

Materials and Methods

Construction of plasmids with truncated RdRp genes fused to eGFP gene. The truncated CSFV RdRp gene (located at nt 10587–11631, GenBank, Acc. No. AY663656) was amplified by PCR from a full-length genomic plasmid pOKCWH using the primers CRP-up (5'-TTTAAGCTTATGTTCGAACGCAAAAACGTA-3') and CRP-down (5'-AAAGGATCCTTGCAGTTCAGTTGAT-3'). The HCV RdRp fragment (located at nt 7970–9044, Acc. No. AY587016) was amplified from plasmid pTagNS5b (kindly provided by Dr. Guiqing Peng, Wuhan, China) using the primers HRP-up (5'-TTTAAGCTTATGGACTTGCTGGAAGACAC-3') and HRP-down (5'-AAAGGATCCGATCTCACCTGGAGAGT-3'). The truncated RdRp genes were cloned into a reporter plasmid pCDM4 (a gift from Dr. Hongbo Zhou, Wuhan, China) using the *Hind*III and *Bam*HI sites. The JEV RdRp target sequence (located at nt 9063–10044 in genome, Acc. No. AF315119) was amplified from JEV SA14-14-2 with RT-PCR Kit with Avian myeloblastosis virus (TaKaRa) using the following primers JRP-up (5'-TTTGGATCCATGAAGCCTGGAGAGTTTGGA-3') and JRP-down (5'-AAAGGATCCTGGCACTGCTGAGCAAA-3'). The cDNA products were inserted into pCDM4 plasmid with the single *Bam*HI site. Plasmid pCDM4 contains an enhanced green fluorescent protein (eGFP) downstream of the immediate early promoter of the Human cytomegalovirus (HCMV). The RdRp gene fragments without stop codon were fused upstream of eGFP gene, and the resulting plasmids were designated pCRGFP, pHRGFP and pJRGFP, respectively (Fig. 1A).

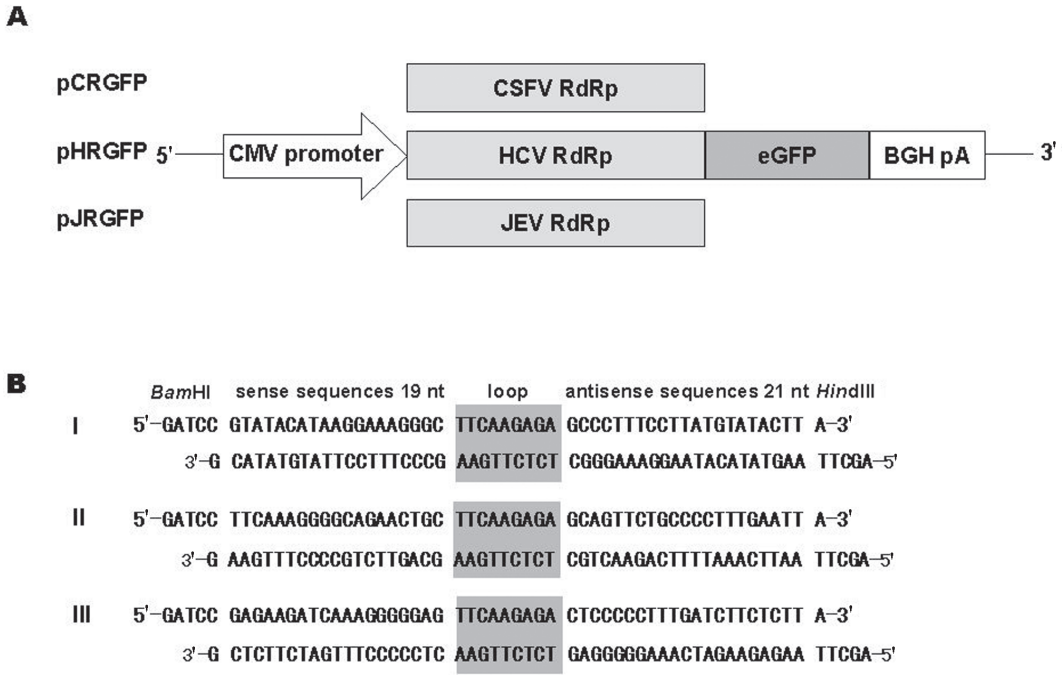

Fig. 1

Schematic representation of the RdRp and siRNA plasmid system

A. Plasmids containing the fragments of RdRp and eGFP genes under the control of HCMV. B. 55-mer oligodeoxynucleotide sequences encoding CSFV siRNA (I), HCV siRNA (II), and JEV siRNA (III).

