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Long-term RNA interference from optimized siRNA expression constructs in adult mice

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Abstract

DNA constructs for small interfering RNA (siRNA) expression in mammalian cells have the potential for longer-term target gene knockdown than synthetic siRNAs. We compared in adult mice the efficacy and longevity of target gene knockdown from siRNA expression cassettes contained in plasmids, PCR-generated linear constructs or PCR constructs containing ''dumbbell'' ends using the hydrodynamic delivery method. Plasmid siRNA expression constructs were more effective than PCR constructs for target gene knockdown. The efficacy of the PCR constructs was improved by addition of short extensions beyond the transcription termination signal and greatly improved by addition of dumbbell ends. Constructs containing the H1 promoter were significantly less effective in mice than those containing the U6 promoter, whereas both promoters functioned equally well in cultured cells. Target gene knockdown perdured for at least 20 weeks in mice after delivery of either PCR or plasmid siRNA expression cassettes. These results will help guide RNAi vector design.

Keywords: RNA interference; siRNA expression vectors; RNA polymerase III promoter; H1 promoter; U6 promoter; Longevity of expression; Non-viral gene transfer; PCR product; In vivo gene transfer

RNA interference (RNAi) has revolutionized the study of gene function by target gene knockdown and has the potential to be used therapeutically (reviewed in [1,2]). RNAi can be induced in mammalian cells by introduction of small interfering RNA (siRNA) [3,4]. These are double-stranded RNA molecules 21–23 nucleotides in length and resemble RNA cleavage products resulting from the action of the Dicer enzyme. siRNA can be prepared synthetically or enzymatically in vitro and then delivered directly to cells. Alternatively, siRNA can be expressed within cells by delivery of siRNA expression constructs. These have the potential to mediate longer-term RNAi than synthetic siRNA by virtue of their continual siRNA production. The ability to use siRNA expression vectors greatly increases the range of applications in which RNAi can be used. For example, stable cell lines as well as transgenic mice can be generated using constructs that express siRNA [5–8]. Furthermore, vectors containing siRNA expression cassettes could be developed for gene therapy applications.

The use of DNA-based siRNA expression vectors was originally documented in a flurry of publications describing the use of RNA polymerase III (pol III) promoters to drive expression of siRNA transcripts. siRNAs were generated either as separate sense and antisense strands, which then annealed in the cell, or as a single short hairpin RNA (shRNA) containing both siRNA strands [9–17]. Pol III promoters are ideal for expression of siRNAs, as the sites of transcription initiation and termination are well defined. A typical pol III-driven siRNA expression cassette is composed of a

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promoter 100–300 bp in length, an siRNA template sequence, and a polythymidine tract of 4–6 nucleotides that functions as the transcription termination signal. The most frequently used siRNA expression cassettes utilize the human U6 (hU6), human H1 (hH1), and mouse U6 (mU6) pol III promoters.

Because of the small size of pol III-driven siRNA expression cassettes, PCR constructs that contain the entire expression cassette can easily be generated [18,19]. These small DNA constructs have some advantages over plasmid constructs: they can be rapidly generated in order to screen for effective siRNA expression prior to cloning into plasmid or viral vectors, the small size of PCR constructs may allow for more efficient nuclear delivery [20], and they may be more easily released than plasmids from synthetic nanoparticles used to deliver DNA in mammals (unpublished data). Additionally, PCR constructs can be generated with termini that are amenable for attachment of moieties that aid in delivery, ligands designed to promote cell-type specific targeting in vivo, or labels to aid in tracking and uptake studies [20,21].

In these studies, we compared the ability of different pol III promoters harbored in plasmids or PCR constructs to express siRNA and induce RNAi in cultured cells and in mice, optimized PCR constructs for siRNA expression, and determined the longevity of target gene knockdown in mice. We and others have previously shown that siRNA can be delivered to adult mice using the hydrodynamic tail vein injection procedure [22–25]. This simple injection method allows delivery of nucleic acids to various organs, but is most effective for delivery to the liver where 10–40% of hepatocytes are transfected [26,27]. In these studies, we co-delivered by hydrodynamic tail vein injection a plasmid expressing either luciferase or secreted alkaline phosphatase (SEAP) together with shRNA or siRNA expression cassette constructs targeting the reporter gene. This co-delivery strategy allowed us to accurately measure target gene knockdown over a wide dynamic range independent of delivery efficiency.

Materials and methods

Generating shRNA and siRNA expression cassettes by polymerase chain reaction. Construction of RNA polymerase III promoter plasmids is described in Supplementary Data. The hU6 plasmid included promoter region −264 to +1; the mU6 plasmid included −313 to +1 of the promoter; and the hH1 plasmid contained −100 to −1 of the promoter. The shRNA-Luc$^+$ hairpin used in these studies targeted nucleotides 1340–1368 of the *luciferase*$^+$ coding sequence (Promega, Madison, WI; GenBank Accession No. U47296). It had a stem of 29 bp and the sense strand contained three wobble bases. The hU6shRNA-Luc$^+$ expression cassettes were initially generated by polymerase chain reaction (PCR) of the human U6 promoter plasmid phU6 using the PCR-SHAGging procedure [13] with forward primer hU6-*Eco*RI (5′-agggaattCAAGGTCGGGCAGGAAGAGGG-3′), which included additional sequences for cloning (shown in lower case), and reverse primer hU6/sh1-luc encoding the entire shRNA-Luc$^+$ hairpin sequence and pol III termination signal (5′-AAAAATGGATTCCAACTCAGCGAGAGCCACCCGATCAAGCTTCATCAGGTGGCTCCCGCTGAATTGGAATCCggtgtttcgtcctttccacaa-3′). The segment of the reverse primer that anneals to the promoter region is shown in lower case. The expression cassette was amplified in a 100 μl reaction containing 1× PCR buffer with 0.2 mM of each dNTP, 2.5 U of ExTaq DNA polymerase (Takara Mirus Bio, Madison, WI), 50 pmol of each primer, and 10 ng phU6. To generate the hU6shRNA-Luc$^+$ expression cassettes with extensions of up to 25 bp downstream of the termination signal, a second PCR was used to amplify the first product. In the second PCR, 0.1 ng of the first PCR product was amplified with 50 pmol of forward primer hU6-*Eco*RI and 50 pmol of the extension primer in 1× PCR buffer with dNTPs, as for the first PCR. Extension primers were 5′-AAAAATGGATTCCAACTCAG-3′ for the +0 extension, 5′-ACACTAAAAATGGATTCCAACTCAG-3′ for +5, 5′-GGGCAACACTAAAAATGGATTCCAACTCAG-3′ for +10, 5′-ACAATGGGCAACACTAAAAATGGATTCCAACTCAG-3′ for +15, 5′-ACAGGACAATGGGCAACACTAAAAATGGATTCCAACTCAG-3′ for +20, and 5′-TCAGAACAGGACAATGGGCAACACTAAAAATGGATTCCAACTCAG-3′ for +25. The cycling conditions to generate shRNA and siRNA (separate sense and antisense strands) expression cassettes were 95 °C for 3 min; 30 cycles of 95 °C, 10 s, 62 °C, 20 s, 68 °C, 1 min; and a final extension of 68 °C for 10 min. The hU6 *luc*$^+$ siRNA expression cassettes (hU6s/asRNA-Luc$^+$) were designed to target nucleotides 850–869 of the *luc*$^+$ coding sequence [12]. These were generated with forward primer hU6-*Eco*RI and reverse primer 5′-AAAAAGTTGGCACCAGCAGCGCACggtgttcgtcctttccacaa-3′ for the sense strand cassette (hU6sRNA-Luc$^+$) or 5′-AAAAAGTGCGCTGCTGGTGCCAACggtgtttcgtcctttccacaa-3′ for the antisense strand cassette (hU6asRNA-Luc$^+$). The shRNA-GFP and hU6s/asRNA-SEAP expression cassettes were generated similarly with the siXpress Kit (Mirus Bio, Madison, WI) and are described further in Supplementary Data. The hU6s/asRNA-SEAP cassettes target nucleotides 868–894 in the coding region of human placental SEAP (Clontech, Mountain View, CA, GenBank Accession No. U89937). After cycling and verification of product size by agarose gel electrophoresis, PCR products larger than 300 bp were purified with the Montage PCR 96 Filter Plate (Millipore, Bedford, MA). PCR products smaller than 300 bp were purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Following either purification procedure, the PCR products were resuspended or eluted in 5 mM Hepes, pH 8.5. These pol III shRNA and siRNA expression cassettes were tested in cultured cells and were also cloned into plasmid vectors as described in Supplementary Data.

