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Inhibition of SARS-CoV replication cycle by small interference RNAs silencing specific SARS proteins, 7a/7b, 3a/3b and S

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Abstract

The severe acute respiratory syndrome coronavirus (SARS CoV) genome has 14 potential open reading frames (ORFs). The first ORF is translated from the full-length genomic mRNA while the remaining ORFs are translated from eight subgeomic RNAs (sgRNAs). In this study, we designed small interference RNAs (siRNAs) targeting sgRNA 2, 3 and 7 and tested their efficiency and specificity in silencing the protein translated from the targeted sgRNA. Our results demonstrated that siRNA 7 could inhibit sgRNA 7, which showed 19/19 nucleotides (nt) matching, and sgRNA 8, which showed 18/19 nt matching; but, it did not inhibit the full-length genomic mRNA which showed 17/19 nt matching. Overall, each of the siRNAs can inhibit the targeted sgRNA without affecting the full-length genomic mRNA or the other sgRNAs that showed mismatch of two or more nt. Thus, siRNA could be designed so as to knockdown the expression of viral protein(s) from a targeted sgRNA during viral infection, thereby allowing the contribution of individual viral proteins to viral infection to be delineated. When Vero E6 cells expressing siRNA 2, 3 or 7 were infected with SARS-CoV, a significant reduction in the yield of progeny virus was observed. Indirect immunofluorescence assays showed that in the infected cells expressing each of the siRNAs, there was aspecific silencing of S, 3a and 7a, respectively, but the expression of nucleocapsid protein was not affected. Thus, our data suggests that the accessory proteins, i.e. 3a and 7a, could play an important role during the replication cycle of the SARS-CoV.

Keywords: siRNA; SARS corona virus; 3a; 7a

1. Introduction

A novel coronavirus was identified as the causative agent of SARS, which affected over 30 countries world wide, and over 8000 cases reported with over 800 fatalities (World Health Organization, http://www.who.int/csr/sars/country/en/). SARS-CoV is a positive sense RNA virus with a genome about 30 kb and 14 potential open reading frames (ORFs) (Marra et al., 2003; Rota et al., 2003). SARS-CoV has four structural proteins, spike (S), membrane, nucleocapsid (N) and envelope, which are common to all members of the genus coronavirus (Marra et al., 2003; Rota et al., 2003). The genome contains another nine ORFs, excluding the four structural proteins and the replicase gene 1a/1b, and the length of these ORFs varies from 39 to 274 amino acids (Marra et al., 2003; Rota et al., 2003). These accessory proteins are specific for the SARS CoV but their functions are still not clear (see recent review by Tan et al., 2006).

Like other coronaviruses, SARS-CoV uses a transcription attenuation mechanism to synthesize both full-length and subgenomic-length negative-strand RNAs which then function as templates for synthesis of full-length genomic mRNA and subgenomic mRNAs (sgRNAs) (Fig. 1). The first ORF is translated from the full-length genomic mRNA (RNA 1), while the remaining ORFs are translated from eight sgRNAs (RNA two to nine) synthesized as a nested set of 3′ co-terminal RNA species in which the leader RNA sequences on the 5′ end of the genome are joined to the body sequences at distinct transcription regulatory sequences containing a highly conserved consensus sequence (CS) (Marra et al., 2003; Rota et al., 2003; Snijder et al., 2003; Thiel et al., 2003; Yount et al., 2003). As illustrated in Fig. 1, the full-length genomic contains nearly all the sequences found in any of the sgRNAs, hence it may be expected that siRNA targeting any of the sgRNAs will also affect the translation of the full-length genomic mRNA. Indeed, it was recently reported

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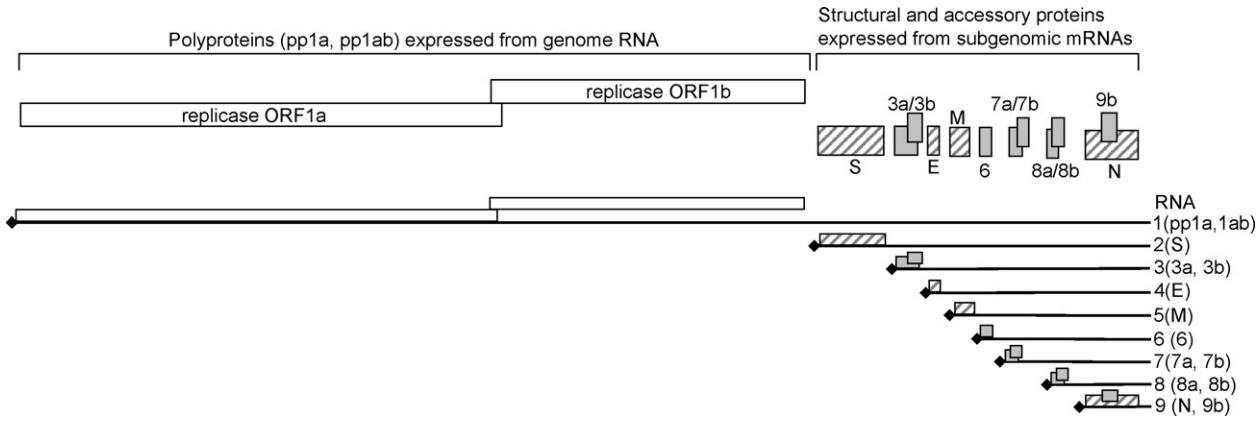

