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Gene silencing in mammalian cells by PCR-based short hairpin RNA

Deming Gou, Nili Jin, Lin Liu*

Department of Physiological Sciences, Oklahoma State University, 264 McElroy Hall, Stillwater, OK 74078, USA

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Abstract RNA interference (RNAi) provides a powerful tool to silence genes in a sequence-specific manner in a variety of systems. However, not all sequences are effective in the RNAi-mediated gene silencing. In this study, we developed a polymerase chain reaction (PCR)-based RNAi strategy for a quick screening of small interfering RNA (siRNA) efficiency. This method utilized a two-step PCR to generate a chimeric DNA template containing the U6 promoter or cytomegalovirus promoter and short hairpin DNA. We demonstrated that the transfection of the PCR products into mammalian cells resulted in specific depressions of exogenous (luciferase, green fluorescent protein and β-galactosidase) and endogenous (annexin II) gene expressions. This PCR strategy provides a rapid, easy and cheap approach for testing candidates siRNA sequences and is an attractive alternative to subcloning.

Key words: RNA interference; Gene silencing; Annexin II; RNA polymerase promoter; Small interfering RNA

1. Introduction

One of the most frequently asked questions in biological research is what is the function of a particular gene product. A direct approach for answering the question is the loss-of-function study. In mice, gene knock-out is widely used, but it is time-consuming and costly. Other methods such as antisense oligonucleotides and dominant-negative have limited success because of insufficiency and inconsistency. Recently, a rapidly developed technique, RNA interference (RNAi), has been showing great promise in depressing gene expression in a variety of systems including *Caenorhabditis elegans*, *Drosophila*, fungi, plants and animals [1]. RNAi is a process of sequence-specific post-transcriptional gene silencing via double-stranded RNA (dsRNA). It is believed to have evolved as a host defense mechanism directed at transposable elements and infecting viruses. Naturally occurring RNAi proceeds via a two-step mechanism. In the first step, long dsRNAs are recognized by a nuclease in the RNase III family known as Dicer, which cleaves long dsRNA into double-strand fragments between 21 and 23 nt in length, termed small interfering RNA (siRNA). These siRNAs are incorporated into the RNA-induced silencing complex, which recognizes and cleaves the corresponding mRNA. Introduction of 21-nt RNA duplexes into mammalian cells leads to specific depression of target genes without triggering interferon responses [2,3]. This discovery significantly advances the field and provides a powerful tool for studying protein functions. Since then, RNAi has been applied to many different cell cultures including mammalian cells [2–4], primary cell culture [5–7], embryonic stem cells [8,9], and animals [10–14].

To knock down target genes in mammalian cells, chemically synthesized 21-nt siRNA duplexes were first used [2,3]. siRNA can also be in vitro transcribed with T7 RNA polymerase, and such siRNA was as effective as chemically synthesized siRNA [15,16]. To obtain stable transfection in cells and animals, DNA expression vector-based siRNA has been developed. In this strategy, a short hairpin RNA (shRNA) or antisense and sense RNAs are generated in cells under the control of an RNA polymerase II (cytomegalovirus, CMV) [11,17] or III (U6 or H1) promoter [15,18–22]. To increase transfection efficiency or integrate shDNA into the genome, a retroviral or lentiviral vector-based siRNA has been used [7,11,23–27].

Much progress has been made in perfecting the RNAi technique in a short period of time. However, the efficiency of siRNA is dependent on the identification of specific target sites because not all sequences are effective in RNAi-mediated gene silencing. To date, choosing of siRNA sequences is still empirical although a few rules can be followed. Generally, several siRNAs derived from different regions of a target mRNA need to be tested in order to achieve an efficient siRNA, which is costly and time-consuming. Therefore, a quick and easy method to obtain siRNAs for the screening of efficiency is warranted. In the current study, we described a polymerase chain reaction (PCR)-based shRNA strategy that eliminates the requirement to subclone the U6–shDNA cassette into an expression vector. The siRNA expression cassette is formed by two-step PCR from a plasmid-encoded U6 promoter as the template. The transfection of U6–shDNA PCR products into mammalian cells specifically depressed expression of exogenous and endogenous genes. Furthermore, the replacement of U6 promoter with CMV promoter had a similar result in gene silencing.

2. Materials and methods

2.1. Target sites for silencing gene expression

Firefly luciferase (pGL3), green fluorescent protein (GFP), β-galactosidase (LacZ) and an endogenous gene, annexin II, were used to test the efficiency of gene silencing by PCR products, which contain the U6 or CMV promoter and the corresponding targeted sequences. The sequences corresponding to target sites are as follows: coding regions 153–173 (5′-AACTTACGCTGAGTACTTCGA-3′) for pGL3; 104–127 (5′-AAGGTGATGCTACATACGGAAAGC-3′) for GFP; 427–447 (5′-AACTCGGCGTTTCATCTGTGG-3′) for LacZ; 495–495 (5′-AAGGACATCATCTCTGACACA-3′) or 876–896 (5′-AATCATGGTCTCTCGCAGTGA-3′) for mouse annexin II. Some of these

*Corresponding author. Fax: (1)-405-744 8263.
E-mail address: liulin@okstate.edu (L. Liu).

