Primer extension-based method for the generation of a siRNA/miRNA expression vector

Deming Gou, Honghao Zhang, Pradyumna S. Baviskar, and Lin Liu

Department of Physiological Sciences, Oklahoma State University, Stillwater, Oklahoma

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Gou D, Zhang H, Baviskar PS, Liu L. Primer extension-based method for the generation of a siRNA/miRNA expression vector. Physiol Genomics 31: 554–562, 2007. First published September 5, 2007; doi:10.1152/physiolgenomics.00005.2007.—RNA interference (RNAi) has become a powerful technique for studying gene function, biological pathways, and the physiology of diseases. Typically, the RNAi response in mammalian cells is mediated by small interfering RNA (siRNA). The use of synthesized siRNA to silence gene is relatively quick and easy, but it is costly with transient effects. A short hairpin RNA (shRNA) with complementary sense and antisense sequences of a target gene separated by a loop structure results in gene silencing that is as effective as chemically synthesized siRNA with fewer limitations. However, current methods for constructing shRNA vectors require the synthesis of long oligonucleotides, which is costly and often suffers from mutation problems during synthesis. Here, we report an alternative approach to generate a shRNA expression vector with high efficacy. We utilized shorter (<50-nucleotide) primers to generate a shRNA insert by the primer extension method. Our new approach for the construction of shRNA expression vectors dramatically reduced the possibility of mutations. Using this method, we constructed a microRNA (miRNA) library, which facilitates the expression of 254 matured miRNAs. We also performed high-throughput screening of miRNAs involved in the regulation of human Survivin promoter activity in lung A549 cells. We found that the expression of miR-192, 199a, 19a, 20a, 213, and 371 caused the activation of the Survivin promoter whereas miR-302b*, 34a, 98, 381, 463 and 471 decreased the Survivin promoter activity.

RNA interference; short-hairpin RNA; Survivin promoter; microRNAs; high-throughput assay

OVER THE LAST FEW YEARS, RNA interference (RNAi) has emerged as an effective method of silencing gene expression in a variety of organisms, particularly mammals (19). Among its many applications are the characterization and regulation of gene function, analysis of signaling pathway, and target validation. Another intriguing aspect of RNAi is its potential therapeutic value. The RNAi response in mammalian cells mediated by double-stranded RNA (dsRNA) is a well-defined two-step process. Initially, the dsRNA is cleaved into small interfering RNAs (siRNA) of ~19 to 25 nucleotide (nt) by an RNase III-like enzyme known as Dicer. Then, the siRNA is incorporated into a RNA-induced silencing complex, which destroys mRNAs that are homologous to the integral siRNA (45). In mammalian cells, interferon-mediated antiviral response to long dsRNA (>30 bp) causes the global shutdown of protein synthesis. To bypass this nonspecific effect, siRNA (<30 nt) has been used to induce reliable and efficient knock-down of target genes while evading the interferon response (13).

