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Inhibition of Severe Acute Respiratory Syndrome Virus Replication by Small Interfering RNAs in Mammalian Cells

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Severe acute respiratory syndrome (SARS) is an acute respiratory infectious disease that spread worldwide in early 2003. The cause was determined as a novel coronavirus (CoV), SARS-associated CoV (SARS-CoV), with a single-stranded, plus-sense RNA. To date, no effective specific treatment has been identified. To exploit the possibility of using RNA interference as a therapeutic approach to fight the disease, plasmid-mediated small interfering RNAs (siRNAs) were generated to target the SARS-CoV genome. The expression of siRNAs from two plasmids, which specifically target the viral RNA polymerase, effectively blocked the cytopathic effects of SARS-CoV on Vero cells. These two plasmids also inhibited viral replication as shown by titer assays and by an examination of viral RNA and protein levels. Thus, our results demonstrated the feasibility of developing siRNAs as effective anti-SARS drugs.

Severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) is an enveloped, single-stranded, plus-sense RNA virus, whose genome is about 30 kb in length. The genome organization of SARS-CoV is similar to that of other CoVs. Although the early studies of the sequence comparisons showed that SARS-CoV does not closely resemble any of the previously characterized CoVs (18, 23), the recent phylogenetic analyses indicated that SARS-CoV and the group 2 CoVs are closely related and may share a common ancestor (7, 30). The SARS-CoV replicase gene (open reading frame [ORF]1a and ORF1b) comprises about two-thirds of the genome, and its products are translated directly from the genomic RNA and then undergo cotranslational proteolytic processing to produce a number of proteins. The remaining viral proteins are translated from subgenomic mRNAs, all of which share the genomic 5′ leader sequence through a discontinuous transcription process (23). The SARS-CoV genome encodes at least four major structural proteins: nucleocapsid (N), spike (S), membrane, and small envelope (32). As in other enveloped viruses, S protein, a glycoprotein on the viral surface, is crucial for viral attachment and entry into the host cell (16). In addition, variations of S protein among strains of CoVs are responsible for host range and tissue tropism. S, membrane, and N proteins all contribute to evoking the host immune response (11, 24).

RNA-mediated interference (RNAi) as an antiviral mechanism was originally discovered in plants and then found in other organisms such as *Caenorhabditis elegans*, *Drosophila*, and vertebrates (1, 3, 15). It is an evolutionarily conserved process for the specific suppression of gene expression (for a review, see references 3, 6, 12, and 26). In this process, recognition of double-stranded RNA leads to the production of small interfering RNAs (siRNAs) of 21 to 22 nucleotides (nt), which associate with a multiprotein complex known as the RNA-induced silencing complex and ultimately target homologous mRNA for degradation based on complementary base pairing.

RNAi has been successfully used in blocking the replication of human immunodeficiency virus and human hepatitis C viruses in human cells and may provide a new therapeutic approach to certain diseases (5, 8, 10, 19, 28, 29). As SARS-CoV is an RNA virus, RNAi could be a reasonable approach for therapeutic purposes to use against SARS. In this study, we provide evidence that SARS-CoV replication can be efficiently inhibited by vector-derived siRNA-mediated RNAi in Vero cells.

MATERIALS AND METHODS

Selection of siRNA target sequences and vector construction. The mammalian expression vector pSUPER.retro (pSR) (OligoEngine) was used for the expression of siRNA. Six specific anti-SARS siRNA expression plasmids were constructed to target different sites of the SARS-CoV (strain HKU-39849; GenBank accession no. AY278491) genome (14): pSR01 (targeting sequence, nt 58 to 76), 5′-GTTCTCTAAACGAACTTTA-3′; pSR02 (nt 14450 to 14468), 5′-CTTACA TAGCTCGCGTCTC-3′; pSR03 (nt 15877 to 15895), 5′-GAATATTAGGCGC AGGCTG-3′; pSR04 (nt 9771 to 9789), 5′-ATTGCGTAGCGAGACACTG-3′; pSR05 (nt 12714 to 12732), 5′-CAATTCGAAGGGAGGTAGG-3′; and pSR06 (nt 27767 to 27785), 5′-GTCTAAACGAACATGAAAC-3′ (Fig. 1). A nonspecific siRNA expression vector, pNS (5′-AGCGGACTAAGTCCATTGC-3′), was constructed as a negative control. These sequences were all analyzed by a BLAST search of the GenBank database to avoid similar sequences found in the human genome. Oligonucleotides were synthesized (Sangon, Shanghai, China) and inserted into the pSR vector in the BglII and HindIII sites according to the manufacturer's instructions.