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sequences have been tested by other investigators using different methods and were effective in silencing gene expression [2,28].

2.2. Preparation of PCR products containing U6 promoter and shDNA

Using *Apa*I-digested plasmid pSilence1.0-U6 (Ambion) as a template, U6 promoter followed by a 19–22-nt sense strand of siRNA, a 9-nt loop (5′-TTCAAGAGA-3′), a 19–22-nt antisense strand of siRNA, and a stretch of six deoxythymidines (poly(T)$_6$) were amplified by two-step PCR. The forward primer, 5′ P_{U6}F (5′-CACCGAATTGGGTACCCGCTC-3′), is complementary to the 18 nt at the 5′-end of the U6 promoter and was used for all PCR steps. 5′ P_{U6}F contains four nucleotides, CACC, which will enable directional cloning of PCR products into adenovirus expression systems in future. In the first round of PCR, the reverse primer, 3′ Primer 1 consisted of (5′) the 9-nt loop, a 19–22-nt antisense sequence and GGCC (3′). The reaction conditions were 95°C, 3 min; 95°C for 30 s, 60°C for 40 s, 72°C for 40 s, 35 cycles; 72°C, 10 min. One microliter of this first PCR reaction mixture was re-amplified in the second round of PCR reaction using the same forward primer 5′ P_{U6}F and a new designed reverse primer, 3′ Primer 2, which contains (5′) poly(A)$_6$, a 19–22-nt sense strand of siRNA, and a complementary sequence to the 9-nt loop (3′). The optimal annealing temperature was increased to 62°C, 40 s. The PCR products were purified using QIAquick PCR purification kit (Qiagen).

2.3. Preparation of PCR products containing CMV promoter and shDNA

The CMV promoter was amplified using the plasmid pcDNA3.1/CT-GFP (Invitrogen) as a template by a CMV forward primer, 5′ P_{CMV}F (5′-CACCGATGTACGGGCCAGATATACG-3′), and a reverse primer complementary to the 3′-end of the CMV promoter (5′-TCTCTTGAAGGGCCCTAGTTAGCCAGAGAGCTCTGC-3′). The *Apa*I restriction site (GGGCCC) was added at the 5′-end of the reverse primer. After digestion of the CMV promoter with *Apa*I, we used the same two-step PCR as described above to make the PCR products containing CMV promoter and shDNA except that 5′ P_{CMV}F was used as the forward primer. To generate the PCR products containing the CMV promoter and a sense or an antisense strand of siRNA, one-step PCR was performed using the CMV forward primer 5′ P_{CMV}F and the reverse primer containing the antisense or sense sequence and a complementary sequence (5′-AGTTAGCCAGAG-3′) to the last 12 nt of the CMV promoter (3′ primer A or 3′ primer B). The PCR conditions were as follows: 30 s at 95°C, 30 s at 58°C, 45 s at 72°C for 35 cycles.

2.4. Cell culture and transfection

NIH/3T3 and L-2 cells, and 293A cells were purchased from ATCC and Invitrogen, respectively. L929 cells were a generous gift from Dr. R. Eberle (Oklahoma State University). NIH/3T3, 293A, and L929 cells were cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose, 44 mM $NaHCO_3$ and 10% fetal bovine serum (FBS). L-2 cells were maintained in F-12K medium (ATCC) supplemented with 10% FBS. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C. All media were supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). FBS was heat-inactivated. Twenty-four hours before transfection, the cells at 50–80% confluence were trypsinized, diluted 1:5 with fresh medium without antibiotics ($1–3\times10^5$/ml), and transferred to 24-well plates (500 μl/well). Reporter gene plasmids, pcDNA3.1/CT-GFP and pLenti6/V5-GW/LacZ, and pGL3 and pRL were obtained from Invitrogen and Promega, respectively. Co-transfection of 0.5 μg of reporter gene plasmids and 0.3 μg of PCR products was carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection, the cells were incubated for 24 h for reporter gene expression and for 48 h for endogenous gene expression. Luciferase expression was subsequently monitored with the dual luciferase assay (Promega). To detect the expression of the LacZ gene, the fixed cells were incubated at 37°C overnight with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) at pH 8.2. Staining for X-gal at this pH gave no non-specific staining in the control culture. The expression of GFP was directly detected by an inverted fluorescence microscope or Western blot.

2.5. Western blot

The cells were harvested and lysed with the lysis buffer (250 mM sucrose, 1% Triton X-100, 10 mM EGTA, 2 mM EDTA, 50 mM Tris–HCl, pH 7.4, 200 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4). The cell lysate (10–20 μg protein) was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking non-specific binding sites with 5% non-fat milk, the membrane was incubated with primary antibodies for 1 h at room temperature. The primary antibodies used in this experiment were as follows: anti-GFP antibody (BD Biosciences, 1:1000 dilution) and anti-annexin II polyclonal antibody (Santa Cruz Biotechnology, 1:3000 dilution). After washing, the blot was incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG, and immunoreactive bands were visualized with the ECL kit (Amersham Biosciences).

