Journal of Biochemistry and Molecular Biology, Vol. 39, No. 2, March 2006, pp. 329-334

JOURNAL OF Biochemistry and Molecular Biology

Short communication

A Simple and Economical Short-oligonucleotide-based Approach to shRNA Generation

Jinsu Kim, Hyukmin Kim, Yoonsoo Lee, Kyungbae Yang, Sangwon Byun and Kyuhyung Han*
Department of Biomedical Science and Institute of Bioscience and Biotechnology, Hallym University, Chunchon 200-702, Korea

Received 10 November 2005, Accepted 19 January 2006

RNAi (RNA interference) has become a popular means of knocking down a specific gene *in vivo*. The most common approach involves the use of chemically synthesized short interfering RNAs (siRNAs), which are relatively easy and fast to use, but which are costly and have only transient effects. These limitations can be overcome by using short hairpin RNA (shRNA) expression vectors. However, current methods of generating shRNA expression vectors require either the synthesis of long (50-70 nt) costly oligonucleotides or multi-step processes. To overcome this drawback, we have developed a one-step short-oligonucleotides-based method with preparation costs of only 15% of those of the conventional methods used to obtain essentially the same DNA fragment encoding shRNA. Sequences containing 19 bases homologous to target genes were synthesized as 17- and 31-nt DNA oligonucleotides and used to construct shRNA expression vectors. Using these plasmids, we were able to effectively silence target genes. Because our method relies on the one-step ligation of short oligonucleotides, it is simple, less error-prone, and economical.

Keywords: Oligonucleotides, RNAi, shRNA, Vector

Introduction

RNA interference (RNAi) is a process whereby the presence or introduction of double-stranded RNA (dsRNA) into cells results in the degradation of homologous mRNA (Fire *et al*., 1998; Caplen *et al*., 2001). In mammalian cells, the antisense strand of synthetic short interfering RNA (siRNA) serves as a template for the RNA-induced silencing complex (RISC) to recognize and cleave complementary messenger RNA (mRNA), which is then rapidly degraded (Meister and Tuschl, 2004). RNAi has recently become a method of choice for mammalian cell genetic analysis and has the potential to be used to treat a variety of acquired and hereditary diseases (Cotrell and Doering, 2003; Caplen, 2004; Hannon and Rossi, 2004).

RNAi can be induced using either short interfering RNA (siRNA) or the endogenous expression of short hairpin RNAs (shRNAs) (Sandy *et al*., 2005). siRNAs are 21- to 22-nucleotide-long dsRNA molecules that contain a 19-bp core sequence and two unpaired nucleotides at each 3' end (Zamore *et al*., 2000). The most common approach used to generate siRNA is chemical synthesis, which is relatively easy and fast. However, the main drawbacks are that it is costly and that the silencing effect is transient; it usually lasts for a few days at most. These limitations can be overcome by using shRNAs, which are most commonly generated by the RNA polymerase III (Pol III)-mediated transcription of single-stranded RNAs of 50-70 nucleotides (nt) in length that contain two complementary 19-29 nt long RNAs separated by a short loop of 4-10 nt (Brummelkamp *et al*., 2002; Sui *et al*., 2002). Therefore, the transcript form of shRNA, with a double-stranded stem of 19-29 base pairs (bp), which is recognized and cleaved at the loop by Dicer, enters the RISC as siRNA *in vivo*. shRNA synthesized from vector-based expression is regarded as effective as siRNA synthesized *in vitro* in terms of suppressing the expressions of their corresponding genes (Dinh and Mo, 2005).

Vector-based expressions of shRNA and siRNA are commonly driven by U6 (Sui *et al*., 2002), H1 (Brummelkamp *et al*., 2002), or 7SK (Czauderna *et al*., 2003) promoters. Thus, to express an shRNA, an expression cassette encoding the following listed items in order, is inserted immediately downstream of the promoter, e.g., the top strand of the hairpin, the hairpin loop, the bottom strand of the hairpin, and the terminator. In the first method, two chemically synthesized 50-70 nt long oligonucleotides are annealed and then ligated into a vector. This is a relatively straightforward method, although the synthesis of such long oligonucleotides is error