Cell culture and virus infection. African green monkey kidney Vero cells were maintained in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) and antibiotics. SARS-CoV was isolated from

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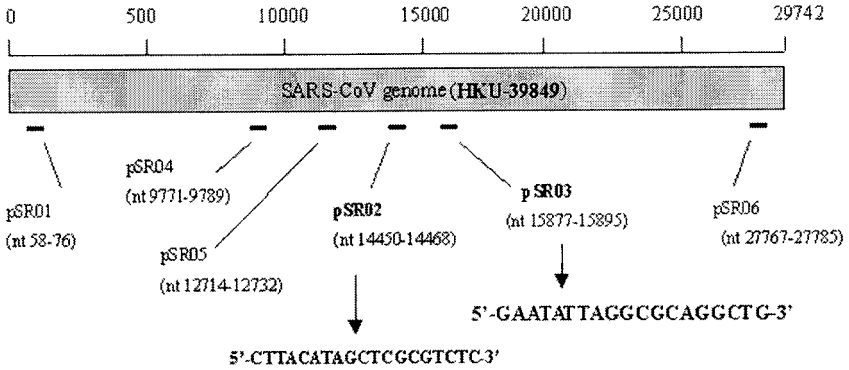

FIG. 1. Schematic representation of the sites of the six siRNA target sequences against genomic RNA of SARS-CoV (strain HKU-39849).

a SARS patient. The virus stock has a titer of 3.2×10^5 of the 50% tissue culture infective doses ($TCID_{50}$)/ml of stock as measured in Vero cells.

Vero cells were seeded in a T25 flask and grew to 60 to 70% confluence at the time of transfection. The cells were transfected with 1.5 or 3 μg of siRNA expression plasmids with Lipofectamine reagent (GIBCO) overnight. Then, the cells were cultured in growth medium for 8 h and subjected to viral infection. The cells were rinsed once with phosphate-buffered saline and incubated with the infection mixture, which contained 1×10^2 $TCID_{50}$ of SARS-CoV. Cytopathic effects (CPE) were examined after 24 h. At various times, cells were harvested for Western blotting and semiquantitative real-time PCR analysis.

Virus titration. Vero cells were seeded in 96-well plates 1 day before infection. Then, supernatants collected from the cells that had been infected with SARS virus for 24 h were serially diluted and added to wells in triplicate. After 3 days of infection, the $TCID_{50}$ values were calculated as previously described (4). The data were analyzed by a Student's two-tailed *t* test.

Western blotting. At 24 h postviral infection, cells were harvested, and total proteins were extracted. The protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce). Proteins were separated by 10% sodium dodecyl sulfate gels, transferred to nitrocellulose membranes, and blotted with mouse anti-N protein or rabbit anti-3CL-proteinase antisera, which were generated by injecting purified N protein into mice and 3CL-proteinase into a New Zealand White rabbit, respectively, followed by incubation with anti-mouse secondary antibody conjugated to alkaline phosphatase (Zymed) or anti-rabbit secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia). Proteins were detected by using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP; Pierce) or enhanced chemiluminescence (Amersham Pharmacia). These blots were reprobed with anti-β-actin antibodies (Sigma) for loading control after the blots were stripped.

Reverse transcription and real-time PCR analysis. At 18 h postviral infection, total RNA was extracted by using Trizol reagent (GIBCO) and then treated with DNase 1 (Promega) according to the manufacturer's recommendations. A reverse transcription reaction was carried out by using a reverse transcription system (Promega) according to the manufacturer's recommendations with a 30-μl reaction mixture containing 1.5 μg of total RNA. The final volume was brought up to 100 μl with H_2O for further use.

In real-time PCR, 5 μl of cDNA was amplified in a 25-μl reaction volume containing a 0.4 mM concentration of each deoxynucleoside triphosphate, 2.5 μl of 10× LA *Taq* buffer, 1.25 μl of 20× SYBR green I buffer (OPE, Shanghai, China), 1.25 U of *Taq* DNA polymerase (Takara), and a 0.4 pM concentration of each primer. The following primers were used: sense, 5′-AAACCAGGTGGAA CATCATCCG-3′, and antisense, 5′-ACTCAGGTTCCCAGTACCGTGAG-3′, for SARS-CoV; and sense, 5′-CATCACTGCCACCCAGAAGA-3′, and antisense, 5′-GCTGTAGCCAAATTCGTTGT-3′, for GAPDH (glyceraldehyde-3-phosphate dehydrogenase). The PCR product of SARS-CoV corresponds to the region of nt 15396 to 16128. The reaction was performed in a DNA Engine Opticon continuous fluorescence detection system (MJ Research). Following a denaturation step at 94°C for 2 min, 35 cycles of amplification were performed at 94°C for 20 sec, 57°C for 25 sec, and 72°C for 40 sec. Each sample was run in triplicate. Data were analyzed according to the comparative threshold cycle (C_t) method, where the amount of target (SARS-CoV), normalized to an endogenous reference (GAPDH) and relative to an experimental control (virus), is given by $2^{-\Delta\Delta Ct}$ (17). C_t indicates the PCR cycle number at which the amount of amplified target reaches a fixed threshold. The ΔC_t value is determined by subtracting the average reference C_t value from the average target C_t value. The $\Delta\Delta C_t$ value involves subtraction by the ΔCt experimental control value.

