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Generation of variable and fixed length siRNA from a novel siRNA expression vector

Cheng Du [a,*], Baosheng Ge [b], Xiaoyan Feng [b], Wing C. Chan [a], Timothy W. McKeithan [b]

[a] Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE 68198, USA
[b] Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE 68198, USA

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Abstract

Small interfering RNA (siRNA) expression vectors using a Pol III promoter fall into two categories, vectors with a single Pol III promoter that express small hairpin RNA (shRNA) and vectors with two head-to-head (convergent) Pol III promoters that express siRNA. There are technical difficulties in preparing convergent siRNA vectors from cDNA. Here, we report construction of a novel convergent siRNA expression vector, pTHUB. Two *Xcm*I sites were inserted between opposing Pol III promoters. After linearization with *Xcm*I, pTHUB has a single 3′ A overhang at each end that allows direct cloning of partially DNase I digested cDNA fragments (20–30 bp) tailed with ddT. A derivative method for generating 19 bp siRNA in pTHUB is also described. The suppression efficiency of the pTHUB vector is comparable to those of conventional shRNA vectors. We have made a siRNA library from a single cDNA. The same approach can be used to construct whole-genome siRNA libraries from cellular cDNA.

Keywords: siRNA; Expression; Vector; Library; Convergent

RNA interference (RNAi) is a conserved mechanism in eukaryotes for post-transcriptional gene silencing. In practice, 19-bp dsRNAs with 2-nt 3′ overhangs are the most effective form in suppression [1]. Most approaches to the preparation of siRNA expression vectors have used a single Pol III promoter driving the expression of short hairpin (sh)RNAs. The shRNAs are transcribed from 19 to 29 bp inverted DNA repeats connected by a loop [2,3]. Typically, each shRNA cassette has been generated using chemically synthesized double-stranded DNA oligonucleotides. The transcript from such a shRNA vector is a single-stranded RNA, which folds back to form a hairpin and is then processed to form a RNA duplex. Strategies have been developed for converting cellular cDNAs into shRNA libraries through a series of enzymatic steps [4–7]. Unfortunately, cassettes expressing shRNAs can be difficult to sequence and are unstable when transformed into *Escherichia coli* [8].

Alternatively, siRNA can be produced from head-to-head (convergent) dual Pol III promoters [9–13]. In this system, the linear dsDNA (19–29 bp) that is the template for a siRNA is transcribed from the two opposing promoters, thus eliminating the need for the hairpin structure in a shRNA construct and its associated problems.

The major technical problem in using the convergent system to construct siRNA libraries from cDNAs is cloning site compatibility. In general, each promoter in this system has an A_5 stretch at the transcription start site to provide the T_5 transcription termination signal for the opposing promoter (Fig. 1). At the same time, there must be restriction sites in the A_5 region for cloning. In previous reports [9–12], the restriction sites used in the convergent vectors generated 4-bp cohesive overhangs for cloning. The siRNA sequence, the T_5 termination signal, and the restriction sites were provided by chemical synthesis. In one report [13], two different unphosphorylated oligonu-

* Corresponding author.
E-mail address: cdu@unmc.edu (C. Du).

