PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2007, 34(10): 1092~1100
www.pibb.ac.cn

Efficient Depletion of Multiple SARS-CoV mRNAs by a Single Small Interfering RNA Targeting The Leader Sequence*

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Abstract Small interfering RNAs (siRNAs) can efficiently inhibit gene expression by sequence-specific RNA interference (RNAi). A common 5′ leader sequence exists in the genomic RNA and all subgenomic RNAs of SARS-CoV, and is well conserved among various SARS-CoV strains, thus providing a preferable target for RNAi of SARS-CoV replication. Here efficient depletion of the SARS-CoV mRNAs by either a synthetic siRNA or DNA vector-derived short hairpin RNAs (shRNAs) targeting the leader sequence in mammalian cell lines were reported. The siRNA or shRNAs efficiently suppressed the expression of an EGFP reporter gene which contains the leader sequence at the 5′ end. Both the siRNA and shRNAs efficiently knocked down the levels of leader-containing transcripts of three SARS-CoV genes encoding the spike protein, membrane protein and nucleocapsid protein were demonstrated. The results suggest that RNAi targeting the leader sequence is a potential efficient strategy for anti-SARS-CoV therapy.

Key words leader RNA, RNAi, SARS-CoV, shRNA, siRNA

Severe Acute Respiratory Syndrome (SARS) imperiled the health of human in more than 33 nations with over 8 450 infected cases and a fatality rate of 9.6% in an epidemic in 2002～2003. The causative pathogen, SARS coronavirus (SARS-CoV), and SARS-like coronaviruses form a distinct cluster within the *Coronavirus* genus[1~3]. SARS-CoV is believed to be an independent viral invasion from animal to human and to continue to circulate in civets, bats and perhaps other animals to cause sporadic human cases. The re-emergence of isolated SARS cases in Asia have confirmed a wide-spread conjecture that the SARS-CoV will likely be with humans for years to come[2, 3]. However, there is no specific therapy and effective vaccines available although much scientific progress has been made[4, 5]. The high mortality of SARS-CoV infections, its enormous economic and social impact, as well as fears of renewed outbreaks, make it paramount to endeavor to work out the efficacious treatments of SARS-CoV.

SARS-CoV and other coronaviruses more likely use a strategy, called discontinuous transcription, to synthesize a 3′-coterminal nested set of subgenomic mRNAs (sgmRNAs) with a common 5′ leader sequence derived from the genomic RNA[6~8]. Several groups have experimentally shown the presence of the leader sequence in each of the SARS-CoV sgmRNAs and determined the leader-to-body fusion sites precisely[1, 9]. The leader sequence of coronaviruses is approximately 65～98 nucleotides (nt) long, which includes a crucial signal, transcription regulating sequences (TRS), for the synthesis of sgmRNAs. The body TRSs on the genomic RNA serve as termination or pausing signals for negative strand synthesis, and the nascent negative-stranded RNAs then jump to the leader TRS sequence at the 5′ end of the genomic RNA template, generating subgenomic negative-stranded RNAs with an antisense leader sequence at

*This work was supported by a grant from SARS-CoV study from The Chinese Academy of Sciences.
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Received: March 2, 2007 Accepted: April 29, 2007

their 3′-ends, which in turn serve as templates for synthesis of viral sgmRNAs[6].

RNAi is a natural evolutionarily conserved sequence-specific post-transcriptional gene silencing defence mechanism in plants [10] and animals [11]. The siRNA-triggered RNAi is effective in degrading target RNAs with sequences homologous to that of siRNA [12]. Target RNA cleavage is catalyzed by the RNA-induced silencing complex (RISC), which functions as a siRNA-directed nuclease [13]. RNAi has attracted considerable attention as a potential therapeutic strategy against a number of human viruses such as HIV[14], HCV[15] and SARS-CoV[16]. On RNAi of SARS-CoV, efforts have been made by targeting different viral protein-coding sequences, such as those for replicase [17~20], RNA dependent RNA polymerase [21, 22], spike protein (S) [23~25], non-structural protein [18, 26] and nucleocapsid protein (N) [27~29]. However, the viral non-coding sequences, such as the 5′- and 3′-UTRs and the leader sequence, have received less attention as RNAi targets. For the leader sequence, there are several reasons to choose it as a target of RNAi. First, the leader sequence plays important roles in SARS-CoV RNA transcription process. Second, the leader sequence is present in almost all of the SARS-CoV sgmRNAs as well as in the viral genomic RNA. Third, the leader sequence is well conserved among various SARS-CoV strains compared to the viral coding sequences so that RNAi targeting the leader sequence will reduce the rate of escaped virus variants [30, 31]. Indeed there was a report that one leader-targeting shRNA expressed from a DNA vector could decrease the abundance of SARS-CoV mRNAs and inhibit the virus replication in Vero E6 cells, more effectively than an shRNA targeting the S gene region [32]. Here we present a study to evaluate the effect of both synthetic siRNA and shRNAs targeting two other regions in the leader sequence on down-regulation of SARS-CoV RNAs. The Enhanced Green Fluorescent Protein (EGFP) reporter gene or the SARS-CoV S, N or Membrane (M) gene, each fused with a SARS-CoV leader sequence at the 5′ end, was transfected into human HeLa or 293T cells. By co-transfection with the leader sequence-specific synthetic siRNA or shRNA-expressing DNA vector, we observed over 80% reduction in expression of the target genes. These results suggest that RNAi targeting the leader sequence either by synthetic siRNA or shRNAs could be a useful strategy for treatment of SARS-CoV infection.