RESULTS

Inhibition of CPE of SARS-CoV by siRNA. To study the CPE of SARS-CoV, monkey kidney Vero cells were infected with SARS-CoV isolated from a patient, and the cells were examined with phase-contrast microscopy 24 h later. In contrast to the normal cells that were flat and tightly stuck to the plate, the cells infected with SARS-CoV exhibited evident morphological changes, as shown by the rounded cell bodies and their elongated shape (Fig. 2; compare A and B). These CPE-positive cells finally detached from the plate. CPE began at 20 h postviral infection, and all the cells dropped off the plate after 30 h of infection.

To examine whether the expression of SARS-CoV-specific siRNA has an inhibitory effect on virus-induced CPE, we generated six siRNA-expressing plasmids to target the SARS-CoV genome. The cells were transfected with siRNA-expressing plasmids and then infected with SARS-CoV. An examination of cell morphology showed that the empty vector (pSR) and nonspecific siRNA expression vector (pNS) had no evident influence on SARS-CoV-induced CPE (Fig. 2C-D). In contrast, pretransfection of siRNA plasmids pSR02 and pSR03, both of which target the RNA polymerase, remarkably inhibited the CPE of SARS-CoV on the cells (Fig. 2F to I). Pretransfection of pSR01 and three other plasmids had weak or no effects on SARS-CoV-induced CPE (Fig. 1E and data not shown). Thus, pSR02 and pSR03 were selected for further analysis.

Interference of SARS-CoV replication by siRNA. To study the specific inhibitory effects of the siRNA-expressing plasmids on SARS-CoV replication in Vero cells, we collected the media from the infected cells and determined by titer assay the amounts of infectious virus produced. The viral production from the infected cells decreased significantly when the cells were pretransfected with siRNA-expressing plasmids pSR02 and pSR03, compared to levels of viral production in the samples without plasmid transfection. (Fig. 3). The activities of the siRNAs derived from pSR02 and pSR03 in blocking SARS-CoV replication are consistent with their functions in inhibiting virus-induced CPE. However, it is notable that the empty

