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RNA Interference-Mediated Inhibition of Semliki Forest Virus Replication in Mammalian Cells

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ABSTRACT

RNA interference (RNAi) has recently shown promise as a mode of inhibition of slowly replicating viruses causing chronic diseases such as hepatitis C. To investigate whether RNAi is also feasible for rapidly growing RNA viruses such as alphaviruses, we tested the ability of expressed short hairpin RNAs (shRNAs) to inhibit the Semliki Forest virus (SFV), a rapidly replicating positive-strand RNA virus. Plasmids expressing shRNAs targeting SFV target sequences under the control of a human U6 promoter were introduced into BHK-21 cells. The targets included sequences encoding nonstructural (nsP1, 2, and 4) and structural (capsid) proteins as well as nonviral sequences serving as control targets. Twenty-four to 48 hours following transfection with shRNA plasmids, the cells were infected with replication-competent or replication-deficient recombinant SFV expressing green fluorescent protein (GFP) at a multiplicity of infection (MOI) of ~5. Viral replication was monitored by fluorescence microscopy and flow cytometry. Specific and marked reduction of viral replication was observed with shRNAs targeting nsP1 and nsP4. The degree of inhibition of the replication-deficient SFV was ≥70% over a 5-day period, a level similar to the transfection efficiency, suggesting complete inhibition of nonreplicating virus in the transfected cell population. However, only nsP1 shRNA was inhibitory against replication-competent SFV (~30%–50% reduction), and this effect was transient. No inhibition was observed with control shRNAs. In contrast to the recent success of RNAi approaches for slowly growing viruses, these results illustrate the challenge of inhibiting very rapidly replicating RNA viruses by RNAi. However, the addition of RNAi approaches to other antiviral modalities might improve the response to acute infections.

INTRODUCTION

RNA INTERFERENCE (RNAi) is a sequence-specific gene silencing process triggered by double-stranded RNA (dsRNA) that can operate at both transcriptional and posttranscriptional levels (Dykxhoorn et al., 2003; Meister and Tuschl, 2004; Zamore and Haley, 2005). The phenomenon is widespread in nature, having been observed in protozoa, plants, fungi, and animals. In the case of plants, there is clear evidence for a protective role against viral infection through recognition of dsRNA intermediates (Tijsterman et al., 2002). When dsRNA longer than about 20 bp is introduced into a cell, the proximate effectors of RNAi are short (~21–23 nucleo-

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tide [nt]) dsRNA molecules, termed small interfering RNAs (siRNAs), which are processed from longer precursors by the enzyme Dicer. The siRNA becomes incorporated into the RNA-induced silencing complex (RISC) with release of one of the strands and targeting by the other, retained (guide) strand to complementary mRNAs. In many cases, the degree of pairing between the guide (antisense) strand and the target mRNA determines whether mRNA silencing is achieved via site-specific cleavage of the message or, as in the case of microRNAs (miRNAs), largely through translational repression. For cleavage-mediated silencing, cleavage products are released and degraded, allowing the siRNA–RISC to cleave additional target mRNA molecules.

The discovery that introducing exogenous siRNAs can specifically silence genes in mammalian cells (Caplen et al., 2001; Elbashir et al., 2001) led to widespread use of siRNAs for gene function analysis and target validation. Although many RNAi studies relied on chemically synthesized or *in vitro*-transcribed siRNAs that were delivered to target cells by transfection, the use of pol III promoter and terminator sequences has allowed the efficient intracellular expression of short hairpin RNAs (shRNAs) from unidirectional promoters (Tuschl, 2001; Cullen, 2002; Devroe and Silver, 2002; Hannon, 2002; Ramaswamy and Slack, 2002), or separate strand siRNA duplexes from opposing (Tran et al., 2003; Zheng et al., 2004; Seyhan et al., 2005) or tandem promoters (Lee et al., 2002; Lee and Rossi, 2004). shRNAs can also be transcribed from pol II promoters, as occurs in the natural synthesis of precursor miRNAs (Cullen, 2005; Dickins et al., 2005; Stegmeier et al., 2005). Promoter-expressed shRNAs usually elicit more robust silencing than siRNAs expressed from separate promoters. Although shRNAs and miRNAs are of different origins, both are predicted to fold into RNA hairpins bearing 2-nt 3′-overhangs and are translocated into the cytoplasm by Exportin 5, which recognizes short RNA stem–loop structures bearing short 3′-overhangs (Cullen, 2005; Dickins et al., 2005; Stegmeier et al., 2005).

Given that RNAi is a natural antiviral mechanism in plants, RNAi technology is potentially well suited to treating viral infection, and numerous examples now illustrate that a wide range of viruses can be inhibited by this approach (Caplen et al., 2002; Radhakrishnan et al., 2004; Bitko et al., 2005; Morrissey et al., 2005a; Wang et al., 2005; Dykxhoorn and Lieberman, 2006; Geisbert et al., 2006; Ilves et al., 2006; O'Brien, 2006; Reuter et al., 2006). Effective siRNA target sites can be identified either by testing individual siRNA candidates generated by computer algorithms (Elbashir et al., 2001; Reynolds et al., 2004) or by selection from a library of siRNAs (Seyhan et al., 2005). In the case of viruses, however, the effectiveness of siRNAs can be lost over time as a result of mutation, leading to the development of viral resistance. RNA viruses are particularly prone to mutational escape due to the error-prone nature of the RNA-dependent RNA polymerases or reverse transcriptases that they use in replicating their genomes. An example is HIV-1 (Bennasser et al., 2005). When rapid mutation is combined with rapid growth, as in the case of alphaviruses, the question arises as to whether RNAi approaches can be effective at all. We have addressed this question using a model alphavirus, the Semliki Forest virus (SFV).

Alphaviruses are arthropod-borne, and can cause acute encephalitis and arthritis in humans and animals (Strauss and Strauss, 1994). Some alphaviruses (Eastern and Western Equine Encephalitis viruses) cause fatal encephalitis in humans in both North and South America (Strauss and Strauss, 1994). SFV itself has a broad natural host range, and can infect a wide variety of higher eukaryotes (Lundstrom, 1999). Although SFV is usually considered nonvirulent for humans, variants of SFV in Africa have caused a disease characterized by severe headache, fever, myalgia, and arthralgia (Strauss and Strauss, 1994).

