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shRNAs Targeting Hepatitis C: Effects of Sequence and Structural Features, and Comparison with siRNA

ALEXANDER V. VLASSOV,[1,3,*] BRENT KORBA,[2] KRISTINE FARRAR,[2] SAMPA MUKERJEE,[2] ATTILA A. SEYHAN,[1,†] HEINI ILVES,[1] ROGER L. KASPAR,[1,‡] DEVIN LEAKE,[5] SERGEI A. KAZAKOV,[1] and BRIAN H. JOHNSTON[1,4]

ABSTRACT

Hepatitis C virus (HCV) is a leading cause of liver cirrhosis and hepatocellular carcinoma worldwide. Currently available treatment options are of limited efficacy, and there is an urgent need for development of alternative therapies. RNA interference (RNAi) is a natural mechanism by which small interfering RNA (siRNA) or short hairpin RNA (shRNA) can mediate degradation of a target RNA molecule in a sequence-specific manner. In this study, we screened *in vitro*-transcribed 25-bp shRNAs targeting the internal ribosome entry site (IRES) of HCV for the ability to inhibit IRES-driven gene expression in cultured cells. We identified a 44-nt region at the 3′-end of the IRES within which all shRNAs efficiently inhibited expression of an IRES-linked reporter gene. Subsequent scans within this region with 19-bp shRNAs identified even more potent molecules, providing effective inhibition at concentrations of 0.1 nM. Experiments varying features of the shRNA design showed that, for 25-bp shRNAs, neither the size of the loop (4–10 nt) nor the sequence or pairing status of the ends affects activity, whereas in the case of 19-bp shRNAs, larger loops and the presence of a 3′-UU overhang increase efficacy. A comparison of shRNAs and siRNAs targeting the same sequence revealed that shRNAs were of comparable or greater potency than the corresponding siRNAs. Anti-HCV activity was confirmed with HCV subgenomic replicons in a human hepatocyte line. The results indicate that shRNAs, which can be prepared by either transcription or chemical synthesis, may be effective agents for the control of HCV.

INTRODUCTION

HEPATITIS C afflicts 3.9 million people in the United States (175 million worldwide), and is the primary indication for liver transplants in the United States. Of patients initially infected by the hepatitis C virus (HCV), 70% develop chronic liver disease, including in some cases cirrhosis and hepatocellular carcinoma (Alter et al., 1999). Existing standards of care, using ribavirin and pegylated interferon alpha, are effective only in approximately 50% of patients with the most common genotype in the United States (genotype 1), and have substantial side effects. Currently, much effort is focused on the identification of small-molecule agents that inhibit specific steps in the life cycle of the virus. Many of these efforts target HCV-encoded enzymes, and others aim to modu-

[1]SomaGenics, Inc., Santa Cruz, CA 95060.
[2]Department of Microbiology and Immunology, Georgetown University Medical Center, Rockville, MD 20850.
[3]Institute of Chemical Biology and Fundamental Medicine, Novosibirsk, 630090, Russia.
[4]Department of Pediatrics, Stanford University School of Medicine, Stanford, CA 94305.
[5]Thermo Fisher Scientific, Lafayette, CO 80026.
*Current address: Ambion/Applied Biosystems, Austin, TX 78744.
†Current address: Open Biosystems, Huntsville, AL 35806.
‡Current address: TransDerm, Inc., Santa Cruz, CA 95060.

late the host immune response (DeFrancesco and Migliaccio, 2005; McHutchison and Patel, 2002; Sookoian, 2003). The development of more effective HCV treatments has been hampered by the lack of a good small animal model, the inability, until very recently, to stably culture the virus in tissue culture cells, and the high viral mutation rate (Radhakrishnan et al., 2004; Randall and Rice, 2004). However, the availability of an HCV subgenomic replicon system has allowed the study of HCV replication, host–cell interactions, and evaluation of antiviral agents in cultured cells (Hugle and Cerny, 2003; Pietschmann and Bartenschlager, 2003), and recently an HCV isolate has been finally identified that produces infectious virus in cell culture (Lindenbach et al., 2006).

In addition to small molecule searches, a substantial effort has been made to find effective nucleic acid-based inhibitors of HCV (Hugle and Cerny, 2003; Martinand-Mari et al., 2003), including antisense oligonucleotides, ribozymes, and more recently small interfering RNAs (siRNAs) and small hairpin RNAs (shRNAs). siRNAs and shRNAs act through the evolutionarily conserved RNA interference (RNAi) mechanism to cause degradation of mRNAs having complementary sequences (Dorsett and Tuschl, 2004; Hannon and Rossi, 2004). Short (19 bp) siRNAs are directly incorporated into the multicomponent RNA-induced silencing complex (RISC), which then releases the sense strand and uses the antisense strand as a guide to seek out and degrade homologous mRNAs. shRNAs need first to be processed in order to cleave the hairpin loop (Paddison and Hannon, 2003). Chemically synthesized siRNAs have shown robust inhibition of target genes in mammalian cells and animals, although in the latter case siRNAs are usually modified for improved pharmokinetic properties (Dykxhoorn and Lieberman, 2005). Small hairpin RNAs (shRNA) display similar potency, and can easily be expressed from bacteriophage (e.g., T7, T3, or SP6) or mammalian pol III promoters (e.g., U6 or H1), making them excellent candidates for viral delivery (Hannon and Rossi, 2004).

A number of research groups have shown that si/shRNAs can effectively target HCV in human tissue culture cells (Kapadia et al., 2003; Korf et al., 2005; Kronke et al., 2004; Randall et al., 2003; Sen et al., 2003; Seo et al., 2003; Wilson et al., 2003; Yokota et al., 2003; Zhang et al., 2004). The only experiments performed to date in mice used HCV IRES-dependent reporter systems, and si/shRNAs were delivered by high-pressure injection (McCaffrey et al., 2002). However, for hepatitis B virus (HBV), for which an animal model does exist, such experiments were successfully performed; stabilized siRNA, incorporated into specialized liposomes and administered by intravenous injection into mice carrying replicating HBV, significantly reduced serum HBV DNA (Morrissey et al., 2005). These and other promising examples of RNAi tests in rodents (reviewed in Behlke, 2006; Dallas and Vlassov, 2006) provide confidence that siRNA drugs are feasible. Finding siRNAs that are active at low doses (to minimize toxicity and off-target effects) and solving delivery problems are the main challenges in the development of nucleic acid-based therapeutics (Dykxhoorn and Lieberman, 2005).

In the first report of the effects of direct delivery of shRNA on target gene inhibition in animals (Wang et al., 2005), we showed that 25-bp shRNAs targeting the HCV internal ribosome entry site (IRES) caused potent inhibition of HCV IRES-dependent reporter expression in cultured human cells, and in mouse liver as analyzed by *in vivo* bioluminescent imaging. Recently another group also reported successful use of directly delivered shRNA (Hamazaki et al., 2006). Both of these studies used *in vitro* transcription by T7 RNA polymerase to generate the shRNAs. In the present report, we describe an extensive search for shRNAs capable of inhibiting HCV IRES-dependent gene expression at very low concentrations (~0.1 nM). We examined the effects of varying shRNA features such as target site, stem length, loop size, and the presence or absence or terminal single-stranded overhangs, and compared shRNAs side by side with corresponding siRNAs. The potency of selected molecules was confirmed using HCV replicons in Huh7 cells. Our results boost the prospects for the eventual use of chemically synthesized or transcribed shRNAs for the control of HCV.