shRNA and siRNA expression cassettes generated by PCR from plasmids. The shRNA and siRNA expression cassettes were cloned into a uniform vector backbone that included either the hU6, mU6 or hH1 promoter. PCR constructs of the siRNA expression cassette could also include extensions of vector sequence either upstream of the promoter or downstream of the transcription termination signal, depending on the primers used. Methods and primers are described in Supplementary Data.

Forming dumbbell PCR constructs. Dumbbells were generated based on methods described by Kamiya et al. [28]. The hU6s/asRNA-SEAP +75 PCR products generated with primers 5′-GAGTCTGAGCCATAAGCTTCAAGGTCGGGCAGGAAGAGGG-3′ and 5′-GAGTCTGAGCCATAAGCTTGATCCTATCTCGAGAGCTGGCAAGG-3′ from plasmids encoding the expression cassettes were digested with *Hin*dIII, re-purified with the Montage Kit, added to a 10-fold excess of 5′ phosphate-labeled sticky end stem and loop-forming oligonucleotide 5′-pAGCTTATGGCGAAGCCATA-3′ in 1× T4 DNA ligase buffer (Takara Mirus Bio), and annealed by incubating at 60 °C for 5 min and snap cooling. T4 DNA ligase was added to the mixture that was then incubated overnight at room temperature, and

subsequently purified with the Montage Kit. The presence of dumbbell ends was verified by resistance to Bal31 nuclease digestion (1.5 U Bal31 in 5 μg DNA, incubated at ambient temperature for 10 min).

Reporter gene vectors. The *Photinus pyralis* (firefly) *luciferase*$^+$ plasmid pGL3-Control and the *Renilla reniformis* (sea pansy) *luciferase* plasmid pRL-SV40 were obtained from Promega. The firefly *luc*$^+$ reporter plasmid pHCR/UbC-*luc*$^+$ for prolonged expression in mice was constructed with the 774 bp human ApoE hepatic control region (HCR, as in [29]) upstream of the human ubiquitin C (UbC) promoter and first intron (from −333 to the second base of exon 2), followed by the firefly *luc*$^+$ cDNA and the SV40 polyadenylation signal. The HCR and UbC fragments were amplified from human genomic DNA (Promega). The *Renilla luciferase* plasmid for extended expression in vivo, pHCR/UbC-*Renilla*, was generated like pHCR/UbC-*luc*$^+$, except with the *Renilla luciferase* coding sequence from pRL-SV40 replacing the firefly *luc*$^+$ cDNA. The vector for long-term SEAP expression in vivo, pHCR/UbC-SEAP, was constructed like pHCR/UbC-*luc*$^+$ except that the human placental SEAP from pSEAP2-Basic (Clontech) replaced the *luc*$^+$.

Transfection of cells in culture. HeLa cells in 24-well plates were transfected in triplicate at 45–65% confluency. Each well received 50 μl Opti-MEM (Invitrogen, Carlsbad, CA), 1.5 μl *Trans*IT-LT1 (Mirus Bio), 249 ng pGL3-Control, 1 ng pRL-SV40, and 250 ng of siRNA expression cassettes (PCR constructs or plasmids). A master mix of pGL3-Control and pRL-SV40 was prepared in half of the Opti-MEM and then distributed to tubes that contained the siRNA cassette DNA. Another master mix of *Trans*IT-LT1 in the other half of the Opti-MEM was incubated at ambient temperature for 10 min and then distributed to the tubes containing the DNA. After a 10 min incubation, the mixtures were added drop-wise to cells in normal serum-containing medium. CHO-LUC cells were transfected in triplicate at 50% confluency in 24-well plates. For each well, 2 μl *Trans*IT-LT1 in 50 μl Opti-MEM were incubated 10 min at ambient temperature. Then, 500 ng of hU6shRNA expression cassette DNA (PCR product or plasmid) was added, mixed gently, incubated 10 min more at ambient temperature, and then the mixture was distributed drop-wise to the wells. After transfection, cells were incubated at 37 °C, 5% CO_2 for 48 h, and then harvested and evaluated for luciferase expression.

Delivery of naked DNA to mice by hydrodynamic tail vein injection. Plasmids and PCR constructs in 2 ml of 1× Ringer's solution were injected into the tail vein of 20 g C57Bl/6 mice (Harlan Sprague Dawley, Indianapolis, IN) in 5–7 s as described previously [27].