The genomic RNA of SFV (42S, 11,442 nt) has a positive polarity and acts as an mRNA. Following infection, the 5′-two-thirds of the 42S genomic RNA is translated into a polyprotein, with subsequent proteolytic cleavage into four nonstructural proteins (nsP1, nsP2, nsP3, and nsP4) that form an RNA replicase complex (Fig. 1). In the cytoplasm, the replicase synthesizes first full-length minus-strand RNA from the plus-strand genomic RNA, and then additional plus-strand copies from the minus strand (Strauss and Strauss, 1994). Production of plus- and minus-strand genomic RNAs begins during the first 4 hours of infection and peaks at 24 hours (Liljestrom and Garoff, 1991; Seyhan et al., 2002). The structural proteins required to make mature virions (capsid and envelope glycoproteins) are encoded by the 3′-third of the genomic (26S) RNA (Fig. 1). The capsid protein recognizes a packaging signal within the nsP2 gene leading to formation of the nucleocapsid (Frolova et al., 1997), after which infectious virions are released through the cell membrane at the prodigious rate of 2000 PFU/h (Strauss and Strauss, 1994).

In this study, we used an algorithm-based approach to design siRNAs targeting regions of several different genes that are highly conserved among strains of SFV. These siRNAs were then expressed in cultured cells to identify effective target sites. We show that these expressed shRNAs can provide marked reduction in viral RNA synthesis, but that over time, replication-competent SFV can overwhelm their inhibitory effects in mammalian cells. However, when combined with other antiviral treatments and a normal immune response, RNAi approaches may find a useful role even for such challenging pathogens as alphaviruses.

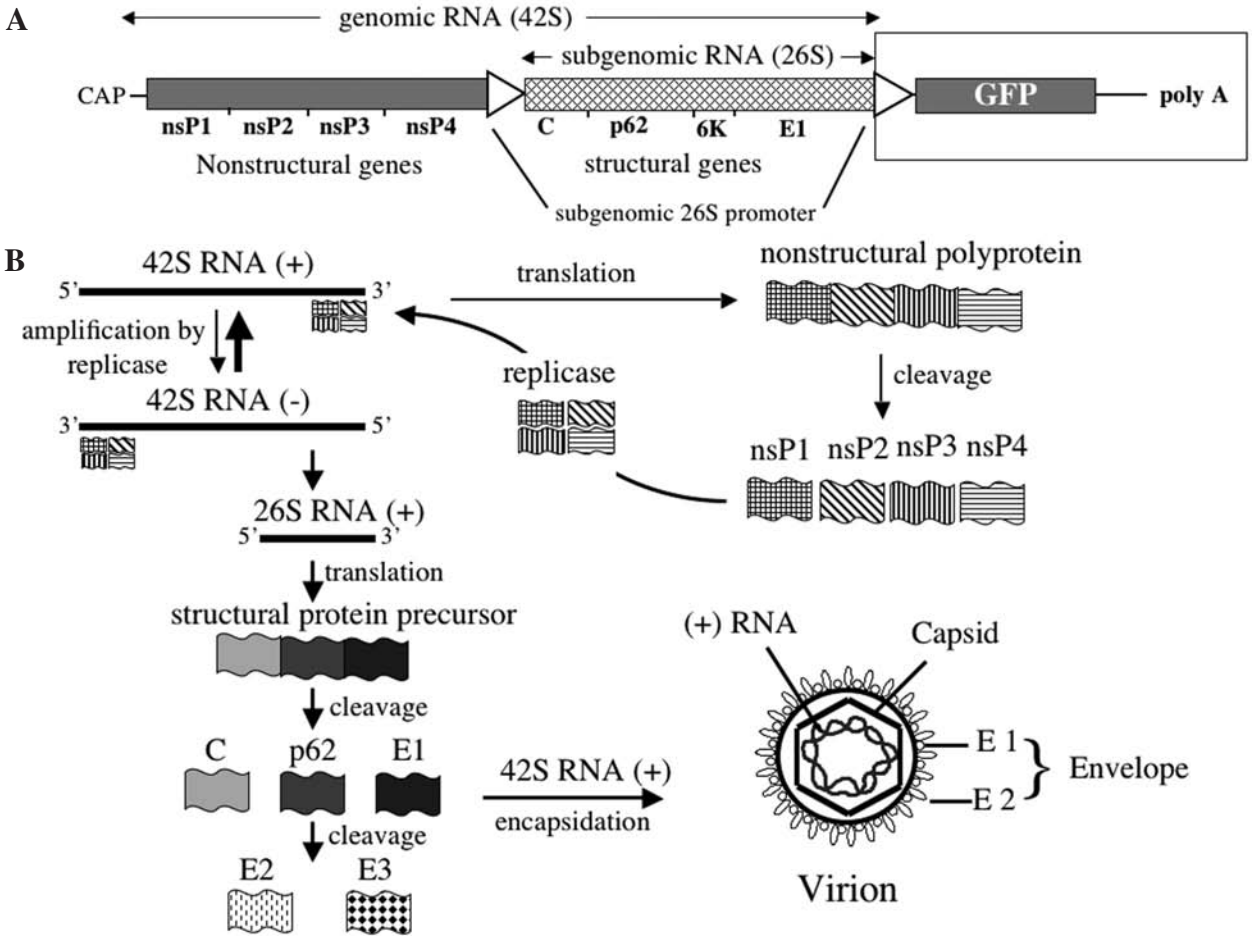

FIG. 1. Schematic presentation of RNA and protein products involved in SFV virus replication. (**A**) In the wild-type SFV, the positive (+) genomic RNA contains two open reading frames (ORF); one encodes nonstructural proteins (replicase complex) while the second encodes structural proteins. The replication-competent recombinant SFV–VA7–GFP, in addition to those two ORFs, also encodes a GFP reporter gene from a second SFV 26S subgenomic promoter (box). In the replication-deficient recombinant SFV–GFP, the subgenomic RNA encoding structural proteins have been replaced with GFP. To produce replication-deficient infectious viral particles, a helper RNA encoding structural proteins must be provided *in trans* with the viral RNA transcript encoding the nonstructural proteins during transfection. (**B**) SFV has a (+) strand RNA genome of 11,442 nt (42S) that functions as an mRNA. Upon infection, replication commences with translation of the 5′-two-thirds of the 42S genomic RNA into a polyprotein, with subsequent autoproteolytic cleavage into four nonstructural proteins (nsPs), nsP1–4, forming an RNA replicase complex. Next, the replicase complex binds to the 3′-end of the (+) strand RNA and synthesizes a complementary full-length negative (−) strand RNA. The reverse reaction [(−) strand as template and (+) strand as product] is also catalyzed by replicase resulting in massive amplification (heavy arrow) of full length (+) and thence more (−) strand RNAs. Once the (−) strand is available, the replicase binds to a subgenomic promoter, synthesizing a subgenomic 26S (+) strand RNA that contains the 3′ ORF. This 3′ ORF encodes a polyprotein that is self-cleaved into the structural proteins: capsid, E3, E2, 6K, and E1. The capsid forms the nucleocapsid with the (+) strand RNA genome, and is also a protease that cleaves itself from the rest of the polypeptide. The remainder of the polyprotein moves into the ER membrane (the N-terminal section of E3 serves as a signal sequence). Posttranslational modifications (glycosylation and acylation) and further cleavage by signal peptidase occurs, yielding four separate proteins, p62 (E2–E3 precursor), 6K, and E1. The envelope proteins form spikes on the viral surface and the C-terminal region of the E2 protein interacts with the nucleocapsid; 6K is important for viral release.