*To whom correspondence should be addressed.
Tel: 82-33-248-2115; Fax: 82-33-256-3420
E-mail : khan@hallym.ac.kr

prone and rather costly. Alternatively, a Pol III promoter-shRNA template cassette can be assembled in a two-step PCR using shorter primers, which is less straightforward than the first method (Castanotto *et al*., 2002). The PCR product can be either cloned into a vector or directly transfected into cells. shRNAs can also be generated from target cDNA by enzymatic digest (Dinh and Mo, 2005). This method involves a multi-step processes that includes two digestion and ligation steps followed by amplification and cloning steps. However, it is likely that not all potentially effective target sequences will be incorporated into shRNA templates by this method, and in addition, some constructs may have non-specific effects. In the present study, we describe another approach, which is basically the same as the first method except that the two 50-70 nt-long oligonucleotides are replaced by two short oligonucleotides, of 17- and 31-nt in length, respectively. Since our method relies on a one-step cloning of dimeric dsDNA comprised of shorter oligonucleotides, it is more straightforward, less error-prone, and more cost-effective.

Experimental Procedures

Recombinant plasmids. A shRNA expression plasmid pI was constructed by ligating three DNA fragments, namely, a 2.9-kb *Hind* III-*Eco* RI fragment of pBluescript II KS<+>/LIC, a 0.23-kb *Eco* RI-*Spe* I fragment of PCR-amplified H1 promoter region, and 34-bp annealed oligonucleotides with *Spe* I and *Hind* III cohesive ends. The primers used for amplification of H1 promoter regions were 5'-catggaattcgaacgctgac-3' and 5'-ccgcactagttgaaagagtggtctcat ac-3', and the oligonucleotides inserted between H1 promoter and terminator were 5'-ctagtgagctcctcgaggaggcctgcagttttta-3' and 5'-agct taaaaactgcaggcctcctcgaggagctca-3'. pSUPER-Luc was constructed by inserting a short dsDNA made of two chemically synthesized 62-mer oligonucleotides into pSUPER (Brummelkamp *et al*., 2002). The oligonucleotides inserted were 5'-gatctttGCTCAACAGTATG AACATTactcaagAATGTTCATACTGTTGAGCtttttggaaa-3' and 5'-agcttttccaaaaaGCTCAACAGTATGAACATTcttgagtAATGTTCAT ACTGTTGAGCaaa-3'. pI-Luc1 and pI-Luc2 were constructed by ligating appropriate oligonucleotides and the pI fragment was prepared by *Bse* RI digestion The oligonucleotides used for pI-Luc1 construction were 5'-GCTCAACAGTATGAACA-3' and 5'-TTcagatctgAATGTTCATACTGTTGAGCTT-3', and the oligonucleotides for pI-Luc2 were 5'-AGTTGCGCGGAGGAGTT-3' and 5'-GTcagat ctgACAACTCCTCCGCGCAACTTT-3'. pI-PPARγ was constructed by ligating a pair of oligonucleotides and a *Bse* RI fragment of pI-stuff vector, which was constructed usaing pGem3 and pI plasmids. The oligonucleotides for pI-PPARγ construction were 5'-GGCTTC ATGACAAGGGA-3' and 5'-GTtcagatctgaACTCCCTTGTCATGA AGCCTT-3'. pT-CMV-LUC was constructed using pT-TATA-LUC reporter plasmid and a 0.13-kb *Aat* II-*Spe* I CMV promoter fragment of pcDNA plasmid. pT-4XPPRE-CMV-LUC plasmid was constructed using a 5.17-kb *Spe* I-*Pst* I fragment of pT-CMV-luc plasmid and a 0.11 kb *Spe* I-*Pst* I fragment of pBS-4XPPRE. pBS-4XPPRE was constructed from pBS-PPRE, which was prepared by ligating a 2.9-kb *Bam* HI-*Pst* I fragment of pBluescript with two oligonucleotides (5'-GATCCTGAAACTAGGGTAAAGTTCAGA TCTGCA-3' and 5'-GATCTGAACTTTACCCTAGTTTCAG-3'). The ligation of a 1.83-kb *Bgl* II-*Sca* I and a 1.17-kb *Bam* HI-*Sca* I fragment of pBS-PPRE resulted in pBS-2XPPRE, and another round of digestion and ligation resulted in pBS-4XPPRE.