Fig. 1. Schematic diagram showing the expression of viral proteins from the severe acute respiratory syndrome coronavirus (SARS-CoV) genome and subgenomic RNAs. Replicase genes (ORFs 1a and 1b) encode for two large polyproteins, pp1a and pp1ab (white solid boxes). These are expressed from the full-length genomic mRNA (RNA 1). Expression of the ORF1b gene involves ribosomal frameshifting into the −1 frame just upstream of the ORF1a translation termination codon. Open reading frames (ORFs) in the last 1/3 of the SARS-CoV genome are translated from eight subgenomic mRNAs (RNA 2 to RNA 9). Four of these encode the structural proteins (checked boxes), spike (S), membrane (M) and envelope (E) and nucleocapsid (N). Another eight SARS-CoV-unique ORFs (grey solid boxes) encode for accessory proteins (3a, 3b, 6, 7a, 7b, 8a, 8b and 9b) with no significance sequence homology to viral proteins of other coronaviruses. All the mRNAs have a common leader sequence (represented by ♦), which is derived from the 5′ end of the genome.

that major replicative inhibition effects of siRNA targeting the sgRNAs may be due to the disruption of the full-length genomic mRNA rather than viral protein knockdown (Zheng et al., 2004).

However, a careful examination of the sgRNAs sequences revealed that junction between the CS and the other sequences that encode the different ORFs shows significant differences (see Table 1). Based on this, we designed three siRNAs to target sgRNAs 2, 3 and 7, which are required for the expression of S, 3a/3b and 7a/7b proteins, respectively. The sequence similarities between each of these siRNA and the full-length genomic RNA and sgRNAs are shown in Table 1. For example, sgRNA 2 contains all the 21 nucleotides (nt) in siRNA 2, while the full-length genomic RNA (mRNA 1) only contains 15 out of the 21 nt. These hairpin siRNAs are then expressed from the U6 promoter in a mammalian expression vector to determine if they can specifically knockdown genes encoded by targeted sgRNAs. Finally, we used these siRNAs to investigate the importance of the accessory proteins, 3a/3b and 7a/7b, for SARS-CoV replication in Vero E6 cells.

2. Materials and methods

2.1. Cell-line and virus

The Vero E6 cells and SARS-CoV used in this study have been previously described (Åkerström et al., 2005).

2.2. Design and construction of vector-based siRNA

The construction of vector-based siRNA was performed by GenScript Corporation (NJ, USA) and the detailed protocols are available from http://www.genscript.com. The vector used

Table 1
Design of siRNA for knockdown of specific SARS coronavirus subgenomic mRNA in infected cells

mRNA	Sequence[a]	Matching nt for siRNA 2[b]	Matching nt for siRNA 3[c]	Matching nt for siRNA 7[d]
1	GTAGATCTGTTCTCTAA**ACGAAC**TTTAAAATCTGT	15/21	16/21	17/19
2	GTAGATCTGTTCTCTAA**ACGAAC**ATGTTTATTTTC	**21/21**	17/21	16/19
3	GTAGATCTGTTCTCTAA**ACGAAC**TTATGGATTTGT	17/21	**21/21**	14/19
4	GTAGATCTGTTCTCTAA**ACGAAC**TTATGTACTCAT	17/21	18/21	14/19
5	GTAGATCTGTTCTCTAA**ACGAAC**TAACTATTATTA	14/21	15/21	14/19
6	GTAGATCTGTTCTCTAA**ACGAAC**GCTTTCTTATTA	15/21	15/21	13/19
7	GTAGATCTGTTCTCTAA**ACGAAC**ATGAAAAAATTA	16/21	14/21	**19/19**
8	GTAGATCTGTTCTCTAA**ACGAAC**ATGAACTTCTCA	16/21	14/21	18/19
9	GTAGATCTGTTCTCTAA**ACGAAC**AAATTAAAATGT	16/21	15/21	15/19

[a] These sequences represent the leader-containing subgenomic mRNAs found in SARS coronavirus infected cells (Yount et al., 2003). The highly conserved consensus sequences (CS), which are highlighted in bold, form the junction between the leader RNA sequences and the body sequences that encode the different open reading frames. The underlined sequences for mRNA 2, 3 and 7 represented the insert sequences for the siRNA used in this study.

[b] This represents the number of matching nucleotide (nt) between the subgenomic mRNA sequences and the sequences targeted by the siRNAs (2) used in this study over the total number of nucleotide targeted by the respective siRNAs. Perfect matches are highlighted in bold.

[c] This represents the number of matching nucleotide (nt) between the subgenomic mRNA sequences and the sequences targeted by the siRNAs (3) used in this study over the total number of nucleotide targeted by the respective siRNAs. Perfect matches are highlighted in bold.