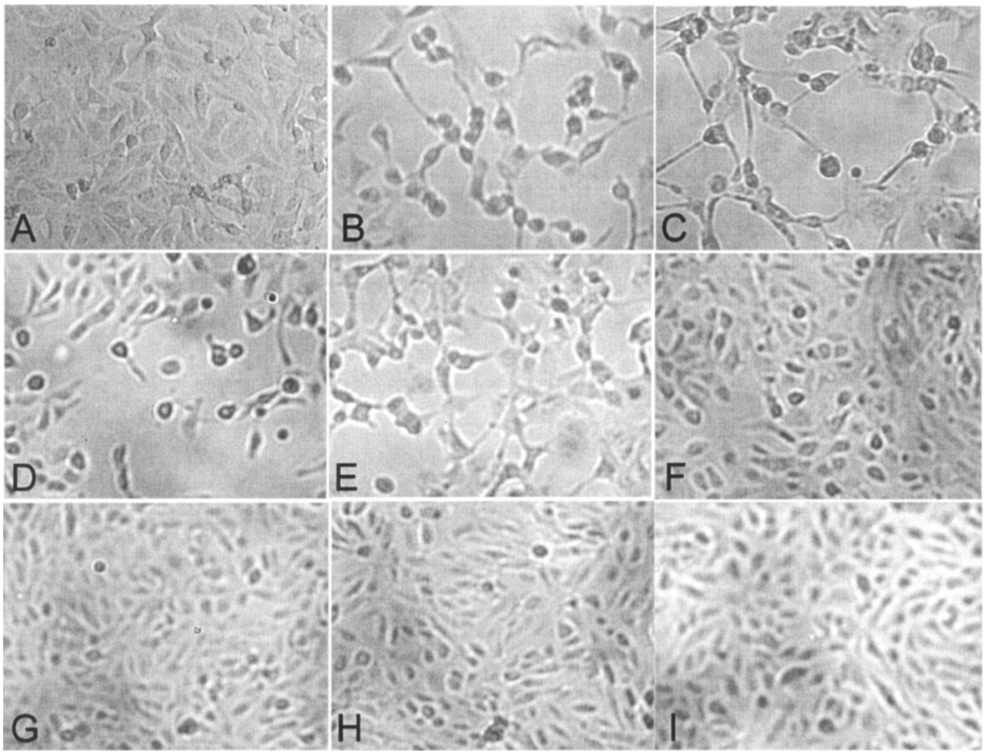

FIG. 2. CPE of SARS-CoV on Vero cells. Vero cells were transfected with various plasmids and then infected with 1×10^2 $TCID_{50}$ of SARS-CoV. CPE were first observed after 20 h, and the pictures were taken at 24 h postinfection. In panels A and B are mock-infected cells without virus and cells infected with SARS-CoV, respectively. In panels C to I, the cells were infected by virus after pretransfection with pSR (C), pNS (D), pSR01 (E), 1.5 μg of pSR02 (F), 3 μg of pSR02 (G), 1.5 μg of pSR03 (H), and 3 μg of pSR03 (I).

vector pSR and nonspecific siRNA-expressing plasmid pNS also had some inhibitory effects on viral production.

Then, we further studied the inhibitory effects of these siRNA-expressing plasmids on SARS-CoV replication at the protein and RNA levels. Vero cells were infected with SARS-CoV after being transfected with various plasmids, and total

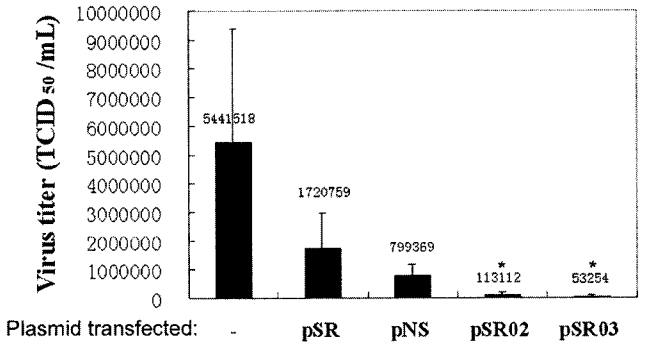

FIG. 3. Effect of siRNA on SARS-CoV replication. The virus-containing supernatants were collected 24 h after virus infection of Vero cells pretransfected with or without 3 μg of various plasmids, as indicated. The supernatants were used for the titer assays. Virus titers were determined from three independent experiments in triplicate, and data shown are the means ± standard deviations. An asterisk indicates that the titer values from pSR02- and pSR03-transfected cells compared to the titer from untreated cells are statistically significant with a P value of 0.0005 (t test). pSR02 and pSR03: siRNAs against SARS-CoV.

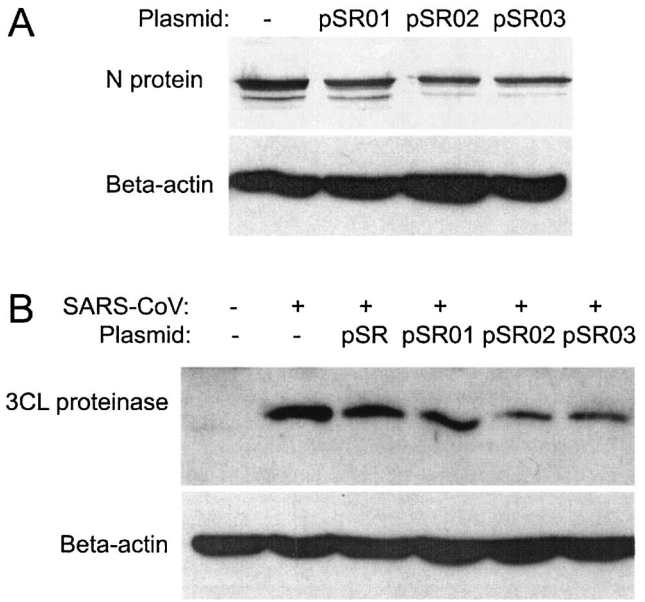

FIG. 4. Reduction of SARS-CoV protein levels by siRNA. Vero cells were transfected with 3 μg of various plasmids and then infected with 1×10^2 $TCID_{50}$ of SARS-CoV. Total cellular proteins were harvested at 24 h postinfection. Western blot analysis was performed with antisera against N protein, 3CL proteinase, or anti-β-actin antibodies. p SR01, pSR02, and pSR03: siRNAs against SARS-CoV. This experiment was repeated three times, and the results of a typical experiment are shown.

