

RNA Interference from Multimeric shRNAs Generated by Rolling Circle Transcription

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ABSTRACT

Methods most commonly used for producing small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) are chemical synthesis and intracellular expression from engineered vectors. For shRNAs, chemical synthesis is very costly and construction of vectors is laborious. Synthesis by phage RNA polymerases from their natural promoters results in a 5'-terminal triphosphate that can trigger an interferon (IFN) response. Moreover, due to the requirement of phage promoters for 5'-GPuPuPu sequences for transcription initiation, shRNA transcripts may have extra 5'-nucleotides that can constrain the sequences that can be targeted. Also, the 3' ends may have an additional $n + 1$ nucleotide not encoded by the template. Here we present a novel approach for synthesizing functional shRNAs via rolling circle transcription (RCT) of small (approximately 70 nt) single-stranded DNA circles using T7 RNA polymerase, which avoids these issues. Due to internal pairing, these circles are dumbbell-shaped. RCT produces large transcripts (>10 kb in length) consisting of multimers (>150 copies) of shRNAs in the absence of promoter, terminator, or primer sequences. Dumbbells targeting red fluorescent protein (DsRed), human tumor necrosis factor- α (TNF- α) and hepatitis C virus (HCV) internal ribosome entry site (IRES) were prepared and transcribed. The resulting long transcripts are substrates for Dicer. When introduced into 293FT and Huh7 cells, the multimeric transcripts inhibited their target genes at levels similar to an equivalent mass of monomeric shRNAs, indicating that they can enter the RNAi pathway. Thus, rolling circle transcription of small DNA dumbbells provides a new source of biologically active interfering RNA.

INTRODUCTION

RNA INTERFERENCE (RNAi) is a biological response to double-stranded RNAs that results in the sequence-specific silencing of gene expression (Dykxhoorn and Lieberman, 2005; Hammond et al., 2000; Novina and Sharp, 2004; Zamore et al., 2000). RNAi is an important mechanism for the regulation of gene expression in a broad range of eukaryotic organisms, including both plants (van der Krol et al., 1990) and animals (Fire et al., 1998). RNAi has also become a powerful tool in functional genomics and shows promise in human therapeutics.

Silencing can be induced in target cells by directly introducing synthetically produced small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs), or alternatively by transfecting cells with engineered vectors expressing siRNAs or shRNAs (Amarzguioui et al., 2005; Barton and Medzhitov, 2002; Cullen, 2002, 2005; Devroe and Silver, 2002; Dykxhoorn and Lieberman, 2005; Grabarek et al., 2003; Hannon, 2002; Kitabwalla and Ruprecht, 2002; Ramaswamy and Slack, 2002; Tuschl, 2001; Zamore and Haley, 2005). Although expression of siRNAs or shRNAs from plasmids or viral vectors is highly effective, generation of the individual vectors is laborious. Synthetic methods generally allow

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faster production of large numbers of RNAs, and there are currently two methods: (1) chemical synthesis, which is relatively costly for use in large-scale screening studies, and (2) *in vitro* transcription of linear “run off” templates by bacteriophage RNA polymerases (RNAP) from their cognate promoters (T7, T3, or SP6). Although widely used, *in vitro* transcription from a T7 promoter by T7 RNAP requires the presence of a G at the 5′ end of target sequence (Donze and Picard, 2002; Yu et al., 2002) due to the preference of T7 RNAP for a guanosine residue for transcription initiation (Milligan and Uhlenbeck, 1989). Furthermore, to increase the RNA yield, additional Gs are often incorporated into the template at the +2 and +3 positions. This approach to maximizing yield somewhat limits the sequences that can be targeted. Another potential drawback of *in vitro* transcription is the recent finding that the 5′-triphosphate present in such transcripts can induce an interferon (IFN) response (Kim et al., 2004). In addition, efficient transcription of siRNAs requires two separate dsDNA templates, and four oligodeoxynucleotides must be synthesized for each siRNA duplex (Donze and Picard, 2002).

We have investigated a simple and novel approach for the enzymatic production of interfering RNA that circumvents many of these problems. It involves the *in vitro* transcription of shRNAs by T7 RNAP using as templates small circular single-stranded DNAs (ssDNA) lacking promoter, primer, or terminator sequences. Several reports have demonstrated that small circular ssDNAs can be efficiently transcribed by phage and bacterial polymerases through rolling-circle transcription (RCT), generating transcripts consisting of repeated sequences in the absence of any known promoter (Daubendiek et al., 1995; Daubendiek and Kool, 1997; Diegelman et al., 1998; Diegelman and Kool, 1999; Seyhan et al., 1999). We hypothesized that circular ssDNA templates in the shape of a dumbbell could also be used to synthesize multimers of shRNAs *in vitro* by phage RNAP. We find that *in vitro* transcription of such promoterless dumbbell templates by T7 RNAP can indeed produce large (>10 kb) multimeric transcripts indicative of production by a rolling circle mechanism. These transcripts can be processed by added recombinant Dicer to generate 19- to 22-bp fragments. When introduced into two cell lines, unprocessed multimeric transcripts caused marked, dose-dependent, and sequence-specific inhibition of their respective targets, including red fluorescent protein (DsRed), human tumor necrosis factor- α (TNF- α), and the internal ribosome entry site (IRES) of hepatitis C virus (HCV). Because each multimeric shRNA transcript contains only a single triphosphate and (sometimes) 5′ block of Gs while encoding hundreds of monomeric shRNAs, the potential disadvantages of those features are minimized.