MATERIALS AND METHODS

Oligonucleotides and plasmid vectors

DNA oligonucleotides were purchased from IDT (Coralville, IA). To create an expression vector with a unidirectional pol III promoter, the promoter region of the human U6 small nuclear RNA was amplified by PCR from genomic DNA of human HT1080 cells by using the following primers (5′-3′): U6 forward, ATCGATGCCCCAGTGGAAAGACGCGCAG, and U6 reverse, GGATCCGAATTCGAAGACCACGGTGTTTCGTCCTTTCCACAA (Qin et al., 2003). The resulting amplification

products were cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA).

Design of SFV-specific shRNAs

To construct the DNA inserts encoding shRNAs, a pair of complementary oligodeoxynucleotides were synthesized such that, after annealing, they formed a duplex with four-base overhangs (compatible with *Bbs*I and *BamH*1) and containing 29-nt gene-specific sense (S) and antisense (AS) sequences separated by a loop (CTTCCTGTCA, taken from miR-23 [Lagos-Quintana et al., 2001] to avoid introducing foreign sequences) and followed by the pol III transcription termination sequence T_6. This duplex was ligated into the U6 expression vector (see previous paragraph).

The oligonucleotide pairs used for shRNAs were the following, shown 5′-3′ with sticky ends for cloning, loop, and terminator sequences in upper case: nsP1 sense (top) strand: ACCG-ggcatttccgtcgttcgaggtggagtcat-CTTCCTGTCA-atgactccacctcgaacgacggaaatgcc-TTTTTTG; nsP1 antisense (bottom) strand: GATCC-AAAAAA-ggcatttccgtcgttcgaggtggagtcat-TGACAGGAAG-atgactccacctcgaacgacggaaatgcc; nsP2 sense (top) strand: ACCG-cgaaagggagttcgtcaacaggaaactat-CTTCCTGTCA-atagtttcctgttgacgaactccctttcg-TTTTTTG; nsP2 antisense (bottom) strand: GATCC-AAAAAA-cgaaagggagttcgtcaacaggaaactat-TGACAGGAAG-atagtttcctgttgacgaactccctttcg; nsP4a sense (top) strand: ACCG-gttgggtaagccgctaacagctgaagaca-CTTCCTGTCA-tgtcttcagctgttagcggcttacccaac-TTTTTTG; nsP4a antisense (bottom) strand: GATCC-AAAAAA-gttgggtaagccgctaacagctgaagaca-TGACAGGAAG-tgtcttcagctgttagcggcttacccaac; nsP4b sense (top) strand: ACCG-gttgggtaagccgttaacagctgaagaca-CTTCCTGTCA-tgtcttcagctgttaacggcttacccaac-TTTTTTG; nsP4b antisense (top) strand: GATCC-AAAAAA-gttgggtaagccgttaacagctgaagaca-TGACAGGAAG-tgtcttcagctgttaacggcttacccaac; Capsid sense (top) strand: ACCG-ccgggagacagtggccggcccatctttga-CTTCCTGTCA-tcaaagatgggccggccactgtctcccgg-TTTTTTG; Capsid antisense (bottom) strand: GATCC-AAAAAA-ccgggagacagtggccggcccatctttga-TGACAGGAAGt-caaagatgggccggccactgtctcccgg. The oligonucleotide pair used for the DsRed control shRNA was as described in (Seyhan et al., 2005); DsRed sense (top) strand: ACCG-tgggagcgcgtgatgaacttcgaggacg-CTTCCTGTCA-cgtcctcgaagttcatcacgcgctcccac-TTTTTTG; DsRed antisense (bottom) strand: GATCC-AAAAAA-gtgggagcgcgtgatgaacttcgaggacg-TGACAGGAAG-cgtcctcgaagttcatcacgcgctccca.

Cell culture and transfection. BHK-21 cells (ATCC) were cultured in D-MEM medium [minimal essential medium (Invitrogen) containing 5% heat-inactivated (56°C, 30 min) fetal bovine serum (Hyclone, Logan, UT), 10% tryptose phosphate (Difco, Detroit, MI), 2 mM L-glutamine (Sigma, St. Louis, MO), penicillin/streptomycin [10,000 U/mL, 10 mg/mL; Invitrogen)]. Transfections were performed using 2 μg of total circular plasmid DNA with Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's recommendations. Twenty-four hours after transfection, the cells were infected with SFV–GFP for viral inhibition studies.

In vitro *synthesis of infectious viral RNAs, transfection, and infection*. To produce a replication-proficient recombinant eGFP-expressing SFV virus, designated SFV–VA7–GFP, plasmid pSFV–VA7–eGFP (Vaha-Koskela et al., 2003) encoding full-length genomic RNA was used. To produce a replication-deficient eGFP-expressing SFV virus, designated SFV1–GFP, plasmids pSFV–86–eGFP encoding the viral replicase and pSFV–Helper2 (Berglund et al., 1993) encoding the structural proteins were used. pSFV–86–eGFP is based on the attenuated strain SFV4 (Accession no. AJ251359). The use of the pSFV–Helper2 vector results in conditionally infectious particles lacking any structural genes, which are therefore replication-deficient. Two micrograms of each cDNA clone were linearized with Spe*I* and transcribed using SP6 RNA polymerase in the presence of the capping analog $m^7G(5')ppp(5')G$ (Ambion, Austin, TX), following the manufacturer's recommendations. RNA transcripts were purified by using a G25 spin column (Pharmacia, Piscataway, NJ) to remove unincorporated nucleotides followed by DNase I-digestion, extraction with phenol-chloroform, and ethanol precipitation. The RNA pellet was dissolved in water and quantified by UV spectrophotometry.