1 Materials and methods

1.1 Design and synthesis of siRNA and shRNA targeting the leader sequence

Complete nucleotide sequences of 142 SARS-CoV isolates were downloaded from the GenBank. Multi-alignment of different regions of the SARS-CoV genome was carried out using ClustalW (version 1.81). The program *mfold* was used for secondary structure prediction and target sequence selection.

Two synthetic 21-nt RNA oligomers (Table 1) for

Table 1 Oligonucleotides for synthetic siRNA and DNA vector-expressed shRNAs

Name	Sequence of oligomer[1)]	Target RNA sequence
shAF	5′ GATCCCC**CCAACCTCGATCTCTTGTA**TTCAAGAGA-**TACAAGAGATCGAGGTTGGTT**TTTTGGAAA 3′	5′ CCAACCUCGAUCUCUUGUA 3′ (nt 19～37)
shAR	5′ AGCTTTTCCAAAAAA**CCAACCTCGATCTCTTGTA**TC-TCTTGAA**TACAAGAGATCGAGGTTGG**GGG 3′	
shBF	5′ GATCCCC**AAGCCAACCAACCTCGATCTC**TTCAAGAG-A**GAGATCGAGGTTGGTTGGCTT**TTTTGGAAA 3′	5′ AAGCCAACCAACCUCGAUCUC 3′ (nt 12～32)
shBR	5′ AGCTTTTCCAAAA**AAGCCAACCAACCTCGATCTC**TCT-CTTGAA**GAGATCGAGGTTGGTTGGCTTGGG** 3′	
shNF	5′ GATCCCC**ACTGTCACTAAGAAATCTG**TTCAAG-AGA**CAGATTTCTTAGTGACAGTT**TTTTGGAAA 3′	5′ ACUGUCACUAAGAAAUCUG 3′ (nt 28 840～28 858)
shNR	5′ AGCTTTTCCAAAA**ACTGTCACTAAGAAATCTG**TCTCTT-GAA**CAGATTTCTTAGTGACAGTGGG** 3′	
siA	Sense: 5′ CCAACCUCGAUCUCUUGUAUU 3′; Antisense: 5′ UACAAGAGAUCGAGGUUGGUU 3′	5′ CCAACCUCGAUCUCUUGUA 3′ (nt 19～37)

[1)] Nucleotides corresponding to the sense and antisense sequence of the target are shadowed.

the leader sequence-specific siRNA (siA) were annealed and the resulting RNA duplex was analyzed on 15% polyacrylamide gels electrophoresis (PAGE). The plasmid pSUPER (OligoEngine) was used to construct DNA vectors to express shRNAs (shA or shB). Complementary DNA oligonucleotides (shAF and shAR, shBF and shBR) corresponding to the sequence of shA or shB, which contain 21-nt sense and antisense target sequences connected by a 9-nt loop (Table 1), were annealed and cloned into the *Bgl* Ⅱ and *Hin*d Ⅲ sites of pSUPER. pSUPER-Ni, which generates the shRNA targeting SARS-CoV N gene (the target site is completely identical in those of eight SARS-CoV isolates whose genome sequences were available when this work initiated in April 2003), was constructed following the same procedure by using complementary DNA oligonucleotides shNF and shNR (Table 1). EGFP knockout plasmid pSUPER-EGFPi (pEGFPi) served as a positive control for EGFP-specific RNAi. The pSUPER-RENTi (pRENTi) was used as an unrelated shRNA control, which can knock out the expression of RENT, a protein component of the nonsence-mediated mRNA decay system.