MATERIALS AND METHODS

Preparation of dumbbell templates for expression of shRNA multimers

The oligodeoxynucleotide (ODN) precursors for DNA dumbbells were chemically synthesized (IDT, Coralville, IA) and 5′-phosphorylated. Dumbbell ODNs contained an interrupted 20–29-nt sense sequence, a micro-RNA-23 (miR23) loop sequence (CTTCCTGTCA), a 20-29-nt antisense sequence, and a AACAA or TGACAGGAAG closing loop sequence. The miR23 sequence was chosen because it is found naturally and is therefore not expected to be toxic (Lagos-Quintana et al., 2001). The antisense sequences included GTGG-GAGCGCGTGATGAACTTCGAGGACG or a truncated version of this sequence for DsRed (Seyhan et al., 2005) and AGCACGAATCCTAAACCTCAAAGA for the HCV IRES (Wang et al., 2005). These ODNs were designed to fold as shown in Figure 1B so that the ends abut each other and together form the sense sequence. Correct folding was facilitated by denaturing the ODNs at 95°C for 2 minutes and slowly cooling to 24°C in T4 DNA ligase buffer (40 mM Tris-HCl [pH 7.8] 10 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM ATP [MBI Fermentas, Hanover, MD]). Addition of T4 DNA Ligase and ATP followed by incubation at 24°C for approximately 1 hour ligated the juxtaposed ends, yielding a covalently closed DNA dumbbell (Fig. 1). Completion of the ligation reaction was verified by denaturing polyacrylamide gel electrophoresis (PAGE). The circularity of the ligated product was confirmed by resistance to end labeling (compared to the unligated ODN) and treatment with T7 DNA polymerase in the absence of dNTPs.

Construction of linear run-off templates for expression of shRNA monomers

Two complementary ODNs were chemically synthesized (IDT, Coralville, IA) having a T7 promoter element (TAATACGACTCACTATAGGG) appended to the sense, miR-23, and antisense sequences used for the dumbbells (but without the second loop), and finally two T residues. ODN pairs were incubated at 95°C for 2 minutes in RNA polymerase buffer (20 μ L of each 100 μ M ODN in 5 μ L 5 \times transcription buffer (Ambion, Austin, TX) and slowly cooled to 24°C over 1 hour. Annealed dsDNA templates were used in subsequent transcription reactions using commercially available transcription kits.

In vitro transcription by T7 RNA polymerase

Various concentrations (1–10 μ M) of template DNAs (covalently-closed or interrupted dumbbells or

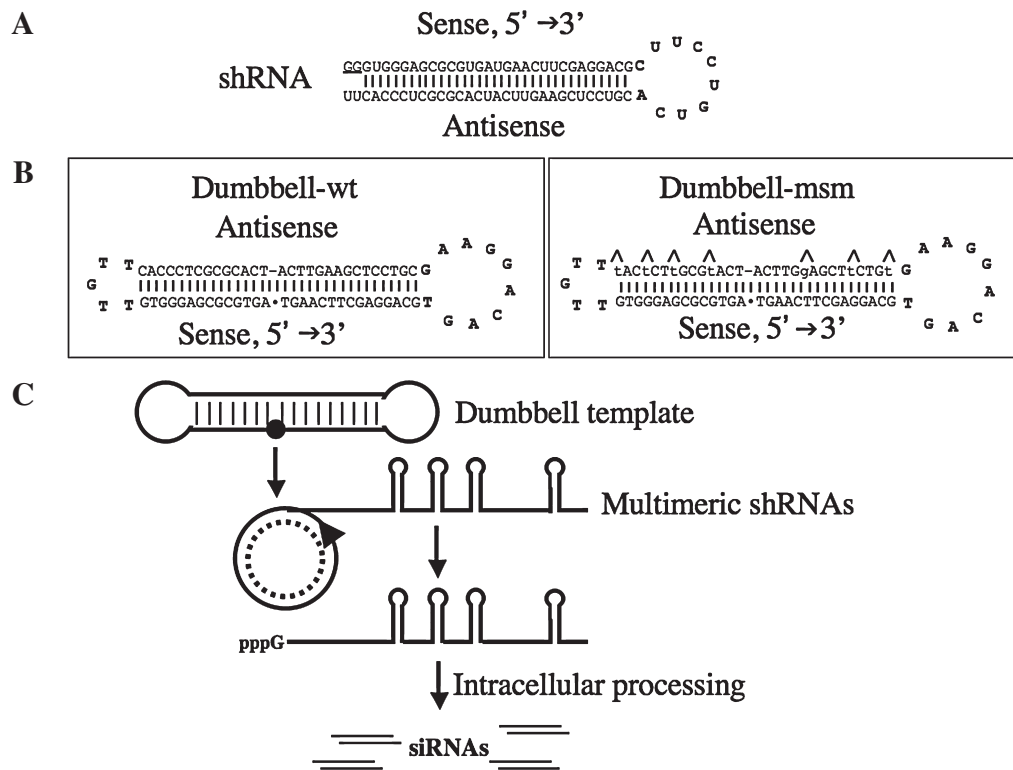


FIG. 1. Scheme for generating long multimeric shRNAs from promoterless single-stranded DNA dumbbells through a rolling circle transcription (RCT) reaction. (A) The shRNA directed against DsRed used as a starting point to design and construct corresponding dumbbell DNA templates. (B) Sequences of DNA dumbbells encoding DsRed specific shRNAs with fully complementary (wt) and mismatched (msm) stem sequences. The latter were made by introducing seven C → T changes within the antisense strand of the dumbbell (i.e., the template for the sense strand of the shRNA), converting G:C base pairs to G:T wobble pairs. (C) RCT of DNA dumbbells by T7 RNA polymerase to generate multimeric units of shRNAs, and their presumed pathway of intracellular processing upon introduction into cells.