BHK-21 cells were grown to 60%–70% confluency in 60-mm dishes and transfected with 2 μg of *in vitro* transcribed SFV–VA7–GFP full-length genomic viral RNA and 4 μL Lipofectin (Invitrogen), in serum-free medium (OPTIMEM, Invitrogen) following the manufacturer's recommendations. For the production of replication-deficient virus, 2 μg of replicase- and structural protein-encoding RNAs (at a 1:1 molar ratio) were used. Transfected cells were incubated for 36 hours at 37°C, 5% CO_2, and culture supernatants were collected, centrifuged at 10,000 rpm for 20 minutes at 4°C to remove any remaining cellular debris, and titered by plaque assays on naive BHK-21 cells (Seyhan et al., 2002).

Viral replication monitored by plaque formation. BHK-21 cells grown in 60-mm dishes (80%–90% confluency) were employed for monitoring viral titer of replication-competent SFV. Cells were infected with serially diluted virus obtained as above. Virions were allowed to adsorb to cells for 1 hour at 37°C, 5% CO_2. The inoculum was removed and cells were overlaid with 0.5% agarose in complete media with 5% serum and fur-

TABLE 1. LOCATION AND SEQUENCE OF shRNA TARGET SITES ON SFV GENOME, SHOWING POSITIONS OF MISMATCHES IN nsP4

shRNA (target position on SFV–VA7–GFP)	*shRNA sense strand sequence*	*SFV–VA7–GFP*[a]	*SFV1–GFP*[a]
nsP1 (148 nt)	ggcatttccgtcgttcgaggtggagtcat	Match	Match
nsP2 (1976 nt)	cgaaagggagttcgtcaacaggaaactat	Match	Match
nsP4a (7127 nt)	gttgggtaagccg**t**taacagctgaagaca	Match	Mismatch
nsP4b (7127 nt)	gttgggtaagccg**c**taacagctgaagaca	Mismatch	Match
capsid (8062 nt)	ccgggagacagtggccggcccatctttga	Match	Match

[a]The SFV strains used to design shRNAs were SFV–VA7 GFP (GenBank accession no. Z48163) and SFV1–GFP. The latter, replication deficient strain was derived from SFV4 (Gene bank accession no. AJ251359). The site used in the *nsP4* gene differs in the two strains; shRNA nsP4a matches SFV–VA7–GFP, while nsP4b matches SFV1–GFP as shown. The shRNA sense strand sequences shown are identical to the target sequence for the matching strain; the underlined residue is the one that is mismatched to the nonmatching strain. The antisense strand of each shRNA is fully complementary to the sense strand shown, and in all cases the loop sequence is the same as that shown in Figure 2D.

SiRNA target sequences in the SFV–VA7 strain 42S genomic RNA were identified using an algorithm available at the Ambion website (www.ambion.com). These sequences are highly conserved across the various strains of SFV. These sites include coding sequences for nonstructural proteins nsP1, nsP2, nsP4, and structural capsid protein. Because the two SFV strains used differ at the center of the target site in nsP4, both versions of nsP4 shRNA were prepared.

ther incubated for 36 hours at 37°C. The agarose overlay was removed and cells were stained with 1% crystal violet solution. Plates were scored for the total number of plaques formed to calculate the number of plaque-forming units (PFU)/mL.

Viral replication monitored by flow cytometry and fluorescence microscopy. BHK-21 cells grown in 60-mm dishes were employed for monitoring viral titers of replication-deficient SFV1–GFP. At 80%–90% confluency, cells were infected with serially diluted virus stocks of replication-deficient SFV1–GFP virus in the presence of 8% PEG (polyethylene glycol 8000, Sigma) in serum-free media for 1 hour at 37°C, 5% CO_2. PEG has been shown to increase transduction efficiency (Arudchandran et al., 1999), and our studies have shown that 8% is optimal (unpublished). The inoculum was replaced with complete media and cells were further incubated for 24 hours at 37°C. For monitoring GFP-expressing cells by fluorescence microscopy (using an Olympus CK40 with a 10× objective and 100× total magnification), the average number of GFP-expressing cells in five random fields of view was used to estimate the total number of fluorescent (i.e., infected) cells per well, which was then multiplied by the dilution factor to determine the titer (infectious units/mL). To score GFP expressing cells by flow cytometry, monolayers of cells were harvested by trypsinization, fixed with formalin, and GFP emission was analyzed by single channel fluorescence in a FACScan flow cytometer (Becton Dickenson, Fullerton, CA). Ten thousand cells were analyzed per sample. The fraction of GFP-expressing cells was used to determine the titer.

Transient transfection of BHK-21 cells with shRNA expression vectors. The day before transfection, BHK-21 cells were seeded at 1.7×10^5 cells per well in a 24-well plate such that they would be 60% confluent at the time of transfection on the next day. For SFV–GFP silencing experiments, cells were transfected with 800 ng shRNA expression vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. They were then incubated for 24–48 hours at 37°C in a 5% CO_2 incubator to allow expression of shRNAs, then washed and used for infection with SFV–GFP.

Viral infection of shRNA-expressing cells. One day after transfection (as described above), BHK-21 cells expressing shRNAs were infected with replication-competent SFV virus at a multiplicity of infection (MOI) of 5 (calculated based on the number of cells per well) in 0.2 mL of serum-free media per well of a 24-well plate. For the replication-deficient SFV1–GFP strain, 8% PEG was also present to enhance infection rates. Infected cells were incubated at 37°C. After 1 hour, an equal volume of complete media containing 10% serum was added to the cells and they were further incubated overnight at 37°C. At various time points, the cells were imaged by fluorescence microscopy. For quantitative analysis, cells were trypsinized and