1.2 Construction of leader-tester fusion gene expression vectors

Two DNA oligonucleotides (LeF and LeR, Table 2) with complementary 3′ ends were annealed and extended by T4 DNA polymerase to form a 69-bp DNA duplex corresponding to the 5′ end of the SARS-CoV (strain CUHK-W1) genomic RNA (AY278554) including 57 nt of the leader sequence and 12 nt of the 5′-UTR. This DNA duplex was used as a template for a series of PCR amplifications to generate fusions of the 57-nt leader sequence to the 5′-UTR sequence of each gene. Primers LeNF and LeNR were used for the N gene, while primers LeSF and LeSR which would result in a direct fusion of the leader sequence to the translation start codon (TSC) of the S gene and primers LeSF and LeMR which will generate a fusion of the leader sequence to the 45-nt 5′-UTR of the M gene were used for the S gene and M gene, respectively. The resulting fusion sequences were separately inserted into the corresponding pcDNA 3.1 vectors harboring a full-length ORF of N, S or M, all His6-tagged at C-termini, either directly in front of TSC of N ORF (via *Nhe* Ⅰ and *Nco* Ⅰ sites) and M ORF (via *Eco*R Ⅰ and *Nco* Ⅰ sites), or using *Eco*R Ⅰ and a *Spe* Ⅰ site which is located 27 nt downstream of TSC of S ORF. The PCR product using primers LeNF and LeNR was also inserted in front of TSC of the EGFP gene residing in plasmid pEGFP-N1 (Clontech). These resulting leader-tester fusion gene expression vectors are named pLe-N-His, pLe-S-His, pLe-M-His and pLe-EGFP, respectively. All of the leader-fusion genes were verified by sequencing.

Table 2 PCR primers used in construction of leader fusion vectors

Name	Primer sequence[1)]
LeF	5′ CTACCCAGGAAAAGCCAACCAACCTCGATCTCTTGTAGATC 3′
LeR	5′ ACAGATTTTAAAGTTCGTTTAGAGAACAGATCTACAAGAGATCGAGG 3′
LeNF	5′ ATTGCTAGCCAGGAAAAGCCAACCAACC 3′
LeNR	5′ ATTCCATGGTAATTTGTTCGTTTAGAGAAC 3′
LeSF	5′ ATTGAATTCTACCCAGGAAAAGCCAA 3′
LeSR	5′ ATACTAGTGAGAGTAAGAAATAATAAGAAAATAAACATGTTCGTTTAGAGAACAGATC 3′
LeMR	5′ CCATGGATAAGAAATGTTAAAGTTCCAAACAGAATAATAATAATAGTTAGTTCGTTTAGAACAGAT 3′

[1)]Nucleotides corresponding to the leader sequence are shadowed. Restriction sites used to construct the leader-tester gene fusion vectors are underlined.

1.3 Cell culture and transfection

HeLa and 293T cells were maintained in DMEM containing 10% FBS at 37℃ with 5% CO_2. For microscopic experiments, cells were seeded in 24-well chamber slides. After incubation for 24 h, when cells reached 70% confluency, co-transfection was performed using FuGene-6 transfection reagent (Roche) with 1 μg of the target gene plasmid and 5 μg of shRNA-expressing vector. Twenty-four hours after transfection, the transfected cells grown on cover-slips were fixed with 4% paraformaldehyde in phosphate buffer (PBS) for 20 min at room temperature, washed

with PBS three times for 5 minutes each. Then the slides were mounted with Vectashield (Vector Laboratories) and the expression of EGFP was observed under a confocal microscope (Leica PCS SP2). For siRNA transfection, cells were trypsinized and plated at 4×10^5 cells per well in six-well plates in DMEM containing 10% FBS 24 h prior to transfection. Ten picomoles (0.15 μg) of the indicated siRNA was co-transfected with 1 μg of the target gene plasmid and 6 μl of Lipofectamine 2000 (Invitrogen).

1.4 RNA extraction and RT-PCR

Total RNA from cells of each well was isolated using RNAeasy kit (QIAGEN) and digested with RQ1 RNase-free DNase (Promega). Reverse transcription was carried out with M-MLV reverse transcriptase (Promega) using oligo $(dT)_{20}$ as a primer at 42℃ for 1 h. The reverse transcriptase was inactivated by heating at 100℃ for 10 min. PCR was performed using 2 μl of cDNA with specific primers (listed in Table 3) in 50 μl reaction with the following conditions: 95℃ 3 min, then 40 cycles of 95℃ 30 s, 56℃ 1 min, and 72℃ 1 min. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA served as an internal control, which was amplified under the same conditions as described above except that only 30 cycles of amplification was performed. Eight microliters of PCR products were run on a 1.0% agarose gel.