Construction of the plasmids encoding siRNAs. Three target sequences in the coding region of truncated RdRp genes were selected on the following basis: software siRNA Target Finder in Ambion (www.ambion.com/techlib) and conservative motif sequence within family *Flaviviridae*. The 21 nt target sequences were selected as a basis for designing 55-mer siRNA template oligonucleotides that were synthesized and inserted into the *Bam*HI and *Hind*III sites of the siRNA expression vector pSilencer 4.1-CMV neo (Ambion) (Fig. 1B). The resulting recombinant plasmids were designated as pSiCSFV, pSiHCV, and pSiJEV, respectively. The plasmid pSilencer 4.1-CMV neo negative control (pS-NC) contains the negative control siRNAs with a sequence that does not target any gene product. All plasmid DNA extraction and purification were carried out using the PureYield™ Plasmid Midiprep System (Promega).

Transfection of cells. Porcine kidney cells (PK-15) were cultivated in DMEM (Sigma) and supplemented with 7% horse serum (Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma) at 37°C and 5% CO_2. Transfection was performed with 6 µg of pSiCSFV or pS-NC and co-transfected with 6 µg of pCRGFP and mixed with 12 µl of Lipofectamine™ 2,000 (Invitrogen). The other plasmids pSiHCV and pSiJEV were treated in similar way. The non-transfected PK-15 cells served as the negative control. All procedures were performed as specified by the manufacturer.

Fluorescent assay. After transfection, the six-well plates were cultivated for 2 days. Images for examining eGFP expression were screened under a fluorescence microscope (OLYMPUS IX70).

Western blot analysis. The cells were harvested at 48 hrs post transfection and the cell pellets were dissolved in 100 µl of sample buffer and heated at 95–100°C for 5mins. After SDS-PAGE and blotting, the nitrocellulose membrane was blocked by incubation in 5% nonfat dry milk in TBST buffer (0.8% NaCl, 0.3% Tris, 0.1% Tween-20 pH 7.6). The blot was washed three times with TBST and incubated in rabbit anti-GFP antibody (Cell Signaling Technology) made in 5% BSA in TBST buffer overnight. The blot was washed 3 times and incubated with HRP-conjugated goat anti-rabbit IgG (Southern Biotech) diluted 1:2,000. After washing, the blot was developed with 3,3-diaminobenzidine tetrahydrochloride (DAB). A parallel experiment was performed using the monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control protein quantifying the specific changes in expression of other proteins.

Flow cytometry assay. The number of cells expressing GFP was measured by flow cytometry assay using the FACSCalibur (Beckton-Dickinson). The data were analyzed by the soft CELLQuest Pro.

Real-time PCR. A total RNA in transfected and control cells was extracted using the EaZy Nucleic Acid Isolation RNA-Solv Reagent (Omega Bio-tek). The amount of RNA in the samples was determined photometrically using an Ultrospec 500/1100 pro UV-Vis Spectrophotometer (Amersham Biosciences). The first-stand cDNA was synthesized using 2 µg of the total RNA for each sample with RNA PCR kit (TaKaRa).

The following primer sets for eGFP gene (142 bp) and housekeeping gene GAPDH (128 bp) were designed for real-time PCR: eGFP-upstream (5'-CGGCCACAAGTTCAGCGTGT-3'), eGFP-downstream (5'-CTGCACGCCGTAGGTCAGG-3'); GAPDH-upstream (5'-CGCCCATCACAAACATGGGG-3'), GAPDH-downstream (5'-CGTATGTTGAGTCCACTGG-3'). The primers were synthesized by AuGCT Biotechnology (Beijing, China). Briefly, reaction volumes of 50 µl contained 25 µl of SYBR® Green Realtime PCR Master Mix (Amersham Biosciences) 10 µmol/l of each primer, 2 µl of sample cDNA and H_2O was added to make the volume up to 50 µl. A master mix containing all the components except the cDNA was prepared and distributed into the respective number of MicroAmp Optical Tubes (Amersham Biosciences). Each sample was set up in four parallels. Real-time PCR was carried out in an ABI PRISM 7,500 (Amersham Biosciences). The PCR reactions were carried out at 95°C for 5 mins followed by 40 cycles of 95°C for 30 secs, 55°C for 30 secs and 72°C for 30 secs. The same cycling profile was used for eGFP and GAPDH real-time PCR.