linear T7 promoter-containing DNAs) were transcribed in 20- μ L reactions using the T7 Megashortscript RNA transcription kit (Ambion) following the manufacturer's protocol. Trace amounts of [α - 32 P]-CTP were included in the reaction to allow monitoring of RNA transcripts by denaturing PAGE and autoradiography. After transcription at 37°C for 4–16 hours, samples were treated with 1 μ L of DNase I (Ambion, 2 U/ μ L) at 37°C for 15 minutes to remove DNA templates and quenched by addition of 1 μ L of 0.5 M EDTA. Transcripts were purified by passage through three successive gel filtration spin columns (Microspin G25, Amersham Biosciences, Piscataway, NJ) and ethanol precipitation. RNA pellets were dissolved in nuclease-free water or TE (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) and RNA concentrations were determined by absorbance at 260 nm. To assess the integrity of transcripts, aliquots from transcription reactions were combined with equal volumes of 2 \times formamide loading buffer (95% formamide, 0.5 mM EDTA, 0.025% xylene cyanol, 0.025% bromphenol blue, and 0.025%

SDS, denatured at 90°C for 2 minutes and analyzed by denaturing 3.5%, 5%, or 10% PAGE.

Treatment of long shRNA multimers with recombinant Dicer

Internally labeled long multimeric shRNA transcripts (approximately 0.5 μ g) were digested with 0.5 unit recombinant Dicer (Stratagene, La Jolla, CA) in 10 μ L of 20 mM Tris-HCl, pH 8.0; 150 mM NaCl; 2.5 mM MgCl₂ for 18 hours at 37°C. Aliquots were collected at time intervals, quenched with equal volumes of formamide loading buffer, heat-denatured (95°C, 2 minutes), and fractionated by denaturing 10% PAGE.

Cell culture, transfection, and assay of target gene silencing

Human 293FT (Invitrogen, Carlsbad, CA) and Huh7 cells (kindly provided by Andrew Simons, Cell Genesys, South San Francisco, CA) were maintained in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker, Walk-

ersville, MD) with 10% fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine, and 1 mM sodium pyruvate. The day before transfection, cells were seeded at 5×10^4 per well in a 48-well plate so that they would be 80% confluent at the time of transfection. To test for the ability of multimeric and monomeric shRNAs to inhibit target expression, cells were cotransfected (in triplicate) with Lipofectamine 2000 (0.75 μ L per well; Invitrogen) complexed with plasmids expressing the target (either 40 ng pDsRed1-C1 (Clontech, Mountain View, CA), 40 ng pCDNA3/HCV-IRES dual luciferase reporter construct (kindly provided by Peter Sarnow, Stanford University, Stanford, CA), or 7.5 ng of pCR3.1-TNF (Seyhan et al. 2005), a control target (25 ng of pSEAP2 (secreted alkaline phosphatase, BD Biosciences Clontech), and various amounts (0, 1, 5, 10 ng per well) of transcripts. (1 ng of either type of transcript in 0.5 mL of media corresponds to 93 pM of 65-nt monomer shRNA.) Plasmid pUC18 was added to the transfection mix to provide a total nucleic acid concentration of 400 ng per transfection per well. Forty-eight to 68 hours after transfection, DsRed protein levels in cells were assessed by fluorescence microscopy (Olympus CK40) and flow cytometry. Supernatants were analyzed for secreted TNF (by enzyme-linked immunosorbent assay [ELISA]) and SEAP (pNPP system (Sigma, St. Louis, MO; Xu et al. 2001). To measure luciferase activity (for the HCV-IRES target), cells were lysed and assayed using the Dual-Luciferase assay system (Promega, Madison, WI) with a MicroLumat LB 96 luminometer (Berthold, Bad Wildbad, Germany).

RESULTS

Construction of circular dumbbell templates

The goal of this study was to determine whether functional small interfering RNAs can be produced by RCT from promoterless circular ssDNA templates. To this end, oligodeoxynucleotides encoding various shRNA sequences (an example is shown in Fig. 1A) were synthesized. They were designed to fold into a dumbbell shape having a duplex stem of 20–29 base pairs (bp) capped by one loop derived from micro-RNA-23 (miR23) and a second loop at the other end. The ODNs were 5'-phosphorylated, heat-denatured, and annealed to ensure the proper folding, with the antisense sequence acting as a splint to juxtapose the ends forming the sense sequence. Subsequent ligation with T4 DNA ligase was highly efficient, converting nearly all of the linear DNAs into covalently closed circles (data not shown).