Table 3 Primers used in RT-PCR experiments

Primer name	Primer sequence	Predicted size of PCR product [1)]
MF	5′ CCTAGCCTGGATTATGTTAC 3′	336 bp (nt 81～446 of M ORF)
MR	5′ CGCAAGTGACCACGAATG 3′	
NF	5′ ATGTCTGATAATGGACCCC 3′	1 116 bp (nt 1～1 116 of N ORF)
NR	5′ GTCCTTTTTAGGCTCTGTTGG 3′	
SF	5′ CTTGACCGGTGCACCACTTTT 3′	590 bp (nt 46～635 of S ORF)
SR	5′ CCAGAAGGTAGATCACGAACTACAT 3′	
G3F	5′ GAAGGTGAAGGTCGGAGT 3′	226 bp (nt 6～231 of human GAPDH
G3R	5′ GAAGATGGTGATGGGATTTC 3′	ORF, GenBank No: BC001601)

[1)]According to strain CUHK-W1.

1.5 Western blot

Cells were harvested and lysed with the lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.02% sodium azide, 1% Triton X-100, 1 g/L aprotinin, and 1 mmol/L PMSF). Total cell protein of 100 μg for each sample was separated by 10% SDS-PAGE and transferred to PVDF membrane. After blocking, the membrane was incubated with the primary antibodies for 1～2 h. The primary antibodies used in the experiments were: anti-GFP mouse monoclonal B-2 antibody (Santa Cruz) at 1∶500 dilution, mouse anti-penta His antibody (QIAGEN) at 1∶2 500 dilution or anti-β-actin goat monoclonal antibody (Santa Cruz) at 1∶500 dilution. After washing, the blot was incubated with alkaline phosphatase-conjugated goat anti-mouse IgG or horseradish peroxidase-conjugated anti-goat IgG. Immunoreactive bands were visualized with the luminol/enhancer chemiluminescent substrate (ECL) kit (PIECE).

2 Results

2.1 SARS-CoV leader sequence is highly conserved among different SARS-CoV strains

To demonstrate the leader sequence is less variable than SARS-CoV coding sequences, we analyzed the mutation rates of the leader sequence, S, M and N genes of different SARS-CoV isolates. The leader from strain CUHK-W1 was chosen as a reference sequence (57 nt) because this sequence was completely identical to those of seven other SARS-CoV isolates whose genome sequences were available when this work initiated in April 2003, while in other viral regions higher mutation rates were observed. When we looked into all the leader sequences available to date, the leader is still the most conserved genomic region among different SARS-CoV strains. The 5′ RACE data indicated that the leader sequences of 36 strains including the SARS-CoV reference strain TOR2 (NC 004718) are all 72 nt long and totally identical. This sequence identity is

extended to other 106 leader sequences shorter than 72 nt with only one different nucleotide found in strain SoD (AY461660.1). Forty-six leader sequences are 57 nt in length, such as that of strain CUHK-W1, lacking the 5′ 15-nt region compared to the 72 nt-long leader sequences. In contrast to the conserved nature of the leader sequence during the 2003 epidemic (total nucleotide mutation rate 1.4% and total mutated-strain rate 0.7%), SARS-CoV genomic sequences coding for structural proteins such as S, M and N exhibited much higher mutation rates: 2.6% ～6.5% for nucleotide mutations and 54.2% ～79.6% for mutated strains (Table 4). Intriguingly, the leader sequence of the SARS-like coronavirus Rp3 (DQ071615) isolated from bats, which are thought as a possible natural reservoir of the SARS cluster of coronaviruses, is completely identical to that of the human SARS-CoV reference strain TOR2. Collectively, these sequence alignments indicated that the leader sequence is highly conserved among strains of the SARS coronavirus cluster and suggest that the leader sequence may be an ideal target for RNAi of SARS-CoV.

Table 4 Mutation rates in different regions of the SARS-CoV genome

Sequence	Mutation rates[1)]	Mutated strains[2)]
Leader	1.4% (1/72)	0.8% (1/142)
Spike gene	2.6% (98/3768)	79.6% (113/142)
Membrane gene	11.0% (73/666)	54.2% (77/142)
N gene	7.5% (95/1269)	56.3% (80/142)

[1)] Figures in parentheses: mutated nts/total length. [2)]Figures in parentheses: mutated strains with at least one mutated nt/142 strains used in this research.