Results

Inhibition of the protein synthesis by siRNAs

In the initial experiments, we compared the silencing ability of three siRNAs targeting different regions of the CSFV RNA polymerase gene and found that the siRNA targeting the conserved Motif V of RNA polymerase (nt 11100–11120 of the genomic RNA) can effectively inhibit expression of RdRp-eGFP fusion protein in the PK-15 cells. Due to this experiment, we designed two siRNAs for HCV (nt 8401–8421 of the genomic RNA) and JEV (nt 9469–9489 of the genomic RNA) at the corresponding sequence in the same motif.

In order to evaluate the inhibitory effect of siRNAs, the expression of protein of the reporter gene eGFP in the cotransfected cells was examined in the fluorescence assay at 36 hrs post transfection. Intensity of fluorescence in PK-15 cells transfected with pHRGFP/pS-NC or pJRGFP/pS-NC was more intense than that of pCRGFP/pS-NC. Intensity of the fluorescence was reduced to a different extent in the cell monolayers cotransfected with three RdRp-eGFP plasmids and relevant siRNAs as compared to the control cells. A few positive cells were observed by inhibition with CSFV siRNA. The cells interfered by JEV siRNA could also emit small amount of fluorescence light, but the fluorescence intensity was prominently weaken. There was no eGFP signal detected in the non-transfected cell control (Fig. 2). The specific silence was confirmed in four parallel experiments.

The inhibitory effects of the siRNAs for the target genes were quantitatively evaluated according the expression of RdRp-eGFP fusion protein by flow cytometry assay 48 hrs after transfection. The down-regulation of eGFP was evaluated by a proportion of eGFP positive cells and an intensity of fluorescence. The proportion of the fluorescent cell population and the mean fluorescence intensity of cells co-transfected with pCRGFP/pSiCSFV decreased by 93.5%

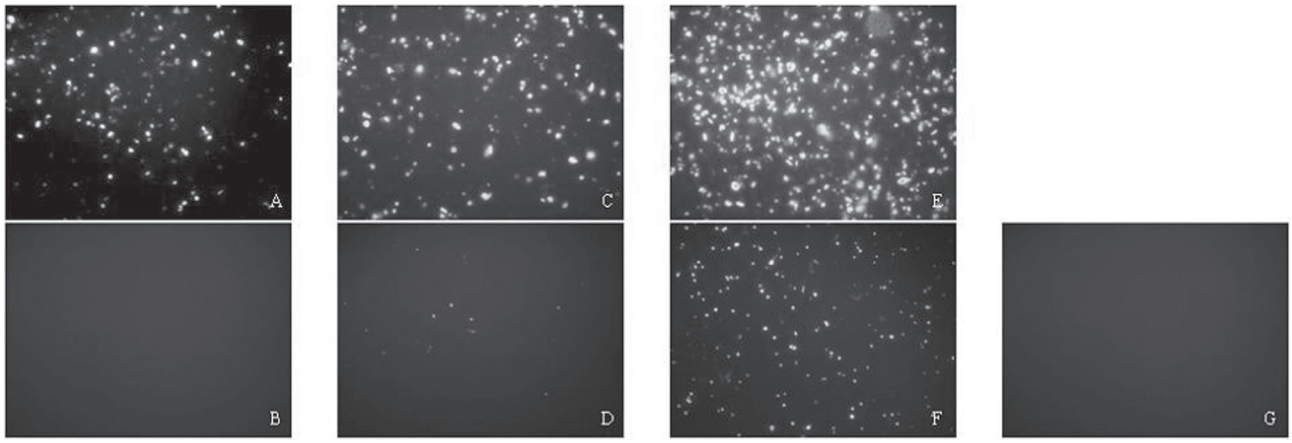

Fig. 2

Fluorescent assay of expression of RdRp-eGFP fusion protein with siRNA in PK-15 cells

pCRGFP and pS-NC (A), pCRGFP and pSiCSFV (B), pHRGFP and pS-NC (C), pHRGFP and pSiHCV (D), pJRGFP and pS-NC (E), pJRGFP and pSiJEV (F), nontransfected cells (G).