MATERIALS AND METHODS

Preparation of shRNAs

shRNAs were generated by *in vitro* transcription of DNA templates having a T7 promoter sequence. Each shRNA was transcribed at 37°C for 1 hour from 5 μM of the annealed dsDNA template using the AmpliScribe T7 Flash transcription kit (Epicentre Technologies, Madison, WI), followed by double purification on a G50 gel filtration spin column (Microspin G-50, Amersham Biosciences, Arlington Heights, IL). DNA oligonucleotides were purchased from IDT (Coralville, IA). Chemically synthesized shRNAs were prepared by Thermo Fisher Scientific, Dharmacon Products (Lafayette, CO).

Transfections and reporter gene assays

The human cell lines 293FT (Invitrogen, Carlsbad, CA) and Huh7 (kindly provided by Andrew Simmons, Cell Genesys) were maintained in DMEM (Cambrex, Walkersville, IN) with 10% fetal bovine serum (HyClone, Logan, UT), supplemented with 2 mM L-glutamine and 1 mM sodium pyruvate. One day prior to transfection, cells were seeded at 1.7×10^5 cells/well in a

48-well plate, resulting in ~80% cell confluency at the time of transfection. Transfections were performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. For the inhibition experiments, 293FT or Huh7 cells were cotransfected (in triplicate) with 40 ng of pHCV, an IRES-linked firefly luciferase reporter construct (Wang et al., 2005; courtesy of P. Sarnow, Stanford University), 25 ng pSEAP2-control plasmid (BD Biosciences Clontech, San Jose, CA) as a transfection and specificity control, and the indicated amounts of T7-generated shRNA (typically 0.5 pmol). pHCV is a dual luciferase expression plasmid in which the HCV IRES is placed between the coding sequences for Renilla and firefly luciferase (fLuc), so that fLuc is dependent on the IRES. We used pSEAP2, which expresses secreted alkaline phosphatase (SEAP), instead of Renilla luciferase expression as a control because shRNA-mediated cleavage in the IRES sequence destabilizes the whole message and Renilla luciferase expression is lost along with that of fLuc. Compensatory pUC18 plasmid was added to the transfection mix to give a total of 400 ng nucleic acid per transfection. Forty-eight hours later, the supernatant was removed and heated at 65°C for 30 minutes. Ten microliters of this supernatant were added to 150 μL *p*-nitrophenyl phosphate liquid substrate system (pNPP, Sigma, St. Louis, MO) to measure SEAP levels. After a 30-minute incubation at room temperature, absorption at 405 nm was quantified using a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA) and SOFTmax software (Molecular Devices). The cells were lysed and luciferase activity measured using the Dual-Luciferase Reporter assay system (Promega, Madison, WI) and a MicroLumat LB 96 P luminometer (Berthold Technologies, Bad Wildbad, Germany).

Assay of shRNA in an HCV replicon system

The antiviral activity of shRNAs was assayed in the stably HCV RNA-replicating cell line AVA5 (derived by transfection of the human hepatoblastoma cell line Huh7.5 with a genotype 1b subgenomic replicon using a previously described assay (Okuse et al., 2005). AVA5 cells maintained on 96-well plates were transfected with HCV shRNA inhibitors using Lipofectamine 2000 (Invitrogen) following the manufacturer's procedure with the following exceptions: cultures were incubated in serum-free medium for 2 hours prior to transfection, and the transfection was performed overnight in complete medium (Blight et al., 2000) without G418. Intracellular HCV RNA levels and cytotoxicity were assessed 72 hours after inhibitor administration. Triplicate cultures were used for the assessment of intracellular HCV RNA (by blot hybridization) and cytotoxicity (by neutral red dye uptake). Untreated cultures, and cultures treated with recombinant human interferon alpha 2b (PBL Interferon Source, Piscataway, NJ), ribavirin (Sigma, St. Louis, MO), or 2′-O-methyl cytidine served as assay controls. Levels of β-actin mRNA present in each culture sample were used to normalize HCV RNA levels.

RESULTS

Identification of shRNAs that inhibit HCV IRES-dependent gene expression in cultured cells

We previously characterized an *in vitro*-transcribed shRNA (designated as sh1 here and as HCVwt in Wang et al., 2005) that targets the 3′ end of the HCV IRES, near the AUG translation start site. A reporter plasmid in which fLuc expression is dependent on the HCV IRES (genotype 1a; GenBank accession #AJ242654) was used to assess the ability of cotransfected shRNA to interfere with the function of the IRES (Wang et al., 2005). Directly delivered sh1 was shown to cause potent inhibition of HCV IRES-dependent reporter expression both in human cell lines and in mouse liver, the latter as analyzed by *in vivo* bioluminescent imaging (Wang et al., 2005).

The goal of the present study was to identify shRNAs having the highest potency by locating the best target site on the IRES and optimizing the shRNA design. Toward this end, we first designed and tested 26 shRNAs targeting various regions of the HCV IRES (Table 1). The target sites were chosen so as to avoid regions that vary among different HCV genotypes. Some were picked by using siRNA selection algorithms such as that provided at http://jura.wi.mit.edu/bioc/siRNAext/. Because the available algorithms are known to miss some effective target sites, we also tested sites that most algorithms would rule out, such as GC-rich or highly structured regions. The shRNAs were generated by *in vitro* transcription using T7 RNA polymerase with dsDNA templates. To maximize transcription efficiency, templates were designed so that the transcripts began with the sequence 5′-pppGGG. All shRNAs in this group had duplex stem lengths of 21–25 bp and a 10-nt loop derived from microRNA-23, as in the original sh1.