Reporter gene assays. HeLa cells transfected with both firefly and *Renilla luciferase* reporter genes were lysed and activity of firefly luc$^+$ and *Renilla* luciferase were measured with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The ratio of relative light units (RLU) from firefly luc$^+$ to RLU from *Renilla* luciferase was determined for each well. The ratios of triplicate wells transfected with a *luc*$^+$ siRNA expression cassette were averaged and scaled to the averaged ratios from the control samples transfected with GFP shRNA or SEAP siRNA expression cassettes. In order to measure firefly and *Renilla* luciferase activity in the livers of mice injected with reporter plasmids pHCR/UbC-*Luc*$^+$ and pHCR/UbC-*Renilla*, each liver was harvested and homogenized in 3 ml of LUX lysis buffer (0.1 M KH_2PO_4, pH 7.8, 1 mM DTT, and 0.1% Triton X-100). This lysate was diluted in Passive Lysis Buffer and assayed according to the manufacturer's protocol for the Dual-Luciferase system. The ratio of relative light units (RLU) from firefly luc$^+$ to RLU from *Renilla* luciferase was determined for each mouse liver and averaged for all the mice in each treatment group. The averaged ratios were scaled to the averaged ratios from the appropriate promoter control expressing shRNA-GFP. SEAP activity in mouse plasma was measured using the Phospha-Light chemiluminescent reporter assay Kit (Tropix, Bedford, MA), according to the manufacturer's instructions.

Results

Comparison of RNA polymerase III promoters in plasmids and PCR fragments for target gene knockdown in cultured cells

Plasmid and PCR-derived hH1, hU6, and mU6 promoter shRNA expression cassettes were assayed for knockdown activity in HeLa cells by co-delivery with a firefly *luciferase*$^+$ (*luc*$^+$) expression plasmid containing the target sequence and a *R. reniformis luciferase* expression plasmid to serve as a delivery control. A schematic of the PCR constructs containing the shRNA expression cassette is shown in Fig. 1A. All the constructs included a 25 bp extension of vector backbone sequence downstream of the transcription termination signal, as we had observed that the addition of nucleotides at this position could affect the construct's knockdown activity. Results indicate that shRNA expression from all three pol III promoters was sufficient to elicit >80% target gene knockdown, whether they were presented as plasmids or PCR fragments (Fig. 1B). However, those constructs harboring the hH1 promoter had slightly higher levels of knockdown (93%) than the mU6 (87%) or hU6 (84%) promoters (comparing plasmids). This trend was true for both PCR and plasmid constructs; however,

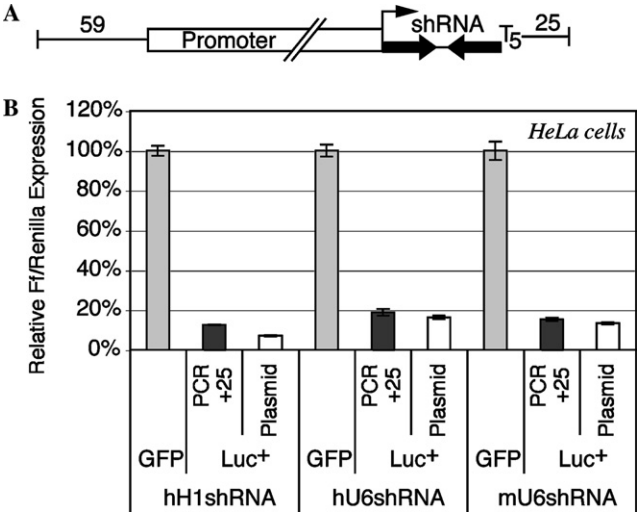

Fig. 1. Comparison of RNA polymerase III promoter constructs for target gene knockdown in HeLa cells. (A) Schematic of shRNA expression cassette PCR constructs that have 59 bp vector sequence upstream of a pol III promoter, shRNA coding sequence (inverted black arrows), a 5-thymidine termination signal (T_5), and a 25 bp extension downstream of the termination signal. (B) HeLa cells were co-transfected with SV40-driven reporter plasmids for firefly luc$^+$ and *Renilla* luciferase expression, and an shRNA-GFP or shRNA-Luc$^+$ expression cassette that contained the hH1, hU6 or mU6 promoter; shRNA-GFP PCR constructs, light grey bars; shRNA-Luc$^+$ +25 PCR constructs, black bars; shRNA-Luc$^+$ plasmids, white bars. Relative firefly luc$^+$ to *Renilla* luciferase activity is shown. Error bars indicate standard deviation (SD).

the PCR constructs performed less well than the plasmid constructs in each case.

Effect of added downstream or upstream length on knockdown activity of PCR-derived shRNA or siRNA expression cassettes in cultured cells

We performed experiments in CHO cells containing a stably integrated CMV-driven *luc*$^+$ gene to determine the number of additional downstream base pairs required for optimal activity of the shRNA expression PCR construct. A series of hU6shRNA-Luc$^+$ PCR constructs that had 5, 10, 15, 20 or 25 additional base pairs downstream of the transcription termination signal were generated. Those that had 10 or more base pair downstream extensions had significantly greater knockdown activity than the products that had either no extension or a 5 bp extension (Fig. 2). We have used a number of sequences in the downstream extension with the same result, suggesting that the length of the DNA extension beyond the termination signal is responsible for the enhanced activity rather than the sequence itself (data not shown). In agreement with results obtained in HeLa cells as described in the previous section, PCR constructs with 25 bp of additional downstream sequence still did not perform as well as plasmid constructs.