and 79.9% respectively, in comparison with pCRGFP/pS-NC; pHRGFP/pSiHCV reduced them by 88.9% and 85.7% respectively, in comparison with pHRGFP/pS-NC; and pJRGFP/pSiJEV reduced them by 34.4% and 54.5% respectively, in comparison with pJRGFP/pS-NC (Fig. 3).

The results of Western blotting showed that HCV siRNA could sharply repress the protein expression of HCV RdRp in cells. The similar results were obtained with CSFV RdRp protein inhibited by its siRNA. On the other hand, the level of the protein expression in cells co-transfected with pJRGFP/pSiJEV was nearly the same as the level in cells co-transfected with pJRGFP/pS-NC. There was no significant difference in the expression of GAPDH among tested groups (Fig. 4).

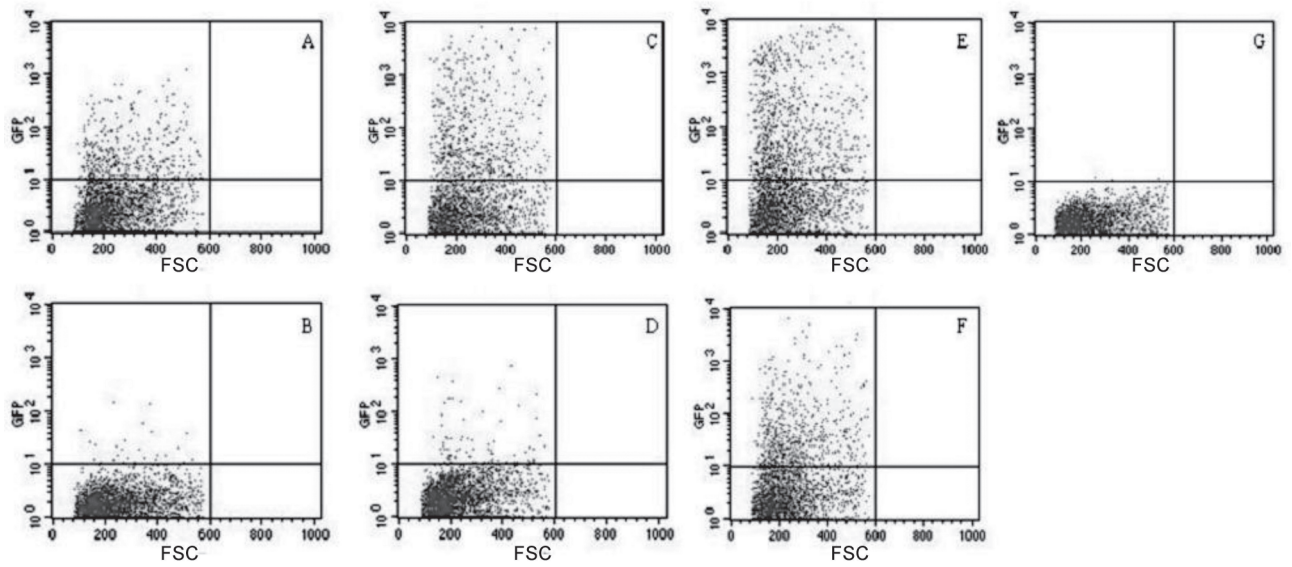

Fig. 3

Flow cytometry of PK-15 cells expressing RdRp-eGFP fusion protein silenced or non-silenced with siRNA

pCRGFP and pS-NC (A), pCRGFP and pSiCSFV (B), pHRGFP and pS-NC (C), pHRGFP and pSiHCV (D), pJRGFP and pS-NC (E), pJRGFP and pSiJEV (F), nontransfected cells (G).