These molecules (27 in all, including the original shRNA sh1) were assayed for activity as previously described (Wang et al., 2005). Briefly, human 293FT cells were cotransfected with pHCV fLuc expression plasmid, a secreted alkaline phosphatase expression plasmid (pSEAP2, to control for efficiency of transfection or off-target effects), and the shRNA to be tested. The results are shown in Table 1. SEAP levels were uniform within experimental error for all samples at 1 nM, but were somewhat reduced for some shRNAs at 5 nM, suggesting that transfection efficiency was relatively consistent and that there were some nonspecific effects at that higher concentration. As can be seen, most of the shRNAs displayed

Table 1. Results of a Screen of 24–25-bp shRNAs for the Ability to Inhibit HCV IRES-Mediated Gene Expression in 293FT Cells

shRNA inhibitor #	*HCV IRES target site, nt*	*fLuc expression (%) at 1 nM shRNA*	*fLuc expression (%) at 1 nM shRNA (C340 → U mutated target)*	*fLuc expression (%) at 5 nM shRNA*
hcv3	29–51	74 ± 8		50 ± 6
sh23	35–59	52 ± 5		39 ± 4
sh24	52–75	44 ± 6		28 ± 3
hcv45	65–86	82 ± 12		62 ± 7
hcv40	73–94	72 ± 9		69 ± 8
sh20	75–99	63 ± 6	65 ± 6	42 ± 3
sh25	82–106	64 ± 8		39 ± 4
sh19	135–159	50 ± 6	52 ± 12	27 ± 4
sh26	152–176	66 ± 11		50 ± 5
hcv44	196–217	99 ± 10		75 ± 8
hcv41	204–224	76 ± 9		51 ± 6
sh27	224–248	75 ± 12		70 ± 8
sh28	253–277	40 ± 8		36 ± 5
sh29	278–302	62 ± 6		21 ± 3
hcv7	288–309	69 ± 8		47 ± 5
sh9	299–323	74 ± 9	72 ± 12	43 ± 5
sh10	318–342	36 ± 4	32 ± 5	17 ± 3
sh17	320–342	41 ± 5	43 ± 8	21 ± 5
hcv30	322–342	59 ± 6	57 ± 7	35 ± 4
sh18	323–346	62 ± 8	65 ± 13	31 ± 8
hcv22	326–346	50 ± 6	76 ± 9	55 ± 7
sh38	328–352	54 ± 4	69 ± 6	12 ± 2
sh39	331–355	13 ± 1	24 ± 3	7 ± 1
sh37	335–359	15 ± 1	16 ± 2	5 ± 1
hcv17	337–359	16 ± 2	24 ± 3	17 ± 2
sh1	344–368	8 ± 1	10 ± 1	4 ± 1
sh11	350–374	10 ± 1	10 ± 1	5 ± 1

only moderate activity (less than 50% inhibition at 1 nM), presumably because the targeted areas on the IRES were highly structured. The only exceptions were shRNAs sh39, sh37, hcv17, and sh11, targeting positions near the sh1 site. These shRNAs caused 85–90% inhibition of IRES-dependent gene expression at 1 nM concentration. Although as expected shRNA activity increases with concentration (Table 1), screening was performed at low concentrations to identify highly active shRNAs and minimize nonspecific effects, which in our experience frequently manifest at concentrations above 10 nM (Wang et al., 2005). Northern blot analysis performed for selected shRNAs confirmed that the target mRNA was being degraded (not shown). This survey revealed a 44-nt region (positions 331–374 on the HCV IRES) within which five overlapping shRNAs displayed high activity; of these five, only one was predicted by algorithms. These data confirm and significantly extend the findings of other studies of the successful targeting of the HCV IRES by shRNAs and siRNAs (Kronke et al., 2004; Randall et al., 2003; Seo et al., 2003; Yokota et al., 2003).

shRNA activity in cells is not significantly affected by point mutations in the target

To test the sensitivity of shRNA to point mutations in the target, a C→U mutation was introduced at position 340 of the HCV IRES. Of the 27 shRNAs initially tested, 9 contained this position within their target sites: sh10, sh17, hcv30, sh18, hcv22, sh38, sh39, sh37, hcv17 (Table 1), and their activities could theoretically be affected by this mutation. These nine shRNAs were assayed with the mutated version of pHCV luciferase construct, along with selected shRNAs targeting other sites as controls. For all nine shRNAs, activity was found to be either unaffected (where the mismatch falls near the pe-

riphery of the target site) or moderately reduced (for mismatches near the center of the target site) in comparison to the original, perfectly matched target (Table 1). These findings are consistent with our previous finding that introduction of mismatches at either of two sites in sh1 also did not significantly affect ability to knock down expression from the wild-type IRES (Wang et al., 2005). Other groups also reported that sh/siRNAs tolerate single mismatches and especially G/U wobble base pairs (Doench et al., 2003; Holen et al., 2005). These results suggest that, if single-point mutations were introduced by the viral polymerase, even in the highly conserved IRES, the virus is likely to remain vulnerable to the shRNAs.

Sequence walking with short shRNAs and siRNAs within the favored site

We next asked whether the HCV-targeting shRNAs could be shortened and still retain their potency. Shorter molecules would presumably be less costly to manufacture, cause fewer off-target effects, and be less likely to induce an interferon response or activate protein kinase R (although, theoretically, in the case of HCV therapy an interferon response might be beneficial, as discussed below). Six shorter shRNAs (sh50-55), having 19-bp stems, 10-nt loops, and 5′-GG and 3′-UU overhangs, were designed to target sequences within the 44-nt region that was found to be "accessible" based on initial screening. Three shRNAs were directed to targets within the region 331–353 and three to within 354–374, in an attempt to find a pair of nonoverlapping shRNA candidates that could be used together to suppress the emergence of viral resistance (Fig. 1A). Three of the six shRNAs (those targeting closer to the middle of the 44-nt region) produced significant inhibition of HCV IRES-dependent gene expression in 293 FT cells (Fig. 1B), and one of the three had similar potency to the 25 bp sh1 encompassing the same target site.