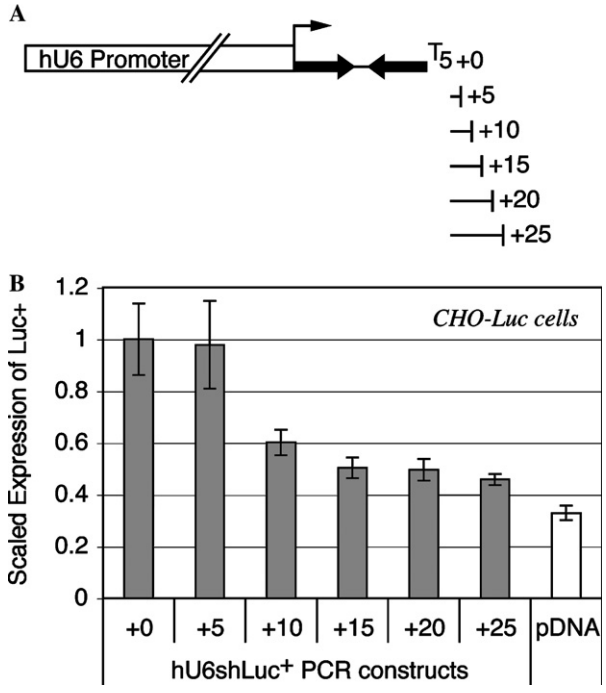

Fig. 2. Effects of varying the length of downstream extensions on shRNA expression constructs. (A) Schematic of U6shRNA-Luc$^+$ constructs with extensions added downstream of the termination signal. Indicated are the hU6 promoter (box), transcription start site (arrow), shRNA (inverted black arrows), termination signal (T_5), and extension lengths. (B) CHO-LUC cells that expressed *luc*$^+$ from stably integrated CMV-*luc*$^+$ were transfected with hU6shRNA-Luc$^+$ expression cassettes with 0, 5, 10, 15, 20 or 25 bp extensions (dark grey bars); or hU6shRNA-Luc$^+$ plasmid, white bar. Averaged relative light units (RLU) of firefly luc$^+$ in each sample were scaled to averaged RLU of luc$^+$ from cells transfected with the hU6shRNA-Luc$^+$ +0 cassette. Error bars indicate SD.

An alternate method for the generation of siRNA in a cell is to express each RNA strand from a separate expression construct. We tested whether these PCR constructs also required sequence extensions beyond the transcription termination signal for optimal activity. PCR products were generated from separate plasmids that encoded the hU6 promoter driving either the sense or antisense strand of the siRNA targeting *luc*$^+$. The length of the expression construct was varied upstream of the promoter and downstream of the termination signal. Upstream extensions were either 9 or 103 bp and downstream extensions were 0, 20, 40 or 148 bp (Fig. 3A). The siRNA expression constructs with downstream extensions of 20 bp or more were much more effective than those with no downstream extensions ($43 \pm 1\%$ remaining firefly luciferase activity from constructs with no extensions and $19 \pm 1\%$ from constructs with a 20 bp extension, Fig. 3B). Addition of even longer downstream extensions of 40 or 148 bp improved activity by only a few percent ($14 \pm 0.2\%$ remaining luc$^+$ activity). Addition of a 103 bp upstream extension only marginally improved the activity of constructs without downstream extensions. These results indicate that addition of at least 20 bp of sequence downstream of the transcriptional termination signal is required for optimal activity of both siRNA and shRNA PCR constructs in the cell lines tested. Again, even addition of the extension did not make PCR constructs as effective as corresponding plasmid constructs.

We tested the possibility that the requirement for additional downstream sequence might be an artifact created by the 5′ exonuclease activity of the thermostable *Taq* DNA polymerase used in the PCR or by the PCR process itself. However, PCR constructs generated with a variety of polymerases, with or without 5′ exonuclease activity, showed enhancement of activity when they possessed 20 bp downstream extensions compared to the constructs with no extensions (data not shown). Furthermore, when an shRNA PCR construct containing a 166 bp downstream extension was truncated by restriction enzyme digestion to an shRNA construct with just a 2 bp extension, it had approximately twofold less activity (data not shown). These results argue against the length requirement being an artifact of the type of polymerase used in the PCR process or of the PCR process itself.

Comparison of RNA polymerase III promoters for shRNA expression and target gene knockdown efficiency in mouse liver

We compared the ability of shRNA expression constructs containing different pol III promoters to elicit

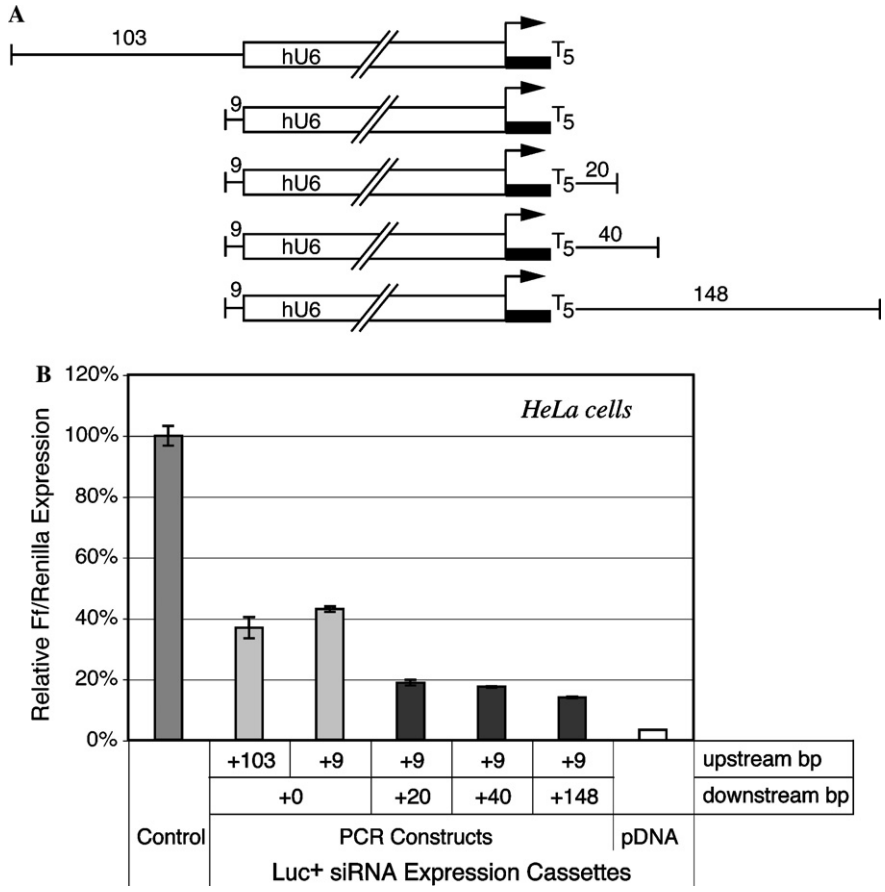

Fig. 3. Effects of adding upstream and downstream extensions on separate strand siRNA expression PCR constructs. (A) Schematic of siRNA expression PCR constructs with varied lengths of vector sequence upstream of the hU6 promoter (box) and downstream of the termination signal (T_5) and one strand of the siRNA sequence indicated by the small black box. (B) HeLa cells were co-transfected with the firefly *luc*$^+$ and *Renilla luciferase* expression plasmids and hU6 siRNA expression PCR constructs (sense and antisense constructs, s/asRNA). The averaged ratios of luc$^+$ to *Renilla* luciferase activity were scaled to the control: hU6s/asRNA-SEAP PCR construct control, dark grey bar; hU6s/asRNA-Luc$^+$ +0 constructs with 9 or 103 bp upstream extension, light grey bars; hU6s/as-Luc$^+$ PCR constructs with downstream extensions as indicated, black bars; plasmids expressing s/asRNA-Luc$^+$ (pDNA), white bar. Error bars indicate SD.