Transcription from DNA dumbbells: effects of duplex length and destabilizing mismatches

Transcription of a DNA dumbbell having a stem length of 29 bp, designated dumbbell-DsRed-29 wt, yielded

RNA products of sufficient length that they barely entered a denaturing 3.5% polyacrylamide gel, suggesting that the reaction proceeded through many rounds of rolling circle transcription (Fig. 1B and Fig. 2A, lane 3). However, the amount of RNA produced by this dumbbell was rather low. We hypothesized that, although T7 RNA polymerase normally transcribes dsDNA and is also able to transcribe ssDNA templates, it might not readily negotiate transitions between fully double-stranded and fully single-stranded regions in the template as would be required to transcribe a dumbbell. We therefore tried destabilizing the duplex region of the DNA dumbbell by introducing seven C \rightarrow T mutations within the antisense strand of the dumbbell (i.e., the template for the sense strand of the shRNA), thereby converting G:C base pairs to G:T wobble pairs and allowing the duplex to open more easily (Fig. 1B). (It has been shown that as many as five such mutations in the sense strand of a DNA encoding a 21-bp shRNA did not affect the siRNA activity [Miyagishi and Taira, 2003].) When this dumbbell (DsRed-29-msm) was transcribed, it produced very long (>10 kb) RNA transcripts in large amounts (Fig. 2A, lane 5), indicating that RCT is more efficient on dumbbells with less stable duplexes. Indeed, comparison of the amount of RNA produced per template for dumbbell DNAs versus standard linear run off templates (Fig. 2A, lanes 1 and 5; equimolar concentrations used), using phosphorimager quantification of the full-length transcription products and spectrophotometric quantification of each RNA transcript, showed that the efficiencies of these templates were essentially the same.

We further examined whether shortening the stem would also increase the efficiency of RCT and lessen the need for mismatches in the stem. Dumbbells containing 25- and 20-bp stems, targeting respectively the hepatitis C virus and murine TNF- α , were constructed. Transcription reactions were performed and the resulting products were analyzed by denaturing PAGE. The results (Fig. 2B) show that shortening the stem length greatly improved the transcription efficiency. The level of RNA transcripts produced from transcription of these short-stem dumbbells was again equivalent to that produced by transcription of T7-driven linear run off templates (Fig. 2B, lanes 1, 3, and 5).

Unligated, interrupted DNA dumbbells produced only transcription products that were shorter than the full-length monomeric shRNA (approximately 70 nt), indicating that some transcription was initiated but subsequently halted by the interruption (Fig. 2A and 2B, lanes 2 and 4). Since the interruption lies within the sense strand of the DNA (the template for the antisense strand of the RNA), transcripts initiating at the interruption will contain only 10–14 nt of the antisense sequence and should not be capable of producing RNA interference in cells (see below).

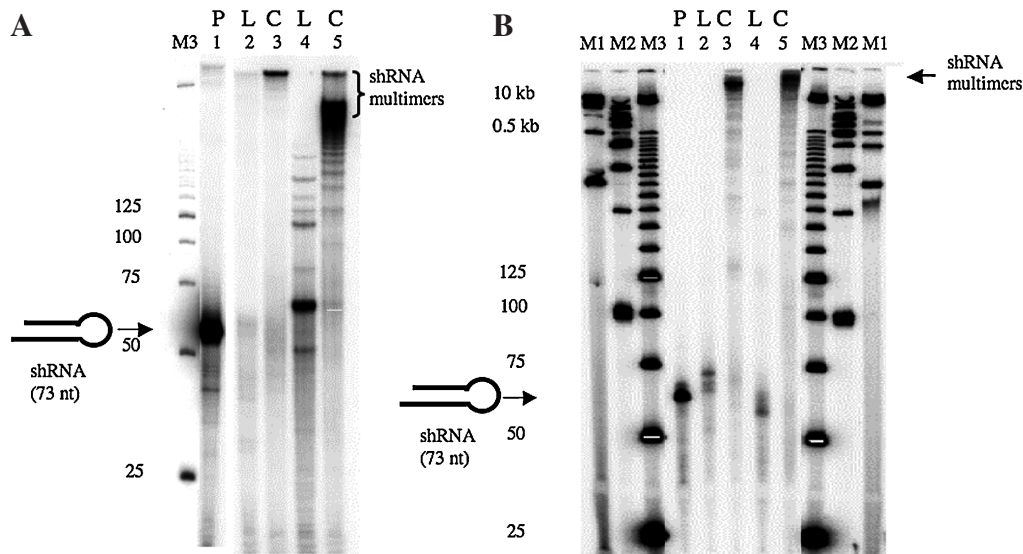


FIG. 2. Transcription of circular DNA dumbbells generates long transcripts containing shRNA multimers through an RCT mechanism. (A) Introducing base mismatches allows efficient RCT of 29-bp dumbbells. Transcription reactions were performed with 2 μ M 29-bp DsRed-specific DNA dumbbells, either after ligation to form covalently closed circles (C) or as unligated linear molecules (L) in the presence of [α ³²P]-CTP using Megashortscript kit (Ambion, Austin, TX) at 37°C for 4–12 hours. Reactions were treated with DNase I and resolved by denaturing 12% PAGE. Lanes 2 and 3: Dumbbell DNA with perfectly matching stem sequences. Lanes 4 and 5: Dumbbell DNA with several mismatched base pairs (see Fig. 1) within the strand that encodes the sense strand of the shRNA. M3: 25-bp DNA ladder as size marker. A double-stranded DNA template containing a T7 RNA polymerase promoter sequence and an inverted repeat sequence encoding shRNA was also used as a control (lane 1). (B) Reducing the stem length of the dumbbell allows efficient RCT without introducing wobble pairs. HCV-IRES and TNF specific linear (L; lanes 2 and 4) and circular (C; lanes 3 and 5) dumbbells with fully complementary stems encoding HCV-IRES (25 bp, lanes 2 and 3) and TNF (20 bp, lanes 4 and 5) specific shRNAs, showing appearance of long multimeric RNAs as well as shorter products. Lane 1, promoter-containing linear template as in (A). RCT reactions were analyzed by denaturing 5% PAGE. DNA size markers: M1, 1 kb ladder; M2, 100 bp ladder, and M3, 25 bp ladder.

