DNA Synthesis

Small-Interfering-RNA Expression in Cells Based on an Efficiently Constructed Dumbbell-Shaped DNA**

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Gene therapy is a promising approach for the treatment of human diseases from both acquired and genetic disorders.[1] The need for improved, effective gene-transfer vector systems has been a roadblock to the successful pharmaceutical application of gene therapy, though both viral and nonviral vectors are currently under evaluation. Viral-vector systems are the most efficient tools for gene delivery into mammalian cells, achieving excellent delivery and expression efficiencies (usually $>90\%$). However, many disadvantages of a viral system, such as cost, immunogenicity, unpredictable integration, and difficulties in manufacturing, have shifted the focus of interest towards nonviral vectors.[2,3] Among nonviral-vector systems, direct injection of naked plasmid DNA into cells is the simplest nonviral-gene-delivery method, and so far one of the most successful methods.[3] However, although this method is the simplest form of gene delivery with plasmid DNA, it still has several disadvantages in its application for gene therapy. For example, these plasmid DNAs usually have extra genes that encode therapeutically unnecessary proteins. Moreover, these extra genes might contain immunostimulatory sequences, such as CpG motifs that can induce immunotoxicity during gene therapy.[4,5] To overcome these problems, a minimal-size gene-transfer vector was invented.[4,6] As shown in Figure 1, this new vector is a circular, covalently closed, dumbbell-shaped DNA molecule[6] that includes only a transcription unit (a promoter and a gene of interest). The dumbbell vector is much smaller (by $\approx 90\%$) than recombinant plasmids routinely used in transfection experiments, thus avoiding the expression of nontherapeutic genes. This vector can also be used to minimize CpG sequences, depending on the gene-transfer application. Another advantage is that circular dumbbell DNAs provide increased stability toward exonucleolytic degradation in a biological environment because of the absence of free termini.[6–8] Dumbbell-shaped DNA is usually generated by a ligation reaction between one transcription unit backbone and two hairpin DNAs.[4,6] However, it is extremely difficult to obtain sufficient dumbbell-DNA molecules through a conventional cloning method. (For a conventional cloning method, see the Supporting Information.)

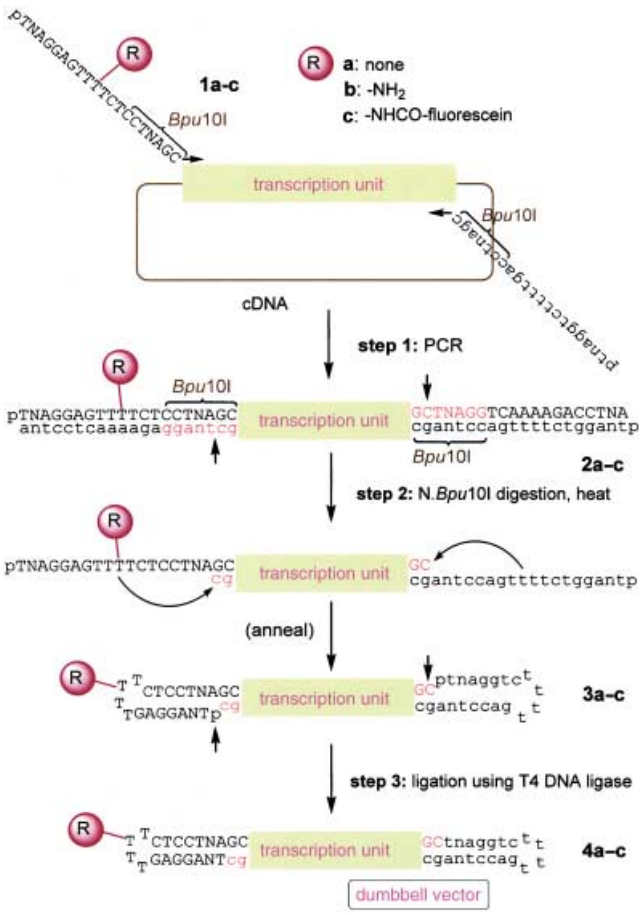

Figure 1. General scheme for the direct and efficient synthesis of dumbbell-shaped DNA by in vitro PCR. a) Plain DNA, b) N-functionalized DNA, c) fluorescein-labeled DNA. For the detailed chemical structures of the oligonucleotides, see the Supporting Information. N. *Bpu*10I is a site- and strand-specific endonuclease artificially engineered from *Bpu*10I that cleaves only one strand of the DNA within its recognition sequence on double-stranded DNA substrates; the capital letter (N) means *nicking* enzyme.