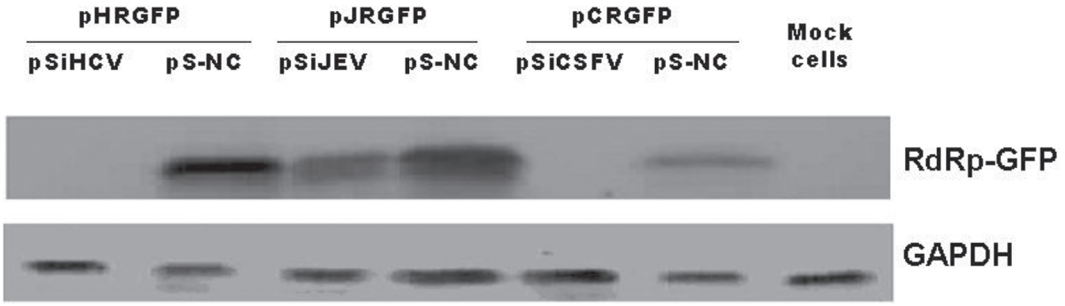

Fig. 4

Western blot analysis of expressed RdRp-eGFP fusion protein silenced or non-silenced with siRNA

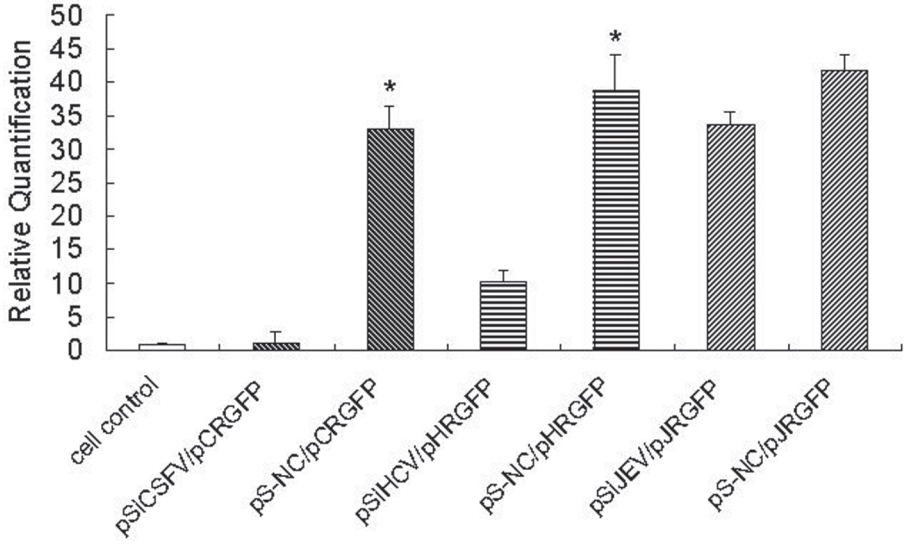

Fig. 5

Real-time PCR of expression of RdRp-eGFP fusion protein silenced or non-silenced with siRNAs in PK-15 cells

The relative values of CSFV, JEV, and HCV RdRp-eGFP mRNA of cotransfected or non-transfected cells were adjusted with values for GAPDH mRNA. *P <0.05

Inhibition of mRNA by siRNAs

The quantitative real-time PCR analysis indicated that the transcriptional level of mRNA of the CSFV RdRp, HCV RdRp and JEV RdRp gene decreased by 96.7%, 74.2% and 19.6% resp. in the presence of corresponding siRNA interference. The transcriptional level of GAPDH was used as an internal reference. The results of real-time PCR were in accordance with those of flow cytometry assay and Western blotting suggesting that the inhibitory effects of siRNAs occurred at the transcriptional or post-transcriptional level. The CSFV siRNA could repress most efficiently the expression of the CSFV RdRp without repressing host cellular gene expression (Fig. 5).

Discussion

RNA interference is a biological process of gene silencing in which small duplexes of RNA specifically target a homologous sequence for cleavage by cellular ribonucleases. Typically, the protein expression is decreased but not completely eliminated (Dykxhoorn and Lieberman, 2006). Previous reports have shown that induction of RNA interference has promising antiviral activity against positive- and negative-stranded RNA viruses and DNA viruses in model system (Haasnoot *et al*., 2003). Recently, RNA interference effectively inhibited mRNA accumulation and protein expression of HCV core (nt 370–390 and 521–541), E2 (nt 1571–1591 and 1983–2003), NS3 (nt 2052–2060)

and NS5B (nt 7326–7344) genes in HEK 293T cells (Liu *et al*., 2006; Takigawa *et al*., 2004). On the other hand, HCV replicons could escape RNA interference induced by a siRNA directed against the NS5B coding region (Wilson and Richardson, 2005).