Transient transfection and reporter assays. HeLa cells grown in DMEM (Invitrogen) containing 10% FBS were plated at a density of 3×10^4 cells/well in a 24-well plate. After 1d of incubation, 0.1 or 0.35 μg of each shRNA expression plasmid, 0 or 0.25 μg of pGem1 carrier, 0.1 μg of a luciferase reporter plasmid pLXR-Luc (containing a 3.4 kb promoter region of human LXRa gene), and 0.05 μg of a control plasmid pCMV-lacZ were transiently cotransfected into HeLa cells using 2.5 μl (0.45%) PEI (40,872-7, Aldrich). HepG2 cells were grown as described above for HeLa cells. After 1d of incubation, 0.1 or 0.2 μg of each shRNA expression plasmid, 0.3 or 0.2 μg of pGem1 carrier, 0.01 μg of a luciferase reporter plasmid pT-CMV-luc or pT-4PPRE-luc, and 0.05 μg of a control plasmid pCMV-lacZ were transiently cotransfected into HepG2 cells using 1.2 μl (0.45%) PEI. Where appropriate, 5 mM troglitazone was added to medium at a final concentration of 10 μM 24 h after transfection. Ratios of firefly luciferase to β-galactosidase activity were determined 48 h after transfection.

Results and Discussion

To simplify the construction of shRNA-generating vectors, we designed a mammalian expression vector (pI) that contains the polymerase III H1-RNA gene promoter, two *Bse* RI restriction sites, and a TTTTT termination signal (Fig 1). *Bse* RI digestion of pI generates 3'-AA overhangs at the end regions of both the H1 promoter and terminator. To test the feasibility of our approach, firefly luciferase gene-specific

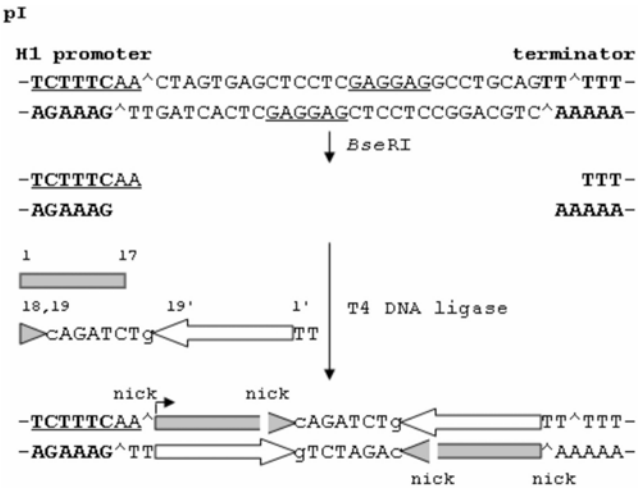

Fig. 1. Strategy used to construct shRNA expression plasmid. Sequences encoding shRNAs with 19 bases of homology to the target gene were synthesized as 17- and 31-nt DNA oligonucleotides and ligated into *Bse* RI sites immediately downstream and upstream of H1 promoter and terminator, respectively. The annealed oligonucleotides then dimerize to form a palindromic 46-bp dsDNA with TT overhangs at 3' ends.