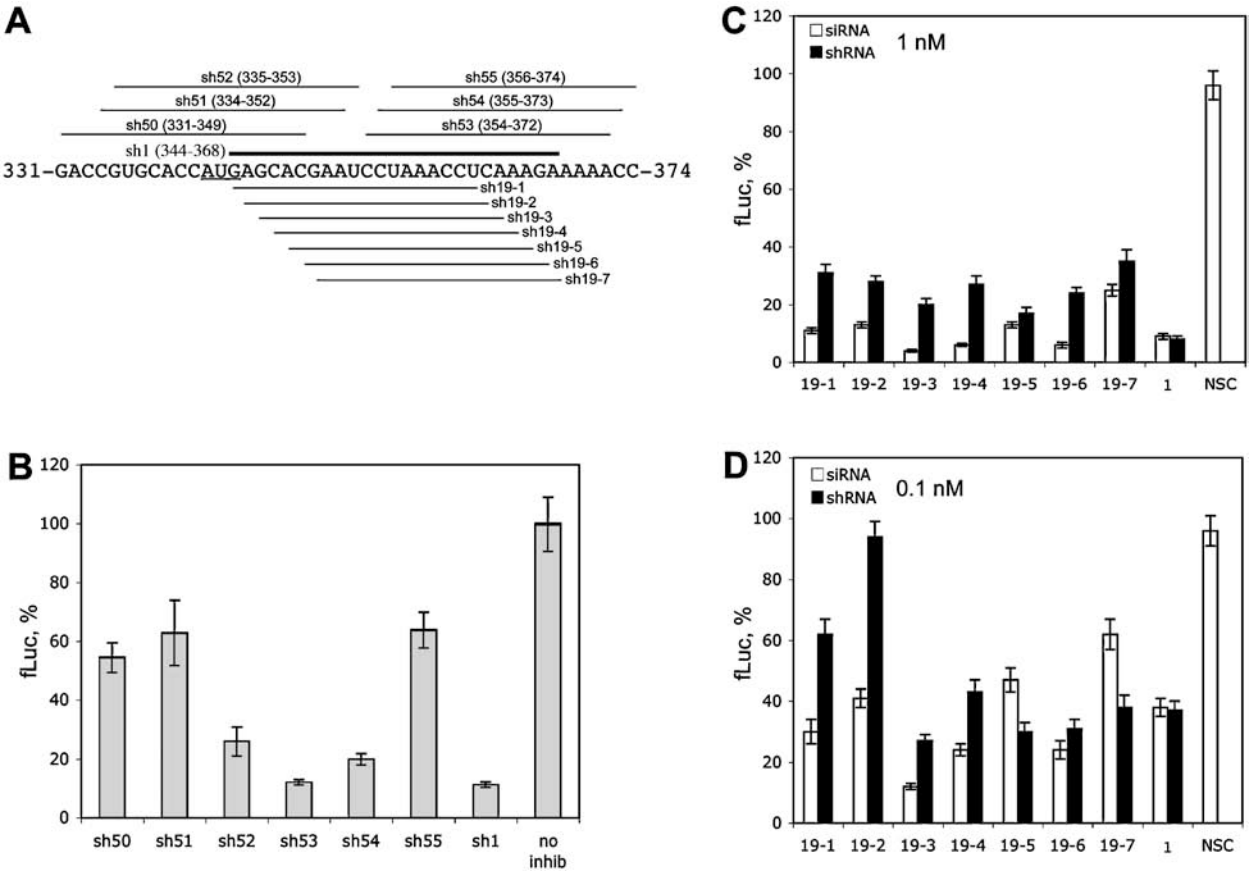

FIG. 1. (**A**) Target sites on the HCV IRES for 19–bp shRNAs tested. (**B**) The results of a test of six shRNAs for the ability to inhibit HCV IRES-mediated gene expression in 293FT cells; shRNA concentration, 1 nM. (**C,D**) Comparison of activity of siRNAs and shRNAs targeting all seven 19–nt subsites within the 25-nt target site for shRNA sh1; sh/siRNA concentration, 1 nM (**C**) and 0.1 nM (**D**). fLuc values are normalized to either no inhibitor (**B**) or a nonspecific control si- or shRNA (NSC) (**C,D**). Raw fLuc values were typically 500,000–700,000 light units for the controls. Error bars indicate standard deviation for averaged results from two to four experiments, each performed in triplicate. Because the errors in SEAP measurements were larger than those in fLuc measurements, the latter were not normalized to SEAP to avoid amplifying fLuc errors. SEAP levels were consistent within replicates and between samples at 0.1 nM concentrations of shRNAs. The same was true at 1 nM except for sh4, where SEAP levels dropped by about 50%, suggesting some nonspecific effects in that case.

To allow a comparison of shRNAs and siRNAs in our search for the most potent inhibitors, for selected in vitro-transcribed shRNAs we also tested the corresponding chemically synthesized siRNAs. First, the "parental" 25-bp T7-transcribed shRNA sh1 and a 25-bp synthetic siRNA targeting the same site were compared for their ability to inhibit HCV IRES-mediated reporter expression. As shown in Figure 1C–D, the siRNA and shRNA produced 91% and 92% inhibition, respectively, at 1 nM concentration, 62% and 63% inhibition at 0.1 nM, and no significant inhibition at 0.01 nM (not shown).

Next, seven synthetic, unmodified 19-bp siRNAs targeting every possible 19-nt sequence within the validated 25-nt site of sh1 (344–368 nt, Fig. 1A) were tested at concentrations ranging from 0.01 to 1 nM (Fig. 1C–D). These siRNAs varied in their potency, which was both less and greater than that of the parental 25-bp shRNA and siRNA. For example, inhibition at 1 nM ranged from 75% for siRNA19-7 to 96% for siRNA19-3. This nucleotide-by-nucleotide position dependence has also been observed for "ordinary" antisense inhibitors, although the best target sites for this class of inhibitors may be different than for RNAi-based inhibitors due to the different mechanisms of action. The most potent molecule, siRNA19-3, showed 88% inhibition at 0.1 nM concentration and 42% at 0.01 nM (data not shown), with siRNAs19-4 and 19-6 also showing some inhibition (29% and 15%, respectively) at 0.01 nM. Thus, 19-bp siRNA sequence walking can reveal even more potent inhibitors than the 25-bp siRNA within its targeting site.

The corresponding *in vitro*-transcribed 19-bp shRNAs also showed significant activity in 293 FT cells, ranging from 65% to 83% at 1 nM concentration (Fig. 1C–D). As with the siRNAs, most shRNA constructs tested were also effective at 0.1 nM, with shRNA19-3 showing the greatest inhibition at 73%. Although the shRNAs were generally somewhat less potent than the siRNAs in this system, some 19-bp shRNAs were more potent than the "parental" 25 bp shRNA at 0.1 nM.

There was a chance that the *in vitro*-transcribed shRNAs contained products of premature termination (Hartvig and Christiansen, 1996) that might have decreased activity, since they were purified only by G-50 spin columns to remove unincorporated nucleoside triphosphates. Analysis of these 19-bp shRNAs by polyacrylamide gel showed purity comparable to that of the synthetic, HPLC-purified siRNAs (Fig. 2), although in the case of the 25-bp shRNAs additional products (longer and/or shorter, comprising up to ~10%) were observed for certain constructs along with the major product of transcription (not shown). When an *in vitro*-transcribed shRNA was compared side by side with a chemically synthesized version of the same shRNA, no difference in potency was observed. We concluded that differences in purity between the siRNAs and shRNAs tested were probably not significant.

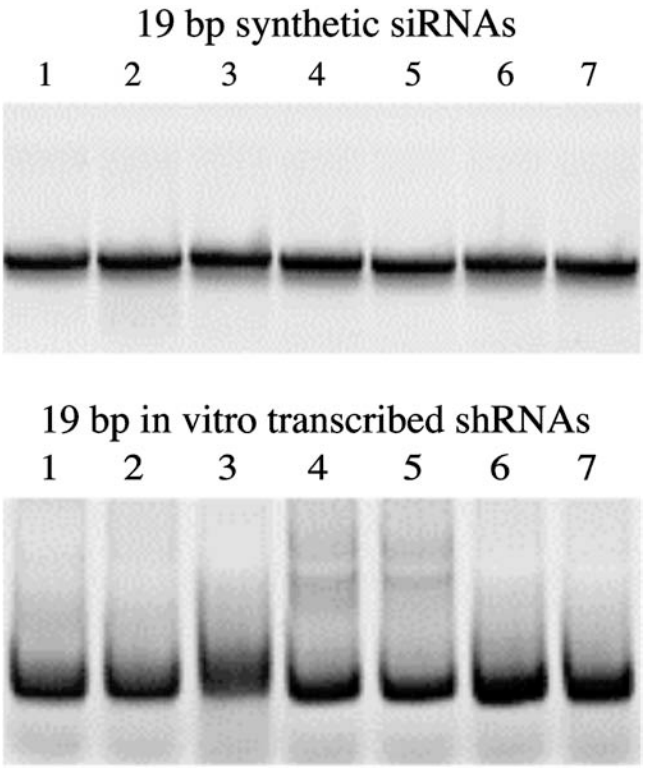

FIG. 2. Analysis of shRNA and siRNA purity by nondenaturing 10% PAGE stained with ethidium bromide. *Top*: Synthetic siRNA (HPLC purified). *Bottom*: *In vitro* transcribed shRNA (purified by G50 spin columns to remove unincorporated NTPs). 60 pmol RNA was loaded per lane.