target gene knockdown in mice. In these experiments, we co-delivered by hydrodynamic tail vein injection shRNA expression cassette PCR constructs in which either the hH1, hU6 or mU6 promoter drives expression of a shRNA targeting the reporter firefly *luc*$^+$ gene, together with a firefly *luc*$^+$ expression plasmid and a control plasmid expressing the *Renilla luciferase* gene. For more stable reporter gene expression in liver, we placed these reporter genes under transcriptional control of the human ApoE hepatic locus control region (HCR) and the human ubiquitin C promoter and first intron (UbC). The shRNA expression cassette PCR constructs used in these experiments were the same as those used in HeLa cells, with downstream extensions consisting of 25 bp (Fig. 1A). Target gene knockdown in the liver was evaluated on day 5 (Fig. 4). Unlike the results in HeLa cells, these in vivo promoter comparison results indicate that PCR constructs containing the hH1 promoter were much less effective at target gene knockdown in mouse liver than those containing the hU6 or mU6 promoters (46 ± 13% versus 89 ± 1% knockdown). Similarly, the hH1 expression cassette contained in a plasmid was less effective than the comparable hU6 plasmid construct evaluated at 3 or 5 days after injection (data not shown).

The requirement for downstream extensions in siRNA expression PCR constructs and the time course of target gene knockdown in mice

We further tested the requirements for downstream extensions on siRNA expression PCR constructs in mice. In these experiments, we used secreted alkaline phosphatase (SEAP) as the target gene. The major advantage of using SEAP rather than *luciferase* as a reporter gene is that SEAP secretion into the serum can be used to monitor expression in the same mouse over time. In addition, SEAP is far less immunogenic in mice than luciferase (data not shown), allowing bona fide long-term reporter gene knockdown studies to be

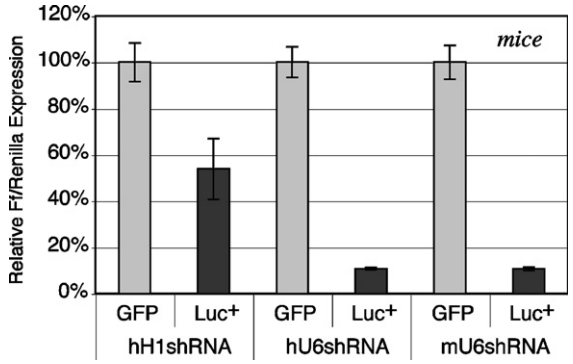

Fig. 4. Comparison of pol III promoter constructs for target gene knockdown in mouse liver. Mice were injected with HCR/UbC-*luc*$^{+}$ (10 μg) and an HCR/UbC plasmid expressing the control gene *Renilla luciferase* (40 μg), together with shRNA PCR constructs (10 μg) as shown in Fig. 1A targeting *luc*$^{+}$ or GFP and containing the hH1, hU6 or mU6 promoter. All shRNA cassettes included a 25 bp extension downstream of the termination signal (+25): shRNA-GFP constructs, grey bars; shRNA-Luc^{+} constructs, black bars. The relative firefly luc^{+} to *Renilla* luciferase activity is shown. $n = 4$, error bars indicate standard error of the mean (SEM).

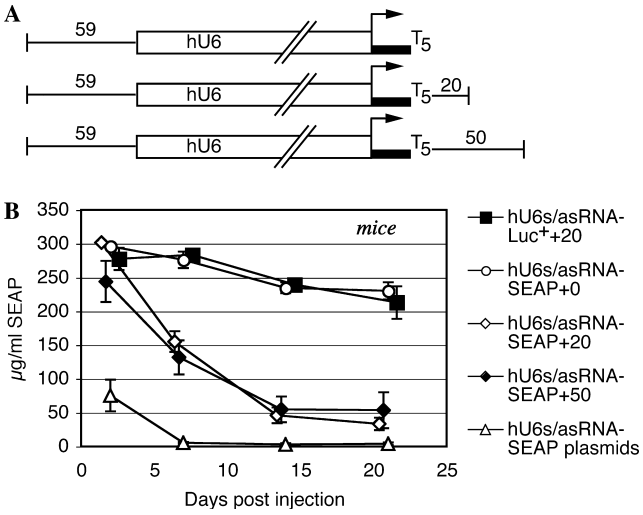

Fig. 5. Activity of siRNA expression PCR constructs in mice. (A) Schematic of separate strand siRNA expression PCR constructs that include vector sequence upstream of the promoter (59 bp), the hU6 promoter (white box), the siRNA sequence (small black box), and the length (in base pairs) of the extensions downstream of the termination signal. (B) Mice were injected on day 0 with 1 μg of pHCR/UbC-SEAP reporter plasmid, 10 μg of carrier plasmid, and 25 μg of combined sense and antisense siRNA expression cassettes in PCR constructs or in plasmids. SEAP activity in plasma was measured on the indicated days post injection, $n = 5$, error bars indicate SEM.

performed. We used the HCR/UbC promoter to drive sustained SEAP expression in the liver. In contrast to viral enhancer/promoters, the HCR/UbC promoter allows for high-levels of SEAP expression in mice for many months after delivery by hydrodynamic tail vein injection (Supplementary Fig. 1).

The pHCR/UbC-SEAP expression vector was co-delivered into mice with pol III constructs coding for either the SEAP siRNA or a control siRNA. hU6 promoter-containing constructs expressing the separate strands of SEAP siRNA (hU6s/asRNA-SEAP) were generated by PCR from a promoter template plasmid and contained a 59 bp upstream extension and downstream extensions of 0, 20 or 50 bp (Fig. 5A). Constructs expressing the sense and antisense strands of siRNA-Luc^{+} (hU6s/asRNA-Luc^{+}) were used as the control. We earlier verified in HeLa cells that the SEAP siRNA construct caused no non-specific knockdown of reporter genes (the control siRNA constructs in Fig. 3B). Two days postdelivery of SEAP expression plasmid and siRNA expression constructs into mice, no knockdown of SEAP from PCR constructs with 0 or 20 bp extensions was evident and only marginal knockdown was evident from constructs containing 50 bp extensions (Fig. 5B). The presence of SEAP in all animals on day 2 in this study shows that the SEAP reporter plasmid was delivered. Similar SEAP expression levels in all mice within a group gave us further confidence that the SEAP expression plasmid was successfully delivered. By day 7, significant knockdown was detected in mice harboring constructs that had extensions of either 20 or 50 bp and the degree of knockdown increased from day 7 to day 14. No knockdown was detected using PCR constructs without downstream extensions (+0) at any time point. At day 14, PCR constructs with extensions of either 20 or 50 bp were similarly effective for knockdown, obtaining 81 ± 10% target gene knockdown using the hU6s/asRNA-SEAP +20 constructs and 77 ± 16% knockdown from the hU6s/asRNA-SEAP +50 constructs.