Long RCT transcripts can be processed into monomeric siRNAs by recombinant Dicer

If RCT of small DNA circles is to have utility as a posttranscriptional silencing tool, one option is to transfect the multimeric products of RCT into cells and rely on intracellular processing to generate monomers that can enter the RNAi pathway. Since the cytoplasm is the first compartment that transfected RNAs will encounter, we wanted to know whether the cytoplasmic dsRNA processing enzyme Dicer could convert multimers to siRNAs. (In the cell, Drosha normally processes primary microRNA transcripts to produce approximately 70-nt hairpins, but this process occurs in the nucleus and it is unlikely that long transfected RNAs would localize there [Kim, 2005]). Internally labeled RCT transcripts generated from various dumbbell DNAs were incubated with recombinant Dicer for various times, and the products were analyzed by denaturing PAGE. Both multimeric and monomeric shRNA transcripts (used as control) yielded approximately 19–22 bp products, the expected size of a monomeric siRNA, by 1 hour of incubation (Fig. 3). (Monomeric shRNAs were generated by transcription using T7 RNA polymerase from a promoter-containing ds-

DNA.) However, processing into siRNAs was slower for the large multimeric shRNAs than for shRNA monomers (Fig. 3). Similar results were seen with use of bacterial RNase III in place of Dicer (not shown)

Silencing of gene expression by large shRNA multimers

Next, we asked whether transfection of cells with RCT-generated multimeric shRNAs can induce silencing in two test targets, the HCV IRES (driving firefly luciferase as a reporter) and murine TNF- α , with secreted alkaline phosphatase (SEAP) used as a nontargeted control. Plasmids expressing these targets (pcDNA3/HCV-IRES-dual Luc or pTNF, each with pSEAP2) along with the corresponding multimeric or monomeric shRNAs were used to transfect 293FT and Huh7 cells. After 48–72 hours, supernatants were removed for SEAP and TNF- α analysis (see Materials and Methods). For HCV-IRES analysis, the cells were lysed and levels of firefly luciferase were measured using the Promega dual luciferase assay.

The results showed a marked, dose-dependent inhibition of HCV-IRES-dependent firefly luciferase reporter