In view of the fact that shRNAs require an additional processing step compared to 19-bp siRNAs (Dorsett and Tuschl, 2004), we considered the possibility that for siRNA the peak of activity may be earlier than for shRNA, in which case the two should not be compared at the same time point. The same results were obtained whether fLuc levels were analyzed 60, 48, or 24 hours posttransfection (data not shown); earlier time points could not be examined due to the low levels of fLuc expressed. However, since the luciferase reporter plasmid is introduced together with the synthetic si/shRNAs, and expression of the target mRNA encoded by the plasmid may take at least as much time as the assembly of a RISC complex from synthetic RNAs, it is not clear whether shRNA processing can be monitored in the context of this assay.

Although 19-bp shRNAs performed somewhat more poorly than siRNAs in 293 FT cells, in the HCV replicon system (see below) and in a mouse model (unpublished), all shRNAs were more potent than the corresponding siRNAs. Most importantly, within the validated site on

the IRES, we have found highly potent siRNAs as well as shRNAs, active at concentrations of 1, 0.1, and even 0.01 nM.

shRNA design: effects of stem length, loop length and sequence, and terminal overhangs

We investigated in more detail how shRNA design affects gene-silencing activity. The original sh1 contained a 25-bp stem with 5′-GG and 3′-UU overhangs (which might form GU base pairs) and a 10-nt miR-23 loop. Each of these features was varied, one at a time (Fig. 3). The microRNA-23 loop sequence was chosen originally because it is found naturally (Lagos-Quintana et al., 2001), and was therefore not expected to be toxic. Two alternative 10-nt loops were tested, along with loops of 6, 5, and 4 nt, each in two sequence versions. Neither loop size nor sequence was found to affect the activity of the 25-bp shRNA. Also, shRNAs of this length lacking the 3′-UU sequence (sh35) had the same efficacy as the corresponding shRNA with this feature. Control shRNA with full-length (25 nt) sense but short (13 nt) antisense regions (sh2) had no activity, as expected. shRNAs having a 3′-CC instead of 3′-UU terminus (allowing the formation of 2 additional Watson-Crick base pairs) produced more potent inhibition of fLuc, but also significantly reduced SEAP levels (not shown). This nonspecific inhibition may have been a consequence of the longer stem (27 bp), which can induce genes of the interferon responsive pathway and activate protein kinase R (PKR). Moving the loop to the other end of the shRNA (sh36) reduced the inhibitory activity from 90% to 15% at 1 nM. Dicer is expected to cleave off the loop about 20 bp from the free end. The resulting siRNA then enters RISC, whereupon one strand of the duplex is released and the remaining strand guides subsequent target cleavage. Depending on whether the loop is at one end or the other, either of two different siRNAs will be produced, with their target sequence shifted by 6 nt relative to each other. These siRNAs might have different inhibitory properties due to their different GC-content (and, therefore, different helical stability) near their termini. Interestingly, use of the antisense strand of the siRNA alone (produced by T7 transcription) also showed

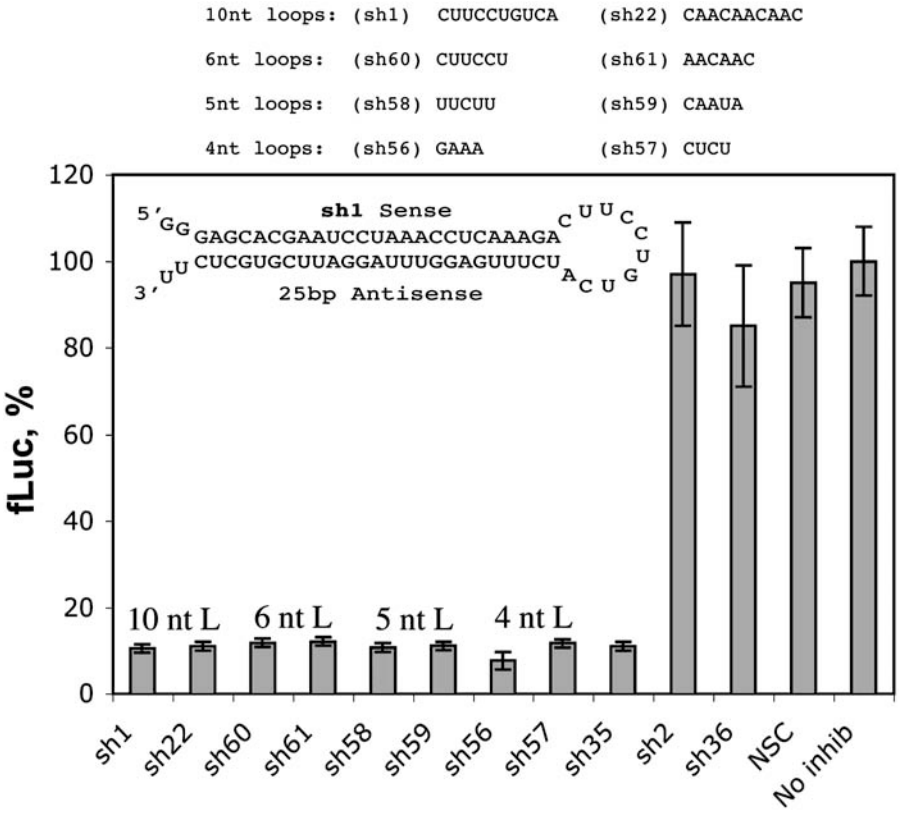

FIG. 3. shRNAs (25 bp): effects of terminal overhangs and loop size and sequence. (*Top*) The variants of the 25 bp shRNA tested (based on sh1), bearing loops of various sizes and sequences. (*Bottom*) The results of a screen of 25-bp shRNAs for the ability to inhibit HCV IRES-mediated gene expression in 293FT cells; shRNA concentration, 1 nM. sh35: sh1 without 3′-UU; sh2: sh1 with its antisense strand shortened to 13 nt; sh36: sh1 with the loop moved to the other end of the duplex. L, loop; members of each pair differ in loop sequence.

significant inhibition: 57% at 1 nM (not shown). The sense strand had no inhibitory effect, as expected. Similar findings have been reported previously (Martinez et al., 2002; Sorensen et al., 2003).