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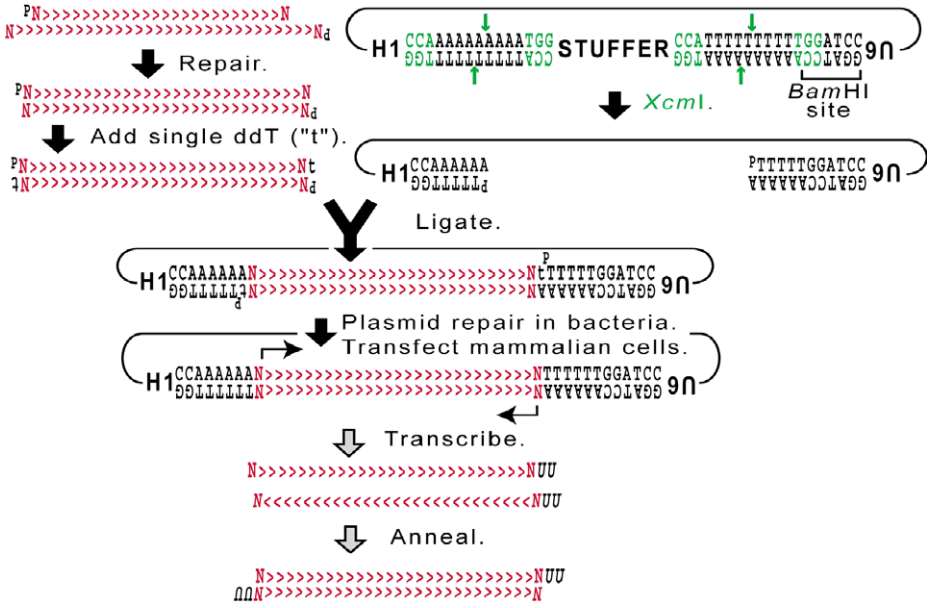

Fig. 1. Scheme for constructing siRNA libraries in pTHUB. DNase I partially digested cDNA fragments (in red) were end-repaired with T4 DNA polymerase, and a single T was added to the 3′ ends with terminal deoxynucleotidyl transferase (TdT) and dideoxyTTP (ddTTP). The 20–30 bp fraction was purified from a PAGE gel and cloned into *Xcm*I-digested pTHUB. The *Bam*HI site is designed to allow for construction of fixed size libraries. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

cleotide adaptors were sequentially ligated to the blunted cDNA fragments (20–30 bp), and PCR was used to amplify the desired ligated products before restriction digestion. However, the blunt-end ligation of oligonucleotides to cDNA and the multiple required PAGE purification steps are inefficient and tedious. We report here a rapid method of generating siRNA vectors from cDNA based on a novel siRNA expression vector, pTHUB, which uses two convergent DNA Pol III promoters. We present two different ways to generate either variable size (20–30 bp) or fixed size (19 bp) siRNA libraries from a single cDNA.

Materials and methods

Plasmids. Human H1 and U6 promoters were amplified from lymphoma cell line DHL16 genomic DNA by PCR with primers H1-5′-CCATGGAATTCGAACGCTGACGTC and H1-3′-AAACTGCAGCCATTTTTTTTTTGGGTGGTCTCATACAGAACTTATAAGATTCCC, U6-5′-TCCCCCGGGCAGGAAGAGGGCCTAT and U6-3′-AAACTGCAGCCATTTTTTTTTTGGATCCTTTCCACAAGATATATAAAGCC, respectively. The H1 PCR product was digested with *Eco*RI and *Pst*I, and the U6 product was digested with *Sma*I and *Pst*I. A three-way ligation of *Eco*RI/*Sma*I linearized pBluescript KS II+ (Stratagene), H1, and U6 was used to generate vector pTHUB (Fig. 1). A single U6 promoter vector (pKS-U6) is similar to pU6 + 27 [14], except that the 27-bp leader sequence was removed and cloning sites were changed to *Cla*I and *Xba*I. The firefly luciferase oligonucleotides are 5′-CGTGCGCTGCTGGTGCCAACTTCAAGAGAGTTGGCACCAGCAGCGCACTTTTT (forward) and 5′-CTAGAAAAAGTGCGCTGCTGGTGCCAACTCTCTTGAAGTTGGCACCAGCAGCGCA (reverse). The resulting plasmid pU6-Luc produces shRNA. The luciferase oligonucleotides for shRNA in pTHUB are 5′-TGCGCTGCTGGTGCCAACTTCAAGAGAGTTGGCACCAGCAGCGCACT and 5′-GTGCGCTGCTGGTGCCAACTCTCTTGAAGTTGGCACCAGCAGCGCT. For 19-bp convergent-type siRNAs, the oligonucleotides are 5′-GTGCGCTGCTGGTGCCAACT and 5′-GTTGGCACCAGCAGCGCACT. The 26-bp oligonucleotides are (1) 5′-GTGCGCTGCTGGTGCCAACCCTATTCT and 5′-GAATAGGGTTGGCACCAGCAGCGCACT and (2) 5′-CGGATTACCAGGGATTTCAGTCGATGT and 5′-CATCGACTGAAATCCCTGGTAATCCGT.