[d] This represents the number of matching nucleotide (nt) between the subgenomic mRNA sequences and the sequences targeted by the siRNAs (7) used in this study over the total number of nucleotide targeted by the respective siRNAs. Perfect matches are highlighted in bold.

is pRNAT-U6.1/Hygro, which is a mammalian expression vector that allows hairpin siRNA to be expressed from the U6 promoter as previously described (Yu et al., 2002). This vector contains a GFP marker under the CMV promoter for easily tracking transfection efficiency. The inserts were chosen to match the sequences of the SARS-CoV sgRNAs 2, 3 and 7 as reported by Yount et al. (2003). For each of the siRNA inserts, the sequence corresponding to the highly conserved CS flanked by five to seven nucleotides (nt) before and 6–10 nt after the CS was chosen because this would give the best differentiation between the different sgRNAs and would therefore, allow specific knockdown of a specific sgRNA (see Table 1). The insert sequences for the siRNAs used in this study were as follows:

- siRNA 2: **GGATCC**CG*AATAAACATGTTCGTTTAGAG*TTGATATCCGCTCTAAACGAACATGTTTATTTTTTTTCCAA**AAGCTT**.
- siRNA 3: **GGATCC**CG*AAATCCATAAGTTCGTTTAGA*TTGATATCCGTCTAAACGAACTTATGGATTTTTTTTTCCAA**AAGCTT**.
- siRNA 7: **GGATCC**CGTCTCTAAACGAACATGAAATTGATATCCG*TTTCATGTTCGTTTAGAGA*TTTTTTCCAA**AAGCTT**.

For each insert, the underlined sequences represent the targeted sequences in the respective subgenomic mRNAs and the underlined italic sequences represent the corresponding reverse complementary sequences. Restrictions sites *Bam*HI and *Hin*dIII (highlighted in bold above) were used for cloning into the pRNAT-U6.1/Hygro vector. The 6T's before *Hin*dIII represent the termination signal.

2.3. Establishment of stable cell-lines expressing siRNAs

Vero E6 cells were transfected with vector-based siRNAs using electroporation as previously described (Tan et al., 2003). Single colonies were isolated after selection in medium containing 0.25 mg/ml of hygromycin B (Roche Molecular Biochemicals, Indianapolis, IN), and the expression of GFP in these clones was analyzed by Western blot analysis, as previously described (Tan et al., 2004b) using anti-GFP antibody (Roche). Control cells were stably transfected with an empty vector. For each of the siRNA constructs, two independent clones with high GFP expression were chosen for infection studies.

2.4. Using plasmids that mimic the sgRNAs to determine the specificity of siRNA

In order to mimic the sgRNAs generated during SARS-CoV infection, standard PCR methods were used to generate CS-containing plasmids with inserts matching the sequences of the full length viral genomic RNA (mRNA 1) and sgRNAs 7 and 8 (see Table 1), respectively. The primers used to generate these inserts are shown in Table 2 and the templates used were cDNA prepared from SARS-CoV infected cells, as previously described (Tan et al., 2004b). The amplicons were digested with *Bam*HI and *Xho*I and cloned into the compatible sites of the pXJ3′HA vector, which allows the expression of C-terminus HA tagged proteins in mammalian cells (Tan et al., 2004b).

Stable cell-lines harboring the different siRNAs were transiently transfected with pXJ-CS-7a, pXJ-CS-8a-HA or pXJ-CS-nsp1-HA using Lipofectamine reagent (Invitrogen, Carlsbad, CA), according to Manufacturer's protocol. After 16 h, the cells were harvested and lysed and subjected to Western blot analysis. Primary antibodies used were anti-HA (Roche), anti-GFP (Roche) and anti-actin (Sigma, St. Louis, MO) monoclonal antibodies and mouse polyclonal anti-7a antibody (Fielding et al., 2004).

2.5. Determination of progeny virus production

The stable cell lines containing empty vector or plasmid with siRNA 2, 3 or 7 inserts were seeded in 24-well plates and incubated at 37 °C overnight. The cells were then infected with SARS-CoV at a multiplicity of infection (MOI) of 0.01, and at 1 h post infection (hpi), the cells were washed twice and fresh DMEM medium containing 10% fetal calf serum was added. Then, at 24 hpi, the supernatant was titrated out on a 96-well plate containing Vero E6 cells. At 48 h after titration, the amount of virus was determined as the 50% cell culture infective dose ($TCID_{50}$). The calculations were made from the cytopathic effect induced in cell culture by different dilutions of the harvested

Table 2
Primers for the construction of CS-containing 7a, 8a and nsp-1 plasmids

Name of plasmid	Sequence[a,b]	Sense
pXJ-CS-7a	5′-CG*GGATCC*TCTCTAA**ACGAAC**ATGAAAATT-3′	Forward
pXJ-CS-7a	5′-CCG*CTCGAG*TCATTCTGTCTT-3′	Reverse
pXJ-CS-8a-HA	5′-CG*GGATCC*TCTCTAA**ACGAAC**ATGAACCTTCTC-3′	Forward
pXJ-CS-8a-HA	5′-CCG*CTCGAG*TTGTGTTGTACC-3′	Reverse
pXJ-CS-nsp1-HA	5′-CG*GGATCC*TCTCTAA**ACGAAC**TTTAAACCATGGAGAG-3′	Forward
pXJ-CS-nsp1-HA	5′-CCG*CTCGAG*TTACCTCCATTGAGCTC-3′	Reverse

[a] These primers are used to construct insert than can mimic the CS-leader-containing full-length genomic mRNA (i.e. mRNA 1) and the subgenomic mRNA 7 and 8 found in SARS coronavirus infected cells (Yount et al., 2003). The highly conserved consensus sequences (CS), which are highlighted in bold, form the junction between the leader RNA sequences and the body sequences that encode the different open reading frames. The sequences that corresponded to the targeted sequence of siRNA 7 (TCTCTAAACGAACATGAAA) are underlined and the number of nt match by pXJ-CS-7a is 19 out of 19, by pXJ-CS-8a-HA is 18/19 and by pXJ-CS-nsp1-HA is 17/19, respectively.