The potency of target gene knockdown mediated by plasmids encoding the same hU6s/asRNA-SEAP expression cassettes was also examined. Interestingly, the plasmid-based siRNA expression cassettes were much more potent for target gene knockdown than the hU6s/asRNA-SEAP +50 PCR constructs, reducing target gene expression by 98.7 ± 0.8% by day 14 (Fig. 5B). As with PCR constructs, there was a delay before maximal target gene knockdown was observed. A delay in target gene knockdown from a U6 shRNA expression plasmid in mice was also noted in other studies [30]. We observed that the degree of knockdown, as well as the time it takes to reach maximal knockdown, depended in part on the choice of target sequence (data not shown).

Increasing the potency of PCR construct siRNA expression cassettes in mice

One possibility for the requirement of downstream extensions on PCR constructs is that exonuclease activity present in cells or in the serum digests the ends of

the PCR constructs to some degree, potentially degrading the siRNA coding sequence. Based on our finding that 50 bp downstream extensions imparted no greater efficacy to the PCR constructs than 20 bp extensions, we hypothesized that the addition of 10–20 bases may be sufficient to protect these critical expression cassette sequences from limited exonuclease digestion. However, PCR constructs with extensions of this length still did not function as well as plasmid vectors, suggesting that there are other factors that limit the activity of these PCR constructs. One of these factors may be the small size of the PCR fragments that makes them poorer templates for pol III transcription than larger constructs. To test this, we asked if the efficacy of the PCR constructs could be improved by making them substantially longer at either the upstream or the downstream end. Long PCR constructs were generated that contain the same sequence as the hU6s/asRNA-SEAP +50 constructs but with an additional 563 bp on the upstream end or 554 bp on the downstream end (Fig. 6A). These were co-delivered to mice with the pHCR/UbC-SEAP target gene expression plasmid using hydrodynamic tail vein injection. Addition of these ~500 bp extensions to either end of the PCR constructs made them as effective as an equal mass of plasmids and 10 times more effective than an equal mass of the short PCR constructs (Fig. 6B).

It is plausible that pol III transcriptional activity is impacted by differences in DNA topology and that this plays a role in determining the activity of the PCR constructs. Kamiya et al. [28] found that transcription of a gene by RNA polymerase II (pol II) was enhanced when the gene was encoded in a linear DNA fragment with "dumbbell" ends. In these constructs, the 5′ phosphate of one strand of the duplex is ligated to the 3′ hydroxyl of the complement strand. To determine if adding dumbbell ends could enhance knockdown activity of the much smaller pol III dependent siRNA expression constructs, we ligated dumbbell DNA fragments onto the ends of the hU6 s/asRNA-SEAP PCR constructs. Dumbbells were generated from hU6s/asRNA-SEAP PCR constructs that included a 75 bp extension to accommodate construction, but otherwise resembled the previously used +50 constructs. The hU6s/asRNA-SEAP dumbbell constructs were co-delivered to mice with a fivefold higher dose of the pHCR/UbC-SEAP target gene expression plasmid (5 μg) than in the previous experiments (1 μg). We had observed that injection of a higher amount of the target gene plasmid led to a decrease in relative target gene knockdown (data not

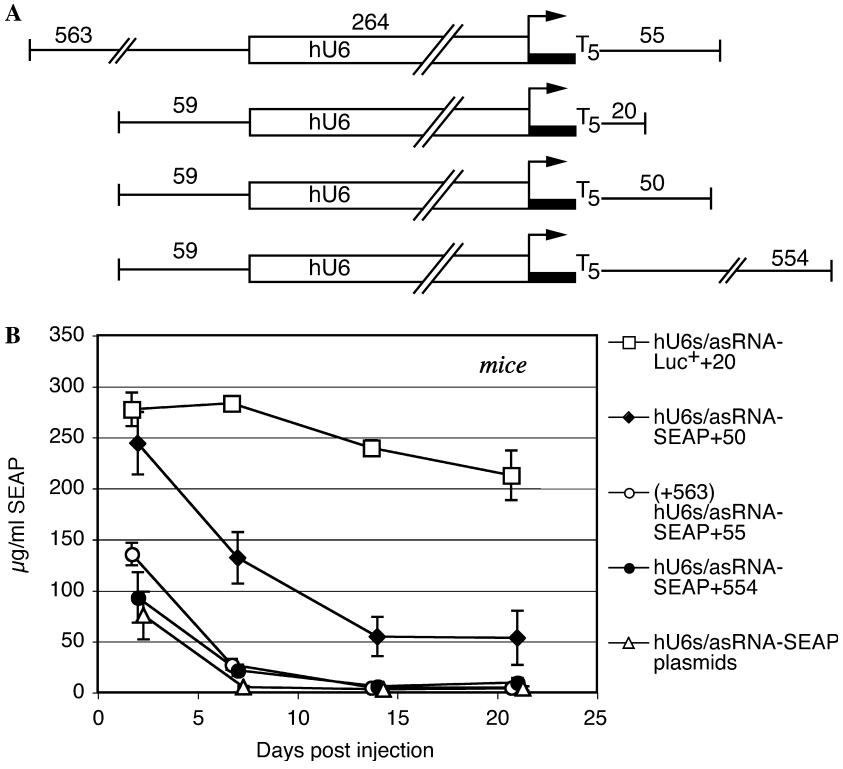

Fig. 6. Activity in mice of siRNA expression PCR products containing long extensions. (A) Schematic of hU6 promoter siRNA expression PCR constructs containing extensions upstream of the promoter or downstream of the termination signal. (B) Mice were injected on day 0 with 1 μg of pHCR/UbC-SEAP reporter plasmid, 10 μg of carrier plasmid, and 25 μg of combined sense and antisense siRNA expression constructs. SEAP activity in plasma was measured on the indicated days post injection and the average concentration is shown, $n = 5$; error bars indicate SEM.