Gene silencing can be induced by direct transfection of cells with chemically synthesized (13) or in vitro transcribed siRNA (24, 30, 33). Alternatively, it can be obtained by transfecting a plasmid or transducing a viral vector encoding a short hairpin RNA (shRNA) driven by a RNA polymerase (pol) III promoter, including U6, H1, 7SK, and tRNA promoters (5, 15, 38, 43), or a pol II promoter such as cytomegalovirus (CMV) or surfactant protein C (16, 42). shRNAs consist of short inverted repeats separated by a small loop sequence. They are processed by the cellular machinery into 19–22 nt siRNA, thereby suppressing the target gene expression. Although siRNA and shRNA elicit comparable results in RNAi experiments, the use of shRNA expression vectors is more appealing with several advantages over chemically synthesized siRNA. First, the use of plasmid to express shRNA is fairly inexpensive and has been shown to achieve long-term target gene suppression in cells and whole organisms. Second, the efficient delivery and stable integration of these shRNA expression cassettes into the host genome can be efficiently achieved by using various viral systems. Third, inducible or cell-specific gene silencing can be obtained in vivo by using a DNA-based shRNA vector. Fourth, vector-based RNAi can be used to rapidly generate knockdown/knockout mice, which would be useful models for unraveling the genetic roots of many human diseases. In the past few years, various groups, including our own, have developed systems for vector-mediated specific RNAi in mammalian cells. Regarding the construction of shRNA vector, the most common strategy requires the synthesis, annealing, and ligation of two complementary oligonucleotides encoding a desired shRNA target sequence into an expression vector (32). The small DNA inserts prepared from the annealed oligonucleotides consist of 19–29 nt complementary to the target sequence followed by its antisense sequence placed in the inverse orientation, separated by a spacer to make the hairpin loop. A terminal signal of 5–6 T and the corresponding overhangs for cloning are also included. Although this method is quick, it often suffers from mutation problems (32, 37). Typically, 20–50% of cloned shRNA constructs contain significant mutations as determined by DNA sequencing. The mutation frequency is close to 75% when the desired siRNA sequence is 29 nt in size (37). The unreliability of this method is in part due to the errors in the synthesis of long oligonucleotides (>50-mer). To verify the shRNA constructs that do not contain any errors, it was advised to pick up at least a few bacterial colonies for sequencing (38). Obviously, this process is time consuming and costly. Another strategy that fewer people use in constructing shRNA vector is a PCR approach. With this approach, a promoter sequence serves as the template with an upstream primer that is complementary to the 5' end of
the promoter region and a downstream primer containing the desired hairpin siRNA target sequence and a region that is complementary to the 3’ end of the promoter (22). Although it allows successful amplification of hairpin structures in a single amplification step, the correct amplicon production is critically dependent on the quality of downstream primer. For this reason, the method requires costly purification of the long downstream primer. shRNA expression vector can also be produced from target cDNA by enzymatic digestion (30). However, this method involves a multistep process and may increase off-target effects. Recently, McIntyre and Fanning (31) reported an alternative approach to construct shRNA expression vector through the primer extension using a long template oligonucleotide and a short universal primer. The mutation rate was decreased by using DNA polymerase Phi29. However, the method still utilizes one long template oligonucleotide (72 nt if the siRNA sequence is 21 nt), which is not a trivial task. The strong secondary structure within this long oligonucleotide led to the difficulty of chain elongation. Kim et al. (25) described another approach of generating shRNA with short oligonucleotides. It is more cost effective and less error prone, but the shRNA vector coming from this method may be less potent because the loop sequences must be palindromic. In the present study, we describe a new design of generating shRNA DNA vectors or template for in vitro transcription. Our design is similar to McIntyre’s method except that the loop sequence is selected for annealing of two oligonucleotides. Since the oligonucleotides for the extension reaction are less than 45 nt, the accuracy of positive clones is dramatically improved. The methods allows for the production of many shRNA vectors in parallel at a greatly reduced cost with high efficacy. By using loop sequence-mediated primer extension, we constructed a microRNA (miRNA) overexpression library and found that a few of miRNAs have important roles in the regulation of Survivin promoter activity.

MATERIALS AND METHODS

Construction of shRNA Expression Vector Using Primer Extension

As a proof of principle, we describe the approach of making human U6 promoter-driven shRNA vector with the most recommended loop sequence of 5’-TTCAAGAGA-3’. The entire procedure involves the following three steps as shown in Fig. 1.

Step 1: primer design. To make a small DNA fragment containing sense-loop-antisense-terminal signal and the corresponding cloning sites, two oligonucleotides were designed for the primer extension. The sequence of upper oligonucleotide includes 5’-CTTGCTTCTCACCC[N19-23]-TTCAAGAGA-3’; the lower oligonucleotide contains 5’-CTTGCTTCTCAAAAN(19-23)TCTCTGAA-3’ (Eco31 I restriction sites underlined). Oligonucleotides were ordered at the University of California, San Diego DNA core facility. The entire procedure involves the following three steps as shown in Fig. 1.

Step 2: primer extension. Twenty-five picomoles of each oligonucleotide was used in the extension reaction including 1 × buffer G (Fermentas), 0.2 mM final concentration of dNTPs, and 1 unit of Klenow fragment (3’ → 5’ exo-). The reaction was carried out at 37°C for 30 min and then the Klenow fragment was inactivated at 75°C for 20 min.