[b] The restriction sites used for cloning purposes are shown in italics.

virus as described previously (Åkerström et al., 2005; Gillim-Ross et al., 2004; Li et al., 2004).

In these experiments where transient transfections of siRNAs constructs were being tested, Vero E6 cells were plated in a 24-well plate and left to attach overnight. Then, the cells were transiently transfected with siRNA plasmid constructs of siRNA 2, 3 or 7 or empty vector as a control using lipofectamine reagent (Invitrogen) according to protocol, with small modifications (1.6 μg of DNA was used for transfection). At 24 h later, the cells were infected as described for the stable cell lines.

2.6. Indirect immunofluorescence assay (IFA)

Vero E6 cells were plated on chamber slides and transiently transfected with siRNA plasmid constructs followed by infection with SARS-CoV as described in Section 2.5 except that an MOI of 1 was used instead. At 24 hpi, supernatant was removed and the cells were washed with PBS and fixed in 4% formaldehyde in PBS and stored at +4 °C until IFA was performed, as previously described (Åkerström et al., 2005). Rabbit polyclonal antibodies against 3a, 7a, and S proteins were previously described (Fielding et al., 2004; Tan et al., 2004b) and a monoclonal antibody against SARS-CoV NP was purchased from Zymed Laboratories (San Francisco, CA). Briefly, The cells were incubated with primary antibodies for 1 h at 37 °C. After rinsing with PBS, the cells were incubated with anti-rabbit or mouse TRITC-conjugated antibodies (Jackson, Baltimore, PA) for 1 h at 37 °C. After three washes the slides were mounted using mounting medium and analyzed by immunofluorescence microscopy (Nikon Eclipse TE300, Tokyo, Japan). Pictures were obtained with a Hamamatsu Digital Camera (Type Wasabi 1, 4 Hamamatsu, Photonics GmbH, Germany).

3. Results

3.1. Design of siRNA targeting specific subgenomic mRNAs of SARS-CoV

During SARS-CoV infection, the first ORF is translated from the full-length genomic mRNA while the remaining ORFs are translated from eight sgRNAs (Snijder et al., 2003; Thiel et al., 2003; Yount et al., 2003). As shown in Table 1, the junction between the leader sequences and the body sequences that encodes for the different ORFs is the highly conserved consensus sequences (CS), which is ACGAAC for SARS-CoV. Because the sequences of the sgRNAs showed the greatest difference in this region, the siRNA sequences were chosen to match the sequences of sgRNAs 2, 3 and 7 of the SARS-CoV as reported by Yount et al. (2003). For each siRNA inserts, the sequence corresponding to CS flanked by five to seven nucleotides (nt) before and 6–10 nt after the CS was chosen because this would give the best differentiation between the different sgRNAs and would therefore, allow knockdown of a specific sgRNA (see Table 1).

The insert sequences for the siRNAs used in this study are shown in Section 2.2. The 21 nt inserts were chosen for the siRNA targeting sgRNAs 2 and 3, which are used for the synthesis of the structural protein S and accessory proteins, 3a and 3b (siRNA 2 and 3). Whereas for sgRNA 7, which is used for the expression of accessory protein, 7a and 7b (siRNA 7), a 19 nt insert was chosen because it has a GC content more suitable for RNA interference (see http://www.genscript.com for details). When comparing the sequences of siRNA 2, 3 and 7 to the full-length genomic mRNA (mRNA 1) and the eight sgRNA (mRNA 2–9), it is clear that there are significant differences between them. For example, the number of mismatches between siRNA 2 and the mRNA 1–9 (except for the targeted mRNA 2) varied from 4 to 7. Similarly, for siRNA 3, the number of mismatches (except for the targeted mRNA 3) varied from 3 to 7, and for siRNA 7, the number of mismatches (except for the targeted mRNA 7) varied from 1 to 6. Whether or not the degree of mismatch between the designed siRNAs and mRNA 1–9 is sufficient to allow specific inhibition of the targeted mRNA is determined experimentally in the following section.

3.2. Determining the specificity of siRNA 2, 3 and 7

In order to determine if the siRNAs that we have designed can specifically inhibit the targeted sgRNAs, we first constructed a cDNA containing the CS and the first ORF of sgRNA 7, i.e. the 7a protein, such that the mRNA transcripted from this plasmid would be matched by siRNA 7. As shown in Fig. 2A, when the pXJ-CS-7a plasmid was transfected into the different stable siRNA cell lines, the expression of 7a protein was specifically knockdown in cells harboring the siRNA 7 and not in the cells harboring siRNA vector, siRNA 2 or siRNA 3. This showed that the siRNAs designed in this study can be used to knockdown specific sgRNAs, even though there is some overlaps between the sequences of the different sgRNAs (Table 1).