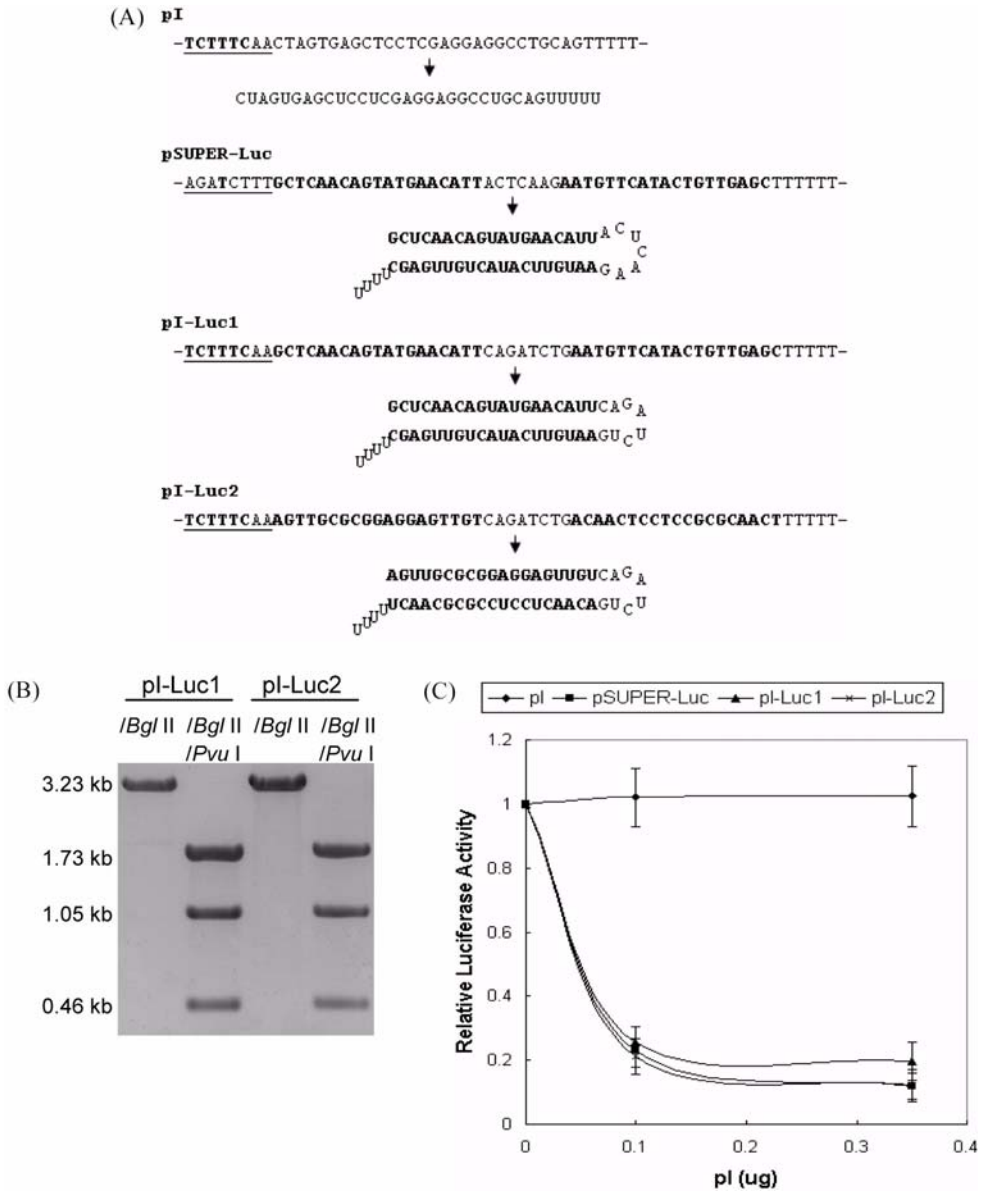

Fig. 2. shRNA-mediated silencing of luciferase reporter gene. (A) Sequences of transcribed regions of shRNA expression plasmids and the predicted secondary structure of shRNA. (B) Confirmation of the shRNA expression plasmids pI-Luc1 and pI-Luc2 by restriction digestion. Restriction digestion at the center of the palindromic region using *Bgl* II generates a 3.23 kb linear DNA fragment. Further digestion with *Pvu* I then generates three DNA fragments as indicated. Both plasmids show the same restriction pattern. (C) shRNAs expressed from expression plasmids suppress luciferase expression in HeLa cells. Each of the three shRNA expression plasmids was cotransfected with a control plasmid pCMV-lacZ and luciferase expression plasmid pLXR-Luc, which contained a 3.4 kb promoter region of human LXRα; gene, into HeLa cells. Firefly luciferase to β-galactosidase activity ratios were determined 48h after transfection. Data represent the average of three independent experiments; error bars indicate standard deviations.