Step 3: digestion, purification, ligation, and transformation. After heat inactivation of Klenow fragment, 5 U of Eco31 I (Fermentas) were directly added into the reaction. After digestion at 37°C for 2 h and purification by QIAEX II gel extraction kit (Qiagen), 100 ng of purified DNA inserts were ligated into the pENTR/U6 vector (Invitrogen). Positive clones were confirmed by automated sequencing using the human U6-sequencing primer (5’-GGACTATCATATGCTTACCG-3’).

Construction of miRNA Overexpression Library

The mi6 vector was amplified from the pSilencer 1.0 vector (Ambion) with the primers 5’-CACCCTGCGGTCTCAGGCGGCGGAGGAGGAGGATCC-3’ and 5’-CTTGCGCTTCCTGCTCTCTTCAAGGTCGAGGTTTCTTGCA-3’. The PCR products were directly cloned into the pENTR/D-Topo vector (Invitrogen), resulting in a pmU6 vector. Four restriction sites, including Bsp119 I, EcoR I, Sma I, and Eco31 I, were introduced at the 3’ end of the mi6 promoter. For the convenience of monitoring transfection efficiency or determining virus titer, a CMV-driven enhanced green fluorescent protein (EGFP) expression cassette was introduced into the downstream of the mi6 promoter through Bsp119 I-Ase I sites (Asc I is in the pENTR vector). This resulted in a ready-to-use vector, pEGFP/mi6. Digestion of the pEGFP/mi6 vector with EcoR I and Bsp119 I left CAAACAAGGCTTTTCTCCAA-3’ and 5’-CTTCGAAGAATTCCCGGGTCTCAAGAGA-3’ with 20-bp oligonucleotides containing the Eco31 I restriction sites.

Fig. 1. Overview of the primer extension method used to produce short hairpin DNA inserts for the construction of shRNA vector. Step 1, rule of two primer design; step 2, primer annealing and extension; step 3, digestion of primer extension product to leave the overhang sequences of 5’-CACC and 5’-AAAA for the ligation with the pENTR/U6 vector.
and lower oligonucleotides, respectively (underlined), the extension product resulted in 5'-TTTG and 5'-GC overhands after digestion with Eco31 I and Bsp119 I and can directly be inserted into pEGFP/mu6 vector through the corresponding sites. The ligation mixtures were transformed into chemically competent cells of GT116. All the inserts were verified by DNA sequencing with the mu6 sequencing primer (5'-ACATGATAGCTGGATTTC-3').

miRNA Library Screen

The 1.1-kb Survivin promoter was amplified from human genomic DNA with a primer set of 5'-CACCGGACCGCGCCGATTAGGCTGA-3' and 5'-TAACTAGTCCATCTGCCCAGTGGCAGTTGGTAC-3' and subcloned into our previously described pENTR/CMV-EGFP vector (16) by switching the CMV promoter through Not I and Spe I sites. Next, we replaced the EGFP fragment with the firefly luciferase (F.Luc), which was amplified from pGL3-control vector (Promega). The new vector, pSurvivin-F.Luc, was used for cotransfection with miRNA overexpression vectors and the normalization vector, pRL-TK (Promega). As an unrelated promoter through Not I and Spe I sites. A549 human lung cancer cells were grown in F12K medium (Invitrogen) supplemented with 10% fetal bovine serum. Transient transfection of plasmids into overexpression vectors and miRNA expression vectors.

RESULTS AND DISCUSSION

Construction of shRNA Vector

To validate our approach, we selected a 21-nt siRNA sequence against the coding region of EGFP at the position of 417 to 437 (5'-'GCACAGCTGCAGTGCTCAACTA-3'). We performed the reaction with 25 pmol of each primer and three DNA polymerases ranging from 0.5 to 2.5 U in 20 μl of total volume. Primer extension products were analyzed on 1.5% sodium boric acid agarose gel. As shown in Fig. 2, primer extension with either Klenow fragment (3'→5' exonuclease) or Bst DNA polymerase large fragment occurs effectively with a sharp DNA band of 76-bp in size. Relatively, Taq DNA polymerase is less efficient, probably because the extension temperature at 72°C is too high for primer annealing. When we checked the amount of each polymerase in a 20 μl of reaction volume, we found that 1.0 U of each polymerase is sufficient in producing enough DNA products.