The target regions within the leader sequence were selected following general rules for designing the siRNA sequence. Based on the predicted structure of the leader RNA, two partially overlapped target sites (A and B) were identified (Figure 1a). When compared with the human EST sequences available in GenBank, no human sequence with homology of consecutive 16 nt to the sequence of A and B was found. Thus, site A was used to design both synthetic siRNA (siA) and the DNA vector pSUPER-shA expressing A-related shRNA (shA), and site B was used to design pSUPER-shB to express B site-related shRNA (shB) (Figure 1b). According to the designing of siRNA expression vectors, shA was predicted to form an RNA duplex with two overhanging uridine residues at the 3′ ends of two strands, while the effector strand of the RNA duplex derived from shB would contain no 3′ overhangs (Figure 1c). The two 3′ overhanging uridines were reported to be necessary for the siRNA-guided mRNA cleavage.

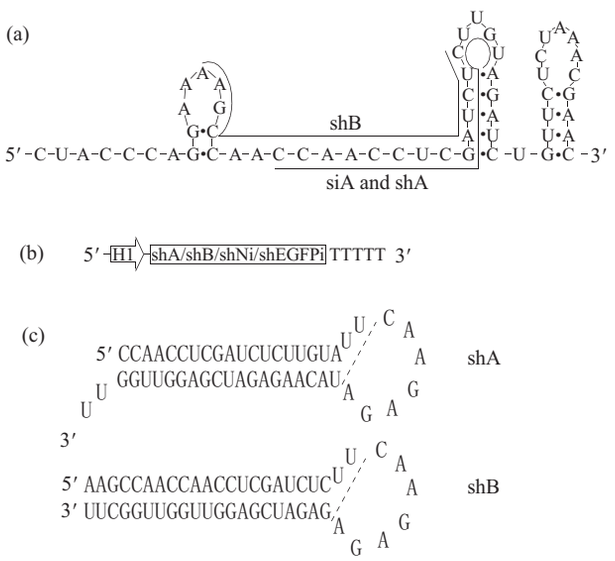

Fig. 1 Design of DNA vector-expressed shRNAs and synthetic siRNA

(a) Predicted secondary structure of the leader RNA of SARS-CoV strain CHUK-W1 and locations of siA/shA- and shB-targeting sites in the leader sequence. (b) Schematic diagram of the pSUPER vectors generating various shRNAs. Human H1 promoter, shRNA coding sequences and the 5-uridine transcription termination signal are indicated. (c) Predicted structures of shA and shB generated from corresponding DNA vectors. Dashed lines indicate the processing of shRNAs by DICER.

2.2 siRNA and shRNAs reduced the expression of SARS leader-EGFP fusion gene in HeLa and 293T cells

To assess the effects of these two H1 promoter-driving shRNAs and the synthetic siRNA on the leader-tester mRNAs, we first used a reporter plasmid pLe-EGFP as a target. The inhibitory effect on the EGFP expression can be easily visualized by fluorescence microscopy. As shown in Figure 2a, when HeLa cells were co-transfected with pEGFP-N1 and the shRNA expression plasmid pEGFPi, much weaker fluorescence was observed than that of cells co-transfected with pEGFP-N1 and the control shRNA vector pRENTi (Figure 2a, panels *1* and *2*). There is no obvious difference in fluorescence found between the cells transfected with pLe-EGFP and pEGFP-N1 in the presence of the control shRNA vector (Figure 2a, panels *1* and *3*), indicating that the leader fusion did

not affect EGFP expression in HeLa cells. When HeLa cells were co-transfected with pLe-EGFP and siA or shA, the EGFP fluorescence from the SARS leader-EGFP fusion was reduced to lower level (Figure 2a, panels *4* and *5*). To our surprise, shB, which would result in the production of an siRNA without 3′ overhanging uridines, even had a better inhibition effect than shA and almost completely abolished the EGFP expression from pLe-EGFP (Figure 2a, panel *6*). EGFP protein could not be detected by Western blotting in all of the three samples treated with leader-targeting small RNAs (data not shown). The different inhibitory effects on SARS leader-EGFP by the three leader-specific reagents were also observed in 293T cells with both fluorescence microscopy (Figure 2b) and Western blotting (Figure 2c). No EGFP fluorescence or EGFP protein expression was detected in the cells co-transfected with pLe-EGFP along with siA or shB (Figure 2b, panels *5* and *6*; Figure 2c, lanes *5* and *6*). In contrast, only 57% inhibition was observed with shA, which targeted the same sequence as siA (Figure 2c, lane *4*). These results collectively illustrated that high efficient interference with SARS-CoV leader sequence can be achieved by either synthetic siRNA or DNA vector-expressed shRNAs.