As shown in Table 1, the mismatch between siRNA 7 and the full length viral genomic RNA (mRNA 1) is 2 nt, and the mismatch between siRNA 7 and sgRNA 8 is 1 nt, so it is possible that siRNA 7 may have some unspecific effects on the translation of the full length viral genomic RNA (mRNA 1) or sgRNA 8. In order to determine if there is such an effect, two cDNAs, pXJ-CS-nsp1-HA and pXJ-CS-8a-HA (see Table 2 for primer sequences), were constructed. The pXJ3′HA vector allows the expressed protein to be easily detected with an anti-HA antibody (Tan et al., 2004a,b). Note that in the case of the full length viral genomic RNA (mRNA 1), the first protein in the polyprotein pp1a, i.e nsp1, was chosen to simplify the detection. As shown in Fig. 2B, the expression of 8a-HA was significantly reduced in siRNA 7 stable cell-line when compared to the vector control cell-line or the siRNA 2 and 3 cell-lines. However, there was no significant difference for the expression of nsp1-HA for all the stable cell-lines. Hence, it may be expected that the siRNA 7 will inhibit both sgRNA 7 and sgRNA 8 but not the full length viral genomic RNA (mRNA 1) or the other sgRNAs.

Our results showed that mismatches of 2 nt and above between the different sgRNAs will allow differentiation inhibition by RNA interference and are consistent with previous reports that if there is a mismatch of more than 1 nt between a 21 nt siRNA and its targeted gene, the gene will not be effi-

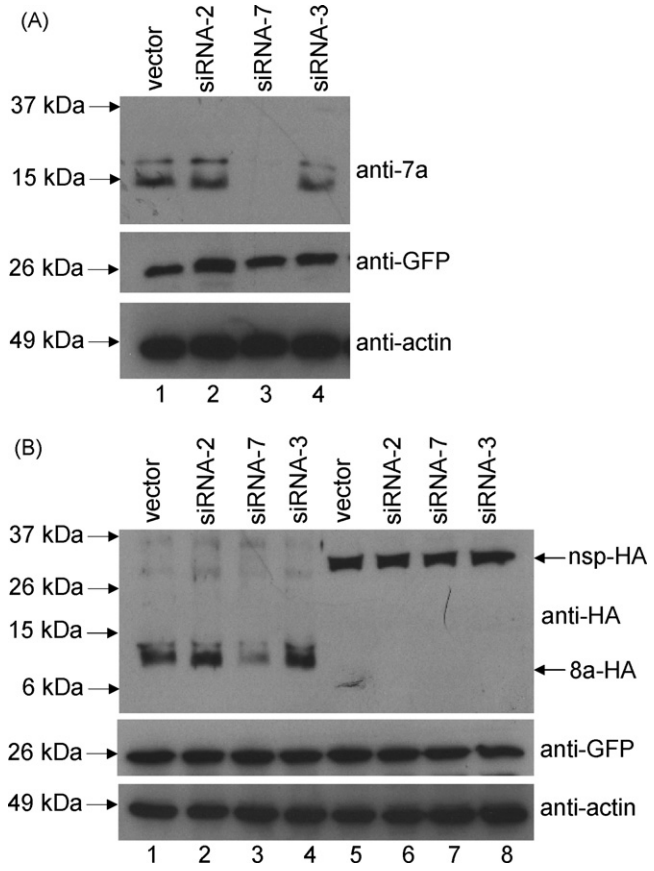

Fig. 2. The specificity of designed siRNAs was demonstrated using cDNA constructs that mimic the subgenomic RNAs of SARS-CoV. (A) Stable cell lines harboring empty vector or siRNA 2, 7 or 3 were transiently transfected with pXJ-CS-7a and harvested after 16 h. Western blot analysis was performed using anti-7a antibody to determine if the expression of 7a is silenced by the siRNA. Anti-GFP antibody was used to determine the amount of siRNA in each cell line and anti-actin antibody was used in the Western Blot to check for equal loading of samples in each well. (B) The same cell lines were transfected with pXJ-CS-8a-HA (lanes 1–4) or pXJ-CS-nsp1-HA (lanes 5–8). Western blot analysis was performed using anti-HA, anti-GFP and anti-actin monoclonal antibodies.