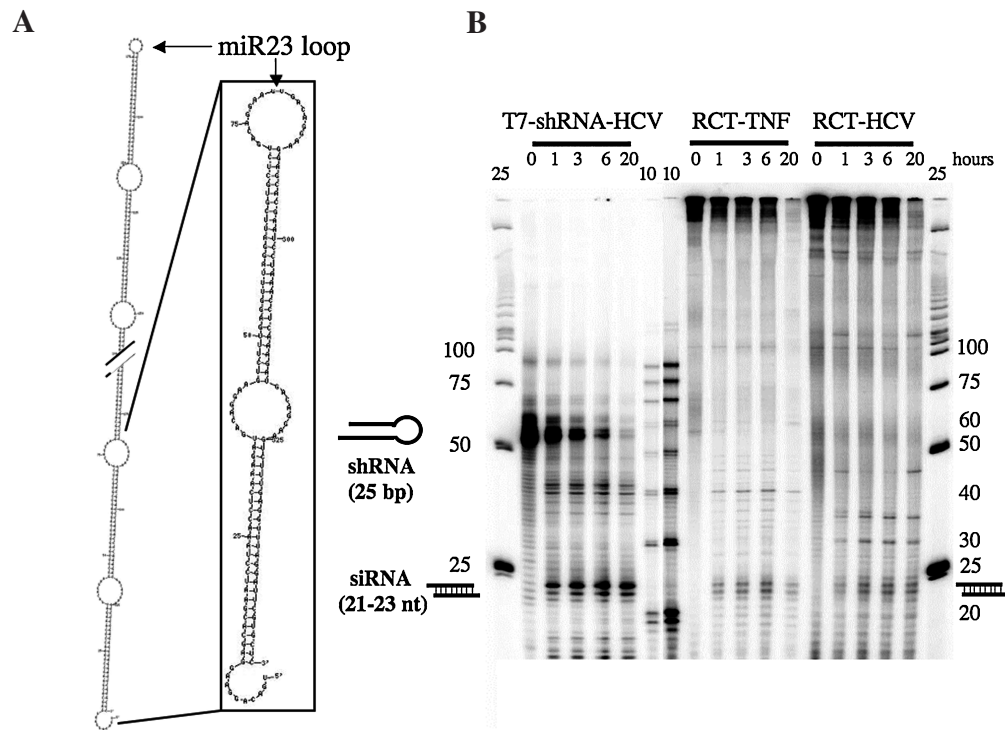


FIG. 3. Recombinant Dicer can process large shRNA multimers into monomeric siRNAs. (A) Example of the secondary structure predicted for an shRNA multimer by the M-fold program. (B) *In vitro* cleavage of long multimeric shRNAs by Dicer. RNA transcripts ($\sim 0.5 \mu\text{g}$) were digested with 0.5 units of recombinant human Dicer ($0.5 \text{ U}/\mu\text{L}$) in $10 \mu\text{L}$ reaction buffer at 37°C . Aliquots were collected at various time intervals, quenched with an equal volume of formamide loading buffer, heat denatured (95°C , 2 minutes) and fractionated by denaturing 10% PAGE. T7-ShRNA-HCV, Dicer processing of monomeric shRNAs transcribed from a linear dsDNA template containing a T7 promoter. RCT-TNF and RCT-HCV, shRNA multimers targeting murine tumor necrosis factor (TNF)- α and the HCV-IRES element generated via RCT of corresponding DNA dumbbells. Lanes labeled 10 and 25 are 10- and 25-bp DNA ladders, respectively.

gene expression by HCV-specific multimeric or monomeric shRNAs, while TNF expression was not affected (Fig. 4). The levels of inhibition achieved by the shRNA multimers were somewhat less on a per-monomer basis to those of the monomeric shRNAs. The TNF analysis showed specific and dose-dependent inhibition of TNF expression by TNF-targeting multimeric and monomeric shRNAs but not by HCV IRES-specific multimers or monomers (Fig. 5). These results suggest that two unrelated genes could be silenced independently and specifically by the corresponding multimeric (or monomeric) shRNAs in the same transfected cells.

In contrast, when HCV IRES- or TNF-specific RNA abortive transcripts produced from the interrupted (linear) DNA dumbbells were used for transfection, far less inhibition of either of the corresponding target gene was seen, indicating that efficient silencing requires uninterrupted antisense strands. The trace activity seen can be explained by the fact that in some cases T7 RNAP can "jump" through gaps in the template strand (Zhou et al., 1995). In all cases tested, the values of SEAP remained

unchanged, indicating that transfection efficiency was consistent and that there was no obvious nonspecific toxicity.

Similar results were seen for multimeric shRNAs targeting DsRed in 293FT cells; multimeric shRNAs with mismatched pairs in their stem (Fig. 2A, lane 5) silenced DsRed expression at levels similar to fully matched shRNAs (Fig. 2A, lane 4 and data not shown). It should be noted that the mismatches were only within the shRNAs; the antisense strand fully matched the target. Again, the levels of inhibition achieved by the shRNA multimers were similar on a per-monomer basis to those of the monomeric shRNAs.

DISCUSSION

Despite the efficacy of T7-based transcription systems to produce siRNAs or shRNAs from linear run off templates, the requirement for 5'-guanosines and the presence of immunostimulatory 5'-triphosphate groups in T7

transcripts prompted us to explore an alternative approach that retains the practicality and low cost of *in vitro* transcription but could circumvent these limitations. This approach takes advantage of the ability of T7 RNAP to initiate transcription in the absence of any promoter.

In normal transcription initiation, RNAP binds to and scans dsDNA. Upon encountering a promoter sequence, RNAP unwinds a region of duplex DNA, allowing transcription to initiate. RNAP can also initiate transcription directly at single-stranded “bubbles” within duplex DNA (Aiyar et al., 1994; Daube and von Hippel, 1992; Mollegaard et al., 1994; Ohmichi et al., 2002). Moreover, circular ssDNAs lacking promoters can also be transcribed by various phage and *Escherichia coli* RNAPs through a rolling circle mechanism (Daubendiek and Kool, 1997, 1999; Seyhan et al., 1999) that produces long RNAs consisting of tandem repeats. In transcription of dumbbell DNAs, RNA synthesis may be initiated at specific structures such as the single-stranded loops, perhaps because they resemble the open transcription complex formed by RNAP (Aiyar et al., 1994; Daube and von Hippel, 1994, 1995; Mollegaard et al., 1994). For transcription to proceed via the rolling circle mechanism, RNAP must establish a processive elongation complex and repeatedly

switch from double-stranded to single-stranded regions and back again as it progresses around the dumbbell. Our findings indicate that the length and stability of the stem strongly influence the efficiency of dumbbell transcription.

We examined several dumbbell DNAs that encode shRNAs targeting three different genes. All dumbbells were transcribed successfully by T7 RNAP to generate long transcripts, indicating that transcription occurred via a rolling circle mechanism. Destabilizing the stems of dumbbells by either shortening them or introducing several wobble pairs markedly improved the transcription efficiency. The ability of T7 polymerase to transcribe dumbbells in the absence of a promoter is even more remarkable than its ability to transcribe circular templates for the hairpin ribozyme (Diegelman and Kool, 1998).