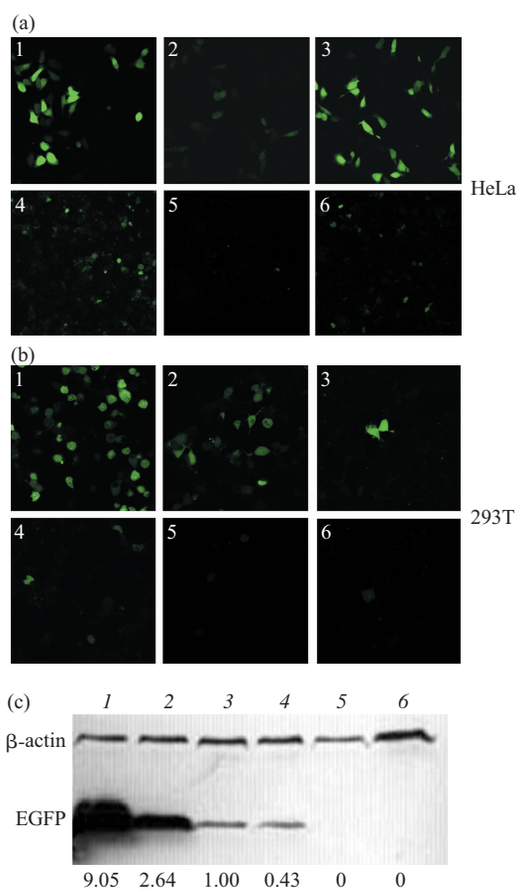

Fig. 2　Inhibition of leader-EGFP protein expression by the leader-specific siRNAs and shRNAs

HeLa cells (a) and 293T cells (b) were co-transfected with pEGFP-N1 and an unrelated control pRENTi (panel *1*) or positive control pEGFPi (panel *2*), pLe-EGFP and pRENTi (panel *3*), shA (panel *4*), siA (panel *5*) or shB (panel *6*). EGFP fluorescence was measured by confocal fluorescence microscope. The expression levels of EGFP protein in 293T cells were analyzed by Western blotting (c). The relative intensity of the bands of EGFP was determined by density scanning as shown on the bottom of the respective lanes. The intensity of the EGFP band in lane *3* is set as *1*.

Unexpectedly, the leader fusion repeatedly reduced the EGFP expression to only 11% of that of pEGFP-N1 in 293T cells (Figure 2b, panels *1* and *3*; Figure 2c, lanes *1* and *3*). However, the expression levels of EGFP were unchanged for leader-EGFP fusion in the *in vitro* TNT ® Rabbit Reticulocyte Lysate System. These results prompted our speculation that the leader-fusion repression of EGFP expression was cell type-dependent. The mechanism underlying the cell type-dependent repression remains unknown.

2.3　Efficient silencing of leader-N fusion gene expression by leader-specific siRNA and shRNAs

To further evaluate the inhibitory effect of the siRNA or shRNAs on the SARS-CoV mRNA level, we constructed the plasmid vector pLe-N-His by fusing the leader sequence to the N gene. As measured by RT-PCR, the siRNA (siA) and two shRNAs (shA and shB) reduced the level of the fusion gene transcript (LeN) in 293T cells (Figure 3a). When 293T cells were transfected with pLe-N-His along with shA or shB, the amount of the fusion gene transcript was reduced to 29.8% or 10.0% in comparison with that produced in the cells co-transfected with pLe-N-His and the unrelated pEGFPi at 48 h after transfection (Figure 3a, lanes *1* ～*3*). Consistent with the strong inhibitory effect of siA on EGFP expression described above, siA reduced the level of the LeN transcript to 5.4% of that observed in cells co-transfected with pLe-N-His and pEGFPi (Figure 3a, lane *4*).

Production of N protein from pLe-N-His in 293T cells was analyzed by Western blotting using the antibody against His6 tag (Figure 3b). siA had a 100% inhibitory effect on N-His expression (comparing lane *1* and lane *4*), while shA and shB inhibited N-His expression by 59.3% (lane *2*) and 90.7% (lane *3*), respectively. Although co-transfection with pSUPER-Ni, which generates an shRNA targeting the SARS-CoV N gene, has resulted in a better inhibitory effect (97.4% , lane *5*) on N-His expression than

leader-targeting shRNA vectors, this target site on the N gene has two variable nucleotides in seven SARS-CoV strains from human and bat hosts isolated during the later phase or after the virus epidemic in 2003. Target sequence variations would dramatically reduce the inhibitory effect by RNAi.