Generation of a variable size (20–30 bp) siRNA expression library. Human cyclin D1 cDNA was used as example. Three micrograms of full-length cyclin D1 cDNA (4.2 kb) was partially digested with 0.1 U DNase I (Invitrogen) in a buffer of 50 mM Tris–HCl, pH 7.5, and 1 mM $MnCl_2$ in a 20 μl volume at room temperature [4,13]. The reaction was stopped at 2, 4, 6, and 8 min by adding 2 μl of 0.1 M EDTA, and the samples were extracted with phenol–chloroform and precipitated with ethanol. The purified DNA was treated with T4 DNA polymerase (New England BioLabs) to repair ends, and the 20–30 bp fraction was separated and excised from a 12% PAGE-TBE gel. The DNA was resuspended in a solution of 1× TdT buffer (New England BioLabs), 1 mM dideoxyTTP (ddTTP, Invitrogen), and 20 U of terminal deoxynucleotidyl transferase (TdT, New England BioLabs) and incubated at 37 °C for 30 min. The product was extracted, precipitated, and resuspended in 10 μl water. The fragments were ligated with pTHUB linearized by *Xcm*I.

Generation of 19-bp siRNA expression libraries. The following oligonucleotides were used: Oligo A-F, 5′-ACGGTGAGTCCAACA, Oligo A-R, 5′-PO_4-TGTTGGACTCACCGTATTACGCGCGCTCACTGGCCGTCGTT. Oligo B-F, 5′-TTTTTTGGATCCCTCTAGCCAGTGTACAA, Oligo B-R, 5′-TTGTACACTGGCTAGAGGGATCCAAAAAANN. PCR primers, 5′-CGACGGCCAGTGAGCGCGCGTAATACG and 5′-ACGGTGAGTCCAACA.

a. DNase I partial digestion. The cDNA of enhanced green fluorescent protein (*EGFP*) was used as an example. The *EGFP* gene was amplified by PCR from plasmid pEGFP-N3 (Clontech). The DNase I partial digestion and end-repair were described above. After T4 DNA polymerase treatment, the blunt-ended DNA fragments were phenol–chloroform extracted, ethanol precipitated, and resuspended in 1× TE buffer.
b. Ligation of EGFP fragments to double-stranded Oligo A. The ligation ratio was 10:1 (oligonucleotide to DNA) in 1× restriction buffer 4 (New England BioLabs) with 10 mM DTT, 1 mM ATP, and 2000 U T4 DNA ligase (final concentrations, New England BioLabs). The ligation reaction was carried out for 3 h to overnight cycling between 30 °C for 30 s and 10 °C for 30 s. The T4 ligase was heat inactivated at 65 °C for 30 min.

c. *Mme*I digestion. The mixture was diluted with an equal volume of 1× buffer 4 and 50 μM *S*-adenosylmethionine (SAM), and 20 U *Mme*I (final concentrations; New England BioLabs). The digestion was carried out at 37 °C for 1–2 h. The reaction mix was phenol–chloroform extracted, ethanol precipitated, and resuspended in 1× TE.

d. Ligation to double-stranded Oligo B. The *Mme*I digested products were ligated to double-stranded Oligo B at a molar ratio of 1:3. The reaction conditions were as described above.