oligonucleotides were designed using the Whitehead siRNA selection web server (Yuan *et al*., 2004). Sequences encoding shRNAs with a 19 base homology with the target gene were synthesized, without phosphorylation at 5' ends, as 17- and 31-nt DNA oligonucleotides. These oligonucleotides anneal to form a 17-bp long dsDNA with a 12-nt long cohesive end and a TT overhang at the 5' and 3' ends, respectively. The annealed oligomers can dimerize to form a palindromic 46-bp dsDNA, which contains two nicks separated by 12-nts in the middle and TT overhangs at 3' ends (Fig. 1). An shRNA expression plasmid was constructed by ligating a pair of oligonucleotides, which had been denatured and annealed, with a purified vector fragment generated by *Bse* RI digestion. The ligated recombinant DNA obtained contained four nicks, two at each 5' end and two in the middle of the palindromic dsDNA. In other words, there are two nicks at both ends of

the 17-nt long oligomers in each strand (Fig. 1). It has been previously found that DNA fragments containing cohesive ends longer than 12-nts anneal stably, such that resultant circular recombinant molecules do not require *in vitro* ligation for efficient bacterial transformation (Aslanidis and de Jong, 1990). The recombinant DNA generated in this study contains not only the *trans* nicks separated by 12-nts, but also *cis* nicks separated by 17-nts. Apparently, the 17-nt long *cis* nicks do not interfere with bacterial transformation, since recombinant molecules have been always successfully recovered.

We designed three vectors that direct the synthesis of the 19-bp double-stranded luciferase target sequence. First, we prepared a positive control vector (pSUPER-Luc) by inserting a short dsDNA comprised of two chemically synthesized 62-mer oligonucleotides into pSUPER (Brummelkamp *et al*., 2002). The second vector pI-Luc1 was constructed using two short oligonucleotides and the shRNA expression plasmid pI prepared in this study. The shRNAs generated by the two vectors were the same, except for the loop region (Fig 2A). The loop composed of pI-Luc1 is palindromic by design. Another vector pI-Luc2, constructed in the same way as pI-Luc1, targets a different region in luciferase cDNA. All three vectors were confirmed by restriction digestion (Fig 2B) and DNA sequencing (data not shown). We compared the abilities of these vectors to inhibit luciferase expression in a transient cotransfection experiment in HeLa cells. As expected, the transfection of pSUPER-Luc resulted in a reproducible reduction in luciferase activity of up to 90% (Fig 2C). Transfection with shRNA expression vector pI had little effect on luciferase expression, and whereas pI-Luc1 was a little less efficient at knocking down luciferase expression, pI-Luc2 was able to knockdown to the same extent as pSUPER-Luc. A similar silencing pattern was observed when shRNA expression vectors (constructed in the same way using *Drosophila* U6 promoter) were tested in *Drosophila* S2 cells (unpublished results).

To test the general applicability of our shRNA generation method, we next attempted to silence an endogenous target gene encoding PPARγ. pI-PPARγ was constructed as described for pI-Luc plasmids, except that the length of the loop region was 10-nt instead of 8-nt, and this was confirmed by restriction digestion (Fig 3A and 3B). To detect silencing of endogenous PPARγ, we constructed two luciferase reporter plasmids pT-CMV-LUC and pT-4XPPRE-CMV-LUC. We then tested the ability of pI-PPARγ to inhibit luciferase expression by transient cotransfection in HepG2 cells. Cotransfection of pI-PPARγ with pT-CMV-LUC, containing about 130 bp of CMV promoter, did not affect luciferase reporter activity (Fig 3C). However, cotransfection with pT-4XPPRE-CMV-LUC, containing four copies of PPAR response element, resulted in reduced reporter activity by about 20%, which is likely to have resulted from the silencing of basal PPARγ activity in HepG2 cells. This silencing activity became pronounced when PPARγ agonist was added to medium to activate endogenous PPARγ. The reporter activity of transfected pT-4XPPRE-CMV-LUC DNA was increased by more than two fold by troglitazone, a PPARγ agonist, while cotransfection with pI-PPARγ almost resulted in the complete abrogation of PPARγ activation (Fig. 3C). This observed silencing was specific to PPARγ since it depended on the presence of PPRE in the reporter plasmid and on troglitazone, but not on agonists of PPARγ and PPARγ (data not shown). These findings demonstrate that effective shRNA expression plasmids may be constructed using a pair of short oligonucleotides, and used