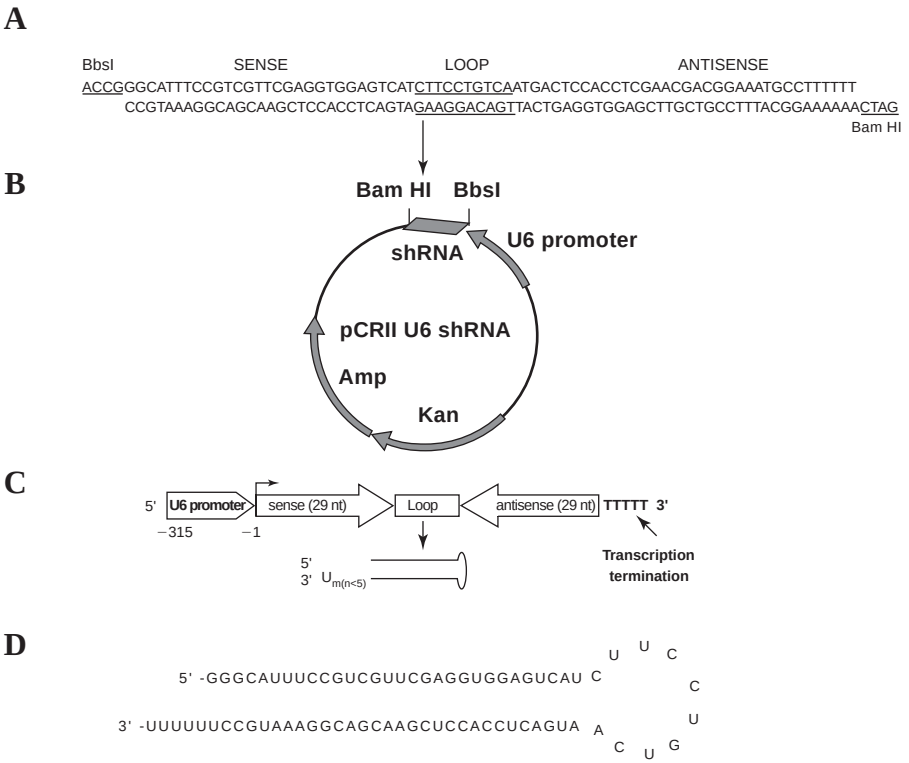

FIG. 2. ShRNA expression construct. (**A**) To construct siRNA hairpin encoding DNA templates, a pair of overlapping chemically synthesized and 5′-phosphorylated DNA oligonucleotides providing four-base overhangs (compatible with *Bbs*I and *Bam*H1) were annealed, forming a duplex DNA. The 29-nt sense and antisense sequences are shown in light type, while a loop sequence derived from native miR-23 is underlined. The sense targeting sequence always starts with G at position 1 (thus, the reverse complementary sequence ends with C), as required for the efficient transcription initiation from the U6 promoter (Paule and White, 2000). All dsRNA template sequences contained an miR-23 loop sequence (Lagos-Quintana et al., 2003) and a TTTTTT terminator at the 3′ end. (**B**) Duplex DNAs with appropriate overhangs were inserted into a plasmid expression vector between *Bbs*I and *Bam*HI sites, immediately downstream of the U6 promoter as described (Seyhan et al., 2005). To generate that vector, a human U6 snRNA gene was subcloned into the PCR II TOPO TA cloning vector (Invitrogen). (**C**) Schematic representation of the human U6 expression cassette and an inverted repeat sequence encoding siRNA hairpins. A block of six thymidines serves as an RNA polymerase III transcription termination signal. The initiation (+1) site and the direction of transcription are indicated. (**D**) Sequence and predicted secondary structure of the nsP1 shRNA transcript. The sense sequence of shRNA always starts with a G (underlined) at position 1. In this case, it may or may not be paired with the opposing U on the other strand.

evaluated for GFP expression by flow cytometry as described above. For the replication-deficient SFV1–GFP, an MOI of 5 provided about 70% infection, while for the replication-competent VA7 virus the same MOI provided 90% infection (measured 9 hours postinfection). (Note that because the two strains were titered using different methods [see above], the titers may not be precisely comparable.) Taking into account the estimated percentage of cells that had taken up shRNA, the percentage that were both transfected and infected was about 50% (0.7 × 0.7) for the replication deficient virus and 63% (0.7 × 0.9) for the replication competent SFV.

Detection of shRNAs by Northern blot analysis. For detecting shRNA transcripts, BHK-21 cells were transfected with an 800-ng shRNA expression vector as described above and incubated for 24–48 hours at 37°C in a 5% CO_2 incubator to allow expression of shRNAs. Cells were then lysed with RNAEasy solution (Qiagen, Chatsworth, CA) and total cellular RNA was isolated. This RNA (10 μg) was electrophoresed through a denaturing 10% polyacrylamide gel, blotted to a positively charged nitrocellulose membrane, and hybridized to DNA probes that were ^{32}P-labeled at their 3′ ends (Paul et al., 2003). Two probes were used: one complementary to the antisense strand of the shRNA, and one complementary to native U6 snRNA, which was used as an internal standard. To provide accuracy within a wide dynamic range, blots were quantified using a Bio-Rad GS-525 Molecular Imager and Molecular Analyst 2.1 software.

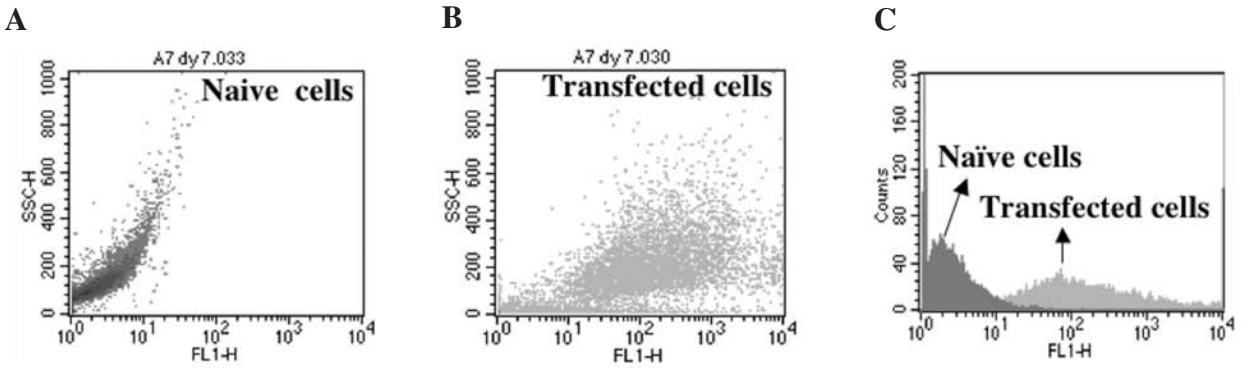

FIG. 3. Transfection of BHK-21 cells with SFV-VA7–GFP mRNA results in replication proficient virus. Cells were transfected with capped SFV-VA7-GFP RNA and assayed for virus production by flow cytometry 1, 3, and 7 days after transfection. Cells were mock-transfected (**A**) or transfected with SFV RNA (**B**) (both **A** and **B** show side scatter vs. GFP fluorescence). For clarity, only the 7-day posttransfection flow cytometry analysis is shown. (**C**) An overlay of one-dimensional histograms of naïve cells and SFV–GFP virus-infected cells.