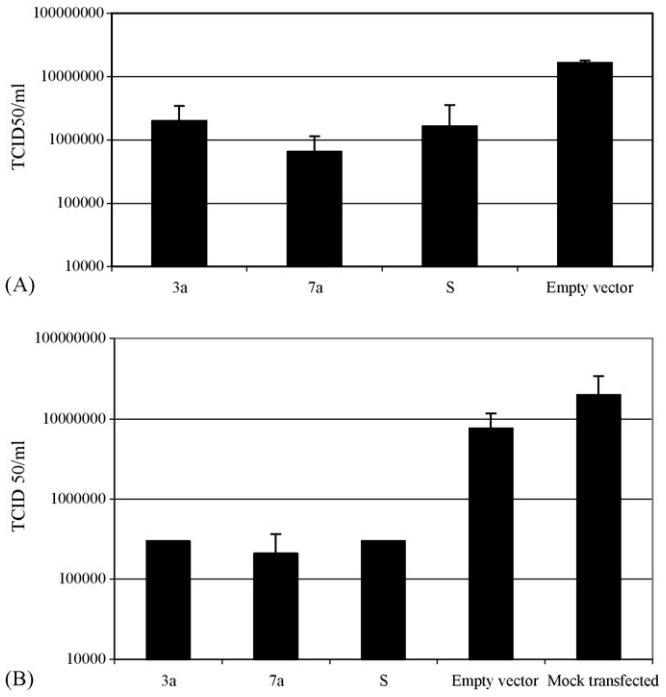

Fig. 3. SARS-CoV subgenomic RNA-specific siRNAs can inhibit the production of progeny virus. (A) Stable cell lines harboring siRNA 2, 3 and 7, which are targeted to subgenomic RNA 2, 3 and 7 of SARS-CoV, were infected with SARS CoV at an MOI of 0.01. Viruses were harvested at 24 hpi and titers were determined by $TCID_{50}$, calculations were made from the CPE induced in cell culture by different dilutions of the harvested virus. Cells transfected with empty vector were used as a control. The values given are average of three independent experiments. Error bars indicate standard deviations. (B) Vero E6 cells transiently transfected with empty vector or plasmid carry siRNA 2, 3 or 7 were infected with SARS CoV at an MOI of 0.01. Viruses were harvested and titers were determined as described above. Mock-infected cells were used as a control. The values given are average of three independent experiments. Error bars indicate standard deviations.

ciently knocked down (Elbashir et al., 2001; Yu et al., 2002). As shown in Table 1, siRNA 2 and 3 showed mismatches to all sgRNAs, except for sgRNA 2 and 3, respectively, by more than 3 nt; therefore, it is not likely that these two siRNAs have any effects on the other sgRNAs.

3.3. Effects of siRNA on the yield of progeny virus

In order to study if silencing of viral proteins by these siRNAs has any effect on the yield of progeny virus, infection of the stable cell lines with SARS-CoV was compared. The stable cell lines for the empty vector or siRNA 2, 3 and 7 were infected with SARS CoV at an MOI of 0.01, and at 24 hpi, the supernatant was titrated out on a 96-well plate containing Vero E6 cells. The amount of virus was deduced by the 50% cell culture infective dose ($CCID_{50}$), calculations were made from the cytopathic effect (CPE) induced in cell culture by different dilutions of the harvested virus [as described previously (Åkerström et al., 2005; Gillim-Ross et al., 2004; Li et al., 2004)]. When compared to vector cells, we could see a reduction of progeny virus in all three stable cell-lines by about 70% (Fig. 3A). Interestingly, the silencing by siRNA 7 appeared to reduce the progeny virus slightly better than the silencing by siRNA 2 or siRNA 3. Possibly, siRNA 7 could inhibit both sgRNA 7 and 8 (see Fig. 2B) such that the expression of four accessory proteins, 7a, 7b, 8a and 8b, would be knocked down.

In an independent experiment, Vero E6 cells were plated in a 24-well plate and transiently transfected with the different siRNA constructs using lipofectamine reagent (Invitrogen). At 24 h after transfection, the cells were infected with SARS-CoV and the amounts of virus produced were determined as described above. Consistent with the results obtained from the stable cell lines, Vero E6 cells that were transiently transfected with siRNA 2, 3 or 7 showed a significantly lower level of progeny virus than cells transfected with siRNA vector or mock-infected cells (Fig. 3B). Again, siRNA 7 reduced the progeny virus slightly better than siRNA 2 and siRNA 3. In fact, the reduction of progeny virus in cells transiently transfected with the siRNA constructs was more when compared to the stable cell-lines, although the typical transfection efficiency was only 80%. By comparing the fluorescence of the GFP signals in these