Because the 19-bp shRNAs displayed potency similar to 25-bp shRNAs and have certain advantages as drug candidates, we also examined the effects of loop variations for 19 bp shRNAs (Fig. 4). Again, loop sizes of 10, 6, 5, and 4 nt were tested, each in two sequence versions. In contrast to what was observed with 25-bp shRNAs, reduction of loop size, especially below 5 nt, resulted in reduced activity for the 19-bp shRNAs for both loop sequences tested. Loops of at least 5–6 nt were required for high activity. This conclusion was confirmed with sh19-1, sh19-5, sh52, sh53, and sh54 (not shown), in addition to the sh19-3 derivatives shown in Figure 4. Removal of the 3′-UU overhang (sh41) also resulted in significant reduction of activity. This overhang effect was seen with 19-bp as well as 20-bp, but not 25 bp, shRNAs. On the other hand, removal of one or both G at the 5′-end (sh68) did not affect the activity. Thus, for 25 bp shRNAs, neither the size of the loop nor the presence of a 3′-UU affect activity, whereas these parameters are important determinants of potency for 19-bp shRNAs.

Recent reports have shown that siRNAs synthesized using a phage RNA polymerase system can trigger potent induction of interferons (IFN) alpha and beta in a variety of cell lines due to the presence of 5′-triphosphates on such transcripts (Hornung et al., 2005; Hornung et al., 2006; Kim et al., 2004; Pichlmair et al., 2006). In the present study, no IFN-beta was induced in 293FT cells at 24, 48, or 96 hours following transfection with sh1 (1 or 3 nM; data not shown) as determined by a human IFN-beta ELISA (R&D Systems, Minneapolis, MN). Also, no induction of 2′-5′ oligoadenylate synthetase levels (OAS) in 293FT cells was seen 48 hours following shRNA administration (data not shown) as determined by a PCR-based protocol using published OAS primer sequences (Fish and Kruithof, 2004). *In vitro*-transcribed sh1 was tested along with dephosphorylated, *in vitro*-transcribed sh1 and a synthetic sh1, and no difference was observed either in the efficacy of fLuc inhibition or the effect on SEAP levels. Also, no obvious cytotoxicity was seen. These results are in accord with a recent study (Hamazaki et al., 2006) in which T7-transcribed shRNAs with 5′-ppp were found not to activate PKR, OAS, or interferon-regulatory factor-3 in cells. While toxic effects might appear at high concentration, at shRNA concentra-

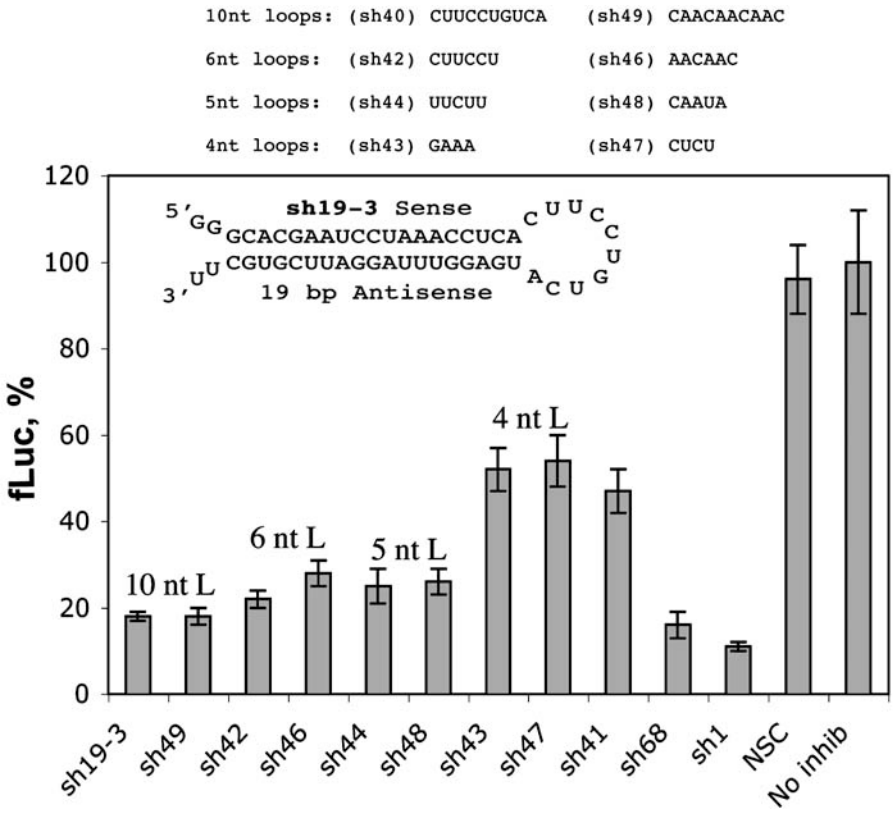

FIG. 4. shRNAs (19 bp): effects of terminal overhangs and loop size and sequence. (*Top*) The variants of the 19 bp shRNA tested (based on sh19-3), bearing loops of various sizes and sequences. (*Bottom*) The results of a screen of 19-bp shRNAs for the ability to inhibit HCV IRES-mediated gene expression in 293FT cells; shRNA concentration, 1 nM. sh41: sh19-3 without 3′-UU; sh68: sh19-3 without 5′-GG. L, loop; members of each pair differ in loop sequence.

tions of 1 nM there appears to be no need to remove the 5′-ppp of T7 transcripts used in cultured cells.

Assay of shRNA in the HCV Replicon system

A number of shRNA and siRNA inhibitors along with negative controls were transfected into the AVA5 cell line which stably expresses an HCV subgenomic genotype 1b replicon (Blight et al., 2000), and the levels of HCV replication were determined by quantitative blot hybridization (Okuse et al., 2005). EC_{50}s from two independent experiments (with each sample assayed in triplicate) are shown side by side in Figure 5. All shRNAs found to be efficacious with the fLuc/IRES system in 293 FT cells showed high potency in the replicon system as well. In addition, the following differences from the reporter system were observed: 19-bp shRNAs were somewhat more potent than 19-bp siRNAs, and 25 bp siRNAs and 25 bp shRNAs were less potent than any of the 19-bp shRNAs or siRNAs tested. No obvious cytotoxicity was observed for any of the shRNA or siRNA samples up to 25 nM (data not shown).

DISCUSSION

The HCV IRES is highly conserved, making it a prime target for nucleic acid-based inhibitors despite a level of secondary and tertiary structure that makes antisense approaches problematic (Kretschmer-Kazemi Far and Sczakiel, 2003; Overhoff et al., 2005). Several groups recently reported some success targeting the HCV IRES in 293FT and Huh7 cell lines (Kapadia et al., 2003; Korf et al., 2005; Kronke et al., 2004; Randall et al., 2003; Sen et al., 2003; Seo et al., 2003; Wilson et al., 2003; Yokota et al., 2003; Zhang et al., 2004). We earlier demonstrated that a T7 RNA polymerase-transcribed shRNA directed against the 3′ end of the HCV IRES, including and downstream of the AUG translation start site, induces ~86% and ~90% knockdown of HCV IRES-dependent luciferase expression at 1 nM concentration in 293FT cells and Huh7 cells, respectively (Wang et al., 2005). Furthermore, direct delivery of shRNA to mouse liver was shown to potently and reproducibly inhibit (by up to 99%) HCV IRES-dependent reporter expression (Wang et al., 2005). This was the first demonstration of RNAi-mediated gene inhibition in an animal model by *in vitro* transcribed RNA hairpins, as opposed to the more common approaches of delivering synthetic siRNAs, or vectors expressing shRNA *in vivo*.