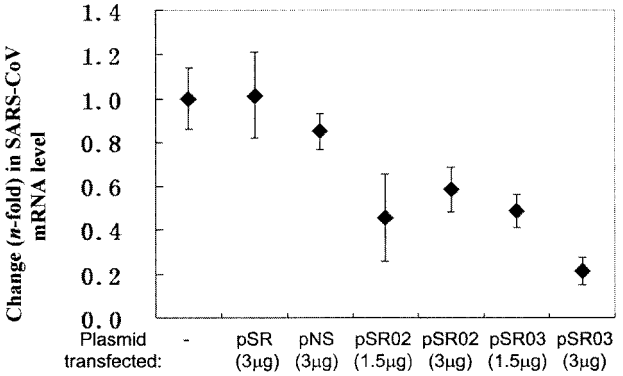

FIG. 5. Attenuation of SARS-CoV mRNA levels by siRNA. Vero cells pretransfected with no vector (-), empty vector (pSR), nonspecific siRNA plasmid (pNS), or plasmids expressing siRNA against SARS-CoV (pSR02 and pSR03) were infected with 1×10^2 $TCID_{50}$ of SARS-CoV. Samples were collected at 18h postinfection; mRNA was extracted and reverse transcribed to cDNA. The cDNA was subjected to real-time PCR analysis by using gene-specific primers for SARS-CoV or GAPDH. The change (*n*-fold) in SARS-CoV mRNA levels were presented as $2^{-\Delta\Delta Ct}$ normalized to GAPDH (an endogenous reference) and relative to a virus sample (an experimental control). Data are from triplicate experiments and are shown as the means ± standard deviations. Three independent experiments were performed, and similar results were obtained.

proteins and RNA were extracted from the cells. As shown in Fig. 4, both pSR02 and pSR03 effectively reduced the synthesis of N protein (Fig. 4A) and 3CL proteinase (Fig. 4B). Consistent with these results, pSR02 and pSR03 also significantly attenuated viral RNA production as examined by semiquantitative real-time PCR (Fig. 5). Taken together, these results suggest that these two siRNAs can effectively block SARS-CoV replication in Vero cells.

DISCUSSION

RNAi has been demonstrated to be a powerful method for gene silencing. Furthermore, the idea of using RNAi for therapeutic purposes has extensively been exploited in treating various diseases such as cancer and dominantly inherited genetic disorders (19, 28). Virus infection-related diseases were among such RNAi applications. For example, human immunodeficiency virus was one of the first targets for such application (13, 21). Other virus-induced diseases considered for RNAi-based therapy include hepatitis C virus- and hepatitis B virus-induced liver disease, human papilloma virus-related tumorigenesis, virus-induced influenza, and others (9, 10, 20, 25, 27, 31).

In this study, we have tested the effect of six vector-based siRNAs on the inhibition of SARS-CoV replication. We found that only two of them can effectively block SARS-CoV replication in Vero cells, and four others had minimal effects. Interestingly, both of the siRNAs that block SARS-CoV replication target the RNA polymerase. We also noticed that the empty vector pSR and nonspecific siRNA-expressing plasmid pSN have some inhibitory effects on SARS-CoV replication. This result could be due to a nonspecific effect of the introduction of plasmids into the cell. It has been demonstrated that exogenously introduced plasmids can lead to the generation of nonspecific double-stranded RNAs, which in turn induce interferon production and inhibit viral replication to some extent (22).

Our results suggest that siRNA has a potential to be developed into anti-SARS drugs. There are many means by which to interfere with viral infection. Vaccine is one of the most effective ways. However, a major concern in the development of vaccines against viral infection is that the viral genes encoding the antigenic proteins often undergo rapid mutations. The variations of S protein among strains of CoV are primarily responsible for host range and tissue tropism (11). Genetic mutation in the segments encoding the major antigenic proteins would pose potential problems for the development of effective vaccines. To avoid the possibility that the sequence variation between different SARS-CoV strains may restrict siRNA inhibitory effects, the siRNA target sequences were chosen with consideration given to sequence conservation between different strains (24, 32). The target sequences of pSR02 and pSR03, which can efficiently inhibit SARS-CoV replication, are conserved in all the SARS-CoV strains published in the GenBank database. Thus, both pSR02 and pSR03, which target the SARS-CoV genome region encoding RNA polymerase, may provide important alternatives for vaccines against the S protein. The vector pSR was selected as an siRNA expression vector as it can be used to generate recombinant retroviruses. Retrovirus-mediated gene delivery has been shown to be effective in most cell lines and many primary cell types and to provide great facility for further clinical application studies (2).

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