Once synthesized, large multimeric shRNAs can fold into highly intricate structures. As an example, one secondary structure predicted by the program M-fold of an approximately 700-nt segment of an expected product of RCT is shown in Fig. 3A. Despite this complex structure, multimeric shRNAs were processed by cloned Dicer to generate approximately 21-bp siRNAs (Fig. 3B), suggesting that they are likely to be substrates for Dicer in

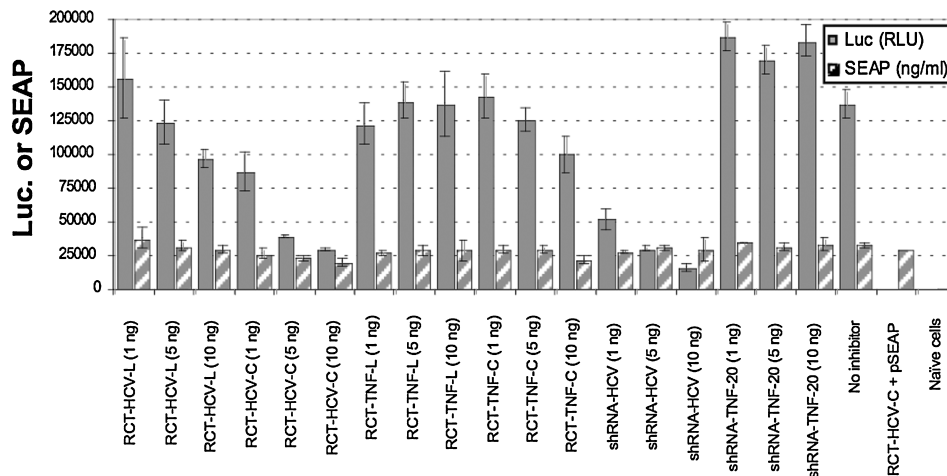


FIG. 4. Multimeric shRNAs targeting the HCV-IRES induce dose-dependent inhibition of HCV-IRES-mediated luciferase expression in mammalian cells. Huh 7 cells were cotransfected with 40 ng pCDNA3-HCV-IRES-*f*-luciferase reporter plasmid, 25 ng secreted alkaline phosphatase expression plasmid (pSEAP2), and the indicated amounts of RCT-generated multimeric shRNAs or control monomeric shRNAs. pUC18 plasmid was added to give a final total nucleic acid concentration of 400 ng per transfection. Sixty-eight hours later, supernatant was removed for SEAP analysis. The cells were lysed and firefly luciferase activity was measured using the Promega luciferase assay and Turner TD 20/20 luminometer. SEAP activity was measured by a colorimetric assay (pNPP system, Sigma, St. Louis, MO). The bars represent average and standard deviation for measurements of Luc and SEAP carried out in triplicate. RCT-HCV-L, linear (interrupted) dumbbell targeting HCV-IRES used as transcription template (no-RCT control). RCT-HCV-C, circular HCV-IRES dumbbell used as template for RCT to generate long shRNA multimers. RCT-TNF-L and RCT-TNF-C, transcripts targeted against TNF- α from linear and circular dumbbells, used as a specificity (negative) control. shRNA-HCV and shRNA-TNF, monomeric shRNAs used as positive and negative controls, respectively. No inhibitor, cells were transfected only with the plasmids expressing HCV-IRES-luciferase and SEAP. RCT-HCV-C+pSEAP, cells were transfected with 10 ng of RCT-HCV-C shRNA multimers and pSEAP plasmid only, without the HCV-IRES-luciferase plasmid. Naïve cells, untransfected Huh7 cells. RLU, relative light units.

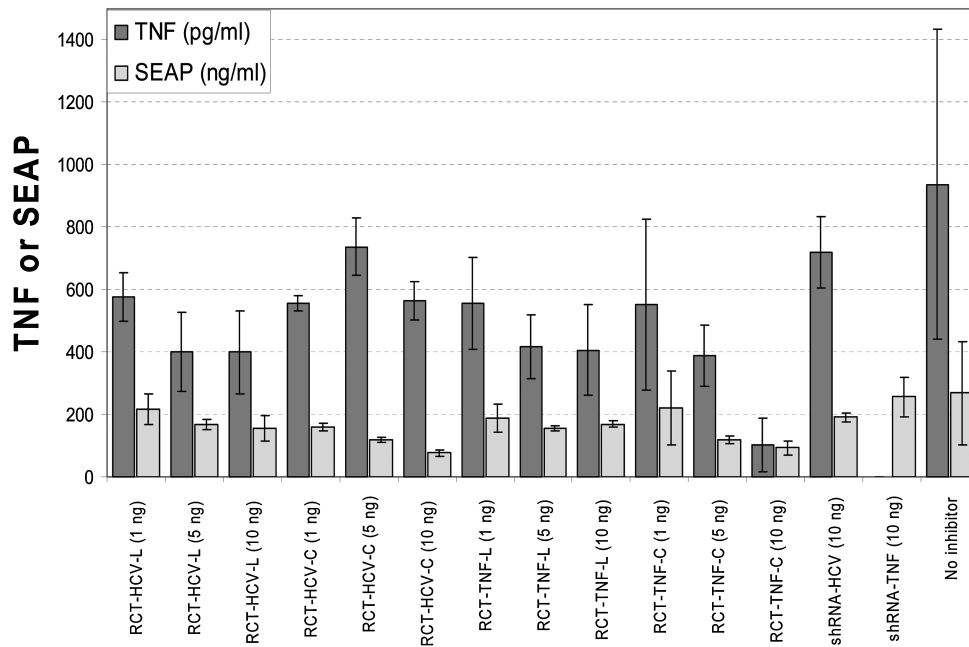


FIG. 5. Dose-dependent inhibition of TNF- α expression by large shRNA multimers. Huh7 cells were cotransfected with 7.5 ng pCR3.1 TNF- α construct, 25 ng pSEAP2, and various amounts of the indicated multimeric or monomeric shRNAs. Plasmid pUC18 was added to give a final total nucleic acid concentration of 400 ng per transfection. Fifty hours posttransfection, cell supernatants were collected and assayed by ELISA for TNF- α and by a colorimetric assay (pNPP system) for SEAP. The bars represent average and standard deviation for measurements of TNF- α (pg/mL) and SEAP (ng/mL) carried out in triplicate. Samples are designated as in Figure 4.