e. PCR amplification. The PCR was carried out with *Taq* DNA polymerase (New England BioLabs) with 1 μl of ligation product in 50 μl volume, with a thermocycle program of 95 °C for 2 min, 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min for 30 cycles [15]. Because product–product annealing competes with product–primer annealing in late PCR cycles, heteroduplexes are generated in which the two strands are derived from different cDNA fragments. Therefore, after the initial PCR, an equal volume of fresh PCR mix (everything except templates) was added into the tube and one additional PCR cycle was carried out to eliminate most of these heteroduplex molecules (Fig. 5).

f. Restriction enzyme digestion. *Bam*HI and *Hph*I were added directly into the PCR mix and incubated at 37 °C for 2 h. The product of the expected size was separated, excised, and purified from a PAGE-TBE gel. Alternatively, PCR primers 5′ end-labeled with biotin were used. After PCR and digestion, streptavidin beads were added to remove byproducts. The fragments remaining in the supernatant were ethanol precipitated.

g. Cloning into pTHUB. The pTHUB vector was linearized with *Bam*HI and *Xcm*I. Cloning and transformation were conducted using standard methods.

siRNA assay. The Promega dual-reporter assay was used following the manufacturer's protocol. Human HEK293 and mouse NIH3T3 cells were seeded 24 h before transfection. Lipofectamine 2000 (Invitrogen) reagent was used for transfection. Luciferase activity was measured 24 h after transfection.

Library screening. NIH3T3 cells were seeded one day before transfection at a density of 2500–5000 per well on 96-well plates. Lipofectamine 2000 (Invitrogen) was mixed with 25 ng EGFP reporter plasmid (pEGFP-N3), 15 ng DsRed-C2 (BD Biosciences), and 60 ng of the individual siRNA plasmid. Fluorescence was examined 24 h later.

Results

Construction and testing pTHUB

We addressed the cloning compatibility limitation of convergent system by constructing the pTHUB vector. In this vector, human U6 and H1 promoters were arranged head-to-head. Two sites for the restriction enzyme *Xcm*I, which recognizes and cleaves the sequence CCANN NNN/NNNNTGG, were introduced at the U6 and H1 transcription start sites (Fig. 1). A stretch of A_9 in the middle of each *Xcm*I site was used in pTHUB. *Xcm*I digestion generates two 3′-single A overhangs on pTHUB. The two 3′-single A overhangs not only prevent vector self-ligation during cloning, but also allow ligation to DNA fragments with a single 3′-T overhang at both ends. In addition, the A_6 stretches provide termination signals for the opposing promoters (Fig. 1).

To test the efficiency of pTHUB as a siRNA expression vector, a hairpin cassette that generates a shRNA directed against firefly luciferase was inserted into pTHUB. The resulting construct, LL-Luc, could express shRNAs from both directions. To test individual promoter activity, the LL-Luc was digested with unique restriction enzymes that destroyed either the H1 or the U6 promoter (see Materials and methods), thereby leaving only one functional promoter. The linearized vectors were transfected into mouse 3T3 or human HEK293 cells. As a positive control, the same shRNA cassette was also cloned into a U6 single promoter expression vector (U6-Luc, similar to pSuper [16]). A luciferase assay was carried out using the Promega dual reporter system. As seen in Fig. 2A, the positive control construct U6-Luc suppressed firefly luciferase activity very well, sug-