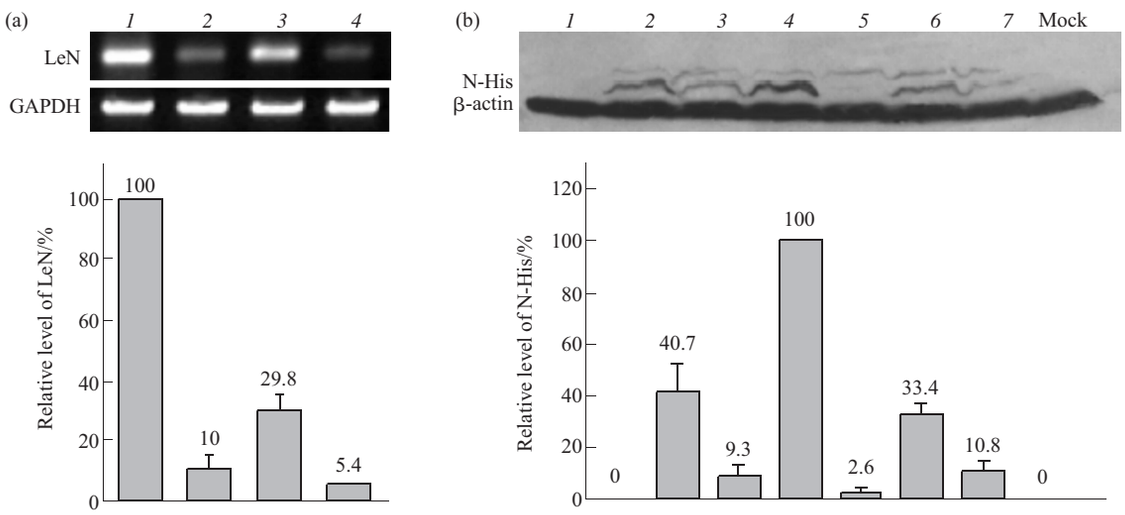

Fig. 3 Silencing of the leader-N fusion gene expression by leader-specific siRNA and shRNAs

(a) 293T cells were transfected by pLe-N-His along with pEGFPi (lane *1*), shB (lane *2*), shA (lane *3*) or siA (lane *4*). The levels of the leader-N fusion gene transcript (LeN) were analyzed by RT-PCR. (b) The expression levels of N-His protein were analyzed by Western blotting using anti-His antibody. 293T cell were co-transfected with the pLe-N-His and siA (lane *1*), shA (lane *2*), shB (lane *3*), pEGFPi control (lane *4*) or pSUPER-Ni (lane *5*). The relative intensities of the bands in (a) and (b) were determined by density scanning as shown in the bottom histograms corresponding to relevant lanes. Error bars represent standard deviation of three independent experiments.

2.4 Efficient silencing of expression of leader-M and leader-S fusion genes by leader-specific siRNA and shRNAs

To test whether the leader-specific siRNA and shRNAs have inhibitory effects also on other SARS-CoV sgmRNAs, the sequences encoding the M protein and S protein were separately fused to the leader sequence at their 5′-ends and the levels of the fusion transcripts expressed in the presence of the leader-specific siRNA or shRNAs were analyzed by RT-PCR. siA and two shRNAs (shA and shB) can efficiently reduced the levels of leader-M (LeM, Figure 4a) and leader-S (Figure 4b) fusion gene transcripts in 293T cells. The level of LeM in 293T cells (Figure 4a, lane *1*) and in HeLa cells (unpublished data) was relatively low and LeM expression was completely destroyed by siA, shA or shB (Figure 4a, lanes *2*～*4*). When 293T cells were transfected with pLe-S-His along with shB (Figure 4b, lane *2*) or shA (Figure 4b, lane *3*), the amount of the fusion gene transcript was reduced to 2% or 10% in comparison with that produced in the cells co-transfected with pLe-S-His and the unrelated pEGFPi at 48 h after transfection (LeS, Figure 4b, lane *1*). Consistent with the results in EGFP and LeN expression described above, siA treatment again had a 100% inhibitory effect on LeS

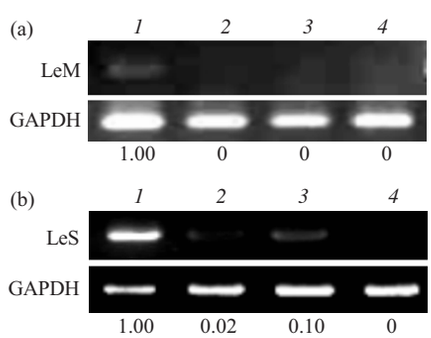

Fig. 4 Silencing of the leader-M fusion and leader-S fusion transcripts by leader-specific siRNA and shRNAs