Because the primer extension products need to be digested with restriction enzymes before cloning into the shRNA vector, we therefore generated those extension products by using Klenow fragment, which is active in all restriction enzyme reaction buffers. Five units of Eco31 I were then added to the reaction mixtures that were heated at 75°C for 20 min to inactivate the Klenow fragment. After allowing the reaction to proceed at 37°C for 2–4 h, we purified the Eco31 I-digested DNA by using a QIAEX II gel extraction kit. The purified DNA was ligated into the pENTR/U6 vector and transformed into GT116 competent cells. For comparison, we also constructed shEGFP vectors by using the conventional method of two longer oligonucleotides. The final sequencing results showed that only 6 plasmids of 80 samples coming from the primer extension method had mutations (Table 1), which is much lower than the annealed oligonucleotide cloning strategy (32 of 80 had mutations), indicating that our approach is less error prone.

Because this method is based on loop sequence for the annealing of two primers, the selection of loop sequence is the key of this procedure. In DNA-based RNAi studies, a short loop sequence is necessary for the construction of shRNA expression vector. Up to now, more than 20 different loop sequences, ranging from 3 to 19 nt, have been reported. For example, Paul et al. (38) used a 4-nt, 5'-UUCG-3', tetra
nucleotide sequence; Sui et al. (43) used 5'-CTCGAG-3' as loop sequence; Agami’s group (5) used a 9-nt loop sequence, 5'-TTCAAGAGA-3'. The loop sequence appears to somewhat influence the RNAi effect. To ensure the efficient annealing of two primers, we recommend using >7-nt loop sequence. In this report, we examined the extension reaction with four respective loop sequences, 8-nt of 5'-CTTGGTTC-3' (loop 1), 9-nt of 5'-TTCAAGAGA-3' (loop 2), 10-nt of 5'-CTTCCCTGTCA-3' (loop 3) and 19-nt of 5'-TAGTGAAGCCACAGATGTA-3' (loop 4) (Fig. 3A). To reduce the primer length during the synthesis of each oligonucleotide at a small scale, only 10 nt in loop 4 (underline) were selected as annealing sequence in the extension reaction. All the aforementioned designs gave successful extension products (Fig. 3B).

To study the possible influence of those loop sequences on the RNAi effect, we transfected 293A cells with each construct in combination with a homologous target expression plasmid pENTR/CMV-EGFP, encoding EGFP and with a nontargeted reporter plasmid pDsRed2-C1, encoding the DsRed2 protein for normalization (12, 41). As shown in Fig. 3C, the first three loop sequences have a similar activity in silencing EGFP. Human miR-30 (loop 4)-mediated RNAi was less effective. This result differs from a previous study, in which the expression of HIV-1-specific shRNA through a miR-375 binding site and 5'-TGCC overhangs that allowed ligation to the 5'-AGCG and 5'-GGCA overhangs of the Eco31 I-digested miR-30-based shRNA inserts. For the preparation of shRNA inserts, two oligonucleotides (~50 nt in length) were designed and used in an extension reaction based on the 10-nt overlap region as shown in Fig. 4. The advantages of the primer extension method in constructing miR-30-based shRNA vectors, compared with the report by Chang et al. (6), include lower cost in synthesizing short oligonucleotides, elimination of the need for PCR amplification, no need for DNA purification before restriction enzyme digestion, and no need to introduce two artificial restriction sites within the miR-30 flanking sequences. Therefore, the resulting primary miRNA sequences are more similar to the original primary miR-30 with the shRNA sequences being 90% correct.

Construction of miRNA Overexpression Library

miRNAs are endogenous ~22 nt RNAs that play important regulatory roles in animals and plants by targeting miRNAs for cleavage or translational repression through components shared with the RNAi pathway (3, 27, 48). Hundreds of miRNAs have been found in animals, plants, and viruses. Overexpression of miRNA may facilitate the study of their functions and permit effective RNAi in vivo. Three different methods have been used to overexpress miRNA, including chemically synthesized mimic miRNA (18), vector-based matured short-hairpin miRNA (50), or flanking sequence-included primary miRNA (47). Relatively, it is cheap and easy to make a DNA vector that expresses a matured miRNA.