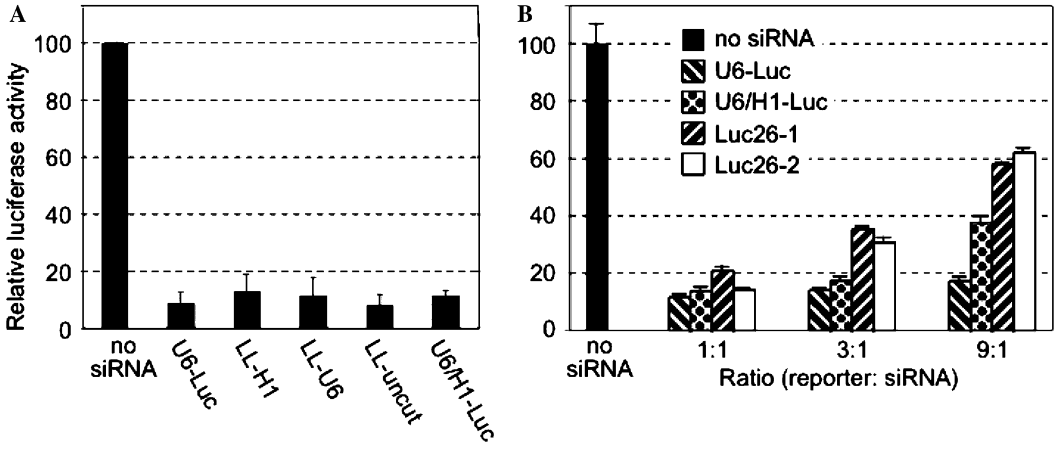

Fig. 2. Characterization of the convergent vector pTHUB as a siRNA expression vector. (A) Determination of promoter activities in pTHUB. "U6-Luc" is a positive control that generates a shRNA from a single U6 promoter. "LL-Luc" has the same shRNA sequence as U6-Luc but constructed in pTHUB. "LL-H1" is LL-Luc linearized by *Nde*I digestion, which destroys the U6 promoter. Similarly, "LL-U6" is LL-Luc DNA linearized by *Psi*I digestion, which destroys the H1 promoter. The "U6/H1-Luc" contains a simple 19-bp insert that yields siRNA by convergent transcription and annealing. All tests were carried out at a molar ratio 3:1 (siRNA vector to reporter vector). (B) Comparison of efficacy of shRNA versus convergent siRNA. Varying molar ratios of siRNA vector to reporter were used to compare the repression efficiency. At high ratios, both systems effectively suppressed luciferase expression. However, at low ratios, the shRNA vector (U6-Luc) was more potent in suppression than the convergent-type vector (U6/H1-Luc). Also shown are the results with two different 26-bp inserts also targeting luciferase. The assay shown was performed on HEK293 cells. Similar results were obtained using NIH3T3 cells.

gesting that the siRNA sequence used was effective. The linearized forms of pTHUB constructs, LL-H1 (with only the H1 promoter functioning) and LL-U6 (with only the U6 promoter functioning), also suppressed luciferase activity effectively. These results showed that the modifications of the U6 and H1 promoters in the pTHUB vector did not affect the transcriptional activities of the two promoters. Furthermore, suppression from the uncut LL-Luc construct (LL-uncut) matched that from the pSuper counterpart, implying that RNA polymerase III transcribed the sense and antisense RNA strands efficiently from opposite directions without a major steric clash.

Next, a convergent-type 19-bp double-stranded oligonucleotide with the same siRNA sequence was cloned into pTHUB (the U6/H1-Luc construct in Fig. 2A). The construct was transfected into NIH3T3 and HEK293 cells, and the suppression efficacy was compared with its shRNA counterpart, U6-Luc. As shown in Fig. 2B, when transfected with high concentrations of siRNA vector (siRNA vector to reporter ratios from 1:1 to 1:3), the suppression mediated by the convergent-type construct was very efficient and reduced luciferase activity by 80–90%. At low siRNA concentration (siRNA vector to reporter ratio 1:9), the shRNA vector (U6-Luc) is more potent in suppression. A previous report [8] described similar findings, and the low potency of convergent siRNA vectors may result from kinetics of annealing two single-stranded RNAs.

Recent studies [17,18] have shown that longer siRNAs (e.g., 25- to 29-bp duplexes) are more potent in suppression at low concentrations without inducing an interferon response. We also tested two 26-bp convergent-type siRNAs directed against firefly luciferase. One contains the 19-bp siRNA sequence used above, whereas the other 26-bp sequence contains a second reported effective 19-bp siRNA [19]. Fig. 2B shows that both 26-bp siRNAs worked almost as well as the 19-bp siRNA at a high ratio (1:1), but less efficiently at a low ratio (1:3); we have not attempted to identify optimal 26-bp sequences, and the compared 19-bp and 26-bp sequences are not equivalent.