The web-based software "siVirus" searches for functional, off-target minimized siRNAs targeting highly conserved regions of divergent viral sequences. These siRNAs are expected to resist viral mutational escape, since their highly conserved targets are likely to contain structurally/functionally-constrained elements (Naito *et al*., 2006). Moreover, many reports have shown that many of siRNAs directed against highly conserved HCV sequence gained effective inhibition for viral replication (Korf *et al*., 2005; Jarczak *et al*., 2005). In addition, a single conserved siRNA designed to target the envelope gene can potently protect mice against lethal encephalitis induced by two neurotropic flaviviruses, JEV and West Nile virus (Kumar *et al*., 2006). As soon as the viral genes are targeted, the related problems of viral sequence diversity and potential escape mutation could be circumvented by choosing highly conserved sequence whose mutation might result in the impaired viral fitness (Dykxhoorn and Lieberman, 2006).

The function of RdRp as the catalytic subunit of the viral replicase is required for the replication of all positive-stranded RNA viruses. Therefore, we wondered whether it would be possible to discover a common siRNA that could repress mRNA transcription and protein expression of RNA polymerase genes of CSFV, HCV and JEV together. Based on the multiple sequence alignment analysis, the selected RNA polymerase target fragments consist of eight conserved domains Pfam00680 (Bateman *et al*., 2002). Finally, the highly conserved Motif V was selected as the target sequence for designing siRNAs. In the initial study, we have proved by fluorescence and flow cytometry assay that siRNAs could not suppress the expression of eGFP (data not shown). Therefore, we could confirm that the reduction of GFP expression was a result of a decrease in the level of RdRp expression in above experiments.

To determine the cell population with positive fluorescence and the mean fluorescence intensity in cells, the flow cytometry assay was used to testify the extent of down-regulation for each siRNA at 48 hrs after transfection. The results corresponded with those of fluorescence imaging. In addition, we used the Western blotting to detect the expression of RdRp-GFP fusion protein in transfected cells. Furthermore, to obtain correct mRNA quantitation of RdRp genes, real-time PCR using specific primers was carried out to verify the down-regulation of mRNA transcription. Based on the obtained results it was concluded that CSFV siRNA could entirely block mRNA transcription and protein expression of CSFV RdRp gene in PK-15 cells and HCV siRNA could cause effective inhibition of relevant RdRp. On the contrary, the insignificant inhibition of JEV RdRp expression was found with JEV siRNA. Interestingly, the content of GC for the targeted sequence was 38% for pSiCSFV, lower than 47% for pSiHCV and pSiJEV. Furthermore, in the JEV siRNA, first 6/7 nucleotides in the 5'-end of the antisense strand were G/C, rather than 4/7 nucleotides in the same place of CSFV and HCV siRNA. The special experiments should be performed to show that the high GC content in vicinity of the loop is a disadvantage to the antisense strand binding to RISC complex.

In summary, plasmid-based CSFV siRNA is quite effective in silencing target-GFP fusion gene expression in PK-15 cells, which may provide favorable prevention and immunity of CSFV infection in swine. The expression of the fusion product of truncated RNA polymerase and eGFP was inhibited by the corresponding siRNA to a different extent at the level of transcription or the posttranscriptional processing. On the other hand, none of these siRNAs based on the design for this highly conserved target site were completely effective for tested viruses within the family *Flaviviridae*.

Acknowledgement. This work was supported by the National Natural Science Foundation of China (NSFC30600447) and by the Hubei Province Natural Science Foundation (2005ABA138). The authors are grateful to Chengdong Ye and Yao Hang for technical assistance.

References

Al-Mutairy B, Walter JE, Pothen A, Mitchell DK (2005): Genome Prediction of Putative Genome-Linked Viral Protein (VPg) of Astroviruses. *Virus Genes* **31**, 21–30.

Bengert P, Dandekar T (2005): Current efforts in the analysis of RNAi and RNAi target genes. *Briefings in Bioinformatics* **6**, 72–85.