To obtain the large amount of dumbbell-shaped DNA needed, we report herein a new strategy for gene construction in vitro by intramolecular ligation to form the hairpin-loop structure. We used this methodology to construct a small-interfering-RNA (siRNA) expression[9] dumbbell-shaped vector, in which sense and antisense sequences against a target gene were placed under the control of a U6

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promoter.[10] The expression of siRNA is expected to trigger the sequence-specific target-gene silencing, called RNA interference (RNAi).[9] Herein we demonstrate the novel synthesis, the enhanced stability , and, most importantly, the greater efficacy of a dumbbell-shaped siRNA expression cassette over a linear-shaped DNA. The three key steps for the construction of the dumbbell DNA are shown in Figure 1:

Step 1: A gene of interest is amplified by polymerase chain reaction (PCR) with a primer pair that can be chemically modified by various substituents.

Step 2: The amplified DNA is cleaved at two site-specific positions to yield two single-stranded DNA species at both ends of the double-stranded gene of interest. The nicking enzyme (N.*Bpu*10I) cleaves only one DNA strand within its quasi-symmetric recognition sequence on its double-stranded DNA substrate, resulting in an intramolecular annealing to form hairpin ends.

Step 3: The product is then treated with T4 DNA ligase, to form circularized dumbbell-shaped DNA. We emphasize that the yield of cyclization was found to be over 90% because the cyclization involves a simple intramolecular ligation reaction (see below), in contrast to the conventional intermolecular reactions among three DNA molecules.

Dumbbell-shaped DNAs are known to be protected against exonuclease degradation because of their hairpin oligodeoxynucleotide structures at the ends.[6,8] To confirm an effective ligation with our strategy, the intermediate linear PCR product (**2c**) and the dumbbell-shaped vector (**4c**) were independently treated with exonuclease III and analyzed by an 8% polyacrylamide gel electrophoresis (PAGE). As shown in Figure 2, DNA with free termini (**2c**, lane 2) was totally degraded after incubation for 1 hour with the nuclease (lane 3), whereas the dumbbell DNA (**4c**, lane 4) clearly resisted nucleolytic degradation under the same conditions (lane 5). Next, we compared the conversion yield of our novel dumbbell-cloning method with that of the conventional dumbbell-cloning method.[4,6] The ratio of band intensities before and after the nuclease digestion reflects the yield of conversion from the linear PCR vector to the dumbbell-shaped vector. As shown in Figure 3, the conversion yield of our dumbbell-cloning method was, under all conditions (concentrations), usually over 90% because of the intramolecular reaction, whereas with the conventional dumbbell-cloning method it was around 40% under our optimized conditions because of the intermolecular nature among three independent molecules (two loops and a gene of interest). Our intramolecular ligation method avoids the use of excess amounts of wasteful capping oligonucleotides (loops). Thus, we can routinely obtain large amounts of dumbbell vectors in a single batch reaction.

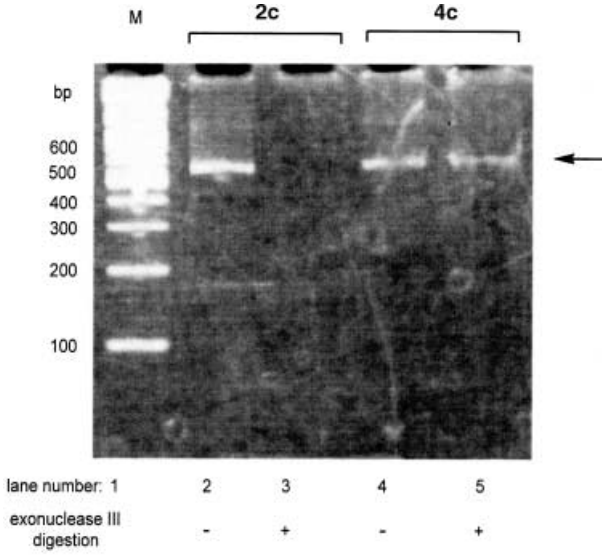

Figure 2. DNA stability toward exonuclease digestion. The same amount of DNA was subjected to 8% PAGE. The gel was stained with ethidium bromide, and the bands were visualized by using a transilluminator at $\lambda_{ex} = 302$ nm. The labels **2c** and **4c** correspond to those in Figure 1. The bands of approximately 600 bp correspond to fluorescein-labeled DNA.