cells. The ability of Dicer to process hairpin repeats is also supported by the finding that tandem, bispecific 19- and 21-bp shRNAs can trigger RNAi in mammalian cells (Anderson et al., 2003; Leirdal and Sioud, 2002). In normal processing of shRNAs and micro-RNAs, Dicer is thought to bind to the end of the stem, with its Paz domain recognizing 3' overhangs, allowing it to cleave at a site 19 bp distant in the direction of the loop (Macrae et al., 2006). Because of the structure of folded multimers, Dicer must cleave each duplex stem twice, once at each end, to reduce a multimer to siRNAs (Vermeulen et al., 2005). In addition, Dicer may function as an exonuclease, binding only to the termini of the multimer as opposed to interior duplex regions. Either of these two features could explain the lower efficiency with which 19- to 23-bp fragments accumulate upon digestion of multimers compared to monomer shRNAs. In any case, the slower dicing does not substantially affect the ability of multimers to inhibit a target message when introduced into cells.

In microRNA (miRNA) biogenesis, many miRNAs are processed from long pol II transcripts (Bartel 2004; Ill and Chiou, 2005; Kim, 2005; Lee et al., 2004) called primary microRNAs (pri-miRNAs). These transcripts, often several kb in length, consist of imperfect double-stranded

stems separated by single-stranded sequences (Lee et al., 2002). The nuclear ribonuclease Drosha processes pri-miRNAs into approximately 70-nt hairpin-shaped precursor miRNAs (pre-miRNAs), which are transported to the cytoplasm for further processing by Dicer (Zeng and Cullen, 2005). Since the products of RCT of dumbbells superficially resemble pri-miRNAs, the question arises as to whether they might enter the RNAi pathway at the stage of Drosha processing. However, the dsRNA stems of the RCT-produced multimers discussed are shorter and have different base-pairing patterns than pri-miRNAs and consensus substrates for Drosha (Han et al., 2004). Moreover, it is unlikely that such large RNAs would enter the nucleus, where Drosha is localized. It seems more likely that the multimers are processed directly by Dicer, perhaps after initial cleavage into smaller fragments by an ssRNA-cutting nuclease. Multimers might also be cleaved by cellular nucleases within the loop regions. A recent report has shown the inability of Dicer to cleave shRNAs with 19-bp stems; these shRNAs were, however, effective at triggering RNAi, suggesting that a separate ribonuclease induces cleavage in the loop (Siolas et al., 2005).

Introducing multimeric shRNAs into cells produced silencing of the target for three ectopically expressed genes

with an efficiency (on a mass basis) slightly less than that of monomeric shRNAs (Figs. 4 and 5). Moreover, at least in the case of DsRed silencing, shRNA multimers having C → U transitions in the sense strand (forming GU wobble pairs) were as effective as multimers containing normal GC pairs. (The antisense strands retained complete Watson-Crick complementarity with their target.) The ability to reduce the stability of the duplex by introducing mismatches or wobble pairs allows use of RCT to generate multimers of longer stem shRNAs, which in monomer form have been reported to be more potent in some cases than 19-bp shRNAs (Siolas et al., 2005).

Dumbbells having 20- or 25-bp stems were transcribed substantially more efficiently than their 29-bp counterparts, avoiding the need to introduce wobble-pairs. The resulting multimeric shRNA transcripts targeting TNF- α (20 bp stem) and the HCV-IRES (25-bp) exhibited dose-dependent inhibition of their respective genes similar to that generated by monomeric shRNAs. The silencing by these RNAs was highly specific, as determined by their lack of effect on the heterologous targets (TNF and SEAP as targets for HCV-IRES, and HCV-IRES and SEAP as targets for TNF).

In mammalian cells, introduction of perfectly base-paired dsRNAs longer than 30-bp activates protein kinase R (PKR), which phosphorylates translation initiation factor eIF-2 α leading to global inhibition of protein synthesis (Der et al., 1997). In the multimeric shRNAs tested, the lengths of the dsRNA segments were less than 30 bp and hence should not be strong activators of PKR. Nonspecific effects have been also reported due to induction of interferon-pathway genes by even shorter RNA duplexes, although these may be due to the presence of certain immunostimulatory sequence features (Bridge et al., 2003; Hornung et al., 2005; Judge et al., 2005). Monitoring of cells after transfection with our RNAs revealed no obvious changes in appearance or growth pattern (data not shown). Together with the lack of effect on two nontarget genes, these results further support the conclusion that inhibition was target-specific and via RNAi, rather than through nonspecific, global inhibition of gene expression. Moreover, multimers have a theoretical advantage over monomers in that the density of immunostimulatory 5'-triphosphate groups is much less (on a mass basis) for multimers than for shRNAs when both are generated by phage polymerases. However, it remains to be seen whether this feature translates into an actual advantage over monomers in terms of nonspecific effects.

In summary, we have shown that, despite their highly structured nature, small DNA dumbbells lacking promoters can be transcribed to generate shRNA multimers that are substrates for Dicer and function as potent and specific gene inhibitors when introduced into cells.

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