In the current studies, systematic screening of shRNA constructs identified a 44-nt site on HCV IRES that is "accessible" to inhibition by RNAi. Within this site (nt 331–374), we found 19–25 bp shRNAs and siRNAs having subnanomolar EC_{50}s as assayed with HCV replicons

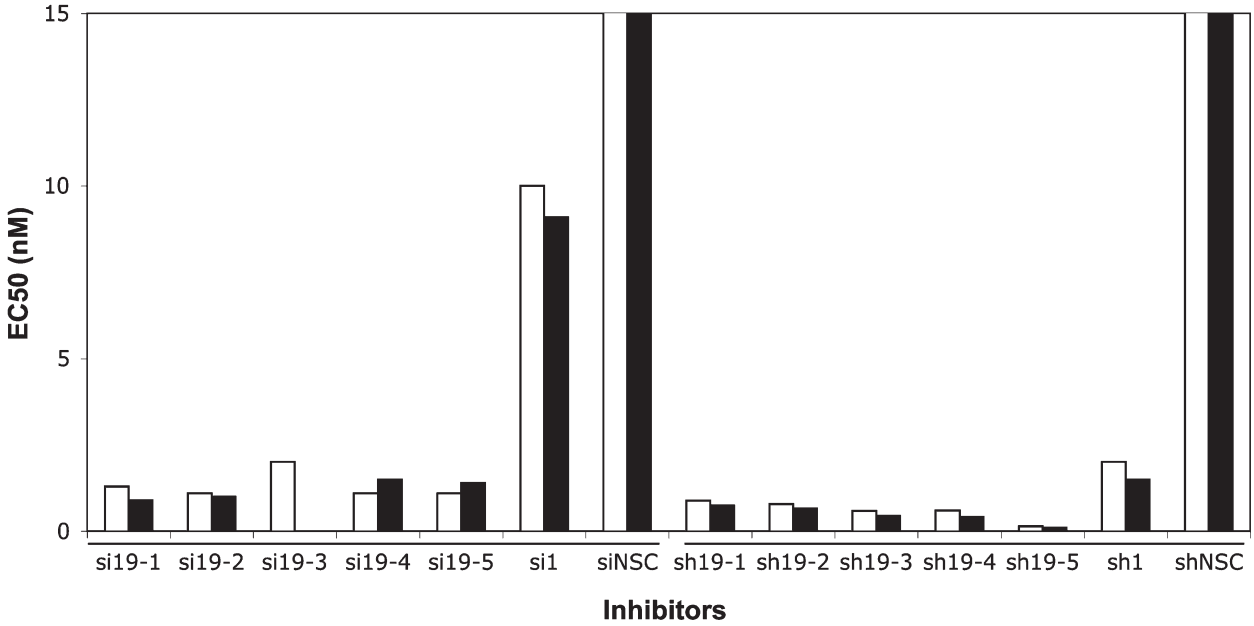

FIG. 5. Inhibition of HCV Replicons by shRNAs and siRNAs. shRNA and corresponding siRNA inhibitors along with negative controls targeting the HCV IRES were assayed in an HCV replicon system (Blight et al., 2000; Okuse et al., 2005). A range of concentrations was tested and the concentration of RNA that resulted in 50% reduction (EC_{50}) of viral RNA was determined. EC_{50}s from two independent experiments are shown in the side-by-side (black and white) bars. The siRNA19-n and shRNA19-n series refer to siRNAs and shRNAs each having 19-bp double-stranded regions and targeting sites designated by $n = 1, 2, 3, 4$, and 5 (see Fig. 1A for sequences). siRNA si1 and shRNA sh1 both target the same 25-bp site and have 25-bp stems. Si/shNSC, nonspecific controls.

and reporter systems. The identification of highly potent sequences potentially enables their usage as drugs at low concentration, reducing the cost of synthesis and potential off-target effects. It should be noted that in the replicon system, the HCV IRES only drives the G-418 resistance gene, and the viral genes are translated via the EMCV IRES. Hence, an sh/siRNA targeting the HCV IRES will only inhibit the replicon if it results in RNA cleavage; translation inhibition will work only in the presence of G-418 (which is normally used only for resistance selection studies). Thus, shRNAs may work better on the actual virus than the replicon, where any microRNA-like translation inhibition activity will not be evident. This might explain why in most cases we observed a higher EC_{50} for the replicon than for the reporter studies.

Certain synthetic shRNAs were previously reported to be more potent than corresponding siRNAs (Harborth et al., 2003; Siolas et al., 2005). Our present and earlier (Wang et al., 2005) data also indicate that in the replicon and mouse models, unmodified shRNAs provide a somewhat higher level of inhibition than corresponding siRNAs (although in the cell culture reporter system this was reversed). As to the optimal length of the molecule, a number of 19-bp si/shRNAs were shown to be superior to a parental 25-bp si/shRNA targeting the same site. (However, it should be noted that on average, longer shRNA molecules are more potent.) Advantages of the shorter 19-bp shRNAs over 25-bp molecules include lower cost of RNA synthesis as well as presumably fewer off-target effects and less induction of interferon response genes.

Typically, shRNAs with a stem length of 19–29 bp and a loop of 4–10 nt have been used in laboratory studies. Our studies on the design of shRNAs have shown that for 25-bp shRNAs, neither the size and sequence of the loop nor the presence of unpaired ends affects activity, in agreement with other reports (Harborth et al., 2003; Paddison and Hannon, 2003). In contrast, we have found that for 19-bp shRNAs, larger loops (regardless of sequence) and the presence of a 3′-UU overhang are crucial for efficacy. The loop of shRNAs must be removed or opened before further processing by RISC can take place. For longer (e.g., 29-bp) shRNAs, Dicer can cleave off the loop within the duplex region (Dorsett and Tuschl, 2004). However, 19-bp shRNAs with 4-nt loops are much poorer substrates for Dicer (Siolas et al., 2005), presumably because their loops lie near or at the Dicer cleavage site (and hence are "felt" as Dicer "measures" from the ends). A larger loop allows more conformational flexibility at the junction between the duplex stem and the single strand of the loop, whereas a small loop may constrain that region to a conformation that is a poor substrate for Dicer. The 3′-UU extension, which provides a docking site for the Paz domain of Dicer (MacRae et al., 2006), may be important to help compensate for the unfavorable position of the loop in the case of the 19-bp shRNAs.