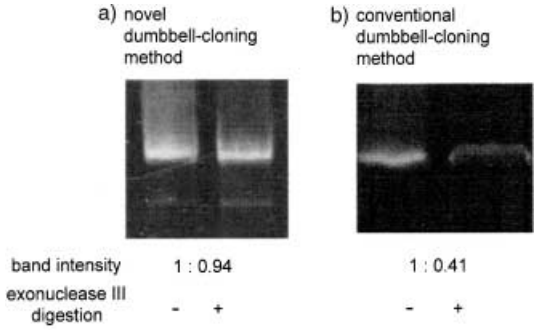

Figure 3. Conversion yields from linear vectors to dumbbell vectors. The same amount of DNA was subjected to 8% PAGE. The gel was then stained with ethidium bromide, and the bands were visualized by using a transilluminator at $\lambda_{ex} = 302$ nm.

When a primary amino group is chemically introduced at a predetermined position of an oligonucleotide, it can be functionalized with succinimidyl ester derivatives; the chemical reaction of the succinimidyl esters is limited only to the primary amino group.[11] As shown in Figure 4, the dumbbell vector (**4b**) with a primary amino group only in the hairpin-loop (lane 1) was successfully converted into a fluorescent dumbbell DNA (yielding **4c**) by reaction with the succinimide ester of fluorescein (lane 2). In contrast, dumbbell DNA (**4a**) without the primary amino group (lane 3) did not react at all with the succinimide ester under the same reaction conditions (lane 4). This means that various substituents can be site-specifically introduced only at the noncoding hairpin-loop region of the dumbbell DNA. Fluorescent dumbbell DNA (**4c**) was also made from the fluorescent primer- (**1c**) mediated direct synthesis by PCR (data not shown).

Using plasmid, linear-, and dumbbell-shaped siRNA expression vectors, the inhibitory efficacies were tested against a target mRNA in cells. These different vectors were individually transfected into HeLa S3 cells that stably expressed EGFP protein. When vectors expressing siRNAs targeted against EGFP were used, a specific and effective

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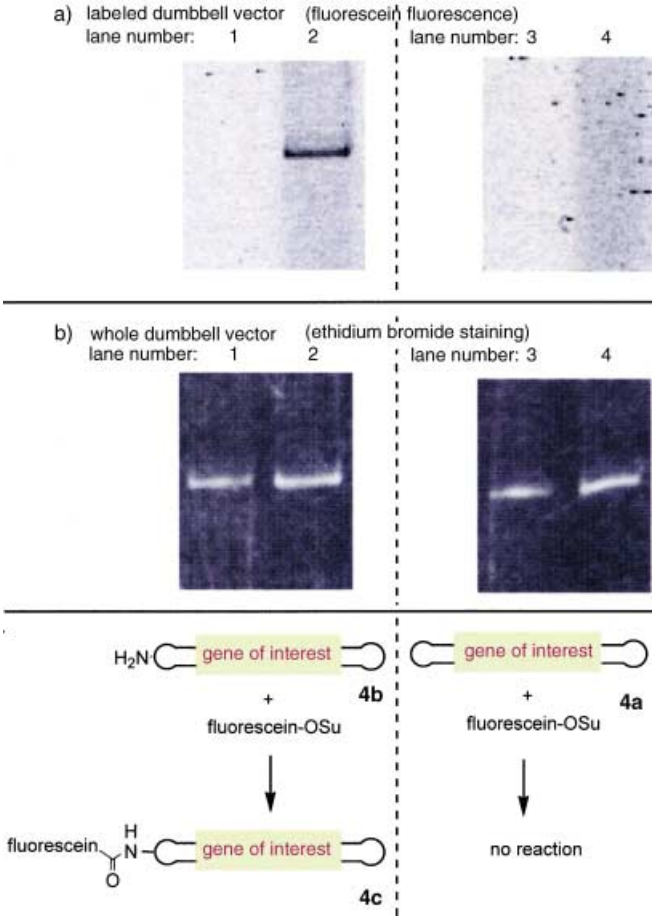