Construction of siRNA libraries

To construct a variable size siRNA library in pTHUB, we developed a strategy for cloning 20–30 bp random cDNA fragments. This strategy was based on the fact that siRNAs of 19–29 bp can suppress gene expression efficiently, but do not provoke the interferon response. In addition, the variable size fragments are much easier to generate than fixed size fragments. Double-stranded cDNA was partially digested by DNase I and end-repaired by T4 DNA polymerase [20]. In order to add a single 3′-T overhang to the blunt-ended fragments, we used terminal deoxynucleotidyl transferase (TdT) and dideoxyTTP (ddTTP), as described by Holton and Graham [21]. This method was successful for cloning short cyclin D1 cDNA fragments into pTHUB. Table 1 shows the sequences from 20 randomly picked clones, all of which contain a cyclin D1 fragment. The fragment sizes, original positions within cyclin D1, and insert orientations are apparently random.

To demonstrate the utility of the pTHUB vector, we also prepared an *EGFP* siRNA library as described above. A screen was successfully performed with mouse 3T3 cells in a 96-well plate. Fig. 3 shows representative results from this screen. Thus, the strategy for constructing siRNA libraries with inserts of variable size is feasible.

A variation of this procedure, allowing the construction of libraries of fixed (19 bp) insert size cDNA fragments, is shown in Fig. 4. DNase I partially digested, blunt-ended cDNAs were ligated to a double-stranded oligonucleotide (Oligo A) that harbors a *Mme*I site. The type IIS enzyme *Mme*I was used as a molecular ruler to cut the cDNAs into random 19 bp fragments [4–6]. A second double-stranded oligonucleotide (Oligo B) with 3′ 2-nt overhangs was ligated to the *Mme*I digested cDNAs. The ligated products were then PCR amplified to increase the yield. After the initial 30-cycle PCR, a high molecular weight smear, in addition of the ideal product, was present in the PCR product (left panel, Fig. 5). Because the various cDNA-derived inserts are flanking by common sequences derived from Oligo A and Oligo B, annealing of single-stranded PCR products derived from different cDNA fragments can occur in late PCR cycles; the partially double-stranded resulting molecules comprise the observed smear. To maximize the yield of fully double-stranded product, an equal volume of fresh PCR mix (including everything except templates) was added after the initial PCR cycles, and one additional PCR cycle was carried out. The quantity of heteroduplex molecules

Table 1
Variable size *CCND1*-pTHUB library (20–30 bp insert)

Clone #	Starting nucleotide #	Orientation	Length of insert (nt)
1	3358	+	21
2	4053	+	21
3	9	+	29
4	4088	−	25
5	4005	−	21
6	1691	+	30
7	918	+	23
8	2701	+	26
9	1798	−	20
10	16	−	21[a]
11	3794	−	21
12	4111	+	20
13	1071	−	24
14	3860	−	17
15	792	−	25
16	1701	+	20
17	205	−	26
18	3320	+	25
19	2864	−	27
20	3988	−	23

Note: Of 73 randomly picked clones analyzed by PCR, 64 clones (88%) contained inserts and 7 clones (10%) were empty. Two clones (3%) contained larger inserts that resulted from blunt-ended ligation of two cyclin D1 fragments due to failure to add a ddT at one end of each fragment; the '+' orientation of insert was defined as the sense sequence was in the same direction of T7 primer.

[a] Five nucleotides were derived from vector sequence.