Several groups have reported the successful use of 19-bp shRNAs with small (3–4 nt) loops for target knockdown (Paul et al., 2002). However, few studies have carefully examined the effects of loop size on the potency of short shRNAs. Brummelkamp et al. (2002) reported that for expressed 19-bp shRNAs, molecules having 9-nt loops had higher activity than those with 7-nt loops, and constructs with 5-nt loops were inactive. Jacque et al. (2002) reported that, for expressed 19-bp shRNAs, activity was about the same for constructs with 3-nt and 7-nt loops, and somewhat less with 5-nt loops. When shRNAs are expressed from vectors, or when synthetic shRNAs are used at high concentration, variation of shRNA efficacy with loop size might not be noticed. Besides, some RNAi studies employ loop sequences such as *UU*CAAGA*GA* that may internally pair (*UU* to *GA*), thus extending the stem of the shRNA by 2 bp and shortening loop from 9 nt to 5 nt. Such alternate structures may make interpretation of results difficult. In general, 19-bp shRNAs are more sensitive to design parameters than longer, 25–29 bp shRNAs, and optimal design may be dependent on the specific assay system, target sequence and delivery route.

The IRES region in the HCV 5′-UTR is highly conserved and has several segments that appear to be invariant. The region around the AUG translation initiation codon is particularly highly conserved, being invariant at positions +8 to −65 (with the exception of a single nucleotide variation at position −2) in over 81 isolates from various geographical locations (Bukh et al., 1992). Despite the conservation of sequence in the IRES motif, it is uncertain whether targeting a single sequence, even if highly conserved, will be sufficient to prevent escape mutants. RNA viruses, including HCV, are notorious for their high mutation rates, which result from the high error rate of the replicase and the lack of proofreading activity. On average, each time HCV RNA is replicated, one error is incorporated into the new strand (Rice, 2003), although not all of these variants are necessarily propagated in subsequent rounds of infection or replication. This error rate is compounded by the prodigious production of viral particles—up to a trillion per day in patients with active infections (Rice, 2003). We have found that in the reporter system a single mismatch in the target mRNA did not significantly affect activity of shRNAs tested (with the mismatch at various positions). Such robustness in the face of viral mutation could be advantageous in terms of targeting evolving viral mutants, although it is established that viruses can readily escape from RNA interference (Gitlin et al., 2005). Also, low sensitivity of si/shRNA to mismatches raises concerns regarding off-target effects of siRNA (Jackson et al.,

2003; Saxena et al., 2003; Scacheri et al., 2004). As with other pharmaceuticals, proper testing of several potential agents will be required to identify those having the highest activities and rejection of those that have unacceptable adverse effects.

Recent reports demonstrated that *in vitro*-synthesized transcripts from bacteriophage promoters can potently induce interferon alpha and beta due to the presence of an "unnatural" 5′-triphosphate (Hornung et al., 2006; Kim et al., 2004; Pichlmair et al., 2006). Interferon can be also induced by si/shRNA features such as the presence of specific sequences (e.g., 5′-UGUGU [Hornung et al., 2005] and 5′-GUCCUUCAA [Judge et al., 2005]), si/shRNA termini (blunt or with certain overhangs) (Marques et al., 2006), and dsRNAs longer than 30 bp (and according to some reports even 21 bp [Bridge et al., 2003; Fish and Kruithof, 2004]). Many of these effects are seen with lipid-mediated delivery of dsRNAs to certain cell types, and are mediated by Toll-like receptors on endosomal membranes (Sioud, 2006). How interferon induction would affect use of shRNAs in a clinical setting for HCV infection is unclear. Current HCV therapy includes treatment with interferon alpha, suggesting that if interferon is induced by shRNA, it may have a positive effect. To date, no interferon-related side effects have been reported in animals following administration of RNAi, although this may simply reflect an absence of proper testing or the short duration of current studies (Radhakrishnan et al., 2004). Clearly, further studies are needed to determine the extent that IFN alpha and beta are induced by shRNAs in animals and how it affects their health.

As with other nucleic acid-based therapies, a major bottleneck in the development of RNAi therapies is the delivery of these molecules to the desired cell type, tissue, or organ (Shoji and Nakashima, 2004; Stein, 1999; Toub et al., 2006). RNAs do not readily cross the cell membrane on their own because of their large molecular mass and their high negative charge. There are two general strategies: use of chemically synthesized RNAs (optionally modified for improved pharmacokinetic properties), and use of viral vectors to express RNA within cells. ShRNA is obviously preferred over siRNA when vectors are used, since it is expressed as a single molecule and efficient intramolecular hybridization occurs to form the intended double-stranded molecule. Viral vectors derived from adenovirus, adeno-associated virus, retrovirus, or lentivirus that are engineered to encode shRNAs can be used for sustained gene knockdown, which would be useful for chronic infections such as hepatitis C and HIV. Although much progress has been made in developing gene-therapy vectors, some obstacles remain (Thomas et al., 2003). These include the possibility of insertional mutagenesis and malignant transformation, as well as the development of a host immune response to RNA or proteins expressed from viral vectors. After a report of cytotoxic effects when shRNA was delivered to tumor cells by lentiviral vectors (Fish and Kruithof, 2004), the long-term effects of sustained high-level shRNA expression (from adeno-associated virus serotype 8, AAV8) in livers of adult mice were investigated (Grimm et al., 2006). An evaluation of 49 distinct AAV/shRNA vectors, unique in length and sequence and directed against six targets, showed that 36 resulted in dose-dependent liver injury, with 23 ultimately causing death.

These concerns about vector-expressed shRNAs have made chemical synthesis of RNA the more attractive approach for RNAi-based therapies. As mentioned above, an important feature of shRNA is its hairpin structure, which ensures that the antisense and sense strands will form a perfect duplex *in vitro* and will stay in this form *in vivo*. For small-scale preparation of shRNA (e.g., for research purposes), *in vitro* transcription is faster and cheaper than chemical synthesis, in contrast to siRNA; however, for large-scale production, chemical synthesis is currently preferable. In general, shRNA is more expensive to synthesize than siRNA, and, until recently, the achievable upper limit for the length of synthetic RNA was ~45 nt. However, longer RNAs with a wide spectrum of possible modifications are becoming more readily available from commercial sources.

It has been reported that siRNAs with minimal modifications, conjugated to cholesterol, can silence an endogenous gene encoding apolipoprotein B after intravenous injection in mice (Soutschek et al., 2004). Recently, the same group reported that synthetic unmodified siRNA, encapsulated in lipid particles and delivered systemically, silenced apolipoprotein B in nonhuman primates (Zimmermann et al., 2006). The efficacy of chemically modified siRNA targeted to hepatitis B virus was examined in an *in vivo* mouse model of HBV replication (Morrissey et al., 2005). SiRNA incorporated into a liposome administered by intravenous injection into mice efficiently reduced the level of serum HBV DNA. The findings of the present study combined with the progress in delivery of RNA to the liver exemplified by these studies suggest that appropriately formulated 19–25 bp shRNAs may be effective agents for the control of HCV.

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Address reprint requests to:
Brian H. Johnston
SomaGenics, Inc.
2161 Delaware Avenue
Santa Cruz, CA 95060

E-mail: bjohnston@somagenics.com