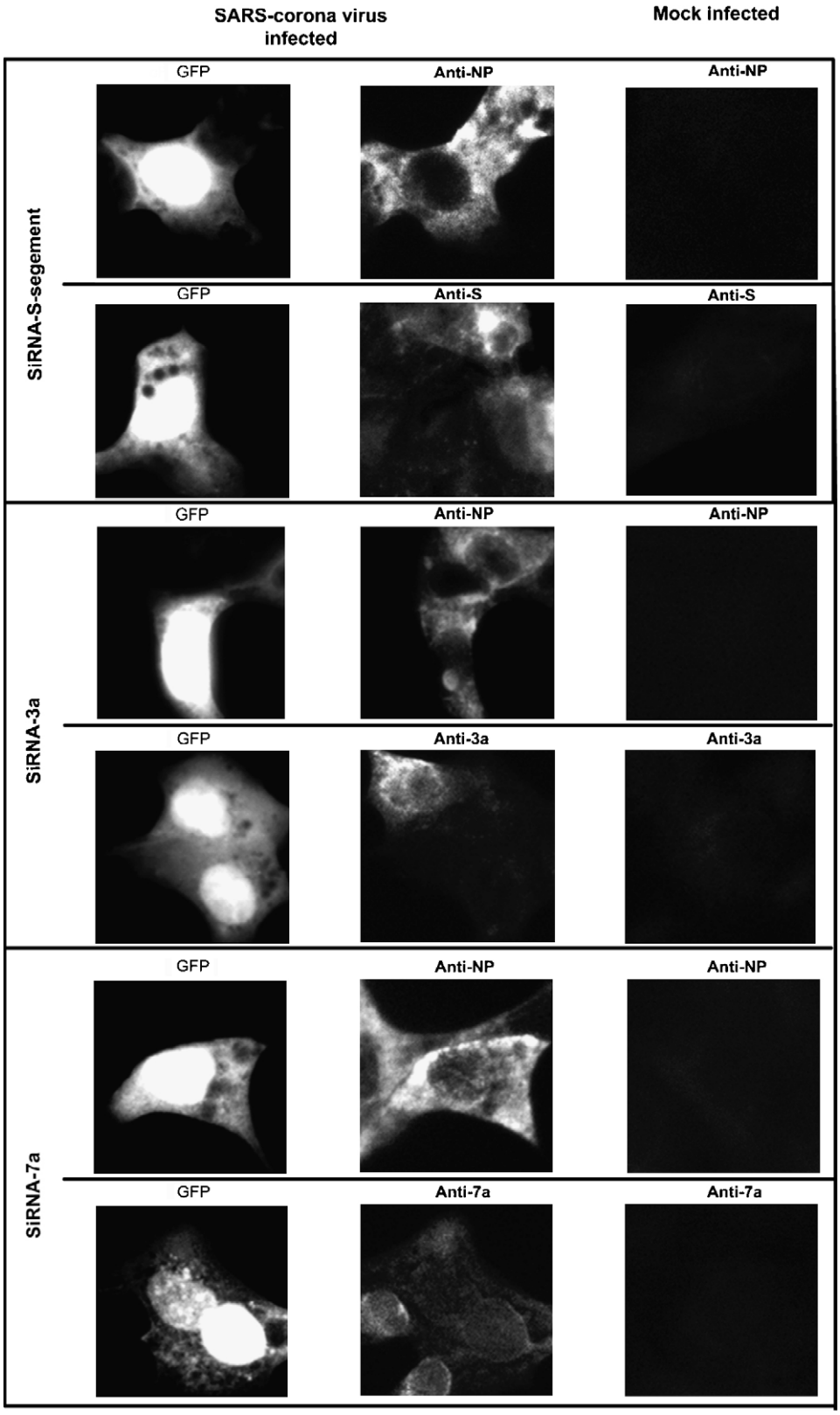

Fig. 4. siRNA 2, 3 and 7, which targeted against subgenomic RNA 2, 3 and 7, can specifically silence the expression of S, 3a and 7a, respectively. Vero E6 cells were transfected with siRNA against S, 3a and 7a as indicated in the figure. Transfected cells were mock-infected or infected with SARS CoV at an MOI of 0.01. At 24 hpi cells were fixed with 4% formaldehyde and then incubated with specific antibodies. The cells were incubated with anti-rabbit or mouse TRITC-conjugated antibodies and analyzed by immunoflourescence assay. GFP expression used for tracking the transfected cells.

cells, we observed that the level of siRNA in the stable cell-lines were somewhat lower than in cells transiently transfected with the plasmid (data not shown), which can explain why we did not observe more pronounced inhibition of yield of progeny virus in stable cell lines expressing these different siRNAs. In the plasmid transfection experiments in Fig. 2, we also observed inefficient silencing of proteins if a high amount of plasmid was used for transfection (data not shown).

3.4. Inhibition of S, 3a and 7a expression by siRNAs in SARS-CoV infected cells

Next, IFA was performed to determine if the proteins translated from the sgRNAs targeted by the siRNAs were efficiently knocked down in infected cells.

Vero E6 cells were transfected with siRNA against S, 3a and 7a as indicated in Fig. 4. These vectors contain a GFP marker under CMV promoter for easy tracking of transfected cells. The cells were mock-infected or infected with SARS CoV. At 24 hpi cells were fixed then analyzed by using specific antibodies against 3a, 7a and S proteins followed by immunofluorescence assay (as described in Section 2.6). The IFA results (Fig. 4) showed that we could specifically silence the expression of 3a, 7a and S in cells expressing siRNA 3, 7 and 2, respectively. In contrast, the expression of N in the infected cells was not affected by any of the siRNAs, demonstrating the specificity of the RNA interference.

4. Discussion

4.1. siRNA targeting the sequence difference at the CS and body sequences junctions of SARS-CoV sgRNAs can specifically knock down the targeted sgRNA

Several laboratories have designed siRNA or shRNA (short hairpin RNA) targeting different regions of the SARS-CoV genome and demonstrated their abilities to inhibit SARS-CoV replication in cell culture (He et al., 2003; Li et al., 2005a,b; Lu et al., 2004; Ni et al., 2005; Wang et al., 2004; Wu et al., 2005; Zhang et al., 2004). These RNAs are targeted at the first 2/3 of the SARS-CoV genome, which encodes the viral replicase genes, the S gene, as well as the leader sequence found at the 5′ end of the genomic and subgenomic mRNAs. A recent study using Rhesus macaque showed that a combination of siRNAs targeted to S and nsp12 genes has both prophylactic and therapeutic effects against SARS-CoV infection (Li et al., 2005a,b).