Figure 4. Loop-specific modification of dumbbell vectors by using the succinimide ester of fluorescein. The same amount of DNA was subjected to 8% PAGE. The bands were first visualized by using FluorImager 595 (Molecular Dynamics) at $\lambda_{ex} = 488$ nm (a). The gel was then stained with ethidium bromide, and the bands were visualized by using a transilluminator at $\lambda_{ex} = 302$ nm (b). Lanes 1 and 2: dumbbell DNA with a primary amino group in the hairpin loop; lanes 3 and 4: dumbbell DNA with no primary amino group. Lanes 2 and 4 contain samples of the reaction mixture with the succinimide ester of fluorescein. Lanes 1 and 3 contain samples before the reaction.

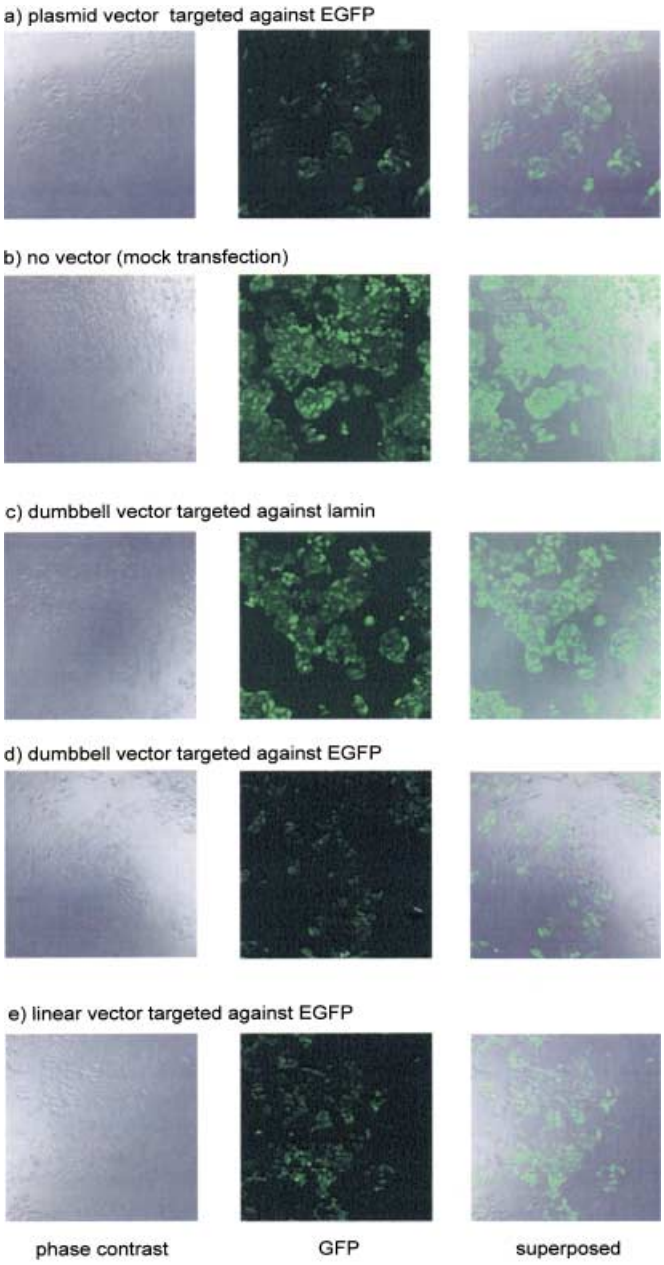

Figure 5. Inhibition of EGFP expression by using various vectors transfected in HeLa S3/EGFP cells.

down-regulation of the target was detected (Figures 5a, d, and e). We found that the best and most reproducible inhibition (more than 70%) was obtained with the dumbbell-shaped cassette (Figure 5d). We also found that the dumbbell-shaped siRNA expression vector was more effective than the linear-shaped PCR product after incubation for 48 h. It is known that RNA interference (RNAi) is one of the best methods for gene suppression.[9,10,12–14] The RNAi effect triggered by the dumbbell-shaped DNA appears to be higher and to last longer than that triggered by the linear-shaped PCR products, as the effect by the latter PCR products decreased after 48 hours in HeLa S3/EGFP. This decrease suggests that the half-life of dumbbell-shaped DNA inside cells is longer than that of linear DNA. This suggestion can be supported by the fact that dumbbell DNA is stable toward exonuclease III digestion, whereas linear DNA is totally degraded within 1 hour (Figure 2). In contrast, mock transfection had no effect on the expression of EGFP (Figure 5b). Similarly, the control dumbbell-shaped U6 expression cassette expressing siRNA targeted against lamin had no effect on the expression of EGFP (Figure 5c), although the lamin level was reduced when using this vector against lamin (see the Supporting Information), which demonstrates the high level of specificity of the vector.