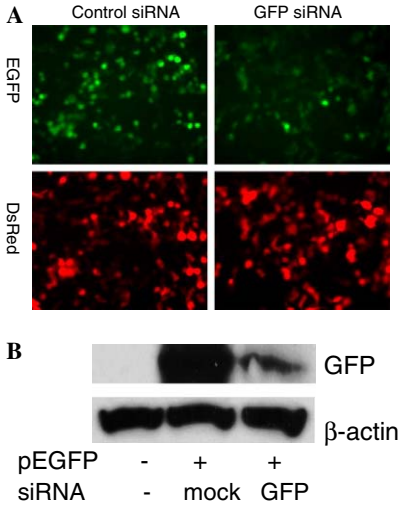

Fig. 3. Selection in mouse 3T3 cells of effective siRNAs targeting GFP from an siRNA library constructed in pTHUB. (A) A selected siRNA against GFP, whose sequence corresponding to EGFP nt 234–256 (TGAAGAAGTCGTGCTGCTTCATG, 23 nt). (B) Results of Western blot. β-Actin was used as loading control. All tests were carried out at a molar ratio 3:1 (siRNA vector to reporter vector).

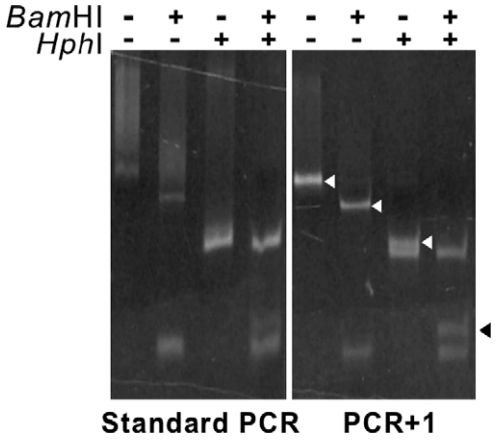

Fig. 5. Conversion of heteroduplex molecules into fully double-stranded duplexes. PCR products from a standard PCR amplification protocol are compared to those from a "PCR + 1" protocol. Left panel, standard PCR products; right panel, "PCR + 1" products. Restriction enzyme digestions were performed as indicated. In the right panel, arrowheads indicate fragments partially derived from cDNA. Note that these bands are much more prominent under "PCR + 1" conditions. The black arrowhead indicates the final desired restriction fragment used in cloning.

was greatly decreased (right panel, Fig. 5). The success of this "PCR+1" protocol was confirmed by restriction digestions; more of the desired product was generated by the "PCR + 1" than that from a standard PCR (compare left panel to right panel in Fig. 5).

Using this method, we made an *EGFP* siRNA library with 19-bp inserts. The quality of the library was determined by random sequencing. As shown in Table 2, all clones were derived from *EGFP* cDNA. The sequences and the orientation of insertion were a random, and inserts