3. Results and discussion

3.1. Design of U6–shDNA PCR products

siRNA can be synthesized in cells using expression vectors containing a short hairpin structure (shDNA) and a RNA polymerase II (CMV) [11,17] or III (U6 or H1 promoter [7,15,18–22]. Although the process can yield large quantities of plasmids, it is time- and labor-intensive, especially when several siRNA designs need to be tested. Since PCR is in routine use in most of the biomedical research laboratories, and is relatively faster than constructing vectors, we attempted to make a DNA template containing U6 promoter and shDNA by PCR for in vivo synthesis of siRNA in cells.

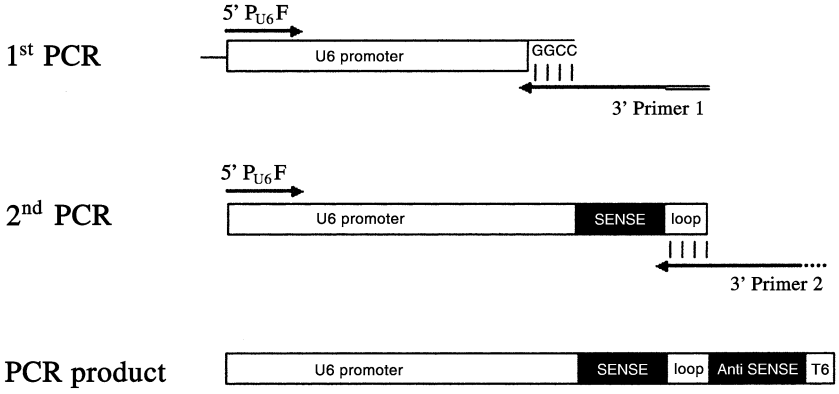

Fig. 1. Schematic representation of the PCR strategy used to yield PCR-based siRNA expression cassettes. Two-step PCR. The first PCR amplified U6 promoter–sense strand–loop (7 nt) using primer pairs 5′ P_{U6}F and 3′ Primer 1 (5′ loop–antisense strand–GGCC 3′). The second PCR amplified U6–promoter–sense strand–loop–antisense strand–poly(T)$_6$ using primer pairs 5′ P_{U6}F and 3′ Primer 2 (5′ poly(A)$_6$–sense strand–the 7-nt loop 3′). The final PCR products consisted of U6 promoter, shDNA and terminator sequence, six thymidines (T6).