In conclusion, we have established a novel and convenient method for creating dumbbell DNA molecules in excellent yield. The size of the dumbbell DNA obtained by this method cannot be obtained by standard solid-phase cyclization procedures.[15] Dumbbell-shaped DNA was found to be more stable than linear DNA both in vitro and in vivo. Recently, our group as well as Castanotto et al. reported a convenient, PCR-based approach for the rapid synthesis of

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U6 promoter-based siRNA gene constructs without carrying out any subsequent cloning, and their products are now commercially available.[14,16] By combining the PCR methodology and the present dumbbell-making technology, we can obtain long-term siRNA expressions without carrying out any time-consuming vector construction. This PCR-mediated minimal siRNA vector-construction system will open up new possibilities for biologically safe and stable gene therapy. Specific gene targeting might also be possible by the attachment of various moieties, such as functional peptides and proteins, through or in other primary amino groups on the hairpin-loop at one or both ends.

Experimental Section[17]

PCR amplification of U6 promoter-driven siRNA coding linear DNA targeted against lamin: 1 μM of each synthetic DNA primer was mixed with an siRNA expression plasmid against lamin (pU6i-lamin), and the PCR reaction was carried out with Ex Taq DNA polymerase (TaKaRa). The sequences of the sense and antisense *universal* primers for the PCR were 5′-pTTA GGA GTT X_nTC TCC TAA GCG TTT TCC CAG TCA CGA CGT TG-3′ and 5′-pTTA GGT CTT TTG ACC TAA GCG AGC GGA TAA CAA TTT CAC ACA GG-3′, respectively. Both primers contain the N.*Bpu*10I recognition sequence (underlined), and the 5′-ends were phosphorylated. The PCR product was isolated by using the Wizard(R) SV Gel and PCR Clean-Up System (Promega). The sense primer contains unmodified (X_1; dT), amino-modified (X_2, amino-modified dT), or fluorescein-modified (X_3, fluorescein-modified dT) deoxythymidine. The HPLC-purified *universal* primers were purchased from Hokkaido System Science Co., Ltd. (Sapporo, Japan).

PCR amplification of U6 promoter-driven siRNA coding linear DNA targeted against EGFP: 1 μM of each synthetic DNA primer was mixed with an siRNA expression plasmid against EGFP (pU6i-EGFP), and then the *first* PCR reaction was carried out with Ex Taq DNA polymerase (TaKaRa). The sequence of the sense and antisense primers for the *first* PCR were 5′-GTT TTC CCA GTC ACG ACG TTG AAG GTC GGG CAG GAA GAG-3′ and 5′-GAG CGG ATA ACA ATT TCA CAC AGG AAA AAG GCT ACG TCC AGG AG-3′, respectively. The *first* PCR product was isolated by 8% polyacrylamide gel electrophoresis (PAGE). The isolated PCR fragment was used as a template DNA in a *second* PCR reaction under the same conditions, using the above *universal* primers. The *second* PCR product was isolated by using the Wizard(R) SV Gel and PCR Clean-Up System (Promega).

Conversion of the siRNA-expressing linear DNA to Dumbbell-shaped DNA: 18.8 μg of each linear PCR product was mixed with 18.8 U of N.*Bpu*10I (MBI Fermentas, Hanover, MD), and the reaction mixture was incubated at 37 °C overnight. The mixture was heated for 1 min at 95 °C, and then brought from 95 °C to room temperature over 1 h. During this step, the terminal hairpin-loop structures were formed. For intramolecular ligation at the hairpin termini, T4 DNA ligase (16000 U) and ligation buffer (TaKaRa) was added directly to the reaction mixture and incubated at 16 °C for 3 h. **Note**: The above conversion processes are part of a one-pot reaction and were performed in one microtube. The ligated product was purified by extraction with phenol/chloroform and precipitated with ethanol.

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[17] All experimental details can be found in the Supporting Information.

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