RESULTS

Design and expression of shRNAs targeting conserved SFV sequences

To identify nonoverlapping target sites in the SFV 42S genomic RNA (Fig. 1), we searched the SFV sequence using an siRNA-finding algorithm (http://www.ambion.com) and identified sites in each of four SFV genes (Table 1) that were highly conserved across various strains of SFV. The siRNA sites are in the coding sequences for nonstructural proteins nsP1, nsP2, nsP4 (replicase) and the structural capsid protein. The shRNA expression vectors were constructed by annealing two synthetic oligonucleotides and cloning into a pCRII plasmid adjacent to a human U6 promoter (Fig. 2) as described previously (Seyhan et al., 2005). An example of a predicted 29-bp shRNA hairpin from one of these vectors is shown in Figure 2D.

Expression and accumulation of shRNA transcripts in BHK-21 cells transfected with shRNA-expressing plasmids were analyzed by Northern blot (data not shown). Transiently transfected cells expressed relatively high levels of shRNA from the U6 promoter, estimated at 3.6×10^4 copies per cell by comparing to levels of native U6 snRNA, which are approximately 5×10^5/cell (Seyhan et al., 2002), and correcting for the estimated 30% of cells that were not transfected (determined by flow cytometry of cells transfected with a similar vector expressing GFP using the same transfection agent). This level of accumulation is in accord with that seen for other small RNAs (shRNAs and ribozymes) expressed from U6 promoters (Seyhan et al., 2002; Paul et al., 2003).

In these cells, expression of shRNA transcripts was measured at 48 hours after transfection; however, silencing of a reporter gene (red fluorescent protein) could be seen as early as 12 hours after transfection (not shown). Because steady-state levels of shRNA expression were reached at approximately 24 hours, that was the time point at which viral infection was initiated.

Inhibition of replication-competent SFV–GFP by expressed shRNA

A fully replication-competent SFV called SFV–VA7–GFP, which contains the entire SFV genome plus a GFP reporter gene expressed from the 26S subgenomic

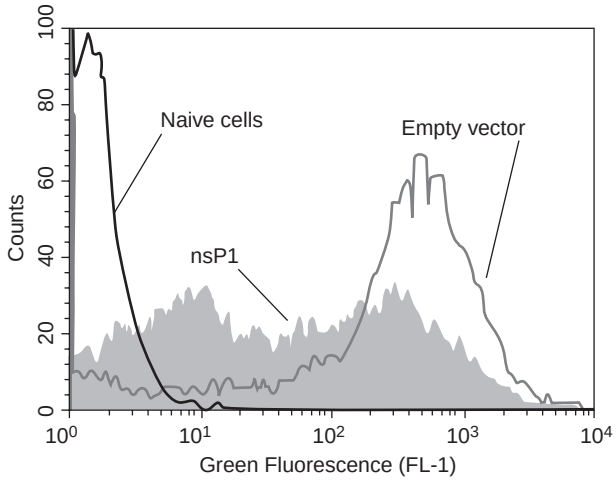

FIG. 4. Inhibition of replication-proficient SFV–VA7–GFP by transiently expressed shRNA targeting nsP1. BHK-21 cells were transiently transfected with the shRNA encoding plasmids. Twenty-four hours after transfection, cells were infected with SFV–GFP recombinant virus at an MOI of 5, and 24 hours after infection they were assayed for virus-mediated GFP expression by flow cytometry. nsP1: shRNA targeting the nsP1 gene; empty vector: pU6 vector with no shRNA insert; naïve cells: uninfected BHK-21 cells.

promoter, was produced by transfecting BHK-21 cells with capped SFV–VA7–GFP RNA. Figure 3 shows that by 7 days posttransfection, virtually all the cells express GFP through either the initial transfection (90%; see Materials and Methods) or infection by secondary virions (the remainder). This virus was then titered and used in the following experiment. First, BHK-21 cells were transiently transfected with the vectors expressing the shRNAs targeting nsP1, nsP2, nsP4, capsid, or a single-base mismatched control for the nsP4 site (see Table 1) as well as a nontargeting control shRNA. These cells were allowed to express shRNAs for 24–48 hours and were then challenged with SFV–VA7–GFP. RNAi-mediated cleavage of the 42S genomic RNA should abolish production of (−) 42S RNA, which is the template for generating the 26S subgenomic and GFP RNAs as well as the (+) 42S genomic RNA. Flow cytometric analysis showed that only one of the SFV-specific shRNA constructs, nsP1, produced a marked reduction—by approximately 30%–50%—in SFV–VA7–GFP replication 24 hours after infection (Fig. 4). However, this inhibitory effect faded thereafter and the cells died within 6 days after infection.

Inhibition of replication-deficient SFV1–GFP by expressed shRNAs

The modest reduction in SFV–VA7–GFP replication by one shRNA in the previous experiment (Fig. 4) raised the question of whether it was the high rate of viral replication or the repeated infection of cells by newly generated viruses that limited the ability of our shRNAs to provide sustained inhibition of SFV replication. To address this question, we used SFV1–GFP, an eGFP-expressing SFV vector lacking the structural genes (Fig. 1), so that although replication of viral RNA occurs at a level similar to that of replication-competent virus, new virions cannot be produced (Ehrengruber et al., 2003). Cells were again transfected with shRNA plasmids, allowed to express shRNAs for 53 hours, and then infected with this replication-deficient virus. Analysis of these cells by fluorescence microscopy showed a marked and sustained reduction in viral RNA replication at 112 hours after infection (Fig. 5A) for shRNAs targeting each of the nsP genes (1, 2, and 4), but not with the capsid gene shRNA (missing in this virus) or an empty vector (pU6, which produces no shRNA) (Fig. 5A). In a parallel experiment, reduction of viral RNA replication was measured quantitatively by flow cytometry. Those results showed that nsP shRNAs reduced viral RNA replication to varying degrees ($p < 0.0005$ for nsP1, nsP2, and nsP4 relative to control shRNAs targeting DsRed or the absent capsid gene; unpaired *t*-test), for up to 5 days (Fig. 5B). The shRNA targeting nsP1 was the most effective, with up to 77% reduction in virus by flow cytometry. The transfection efficiency achieved for BHK-21 cells with the transfection reagent used, Lipofectamine 2000, was 70% for a GFP expression vector as determined using flow cytometry. Adjusting the inhibition level for the proportion of cells transfected by the shRNA vector, inhibition of this replication-deficient SFV–GFP by the nsP1 shRNA must have been closer to 100%, even allowing for the lower expression of this pol II expression vector compared to the pol III-driven shRNA vectors.