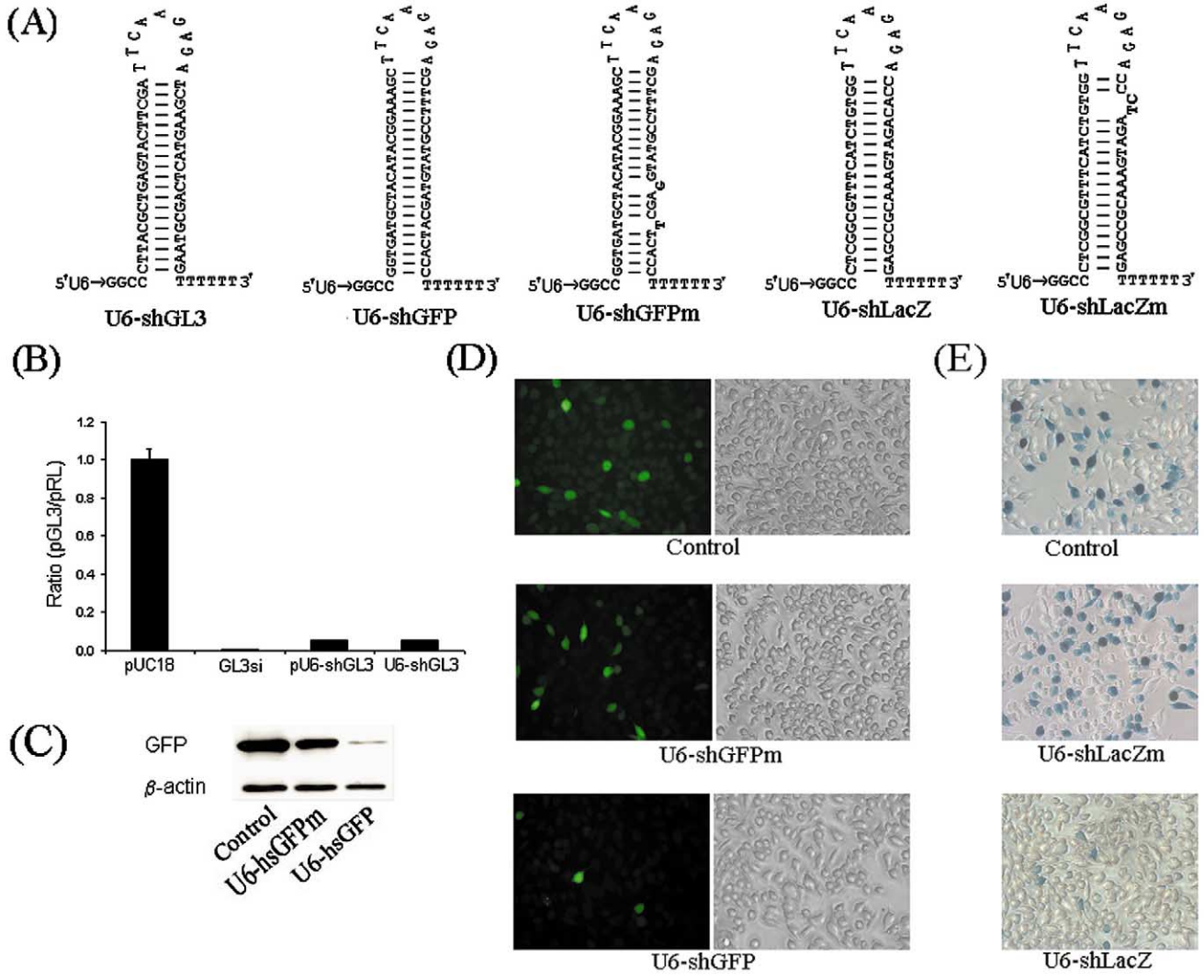

Fig. 2. Inhibition of exogenous genes by U6–shDNA PCR products. A: Sequence and putative folding of short hairpin oligonucleotides used to target the pGL3, GFP and LacZ genes. The 19–22-nt sequence of the target transcript separated by a short spacer (shown as a loop) from the reverse complement of the same sequence is shown. The termination sequence consists of six thymidines (T6). In U6–shGFPm and U6–shLacZm, two-base mismatches only occur in the antisense strands (shown in bold). B: Dual luciferase reporter system: U6–shGL3 PCR products were co-transfected for 24 h with pGL3 and pRL plasmids into L929 cells. pGL3 activity was normalized by pRL. For comparison, chemically synthesized siRNA duplexes (GL3–si) and a shGL3 U6 plasmid (pU6–shGL3) were included. The target sequence for pGL3 is the same. pUC18 plasmid was used as a control. C,D: The PCR products, U6–shGFP or a mutated form (U6–shGFPm), were co-transfected for 24 h with a GFP plasmid into L929 cells. GFP expression was detected by Western blot (C) or a fluorescence microscope (D). U6–shLacZ PCR products were used as a control. In the Western blot, β-actin was used as a loading control. E: The PCR products, U6–shLacZ or a mutated form (U6–shLacZm), were co-transfected for 24 h with a LacZ plasmid into L929 cells. LacZ was detected by staining with X-gal. U6–shGFP PCR products were used a control.

This was accomplished by two rounds of PCR as shown in Fig. 1. We first digested pSilencer™ 1.0-U6 plasmid with *Apa*I to generate a GGCC overhang at the 3′-end of the U6 promoter. The first round of PCR was performed using a forward primer, 5′ P_{U6}F, derived from the very beginning of the U6 promoter and a reverse primer, 3′ Primer 1, containing (5′) a loop sequence (9 nt), an antisense strand of siRNA and four base GGCC (3′), which is complementary to the *Apa*I-digested overhang at the 3′-end of the U6 promoter. In the second round of PCR, we used the same forward primer and a new reverse primer, 3′ Primer 2, containing (5′) a stretch of six deoxyadenosines (A_6), a sense strand of siRNA, and a sequence complementary to the 9-nt loop sequence (3′). The final PCR products (U6–shDNA) contain U6 promoter, sense, loop, antisense and a terminator (T_6) sequence. These PCR products were purified before use. Our design for the U6–shDNA PCR products has the following features. (i) The forward primer 5′ P_{U6}F is universal and can be used for all target genes. (ii) In the first round of PCR, we only used a 4-nt GGCC at the 3′-end of the reverse primer to complement the 3′ overhang of the U6 promoter generated by *Apa*I. In this case, the annealing temperature can be increased to 60°C in order to obtain a single-band PCR product. (iii) All primers used in the PCR reactions are relatively short (≤ 34 nt). This reduces errors that can occur during the synthesis of long primers (>40 nt). Our initial design utilized a one-step PCR reaction using the forward primer 5′ P_{U6}F and a long reverse primer (61 nt) that harbors (5′) terminator, sense, loop, antisense and GGCC. However, we found that more than 70% of inserts have mutations after cloning the PCR

products into a plasmid. These mutations were due to the synthesis of the long primer because all of those mutations were located at the 3′-end of the reverse primer. We therefore chose a two-step PCR to synthesize the sense and antisense strands separately and to link the two strands by inserting the complementary 9-nt loop sequence into each strand. (iv) To avoid the synthesis of long primers, we did not incorporate any other restriction sites into the PCR products, except four bases (CACC) at the 5′-end of the forward primer. Thus, we can clone the final selected PCR products into expression vectors through Topo directional TA cloning after testing their efficiency.

3.2. *Efficient inhibition of both exogenous and endogenous genes by U6–shDNA PCR products*

To determine whether this PCR-based approach can be used to inhibit exogenous gene expression in mammalian cells, we used a dual luciferase reporter system [2]. In this system, firefly luciferase plasmid (pGL3) and sea pansy luciferase plasmid (pRL) were co-transfected into L929 cells (mouse fibroblast) along with U6–shGL3 PCR products, which are predicted to synthesize siRNA targeting to the firefly luciferase mRNA (Fig. 2A). Silencing of pGL3 by the U6–shGL3 products was then determined by the amount of reduction of pGL3 activity after normalization with pRL activity to keep the transfection efficiency at the same level. As shown in Fig. 2B, the U6–shGL3 PCR products reduced pGL3 activity by 95%. The depression of pGL3 gene expression is comparable to the chemically synthesized 21-nt siRNA duplexes (GL3–si) or a U6 plasmid containing the same shDNA (pU6–shGL3). A similar result was obtained in NIH/3T3 cells (mouse fibroblast) and L-2 cells (rat lung epithelial cells) (data not shown).