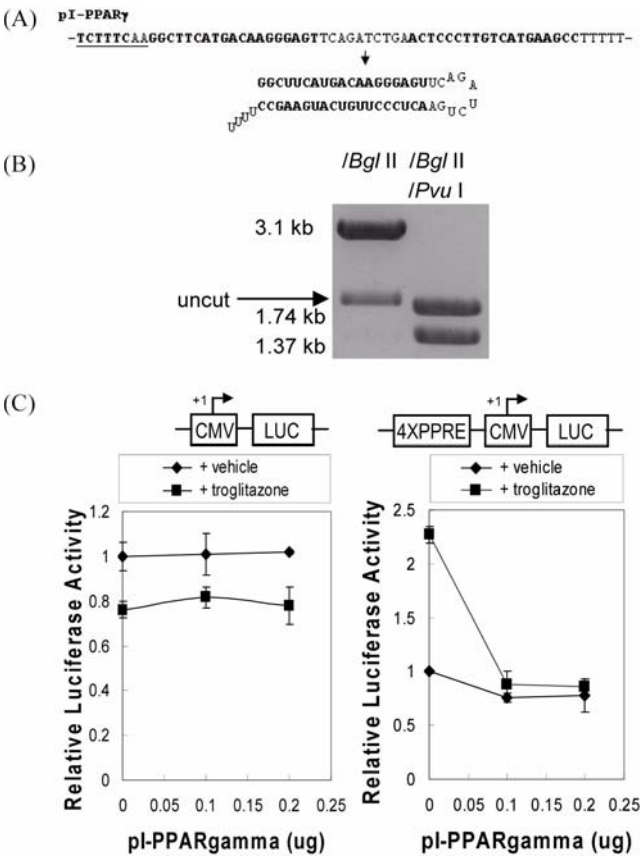

Fig. 3. shRNA-mediated silencing of the endogenous PPARγ gene. (A) Sequence of the transcribed regions of shRNA expression plasmid pI-PPARγ and the predicted secondary structure of the shRNA. (B) Confirmation of pI-PPARγ by restriction digestion. Restriction digestion at the center of the palindromic region using *Bgl* II generated a 3.1 kb linear DNA fragment. Usually uncut DNA (arrowed) was detected when digestion was attempted at the center of the palindromic region. Further digestion with *Pvu* I generated two DNA fragments of 1.74 kb and 1.37 kb. (C) shRNA expressed from the pI-PPARγ suppresses luciferase expression activated by troglitazone, a PPARγ agonist, in HepG2 cells. Each of the two reporter plasmids pT-CMV-Luc and pT-4XPPRE-CMV-Luc were cotransfected with pI-PPARγ and a control plasmid pCMV-lacZ, into HepG2 cells. Firefly luciferase to β-galactosidase activity ratios were determined 48 h after transfection and represent the averages of at least two independent duplicate experiments; error bars indicate standard deviations.

for the specific silencing of endogenous target genes.

The advantages of our method are as follows: First, it costs much less than the conventional method using two long oligonucleotides. For example, when two 62-mer oligonucleotides are used, they need to be synthesized at the 0.2 micromole scale and purified by PAGE. However, 17-mer and 31-mer oligonucleotides may be synthesized at lower than the 0.05 micromole scale without a PAGE purification step. Therefore, the costs of the devised method are only 15% or so of those of the conventional method to prepare essentially the same DNA insert for shRNA expression vector construction. Second, the construction of shRNA expression vector is straightforward, because inserts can be prepared by simply denaturation and annealing two oligonucleotides, and because no special step is needed for ligation. Recombinant shRNA expression plasmids can be generated efficiently because pI cut by *Bse* RI does not self-ligate. Third, a restriction site in the loop may be created because the loop must be palindromic, which may facilitate the screening of recombinant plasmids. Furthermore, the restriction site in the loop may be exploited to evaluate the silencing capability of shRNA expression plasmids by using RNAi reporter plasmids when it is difficult to measure the effectiveness of shRNA directly. RNAi reporter plasmid contains a 19-nt long target DNA, which is recovered by restricting shRNA expression plasmid, downstream of the stop codon of any reporter gene. Reporter activity measured after transient cotransfection, reflects the silencing capability of an shRNA expression plasmid.