An shRNA having a single-base mismatch with the target may retain some antiviral activity

The rapid, error-prone replication of SFV results in the emergence of many sequence variants. This process can rapidly lead to drug resistance. The SFV1–GFP strain differs from the SFV–VA7–GFP strain at the target site of nsP4a shRNA such that pairing to this shRNA results in a mismatch at position 14 in the 29-nt region of complementarity with the target (see Table 1). To control for the consequences of this mismatch, a second shRNA, nsP4b, targeting the same nsP4 site but having a perfect match to SFV1–GFP, was synthesized. As judged by loss of fluorescence in micrographs at 24 hours (Fig. 5A), both the matched nsP4b and the mismatched nsP4a shRNAs provide similar inhibition relative to the controls. When the cells were followed over time using flow cytometry, however, the mismatched shRNA was less efficient than the matched one, as expected (Fig. 5B).

Inhibitory effect of multiple coexpressed shRNAs on SFV–GFP replication

Theoretically, the use of multiple shRNAs targeting separate sites in the virus should help prevent the emergence of resistant mutants. Thus, we pooled plasmids encoding our four active shRNAs (nsP1, nsP2, nsP4a, and nsP4b) to see how well the mixture can inhibit SFV–GFP replication. With each plasmid present at one-fourth the concentration used for a single-vector transfection, significant inhibition was achieved up through the 99-hour time point ($p < 0.002$ relative to DsRed control) (Fig. 5). However, at 125 hours, the inhibitory effect was apparently lost (Fig. 5B).

DISCUSSION

Investigations of the ability of various gene-silencing technologies, including ribozymes and RNAi, to inhibit viral replication have shown that rapidly replicating and mutating viruses are challenging targets (Seyhan et al., 2002; Bagasra, 2005). In this study, we have shown by

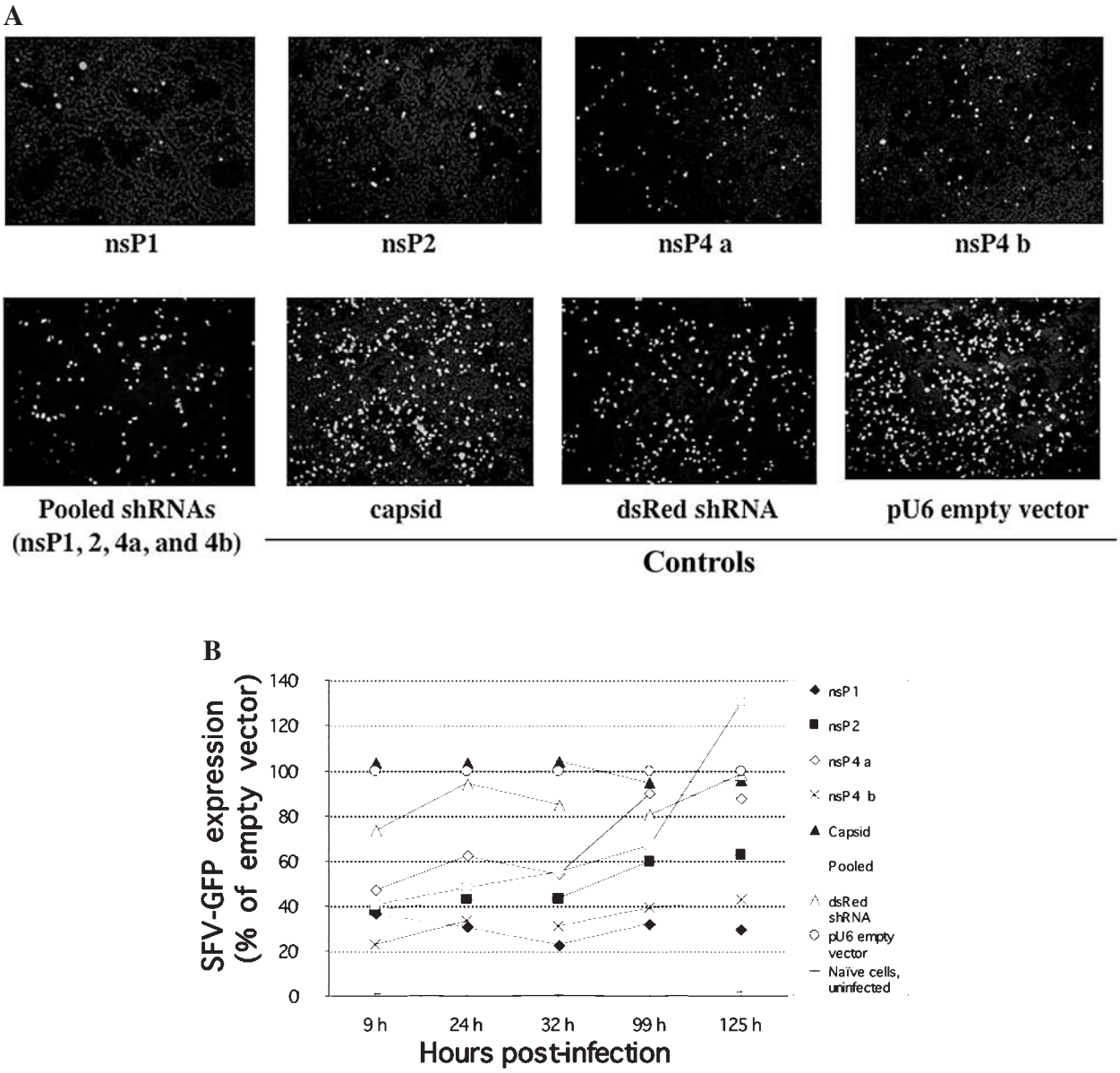

FIG. 5. (**A**) Inhibition of replication-deficient SFV–GFP by transiently expressed shRNAs. BHK-21 cells were transiently transfected with inhibitor plasmids. Fifty-three hours after transfection, cells were infected with replication-deficient SFV–GFP recombinant virus at an MOI of ~5. Cells were imaged by fluorescence microscopy 112 hours after infection. The cell fields show GFP fluorescence indicative of viral replication from BHK-21 cells that had been transfected with plasmids expressing anti-SFV shRNAs prior to virus infection. The applied shRNAs were nsP1, nsP2, nsP4a, nsP4b, and pooled hairpins (nsP1, nsP2, nsP4a, and nsP4b) as well as controls. No inhibition was seen with negative control shRNAs targeting capsid gene or DsRed gene (not present in SFV1–GFP), or with the empty vector. (**B**) Duration of inhibition of replication-deficient SFV–GFP by transiently expressed shRNAs. BHK-21 cells were transiently transfected with the inhibitor plasmids and cells were infected with SFV1–GFP recombinant virus at an MOI of ~5 at 46 hours posttransfection. Cells were analyzed by flow cytometry at 9, 24, 32, 99, and 125 hours after infection. Monolayers of cells were trypsinized, formalin fixed, and analyzed by single channel fluorescence in a flow cytometer. Cell viability vs. time is shown for untreated cells (naïve BHK-21, uninfected), cells transfected with empty vector (pU6; no shRNA insert), and cells transfected with shRNA plasmids (nsP1, nsP2, nsP4a, nsP4, and all four pooled) or nontargeting shRNA plasmids (capsid and DsRed) prior to virus infection. The amount of inhibition of each shRNA was normalized to pU6 empty vector. Because capsid mRNA is not present in this SFV virus, no inhibitory effect was seen by the capsid shRNA. Transfection efficiency for the shRNA expression constructs is estimated to be ~70%, suggesting that actual viral inhibition was considerably higher than the levels indicated. Data points represent averages of duplicate experiments with less than 20% variability.