We next tested other two reporter genes, GFP and LacZ, using the PCR-based RNAi strategy (Fig. 2A). The U6–shDNA PCR product targeted to GFP (U6–shGFP) was co-transfected with pcDNA3.1/CT–GFP plasmid into L929 cells, and Western blot was used to detect GFP protein. After normalizing to β-actin, GFP expression in the U6–shGFP-transfected cells was reduced to 8.5% of the control cells (U6–shLacZ-transfected cells) as determined by densitometry. However, a two-base mismatch (U6–shGFPm) only slightly affected the GFP expression (75% of the control) (Fig. 2C), indicating that the inhibition is highly sequence-specific. A direct visualization under a fluorescence microscope confirmed the result from Western blot (Fig. 2D). A similar result was obtained in 293A cells (data not shown). The U6–shLacZ PCR product specifically depressed LacZ expression in L929 cells (Fig. 2E) and 293A cells (not shown). A two-base mismatch (U6–shLacZm) eliminated the RNAi effect (Fig. 2E). By counting LacZ-positive cells, we estimated that the LacZ expression in the U6–shLacZ- and U–shLacZm-transfected L929 cells were 9.5% and 109% of the control cells (U6–shGFP-transfected cells), respectively.

We further tested whether U6–shDNAs constructed by PCR work on endogenous genes. We chose annexin II, an abundant Ca^{2+}-dependent, phospholipid/actin-binding protein [29]. Two siRNA sequences specifically targeted to mouse annexin II were chosen from the mouse annexin II mRNA se-