The main drawback of our method is that silencing may be less potent in general, because the loop must be palindromic, but even so our approach may be useful when absolute silencing is not needed. However, silencing capability may be improved by changing the sequence and loop region length, because both may affect the silencing activity of shRNA. When a 19-bp stem sequence was used, hairpin RNA with a 9-nt loop sequence was found to have greater silencing activity than the corresponding RNA with a shorter loop sequence (Brummelkamp *et al*., 2002), and when shRNAs with various sequences of 9-nt loop were studied, natural microRNA-derived loop sequences were found to be advantageous (Miyagishi *et al*., 2004). Another problem caused by the palindromic structure is that it is neither digested nor sequenced well. In fact, screening for a recombinant DNA is not straightforward when a unique restriction site, such as a *Bgl* II site in pI-Luc plasmids, in the palindromic loop region is used for digestion. In most cases, restriction does not go to completion (Fig 3B), although sometimes, restriction goes to completion when the plasmid is digested for a few days (Fig. 2B). However, this is not a problem, because complete digestion at the loop is achieved when the recombinant DNA is linearized by digestion with a second enzyme that cuts outside the palindromic region (Fig 3B). Sequencing is not a problem either because recombinant DNAs, such as, the previously mentioned RNAi reporter plasmid containing one copy of 19-nt log target DNA, are easily sequenced. In fact, we have confirmed by sequencing that there was no mutation at the target DNA, which is used to generate antisense RNA and therefore a template for the RISC, of shRNA expression plasmids.

In summary, we report upon the development of a straightforward economical approach to the construction of shRNA expression vectors.

Acknowledgments We would like to thank Dr. Agami for providing us with pSUPER plasmid. This work was supported by the Hallym Academy of Sciences, Hallym University and an Industry-University-Research Institute Consortium Grant from the Korean Ministry of small and Medium Industry.

References

Aslanidis, C. and de Jong, P. J. (1990) Ligation-independent cloning of PCR products (LIC-PCR). *Nucleic Acids Res*. **18**, 6069-6074.

Brummelkamp, T. R., Bernards, R. and Agami, R. (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**, 550-553.

Caplen, N. J., Parrish, S. Imani, F., Fire, A. and Morgan, R. A. (2001) Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl. Acad. Sci. USA* **98**, 9742-9247.

Caplen, N. J. (2004) Gene therapy progress and prospects. Downregulating gene expression: the impact of RNA interference. *Gene Ther.* **11**, 1241-1248.

Castanotto, D., Li, H. and Rossi, J. J. (2002) Functional siRNA expression from transfected PCR products. *RNA* **8**, 1454-1460.

Cotrrell, T. R. and Doering, T. L. (2003) Silence of the strands: RNA interference in eukaryotic pathogens. *Trends Microbiol.* **11**, 37-43.

Czauderna, F., Santel, A., Hinz, M., Fechtner, M., Durieux, B., Fisch, G., Leenders, F., Arnold, W., Giese, K., Klippel, A. and Kauffmann, J. (2003) Inducible shRNA expression for application in a prostate cancer mouse model. *Nucleic Acids Res*. **31**, 127.

Dinh, A. and Mo, Y-Y. (2005) Alternative approach to generate shRNA from cDNA. *Biotechniques* **38**, 629-632.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**, 806-811.

Hannon, G. J. and Rossi, J. J. (2004) Unlocking the potential of the human genome with RNA interference. *Nature* **431**, 371-378.

Meister, G. and Tuschl, T. (2004) Mechanisms of gene silencing by double-stranded RNA. *Nature* **431**, 343-349.

Miyagishi, M., Sumimoto, H., Miyoshi, H., Kawakami, Y. and Taira, K. (2004) Optimization of an siRNA-expression system with an improved hairpin and its significant suppressive effects in mammalian cells. *J. Gene Med.* **6**, 715-723.

Sandy, P., Ventura, A. and Jacks, T. (2005) Mammalian RNAi: a practical guide. *Biotechniques* **39**, 215-224.

Sui, G., Soohoo, C., Affar, E. B., Gay, F., Shi, Y., Forrester, W. C. and Shi, Y. (2002) A DNA vector-based RNAi technology to

suppress gene expression in mammalian cells. *Proc. Natl. Acad. Sci. USA* **99**, 5515-5520.

Yuan, B., Latek, R., Hossbach, M., Tuschl, T. and Lewitter, F. (2004) siRNA Selection Server: an automated siRNA oligonucleotide prediction server. *Nucl. Acids Res.* **32**, 130-134.

Zamore, P. D., Tuschl, T., Sharp, P. A. and Bartel, D. P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25-33.