To test the activity of miRNA expression vectors, we first constructed a F.Luc reporter vector with the miRNA-binding site for miR-375. The binding site was made by annealing two short oligonucleotides containing the miR-375 binding site and inserting into the pGL3 vector at the Xba I restriction site, which is located between the F.Luc gene and the SV40 polyA terminal sequences. We tested the effect of the miR-375 overexpression on suppressing gene expression by cotransflecting the miRNA overexpression vector, the F.Luc reporter vector bearing the miR-375-binding site and a transfection normalization vector, pRL-TK. Using a dual-luciferase assay, we found that the specific inhibition of F.Luc reporter gene was only observed in the cells overexpressing miR-375, but not miR-21 (Fig. 5), indicating that the short-hairpin-based miRNA vector can be used to express functional miRNAs.
By using the primer extension method, we constructed a miRNA library, which facilitates the expression of 254 matured miRNA sequences with following features: 1) the mU6 promoter was selected to drive the expression of short-hairpin miRNA; 2) a 10-nt loop sequence of 5'-CTTCCTGTC-3' was used in primer extension; 3) 5'-overlap sequences (5'-TTTG-3' and 5'-GC-3') for ligation were created by double digestion of Eco31 I and Bsp119 I; and 4) EGFP reporter gene was included for easily tracking the transfection efficiency. Upon the sequencing of all the plasmids, we found that only 17 out of 254 plasmids (6.7%) had mutations, further supporting the high fidelity in constructing shRNA vectors with the primer extension method. We estimate that this method could save over 65% of money from oligonucleotide synthesis, plasmid preparation, and DNA sequencing.

High-Throughput Screening of miRNA Involved in Survivin Promoter Activity

Survivin is a member of the inhibitor of apoptosis family of proteins. It is implicated in two key biological events: control of cell proliferation and regulation of cell lifespan (29). The expression of Survivin is noted in many common tumors, but not in normal adult tissues (1, 2). Overexpression of Survivin inhibits apoptosis and promotes cancer cell survival (17). The inhibition of Survivin expression induces cell death by apoptosis (28, 39). Survivin gene expression in cancer tissue appears to be regulated transcriptionally. Several signaling pathways involved in Survivin modulation have been identified, including Sp1 transcription factor (14), TCF/β catenin (26), tumor suppressor p53 (23), Smad/BMP-7 signaling (49), PI3 kinase/Akt signaling (9), Stat3 signaling (35), and IGF-1/mTOR signaling (46). Although strategies to lower Survivin levels have been pursued for rational cancer therapy, the molecular mechanisms controlling Survivin expression in tumors have not been completely elucidated (46). miRNAs are newly discovered regulators of gene expression that are implicated in many processes, such as cell proliferation, apoptosis, metabolism, cell differentiation, and morphogenesis. Currently, it is not known whether miRNAs are involved in the regulation of Survivin promoter activity via various Survivin transcriptional factors. We therefore screened for the potential miRNAs involved in the activation or inactivation of Survivin promoter activity using a miRNA overexpression library containing 254 miRNAs. To perform a quick high-throughput screen, we constructed a human Survivin promoter-driven F.Luc reporter vector, pSurvivin-F.Luc, and cotransfected A549 human lung cancer cells with the miRNA overexpression library and pRL-TK normalization vector. The cells were lysed 48 h after transfection for the dual-luciferase assay. Compared with the negative control vector of pEGFP/mU6-shCon, any miRNA that changed the Survivin promoter activity by >2-fold was considered as a hit. Each transfection was performed in three replicates and the results are represented in Fig. 6A. miRNAs that had error bars inside the cutoff were not included as hits. Using these criteria, we identified six miRNAs that activated the Survivin promoter activity in A549 cells (miR-192, 199a, 19a, 20a, 213, and 371) and six miRNAs that decreased the Survivin promoter activity (miR-302b*, 34a, 98, 471, 381, and 463).