Bateman A, Birney E, Cerruti L, Durbin R, Etwiller L, Eddy SR, Griffiths-Jones S, Howe KL, Marshall M, Sonnhammer EL (2002): The Pfam protein families database. *Nucleic Acids Res*. **30**, 276–280.

Choi KH, Groarke JM, Young DC, Kuhn RJ, Smith JL, Pevear DC, Rossmann MG (2004): The structure of the RNA-dependent RNA polymerase from bovine viral diarrhea virus establishes the role of GTP in de novo initiation. *Proc. Natl. Acad. Sci. USA* **101**, 4425–4430.

Dykxhoorn DM, Lieberman J (2006): Silencing viral infection. *PLos Med.* **3**, e242.

Ferron F, Bussetta C, Dutartre H, Canard B (2005): The modeled structure of the RNA dependent RNA polymerase of GBV-C virus suggests a role for motif E in Flaviviridae RNA. *BMC Bioinformatics* **6**, 255.

Haasnoot PC, Cupac D, Berkhout B (2003): Inhibition of virus replication by RNA interference. *J. Biomed. Sci*. **10**, 607–616.

Jarczak D, Korf M, Beger C, Manns MP, Kruger M (2005): Hairpin ribozymes in combination with siRNAs against highly conserved hepatitis C virus sequence inhibit RNA replication and protein translation from hepatitis C virus subgenomic replicons. *FEBS J.* **272**, 5910–5922.

Konishi E, Yamaoka M, Kurane I, Mason PW (2000): Japanese encephalitis DNA vaccine candidates expressing premembrane and envelope genes induce virus-specific memory B cells and long-lasting antibodies in swine. *Virology* **268**, 49–55.

Korf M, Jarczak D, Beger C, Manns MP, Kruger M (2005): Inhibition of hepatitis C virus translation and subgenomic replication by siRNAs directed against highly conserved HCV sequence and cellular HCV cofactors. *J. Hepatol.* **43**, 225–234.

Kumar P, Lee SK, Shankar P, Manjunath N (2006): A single siRNA suppresses fatal encephalitis induced by two different flaviviruses. *PLos Med.* **3**, e96.

Li X, Xu Z, He Y, Yao Q, Zhang K, Jin M, Chen H, Qian P (2006): Genome comparison of a novel classical swine fever virus isolated in China in 2004 with other CSFV strains. *Virus Genes* **33**, 133–142.

Lindenbach BD, Rice CM (2001): Flaviridae: The viruses and their replication. In Fields BN, Knipe DM, Howley PM (Eds): *Fields Virology.* 4rd ed. Philadelphia: Lippincott-Raven, pp. 991–1041.

Liu M, Ding H, Zhao P, Qin ZL, Gao J, Cao MM, Luan J, Wu WB, Qi ZT (2006): RNA interference effectively inhibits mRNA accumulation and protein expression of hepatitis C virus core and E2 genes in human cells. *Biosci. Biotechnol. Biochem.* **70**, 2049–2055.

Naito Y, Ui-Tei K, Nishikawa T, Takebe Y, Saigo K (2006): siVirus: web-based antiviral siRNA design software for highly divergent viral sequences. *Nucleic Acid Res.* **34**, 448–450.

Paul AV, Van Boom JH, Filippov D, Wimmer E (1998): Protein-primed RNA synthesis by purified poliovirus RNA polymerase. *Nature* **393**, 280–284.

Selisko B, Dutartre H, Guillemot JC, Debarnot C, Benarroch D, Khromykh A, Despres P, Egloff MP, Canard B (2006): Comparative mechanistic studies of novo RNA synthesis by flavivirus RNA-dependent RNA polymerases. *Virology* **351**, 145–158.

Takigawa Y, Nagano-Fujii M, Deng L, Hidajat R, Tanaka M, Mizuta H, Hotta H (2004): Suppression of hepatitis C virus replicon by RNA interference directed against the NS3 and NS5B region of the viral genome. *Microbiol. Immunol.* **48**, 591–598.

Wilson JA, Richardson CD (2005): Hepatitis C virus replicons escape RNA interference induced by a short interfering RNA directed against the NS5b coding region. *J. Virol.* **79**, 7050–7058.