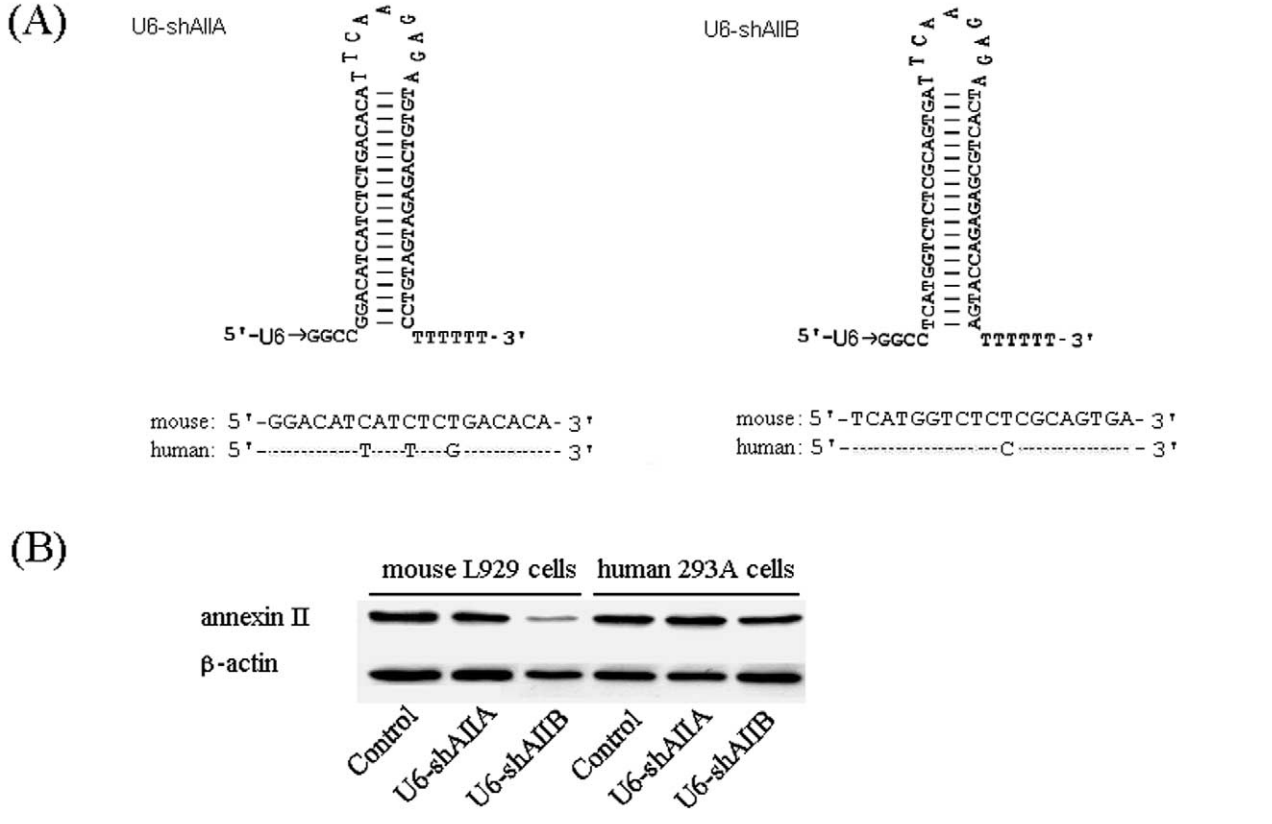

Fig. 3. Inhibition of endogenous gene expression of annexin II by U6–shDNA PCR products. A: Sequence and putative folding of short hairpin oligonucleotides used to target the mouse annexin II gene. Note that there are one- or three-base mismatches in the target sequences of mouse and human. B: The PCR products, U6–shAIIA and U6–shAIIB, were transfected into mouse L929 cells and human 293A cells for 48 h. U6–shGL3 PCR products were used as a control. Annexin II was detected by Western blot. β-Actin was used as a loading control.

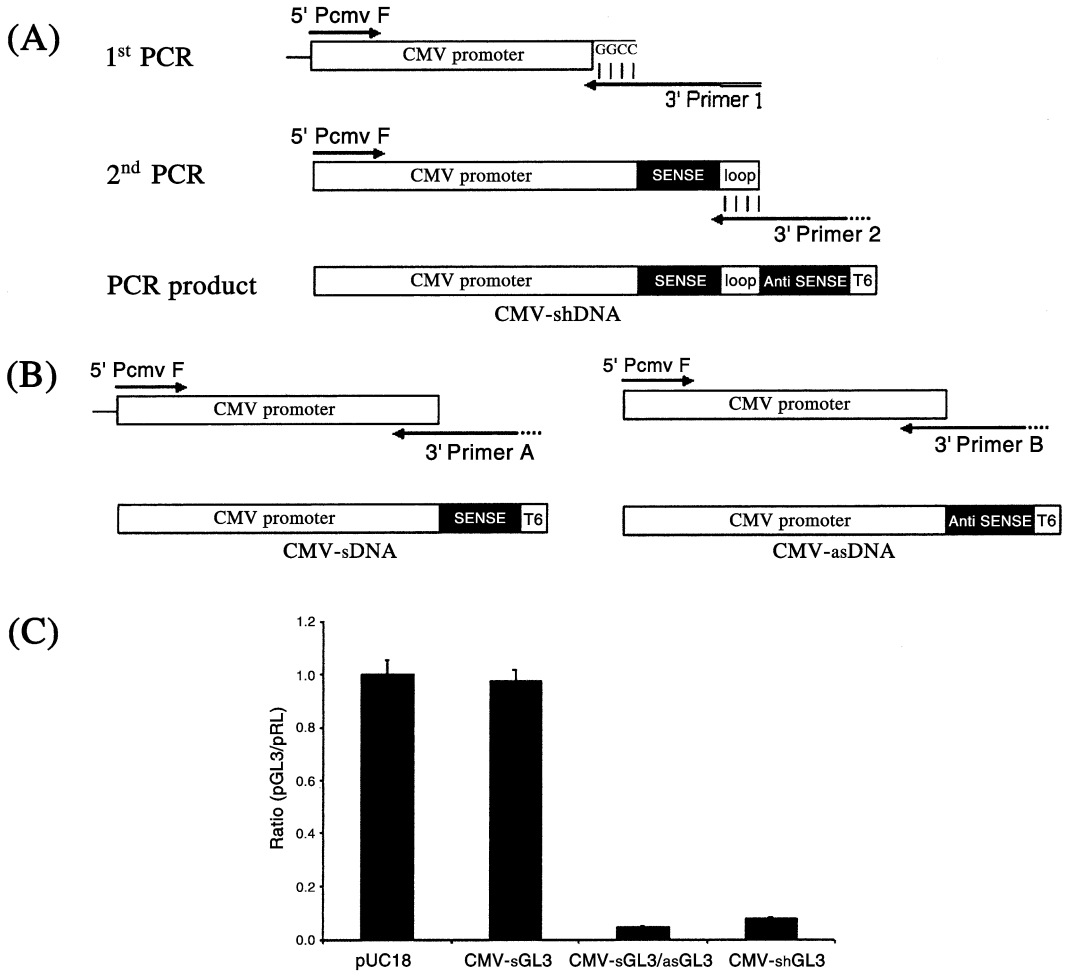

Fig. 4. Inhibition of gene expression by CMV–shDNA PCR products. A: CMV–shDNAs were generated by two rounds of PCR using a forward primer, 5′ P_{CMV}F, and two reverse primers, 3′ Primer 1 (5′ 7-nt loop–antisense strand–GGCC 3′) and 3′ Primer 2 (5′ poly(A)$_6$–sense strand–7-nt loop 3′). A linker containing an *Apa*I restriction site was added to the 3′-end of the CMV promoter. The final PCR products were CMV–shDNA. B: Two PCR products containing CMV–sense strand (CMV–sDNA) and CMV–antisense strand (CMV–asDNA) were generated by one-step PCR using primer pairs: 5′ P_{CMV}F and 3′ Primer A or B (5′ antisense strand or sense strand–a sequence complementary to the last 12 nt of the CMV promoter 3′), respectively. C: Two PCR products targeted to pGL3 were used for gene silencing: CMV–shGL3 (CMV-driven short hairpin DNA) and CMV–sGL3/CMV–asGL3 (CMV-driven sense strand or antisense strand of siRNA). The target sequence for pGL3 is the same as in Fig. 2A. CMV–shGL3, CMV–sGL3, or CMV–sGL3/CMV–asGL3 were co-transfected for 24 h with pGL3 and pRL plasmids into L929 cells. pUC18 plasmid was used as a control. pGL3 activity was normalized to pRL activity.