both fluorescence microscopy and flow cytometry that vector-expressed shRNAs can be used to specifically inhibit RNA replication during infection with one such virus, SFV. Inhibition of RNA replication was sustained for up to 5 days after infection with replication-deficient virus, which was unable to form progeny virions that could initiate additional rounds of infection. Indeed, adjusting for the proportion of cells transfected by shRNA vector (≥70%), the ~30% of cells whose reporter gene was not inhibited may represent mostly nontransfected cells, and therefore the true efficiency of inhibition of transfected cells was probably close to 100%.

Previous work showed that stably expressed hairpin ribozymes that were obtained from a combinatorial library by *in vitro* selection could (over several days) successfully inhibit replication-competent Sindbis virus, which is an alphavirus similar to SFV (Seyhan et al., 2002). In the present study, inhibition of replication-competent virus by shRNAs was less successful, despite the fact that RNAi is generally considered to be a more potent and robust means of target knockdown than ribozyme cleavage. The viral RNA sites targeted by the ribozymes were different from (and shorter than) those of the shRNAs, and might have been more suitable for inhibition of viral replication, either because they were more accessible or because they were more highly conserved and did not permit the emergence of escape mutations. The latter hypothesis may also explain why the nsP1-targeting shRNA was the only shRNA that inhibited replication-competent SFV for up to 36 hours, whereas for the replication-deficient SFV, both nsP1- and nsP4-targeting shRNAs, were effective: the nsP4 site may be more amenable than the nsP1 site to generation of escape mutations with the replication-proficient virus.

In this study, cells were always pretreated with shRNA expression vectors prior to challenge with the virus. Treatment after infection (to mimic a therapeutic as opposed to prophylactic approach) was deemed not feasible because the viral infection tended to render the cells too weak for subsequent transfection with the shRNA vector. Even for the replication-deficient SFV, pretreatment is probably necessary to achieve sufficient time for the accumulation of critical cytoplasmic concentrations of active siRNA/protein complex. Production of plus- and minus-strand genomic RNAs begins during the first 4 hours of infection and peaks at 24 hours (Liljestrom and Garoff, 1991; Seyhan et al., 2002), resulting in a steady-state population of ~200,000 copies of the plus strand RNA species (Wengler, 1980) per cell. Massive replication of the virus leads to the recruitment of nearly all the host's ribosomes for synthesis of viral proteins (Strauss and Strauss, 1994), causing a cytopathology that results in apoptosis within 36 hours.

Protection of cells from infection by replication-competent virus is a still more rigorous challenge. Only the nsP1 shRNA caused a lag in viral replication, apparent at 24 hours as a reduced titer, but at 48 hours, viral titers caught up to the same levels that were seen in cells transfected with plasmids expressing nontargeting control shRNAs. ShRNAs expressed in the nucleus from a U6 promoter are thought to be transported into the cytoplasm by the Exportin 5 pathway used by miRNAs (Lund et al., 2004; Murchison and Hannon, 2004). However, U6-expressed shRNAs show mostly nuclear localization (Paul et al., 2002; Seyhan et al., 2002). If nuclear export is limiting, cytoplasmic concentrations of functional shRNAs may be insufficient to overcome an infection by replication-competent SFV. In addition, repeated infection might overcome RNAi-mediated protection of cells by generating escape mutants or by overwhelming RNAi defenses in other ways.

A number of mammalian viruses have been shown to express proteins that function as suppressors of RNA interference (Kok and Jin, 2006; Zheng et al., 2005). These viral proteins bind double-stranded RNA and are able to protect messenger RNA from degradation by sequestering siRNAs and preventing the formation of an active RISC. In the case of alphaviruses, the capsid protein contains regions that bind specifically to RNA (Strauss and Strauss, 1994). Because replication-competent SFV expresses the capsid, this protein might sequester siRNA, and therefore increase resistance to RNAi.

To help suppress the emergence of resistant mutants under pressure from shRNAs, SFV target sites were restricted to those having a high degree of strain-to-strain conservation. For adeno-associated virus, it has been demonstrated that mutants escaping inhibition by siRNAs through an A-to-U change in the middle of the siRNA target site were detected in less than 20 days (Boden et al., 2003). However, in the present study, silencing by the nsP4-a (mismatched) shRNA indicates that a single base modification in the middle of the 29-nt stem is not particularly disruptive for shRNA activity. Because the mismatched base is in the 14th nt after Dicer cleavage, the mismatched base would be further from the center of the resulting siRNA as well as further from the seed region. Apparently, a mismatch at this position is well tolerated.

An alternative strategy to combat rapidly mutating viruses is to target host factors (e.g., receptors) that are required for the virus but not essential for the host. It is generally hard for a virus to mutate in such a way that it loses the requirement for a host factor. For example, lentivirus-mediated delivery of shRNAs against the HIV-1 coreceptor CCR5 has been shown to provide marked protection from CCR5-tropic HIV-1 infection (Qin et al., 2003).

In summary, our results indicate that silencing of viral genes by expressed shRNAs can inhibit replication of

SFV, a rapidly replicating RNA virus, even when cells are infected at an MOI of ~5. However, when the virus was able to generate progeny virions, subsequent rounds of infection were apparently able to overwhelm the inhibition by RNAi. Considering that MOIs of about 0.01 are often used in testing inhibitors of slower growing viruses such as HIV in cultured cells (J. Rossi, personal communication; O'Brien, 2006), these results should not be regarded as overly discouraging. Although it is unclear whether RNAi will ultimately be useful as a stand-alone treatment for fast-growing viruses, there are many applications where a new antiviral of reasonable potency could be useful, including prophylaxis, combination therapy with other antiviral agents, treatment for needle-stick accidents, and treatment of chronic viral diseases such as hepatitis C (Morrissey et al., 2005b; Wang et al., 2005; Ilves et al., 2006).

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