During SARS-CoV infection, the full-length viral genomic RNA is required for the translation of the ORF 1a/1b, while the sgRNAs are used for the expression of specific viral proteins (Snijder et al., 2003; Thiel et al., 2003; Yount et al., 2003). In this study, we used the short-interfering RNA (siRNA) technology and the difference at the CS and body sequences junctions for the full-length genomic RNA and sgRNAs of SARS-CoV to design hairpin siRNAs to target specifically sgRNAs 2, 3 and 7 in order to silence the expression of the viral proteins S, 3a/3b, 7a/7b, respectively. First, we used consensus sequence(CS)-containing plasmids to mimic the full-length genomic RNA (pXJ-CS-nsp1-HA) and subgenomic RNAs 7 (pXJ-CS-7a) and 8 (pXJ-CS-8a-HA) to determine the specificity of the shRNA constructs. These plasmids contained the CS joined to the initiation codon of the first open reading frame of the full-length genomic RNA (i.e. nsp1) or subgenomic RNAs (i.e. 7a for sgRNA 7 and 8a for sgRNA 8). We found that siRNA 7 could inhibit both sgRNA 7 and 8 (Fig. 2). The siRNA 7 matches sgRNA 7 perfectly well while the mismatch between siRNA 7 and sgRNA 8 is 1 nt (Table 1). In contrast, siRNA 7 did not have an effect on the full length viral genomic RNA (mRNA 1) as the mismatch between siRNA 7 and the full length viral genomic RNA (mRNA 1) is 2 nt. The mismatch between siRNA 2 or 3 and all sgRNAs, except for sgRNA 2 and 3, respectively, is more than 3 nt and, hence, siRNA 2 or 3 are not able to inhibit any other sgRNAs. Hence, we have designed hairpin siRNAs targeting the sequence differences in the CS-body sequence junctions of sgRNA 2, sgRNA 3 or sgRNAs 7 and demonstrated that they can specifically knock down either sgRNA 2, sgRNA 3 or both sgRNAs 7 and 8, respectively, without affecting the full length viral genomic RNA (mRNA 1)or other sgRNAs.

4.2. Knockdowns of the expressions of accessory proteins 3a/3b or 7a/7b and 8a/8b reduce the production of progeny SARS-CoV in Vero E6 cells

To date, two of SARS-CoV accessory proteins, 3a and 7a have been characterized to a great extent (see recent review by Tan et al., 2006). 3a being the first ORF of the sgRNA 3 and anti-3a antibodies having been found in convalescent patients (Guo et al., 2004; Tan et al., 2004a). In addition, 3a has also been found to be expressed in SARS CoV-infected cells (Tan et al., 2004b; Yu et al., 2004; Zeng et al., 2004) and could be detected in tissues obtained from SARS-CoV infected patients (Yu et al., 2004; Law et al., 2005; Chan et al., 2005). The sgRNA 3 contains a second ORF, called 3b, which is mostly likely to be expressed via an internal ribosomal entry mechanism (Marra et al., 2003; Rota et al., 2003; Snijder et al., 2003). 3b has been shown to be localized in the nucleus and to induce apoptosis when overexpressed (Yuan et al., 2005a,b). The 7a protein is encoded by the first ORF of sgRNA 7 (Snijder et al., 2003; Thiel et al., 2003; Yount et al., 2003) and it is expressed in SARS-CoV-infected cells (Fielding et al., 2004; Chen et al., 2005; Nelson et al., 2005). A second protein, termed 7b, may be expressed from sgRNA 7 by "leaky scanning" of ribosomes (Snijder et al., 2003), but to date, there is no characterization of this protein.

Next, we showed that these siRNAs, when introduced into Vero E6 cells by transient or stable transfection, resulted in a significant reduction in the production of progeny virus (Fig. 3). IFA also showed the specific knockdown of S, 3a and 7a proteins in infected cells containing siRNA 2, 3 and 7 (based upon the GFP signal), while the expression of the N protein was not affected (Fig. 4). To our knowledge, this is the first report of inhibition of virus replication by using siRNA against the SARS-CoV accessory proteins. When compared to the inhibition by siRNA against the S protein, siRNA against 3a/3b showed comparable inhibition while siRNA against 7a/7b (and 8a/8b) was slightly more efficient. Our data showed that the siRNA 3 and 7

are able to inhibit the yield of progeny virus, and suggested that the accessory proteins, 7a/7b (and 8a/8b) and 3a/3b, could play important roles during the replication cycle of SARS-CoV.

Yount et al. (2005) recently published results from reverse genetics experiments showing that recombinant virus lacking 7a and 7b can replicate as efficiently as wild-type virus, while the deletion of 3a resulted in about 1 log reduction of virus yield. (Yount et al., 2005). The siRNA 7 was able to reduce the production of progeny viruses in our system, while the recombinant virus with 7a/7b deleted behaved like the wild-type virus. The difference between our data may be explained by the fact that siRNA 7 could inhibit both sgRNA 7 and 8, which are required for the expression of accessory proteins 7a, 7b, 8a and 8b. The simultaneous silencing of these four accessory proteins may also explain why we observed the highest inhibition effect with siRNA 7.

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