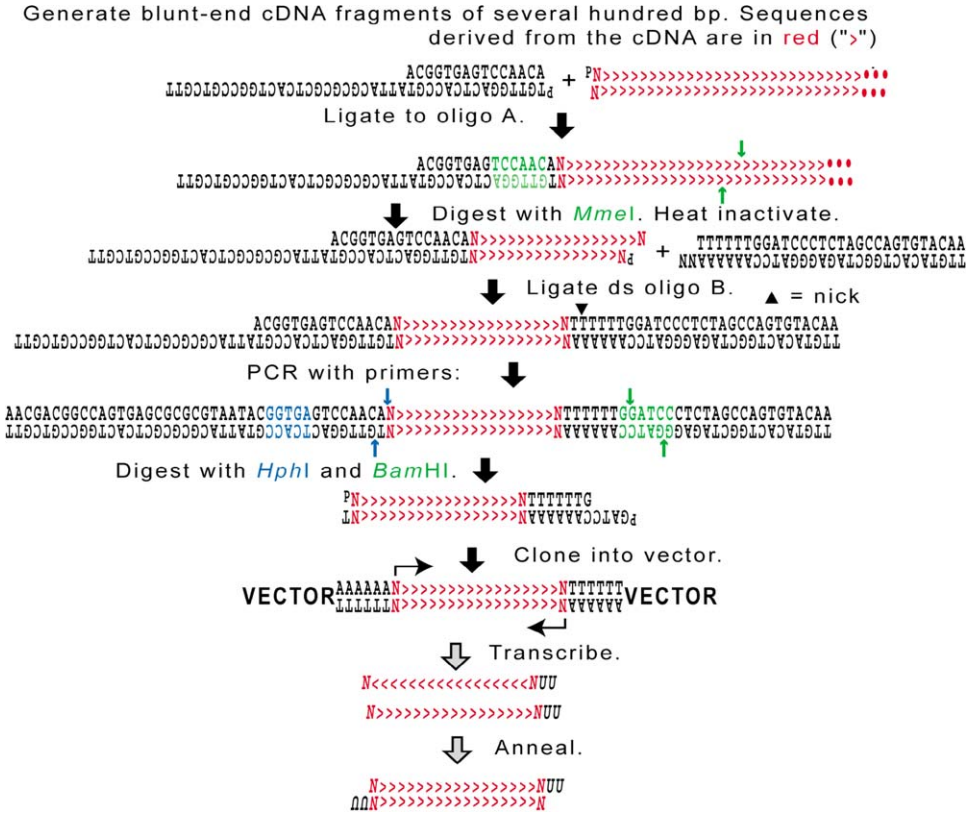

Fig. 4. Flowchart of generation of random 19-bp libraries. cDNA fragments are shown in red. See Materials and methods for details. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

Table 2
EGFP 19-bp siRNA library

Clone #	EGFP position	Length (bp)	Orientation
1	111–130	20	+
2	358–377	20	−
3	395–413	19	+
4	515–533	19	+
5	560–578	19	+
6	37–55	19	−
7	215–234	20	+
8	466–484	19	+
9	477–495	19	+
10	392–410	19	+
11	524–542	19	+
12	595–613	19	+
13	470–488	19	+
14	210–238	19	−
15	505–524	20	+
16	154–172	19	+

were either 19 or 20 bp as expected from the somewhat variable position of *Mme*I cleavage [4].

Discussion

RNA interference (RNAi) provides a powerful tool to study the functions of individual genes as well as to perform genetic screens. The feasibility of applying RNAi as a systematic genome-wide screening tool has been demonstrated in *Caenorhabditis elegans* [22,23], in *Drosophila* cells [24,25], and in mammalian cells [2,3].

Construction of genome-wide siRNA libraries is of general interest. Currently available libraries were constructed by preparing each clone individually using synthetic oligonucleotides. Strategies have been developed for converting cellular cDNAs into shRNA libraries through a series of enzymatic steps [4–7]. Our method represents a novel design for a convergent vector that allows direct cloning of either variable size cDNA fragments (20–30 bp). Although a previous report used the same strategy of inserting 20–30 bp cDNA [13], our method is simpler in practice. We also present a variation of this procedure to construct libraries of fixed (19 bp) insert size by using *Mme*I. Using the same principle, other type IIS enzymes, such as EcoP15I [26], which digests DNA 25/27 nt away from its binding site, can also be used to make libraries of siRNAs with a maximum size of 27 bp.

Tissue- or cell-specific siRNA libraries are preferable to generic synthetic siRNA libraries for several reasons: (a) although the human genome has been completely sequenced, identification of new genes has not been completed yet. Thus, libraries prepared by individual chemical synthesis may miss some genes. (b) Alternative splice forms of some genes may not be affected if only one or a few siRNAs are used. (c) Screening only for genes that are actually expressed in a given tissue reduces the amount of work required. In general, normalized, tissue-specific cDNA libraries from IMAGE collections may be the most cost-effective source of genome-wide cDNA libraries for generating siRNA libraries. Normalized cDNA libraries of cDNAs prepared by individual laboratories [27–29] can be used as well. Nevertheless, the simplified procedure of our method provides opportunities for each laboratory to pursue its specific interests using appropriate tissue-specific siRNA libraries.

In summary, the methods we present greatly simplify the task of construction of siRNA libraries. The siRNA library can be prepared from a single gene for selecting the most effective siRNA, or from genome-wide cDNAs for genetic screening.

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