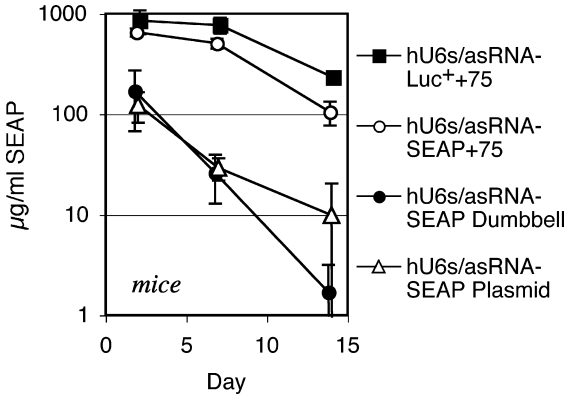

Fig. 7. Ligation of dumbbell termini on siRNA expression PCR constructs increases their efficacy in mice. Mice were injected on day 0 with 5 μg of pHCR/UbC-SEAP reporter plasmid, 20 μg of carrier plasmid, and 25 μg of plasmid or PCR construct hU6 siRNA expression cassettes. The siRNA expression PCR constructs targeting SEAP contained a +75 downstream extension with or without dumbbell termini. Control PCR constructs contained siRNA cassettes targeting firefly *luc*$^{+}$. SEAP activity in plasma was measured on the indicated days post injection and the average value is shown, $n = 5$; error bars indicate SEM.

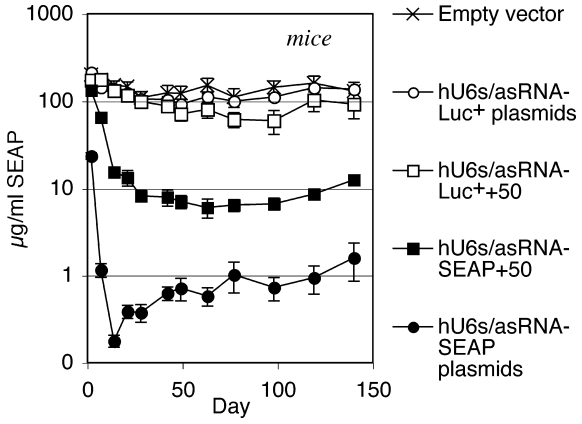

Fig. 8. Long-term RNAi in adult mice after delivery of siRNA expression constructs. The pHCR/UbC-SEAP reporter plasmids (1 μg), carrier plasmid (6 μg), and the experimental sample (25 μg PCR constructs or 40 μg plasmids) were co-delivered to mice on day 0. Empty vector, plasmids without the hU6 promoter and siRNA coding sequences; hU6s/asRNA-Luc^{+} plasmids, hU6 plasmids containing s/asRNA-Luc^{+} sequences; hU6s/asRNA-Luc^{+} +50, hU6 PCR constructs containing s/asRNA-Luc^{+} sequences and a 50 bp extension downstream of the termination signal; hU6s/asRNA-SEAP +50, hU6 PCR constructs containing s/asRNA-SEAP sequences and a 50 bp extension downstream of the termination signal; hU6s/asRNA-SEAP plasmids, hU6 plasmids containing s/asRNA-SEAP sequences. SEAP activity in plasma was measured at the indicated times post injection, $n = 6$; error bars indicate SEM.

shown). Therefore, a higher dose of target gene expression plasmid was used to more easily detect differences in knockdown activity between the highly potent siRNA expression plasmid and dumbbell constructs. As shown in Fig. 7, co-delivery of hU6s/asRNA-SEAP dumbbell constructs resulted in target gene knockdown that was equal to that achieved after co-delivery of hU6s/asRNA-SEAP plasmids. Delivery of hU6s/asRNA-SEAP +75 PCR constructs without dumbbell ends resulted in 7- to 10-fold less knockdown activity at all time points. These results indicate that placing dumbbell ends on the hU6s/asRNA-SEAP PCR constructs made them as effective as plasmid constructs for target gene knockdown when an equal mass was delivered. In this experiment, a notable decrease in SEAP expression over time was observed in the control group. This is likely due to the increased amount of carrier DNA used in this particular experiment, as a similar decrease was not observed in experiments in which lower amounts of carrier DNA were used (Figs. 5, 6 and 8 below). While the SEAP expression levels declined over time, the knockdown effects of SEAP siRNA expression constructs could easily be measured.

Long-term target gene knockdown from PCR constructs and plasmids in mice

Delivery of synthetic siRNA to mouse liver results in target gene knockdown for only a few days [22,24,25]. This time window may not always be sufficient for observing a knockdown-induced phenotype, especially if the targeted gene produces a protein with a long half-life. In order to determine the longevity of target gene knockdown in mice using pol III expression of siRNA, we again used the long-term SEAP expression plasmid, pHCR/UbC-SEAP, in co-delivery experiments with various hU6 siRNA expression constructs. In this experiment, we increased the amount of siRNA expression plasmids injected to 40 μg per mouse. We found that this amount gave maximal knockdown (data not shown). The amount of PCR constructs injected was left unchanged at 25 μg, as we had previously found that this dose gave maximal knockdown (data not shown). After delivery by hydrodynamic tail vein injection, the hU6s/asRNA-SEAP +50 PCR constructs inhibited target gene expression $23 \pm 19\%$ on day 2, $63 \pm 9\%$ on day 7, and $88 \pm 3\%$ on day 14 compared to the hU6s/asRNA-Luc^{+} +50 control (Fig. 8). After day 14, target gene knockdown remained at approximately 90% through week 20, at which time the experiment was terminated. Target gene knockdown using hU6s/asRNA-SEAP plasmid constructs also reached its greatest level by 14 days, but was much more effective, causing greater than 700-fold reduction in SEAP expression compared to the control hU6s/asRNA-Luc^{+} plasmids (99.9% knockdown). High levels of target gene knockdown in mice receiving the phU6s/asRNA-SEAP plasmids were also maintained for the duration of the experiment. These results indicate that >90% target gene knockdown can be achieved for at least several months after a single injection of pol III siRNA-expression constructs into mice.