To address the specificity of the effects of miRNAs on the Survivin promoter assay, an unrelated control promoter, SP-B promoter, was tested to see whether the 12 identified miRNAs affect the SP-B promoter activity in A549 cells. SP-B is a surfactant protein involved in lung surfactant function. Compared with the pEGFP/mU6-shCon vector, most of miRNAs did not change the expression of SP-B promoter-driven F.Luc (Fig. 6B). Only miR-471, which downregulated the Survivin promoter activity, modestly increased the SP-B promoter ac-
tivity. This result suggests that the observed effects of miRNAs on the F.Luc expression are due to the promoter activity, but not due to a direct effect on F.Luc gene.

miRNAs are normally targeted to the 3′-untranslated region (UTR) of a gene. In our experimental design, the 3′-UTR of Survivin was not included in the reporter construct. The effects of miRNAs on the Survivin promoter activity are likely because of the inhibition of Survivin transcriptional activators and repressors or protein(s) regulating those transcriptional factors. E2Fs promote cell death when overexpressed or when activated in response to DNA damage (34). Induction of apoptosis is a unique property of E2F1, E2F2, and E2F3 (11). It has been reported that E2Fs are involved in the Survivin transcriptional regulation, because the Survivin promoter contains an E2F-like binding element. Recent studies indicate that E2F1 is a validated target of miR-17 and miR-20a (36). miR-20a also regulates E2F2 and E2F3 via the binding sites in the 3′-UTR of their respective mRNAs (44). Therefore, it is possible that the activation of Survivin promoter activity by miR-20a may be mediated through the depression of E2F transcriptional factors.

A very interesting finding is that two miRNAs (miR-19 and miR-20) that activate the Survivin promoter belong to the miR-17-92 cluster. The miR-17-92 cluster is composed of seven miRNAs (miR-17-5p, 18, 19a, 20, 19b-1, 920-1, and 17-3p) and resides in intron 3 of the C13orf25 gene at 13q31.3. It has been observed that miR-17-92 cluster is frequently overexpressed in human cancers (20, 21). Recently, another group has reported that miR-17-92 cluster is directly regulated by c-myc (10).

Among six miRNAs that decreased the Survivin promoter activity, miR-34a is commonly deleted in human cancers and directly transactivated by p53 (7, 40). Inactivation of miR-34a strongly attenuates p53-mediated apoptosis, whereas overexpression of miR-34a mildly promotes apoptosis (40). This is consistent with our results that miR-34a decreased the Survivin promoter activity.

Collectively, we demonstrated an alternative approach to construct shRNA/miRNA vectors at a greatly reduced cost with high efficacy. We estimate that this method could save over 65% of money from the synthesis of primers and DNA sequencing. It is extremely useful in generating shRNA libraries at a genome scale. We also reported the construction of a human miRNAs expression library and screening of potential miRNAs involving in Survivin gene regulation. The availability of the miRNA library as well as methods for high-throughput assay makes it possible to identify miRNAs involved in apoptosis, phagocytosis, cell proliferation, cell cycle, p53-
Fig. 6. A: screening for miRNAs involved in the regulation of human Survivin promoter activity in A549 cells. A549 cells were plated 25,000 cells/well in 96-well plates. After overnight culture, cells were transfected with 25 ng of pSurvivin-F.Luc reporter gene vector, 2.5 ng of pRL-TK normalization vector, and 75 ng of each miRNA expression vector by use of Lipofectamine 2000. At 48 h posttransfection, cells were assayed for dual-luciferase activities. The relative luciferase activities were normalized by a GFP control vector expressing unrelated shRNA (shCon). The results shown are means ± SD (n = 3). B: specificity of miRNAs in the promoter activity assay. Human SP-B promoter-driven firefly luciferase vector (pSP-B-F.Luc) was cotransfected with the pRL-TK vector and a miRNA overexpression vector. At 48 h posttransfection, cells were assayed for dual-luciferase activities. The relative firefly luciferase activity in each treatment was normalized by a control vector expressing unrelated shRNA. Error bars indicate SD; n = 3 in each group.
mediated senescence, cell morphogenesis, cytokinesis, and cellular signaling. This will ultimately lead to a greater understanding of the cellular processes as well as reveal key pathways that might be exploited for disease detection, prevention, or treatment (8).

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