quence (Fig. 3A). When the PCR products were transfected into mouse L929 cells, we found that only U6–shAIIB reduced annexin II protein expression. However, U6–shAIIB had little effect in human 293 cells even though there is only one base mismatch in the target sequences of mouse and human (Fig. 3B). After measurement by densitometry and normalizing to β-actin, annexin II protein expression in U6–shAIIB-treated mouse L929 cells and human 293A cells was 20% and 74% of the control (U6–shGL3-treated cells), respectively. Another design of the annexin II siRNA sequence (U6–shAIIA) did not inhibit annexin II protein expression in either cell line (Fig. 3B). These results suggest that (i) U6–shDNA PCR product can be used to reduce endogenous gene expression; (ii) the silencing is dependent on target sequences and is highly specific.

3.3. Inhibition of gene expression by CMV-shDNA PCR products

Most of the vectors currently used for constructing shRNA utilized an RNA polymerase III promoter, U6 or H1 [7,15,18–22]. These promoters have a well-defined termination site of four or five consecutive thymidines and therefore are suitable for the synthesis of small RNA in cells. A modified CMV polymerase II promoter plus a minimal poly(A) cassette has been used to synthesize shRNA [11,17]. We asked whether PCR-based siRNA under the control of a RNA polymerase II promoter could inhibit gene expression. Since the PCR products only contain a promoter and shDNA, there is no need for transcripts to be terminated with a transcriptional termination signal at the 3′-end of the PCR products. However, to preserve some of the features of the siRNA defined by Tuschl and colleagues [2], six T were added at the 3′-end of the PCR products. We used two designs in this experiment. First, a strategy similar to the one used for the U6 promoter was used (Fig. 4A). We first added a linker containing the *Apa*I restriction site at the 3′-end of the CMV promoter. After digestion with *Apa*I, we performed the first round of PCR with a forward primer, 5′ P_{CMV}F, and a reverse primer, 3′ Primer 1, containing (5′) a loop (9 nt)–antisense strand (19 nt)–GGCC (3′) and the second round of PCR with the

5′ P_{CMV}F and another reverse primer, 3′ Primer 2, containing (5′) poly(A)$_6$–sense strand (19 nt)–the 9-nt loop (3′). The final PCR products contain the CMV promoter and shDNA followed by T6. The second design was to generate siRNA from two PCR products: CMV–sense strand–T6 and CMV–antisense strand–T6, using the same forward primer, 5′ P_{CMV}F, and a reverse primer containing A6, antisense or sense strand, and a sequence complementary to the 3′-end of the CMV promoter (3′ Primer A or 3′ Primer B) (Fig. 4B). We tested the CMV-driven siRNA on L929 cells with co-transfection of two reporter plasmids encoding pGL3 and pRL. The results are shown in Fig. 4C. The CMV–shGL3 PCR product depressed pGL3 activity by 92%. There is no quantitative difference between U6-driven shRNA and CMV-driven shRNA products (compare Figs. 2B and 4C). While CMV–sense strand of pGL3 siRNA (CMV–sGL3) had no effect, co-transfection of CMV–sense strand and CMV–antisense strand of pGL3 siRNA (CMV–sGL3/CMV–asGL3) resulted in a 95% inhibition of pGL3 activity. These results indicate that (i) the CMV promoter is as effective as the U6 promoter in the PCR-based RNAi strategy. We speculate that other polymerase II promoters could also be used in the PCR-based RNAi strategy. It will be interesting to knock down gene expression using cell- or tissue-specific promoters based on this PCR method. For example, to achieve cell-specific expression, the SP-C promoter can be chosen to target siRNA to alveolar epithelial type II cells. (ii) The short hairpin structure is not essential for the gene silencing, and sense strand and antisense strand can be synthesized separately using the same promoter. However, for future cloning purposes, the short hairpin design is preferred.

Castanotto et al. [30] recently reported an approach similar to the present study. However, they only gave preliminary qualitative results using a GFP reporter gene and a U6 promoter. In the current study, we performed more detailed studies. (i) Three reporter genes (luciferase, GFP and LacZ) were tested and quantitative results were given. (ii) We also demonstrated that the PCR-based shRNA strategy also works on an endogenous gene. (iii) In addition to the U6 promoter, we showed that other promoters such as the CMV promoter can be used to replace the U6 promoter. This is important because potentially we can use this approach to screen tissue- or cell-specific promoter-driven shRNAs for tissue- or cell-specific targeting of siRNA.