Discussion

In this study, we used the hydrodynamic technique to co-deliver a target reporter gene with siRNA-expression constructs. Simultaneous delivery results in uptake of the various vectors by the same cells. This co-delivery strategy allowed us to perform experiments in mice and enabled accurate assessment of siRNA expression construct activity without complications arising from variable delivery efficiencies or targeting of an endogenous gene. Using this strategy, we first compared the target gene knockdown activity of shRNA-expression constructs containing the commonly used hU6, mU6 or hH1 promoters. In postnatal mice, we found that expression cassettes containing the mouse and human U6 promoters were much more effective for target gene knockdown than those containing the human H1 promoter. In contrast, all three promoters were similarly effective for target gene knockdown in cultured cells. It is unlikely that the low efficiency of the hH1 promoter construct in mice was due to species-specific differences, as shRNA expression cassettes containing this promoter directed high-level target gene knockdown in mouse cell lines from liver and other tissues (data not shown and [31]). Others have used the human or mouse H1 promoter to generate shRNA and knockdown phenotypes in transgenic mice following random transgenesis of the H1 shRNA expression cassette [5–7,32], but the injected embryonic stem cells or blastocysts had to be screened for sufficient siRNA expression before transgenic animals were generated. Indeed, Carmell et al. [6] reported that they were unable to obtain knockdown animals without prior screening. The degree of knockdown in the mice generated by random transgenesis varied with the integration site and number of integrated copies. Recently, Seibler et al. [8] reported they were able to generate transgenic mice in which a single copy of either the hH1 or the hU6 promoter drove expression of shRNA in nearly all tissues. These promoters were similarly effective when integrated into the transcriptionally active *rosa26* locus along with one or more pol II expression cassettes. Interestingly, the small 100 bp hH1 promoter in its genomic context is in very close proximity to a pol II promoter, leading us to speculate whether nearby pol II transcriptional activity is necessary to maintain accessible chromatin for transcription of the H1 promoter. In contrast, the U6 promoter may have the intrinsic capacity to maintain transcriptionally active chromatin by its ability to position nucleosomes. Stunkel et al. [33] have shown that a nucleosome positioned between the proximal sequence element (PSE) and distal sequence element (DSE) of the human U6 promoter activates transcription of this promoter in chromatin. DNA wrapping around this nucleosome brings together transcription factors bound to the PSE and DSE, thereby enhancing transcription from the hU6 promoter. Use of the more position-sensitive H1 promoter-containing constructs may have certain advantages. For example, Kunath et al. [7] reported variable levels of target gene knockdown among transgenic embryos and the level of knockdown correlated with the severity of developmental defect. Thus, use of an H1 promoter may be more suitable than use of the U6 promoter for situations in which isolation of transgenic animals displaying a wider range of hypomorphic phenotypes is desired.

PCR-based shRNA and siRNA expression cassettes have the potential to be utilized in ways that are distinct from plasmid or viral siRNA expression cassettes. Thus, we sought to determine the requirements for highly functional siRNA expression cassettes in small fragments of DNA. First, the effects of adding non-coding extensions to the PCR constructs were examined. For each of the pol III promoter constructs, we found that the promoter region alone provided an adequate length of DNA upstream of the transcription start site for a functional siRNA expression PCR construct. However, a marked increase in activity of the PCR constructs resulted from the addition of 20 bp onto the downstream end of the expression cassette. Therefore, the minimal sizes of hU6 and hH1 promoter-containing hairpin cassettes that showed optimal activity in cultured cells were 322 and 201 bp, respectively. The latter is below the molecular weight cutoff for free diffusion into the nucleus and may allow for greater delivery efficiency [20]. It is possible that the extra bases on PCR constructs protect the siRNA-coding region of the cassette from nucleases present in the cell or extracellular environment. The more dramatic effect of the downstream extensions in mice compared to cultured cells may be due to the fact that the DNA is delivered into mice without a complexing agent and thus is more susceptible to nucleases in the serum and in cells. When delivering DNA to cells in culture, complex formation with cationic transfection reagents helps protect the DNA from nuclease digestion ([34] and our unpublished observations). The degree of protection afforded by the reagent is likely significant, as even PCR constructs without extensions could in some cases elicit reasonable levels of RNAi in cultured cells.

Even with the addition of short downstream extensions, the potency of the PCR construct siRNA expression cassettes in mice was approximately 10-fold less than that of an equal mass of plasmid vectors bearing the same expression cassette, despite there being six times more molecules of PCR constructs. Additionally, there was a longer lag between the time of DNA delivery and the point of maximal inhibition obtained when these PCR constructs were used. The differences in efficacy could be eliminated by further lengthening the PCR constructs on either end (to approximately twice the size of the small construct) or by ligating dumbbell structures to the ends of the small PCR fragment

duplex. For each of the long PCR constructs, the unextended end would still be susceptible to nuclease digestion of sequences critical for siRNA expression. The differences in activity between the small PCR constructs and their much longer counterparts are, therefore, not likely due to further nuclease protection. One possible explanation is that the increased activity of the long PCR constructs is due to a more favorable positioning of nucleosomes. Our much longer PCR constructs would likely have multiple bound nucleosomes, stabilizing the position of the nucleosome bound between the DSE and PSE in the U6 promoter region. The small PCR constructs containing only 20–50 bp downstream extensions and up to 103 bp upstream of the promoter would not be long enough to allow a second nucleosome to form on the DNA fragment without disrupting the position of the nucleosome between the PSE and DSE. Further study would be required to determine if nucleosome remodeling plays a role in transcription of the PCR constructs described in this report.

The differences in efficacy between siRNA expression cassettes in plasmids and in PCR constructs with a short downstream extension could also be eliminated by addition of dumbbell ends to the small PCR construct. The dumbbell ends are expected to protect the PCR construct from exonucleases. They also appear to increase expression by affecting transcription [28]. A possible explanation for this effect is that covalently linking the DNA strands creates topological constraint. Torsional strain is known to affect RNA polymerase activity by helping to separate the DNA strands for transcription initiation [35–38]. Thus, the differences in the kinetics and extent of knockdown observed between plasmid and small PCR construct siRNA expression cassettes without dumbbell ends can be overcome by topologically constraining the small PCR constructs, causing them to be more transcriptionally active.

We demonstrated that high-levels of knockdown mediated by pol III siRNA expression cassettes contained in plasmid or PCR constructs perdured for at least 20 weeks in the livers of adult mice after a single administration. Given that the level of knockdown remained high over the course of the experiment (>99% for plasmid constructs), it is very likely that the siRNA expression constructs would continue to mediate RNAi in liver cells beyond 20 weeks and possibly for the life span of the cell. Maximum knockdown levels were achieved 7–14 days after siRNA expression construct delivery. Long-term gene knockdown after delivery of pol III-based siRNA expression constructs has important implications for the use of RNAi in gene function studies as well as in gene therapy settings. Achieving the desired long-term knockdown after a single administration would minimize the cost and possible side effects of the method used to deliver the expression cassettes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.06.066.

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