(a) 293T cells were transfected by pLe-M-His along with pEGFPi (lane *1*), shB (lane *2*), shA (lane *3*) or siA (lane *4*). The levels of the leader-M fusion gene transcript (LeM) were analyzed by RT-PCR. (b) 293T cells were transfected by pLe-S-His along with pEGFPi (lane *1*), shB (lane *2*), shA (lane *3*) or siA (lane *4*). The levels of the leader-S fusion gene transcript (LeS) were analyzed by RT-PCR.

expression (Figure 4b, lane *4*). Due to the low expression levels, the M-His protein and the S-His protein could not be detected by Western blotting using the His6 tag-specific antibody (data not shown).

The relative intensity of the bands determined by density scanning and normalized against the negative control of silencing (lane *1*) is shown on the bottom of the respective lanes. Similar results were found in three independent experiments.

3 Discussion

Although the SARS disease has faded out since the 2003 epidemic, its re-emergence cannot be excluded [2, 3]. Therefore, the availability of effective prevention and therapeutics against SARS-CoV is crucial for the control of future SARS outbreaks [5]. RNA interference (RNAi) holds considerable promise as a specific therapeutic approach to fight against infectious viral disease such as HIV [14] and HCV [15]. Efficacy and safety of siRNA, as inhibitors of SARS-CoV in the rhesus macaque (*Macaca mulatta*) model have also illustrated the prospects of siRNAs as a new therapeutic agent on SARS-CoV[18]. In this work, we have shown that both the synthetic siRNA and DNA vector-derived shRNAs targeting the leader sequence can efficiently deplete multiple artificially constructed SARS-CoV sgmRNAs in model cell lines, thus adding the leader-specific synthetic siRNA as a potential valid tool to the arsenal against SARS-CoV infection.

For the reason of high morbidity and mortality rates of SARS-CoV, strict limitation has been imposed on experiments using live SARS-CoV in China since the year of 2005 and we have evaluated functions of the anti-SARS-CoV agents only by using SARS-CoV RNA mimics as targets of RNAi. We found that both the siRNA (siA) and shRNA (shB) could effectively destroy multiple SARS-CoV RNAs in 293T cells where SARS-CoV replicated efficiently when ACE2 was provided. Synthetic siRNA had an extremely strong inhibitory effect throughout our experiments. Considering the high conservation of the leader sequence among strains of the SARS coronavirus cluster (Table 4), our RNAi agents can be applied to almost all SARS-CoV strains from different hosts. Collectively, the leader-targeting RNAi seems to be a potent “one bullet, multiple targets” strategy for efficacious prevention and treatment of the SARS disease.

Our data supports the idea that leader sequence may be a better choice of targeting when RNAi is to be applied to other *Nidovirales* viruses which use similar transcription strategies. Not only SARS coronaviruses, but also many other viruses of *Nidovirales* such as arteriviruses can infect both poultry and primates [6]. RNAi targeting on the leader sequence might be an alternative efficacious method for prevention and treatment of diseases caused by these viruses.

Acknowledgements The authors thank Mr. Youhua Xie (Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, The Chinese Academy of Sciences) for providing M, S, N gene expression vectors.

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针对 leader 序列的 siRNA 能够有效抑制 SARS 冠状病毒多个 mRNA 的表达 *

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摘要 小干扰 RNAs (siRNAs) 能够有效降解具有互补序列的 RNA. 在 SARS-CoV 的基因组 RNA 和所有亚基因组 RNA 的 5′端均有一段共同的 leader 序列，而且该 leader 序列在不同的病毒分离物中高度保守，因此 leader 序列可作为一个用于抑制 SARS-CoV 复制的有效靶点. 研究表明，针对 leader 序列化学合成的 siRNA 和 DNA 载体表达的 shRNA 都可以有效抑制 SARS-CoV mRNA 的表达. Leader 序列特异的 siRNA 或 shRNA 不仅可以有效抑制 leader 与报告基因 EGFP 融合基因的表达，而且还可以有效抑制 leader 与刺突蛋白 (spike protein)、膜蛋白(membrane protein)和核衣壳蛋白(nucleocapsid protein) 基因的融合转录产物的表达. 结果表明，针对 leader 序列的 RNA 干扰可以发展成为一种抗 SARS-CoV 治疗的有效策略.

关键词 leader RNA，RNA 干扰，SARS 冠状病毒， 短发夹 RNA， 小干扰 RNA
学科分类号 Q939.4

* 中国科学院 SARS-CoV 专项基金支持项目.
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收稿日期：2007-03-02，接受日期：2007-04-29