In summary, we presented a rapid, easy and cheap PCR method for screening shRNAs for functional studies. This method is composed of two rounds of PCR using one universal primer (for all target genes) and two unique target sequence oligonucleotides. The PCR products provide DNA templates containing a RNA polymerase promoter and a short hairpin DNA or sense/antisense strand for in vivo synthesis of siRNA in cells. The PCR products generated by this method are highly efficient and specific. One- or two-base mismatches eliminated gene silencing effects. Depending on the needs of future cloning, different promoters can be used. Although we only tested two promoters, U6 and CMV, any other promoters should be adapted for this method. Finally, we suggest a general approach for using RNAi in a loss-of-function study: (i) choose four or five siRNA sequences from a target gene based on currently known rules (e.g. http://www.mpibpc.gwdg.de/abteilungen/100/105/index.html); (ii) test the efficiency of selected sequences using the PCR method developed in this study and choose the most efficient sequence for cloning; (iii) clone into an expression plasmid or a virus vector; (iv) test on cells and animals for knocking down the target gene and study phenotypic and functional changes.

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References

[1] Hannon, G.J. (2002) Nature 418, 244–251.
[2] Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Nature 411, 494–498.
[3] Caplen, N.J., Parrish, S., Imani, F., Fire, A. and Morgan, R.A. (2001) Proc. Natl. Acad. Sci. USA 98, 9742–9747.
[4] Chiu, Y.L. and Rana, T.M. (2002) Mol. Cell 10, 549–561.
[5] Gaudilliere, B., Shi, Y. and Bonni, A. (2002) J. Biol. Chem. 277, 46442–46446.
[6] Krichevsky, A.M. and Kosik, K.S. (2002) Proc. Natl. Acad. Sci. USA 99, 11926–11929.
[7] Stewart, S.A., Dykxhoorn, D.M., Palliser, D., Mizuno, H., Yu, E.Y., An, D.S., Sabatini, D.M., Chen, I.S., Hahn, W.C., Sharp, P.A., Weinberg, R.A. and Novina, C.D. (2003) RNA 9, 493–501.
[8] Rubinson, D.A., Dillon, C.P., Kwiatkowski, A.V., Sievers, C., Yang, L., Kopinja, J., Zhang, M., McManus, M.T., Gertler, F.B., Scott, M.L. and Van Parijs, L. (2003) Nat. Genet. 33, 401–406.
[9] Kunath, T., Gish, G., Lickert, H., Jones, N., Pawson, T. and Rossant, J. (2003) Nat. Biotechnol. 21, 559–561.
[10] McCaffrey, A.P., Meuse, L., Pham, T.T., Conklin, D.S., Hannon, G.J. and Kay, M.A. (2002) Nature 418, 38–39.
[11] Xia, H., Mao, Q., Paulson, H.L. and Davidson, B.L. (2002) Nat. Biotechnol. 20, 1006–1010.
[12] Lewis, D.L., Hagstrom, J.E., Loomis, A.G., Wolff, J.A. and Herweijer, H. (2002) Nat. Genet. 32, 107–108.
[13] Hasuwa, H., Kaseda, K., Einarsdottir, T. and Okabe, M. (2002) FEBS Lett. 532, 227–230.
[14] Carmell, M.A., Zhang, L., Conklin, D.S., Hannon, G.J. and Rosenquist, T.A. (2003) Nat. Struct. Biol. 10, 91–92.
[15] Yu, J.Y., DeRuiter, S.L. and Turner, D.L. (2002) Proc. Natl. Acad. Sci. USA 99, 6047–6052.
[16] Donze, O. and Picard, D. (2002) Nucleic Acids Res. 30, e46.
[17] Zeng, Y. and Cullen, B.R. (2003) RNA 9, 112–123.
[18] Sui, G., Soohoo, C., Affar, E.B., Gay, F., Shi, Y. and Forrester, W.C. (2002) Proc. Natl. Acad. Sci. USA 99, 5515–5520.
[19] Brummelkamp, T.R., Bernards, R. and Agami, R. (2002) Science 296, 550–553.
[20] Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J. and Conklin, D.S. (2002) Genes Dev. 16, 948–958.
[21] Miyagishi, M. and Taira, K. (2002) Nat. Biotechnol. 20, 497–500.
[22] McManus, M.T., Petersen, C.P., Haines, B.B., Chen, J. and Sharp, P.A. (2002) RNA 8, 842–850.
[23] Barton, G.M. and Medzhitov, R. (1999) Proc. Natl. Acad. Sci. USA 99, 14943–14945.
[24] Devroe, E. and Silver, P.A. (2002) BMC Biotechnol. 2, 15.
[25] Qin, X.-F., An, D.S., Chen, I.S. and Baltimore, D. (2003) Proc. Natl. Acad. Sci. USA 100, 183–188.
[26] Shen, C., Buck, A.K., Liu, X., Winkler, M. and Reske, S.N. (2003) FEBS Lett. 539, 111–114.
[27] Abbas-Terki, T., Blanco-Bose, W., Deglon, N., Pralong, W. and Aebischer, P. (2002) Hum. Gene Ther. 13, 2197–2201.
[28] Lipardi, C., Wei, Q. and Paterson, B.M. (2001) Cell 107, 297–307.
[29] Gerke, V. and Moss, S.E. (2002) Physiol. Rev. 82, 331–371.
[30] Castanotto, D., Li, H. and Rossi, J.J. (2002) RNA 8, 1454–1460.