Short Communication

RNA Interference With Small Hairpin RNAs Transcribed From a Human U6 Promoter-Driven DNA Vector

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Abstract. RNA interference (RNAi), a process of sequence-specific gene suppression, has been known as a natural gene regulatory mechanism in a wide range of organisms. Recently, a small-interference RNA (siRNA) technology has been reported to produce post-transcriptional gene silencing in mammalian cells. In the present study, we constructed a human U6 promoter-driven mammalian expression vector to produce hairpin double-stranded RNA and transfected this into a human cell line. Using this siRNA system, we were able to knock down the gene expression of an enhanced green fluorescence protein. This result indicates that the plasmid vector-based siRNA system is a promising method to downregulate gene expression in human cells.

Keywords: gene silencing, small interference RNA, human embryonic kidney cell

RNA interference (RNAi) has been reported in plants (1), *Caenorhabditis elegans* (2), and *Drosophila* (3, 4). In 1998, Fire et al. found that double-stranded RNA (dsRNA) was even more potent than either sense or antisense single strands alone, suggesting a new mechanism for gene silencing. They found that articulated RNAi is a highly gene-specific phenomenon that only requires a few molecules per affected cell and that suggested the presence of a catalytic or amplification component in the interference process (5).

Recently, the mechanisms of RNAi have become more defined. When dsRNAs are introduced into an organism, sequence-specific destruction of endogenous target mRNAs occurs. This process involves the cleavage of dsRNA into active 21–23 nt length small-interference RNA (siRNA) by Dicer (6) or another RNase III-like enzyme (7). These siRNAs are incorporated into a RNAi-induced silencing complex (8) and then are used as a guide to degrade the corresponding mRNA (9).

Although siRNA has a great potential to ‘knock down’ specific target genes in lower animals and plants, it has been difficult to apply this technology in the gene silencing of somatic mammalian cells because non-specific reduction of mRNA synthesis may cause cell

death. This side effect is related to antiviral host defense mechanisms in somatic mammalian cells, such as the activation of dsRNA-dependent protein kinase or 2',5'-oligoadenylate synthetases. Transfection of dsRNA (>30 bp) induces a general inhibition of host-cell translation by translation factor inactivation and/or non-specific degradation of the RNA transcript (10, 11).

However, Elbashir et al. recently demonstrated that synthetic 21 nt dsRNA may be able to avoid the host-interferon response, resulting in a specific suppression of endogenous and exogenous gene expression in a variety of mammalian cell lines, including human embryonic kidney (HEK 293T) and HeLa cells (12). Caplen et al. also presented supportive data on RNAi mediated by synthetic siRNAs that can induce gene-specific inhibition in *Caenorhabditis elegans* and in cell lines from humans and mice (13). A key discovery of these studies was that transfection of double-stranded siRNA eliminated nonspecific effects occurring in mammalian cells, suggesting that double-stranded 21 nt siRNA may provide a new tool for specific gene silencing in mammalian cells.

Although gene silencing by transfection of siRNA is effective in mammalian cells, it reduces gene expression for only a short period. To overcome this limitation, transfection of plasmid vectors, which can stably synthesize siRNA in host cells, is a possible alternative tech-

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nology for the short-acting siRNA transfection process.

It has been reported that the siRNAs synthesized by DNA vectors with mouse U6 promoter in the host cells were able to effectively suppress target gene expression (14, 15). The U6 promoter can transcribe small RNAs by RNA polymerase III (RNA-pol III), and this transcription is terminated when RNA-pol III meets 4–5 Ts, located at the 3' end of the gene construct, making it possible to directly produce siRNAs (16). Thus, to apply siRNA technology to human cells, construction of the siRNA plasmid vector human U6 (hU6) promoter (17) would be a most promising strategy.

Miyagishi et al. introduced a hU6 promoter-driven siRNA expression vector system using a different strategy. In this system, they employed a tandem expression system for simultaneous transcription of sense and antisense sequences. After the transcription of individual small single strand RNAs, these strands fused with each other to produce siRNA (18). Although these authors successfully suppressed target gene expression using the tandem siRNA expression system, the construction of this complex vector, which contains two U6 promoters, may be overly time consuming for general use.

Another group has demonstrated that hU6 promoter-driven Lamin A/C hairpin siRNA with a 4-mer spacer can successfully suppress Lamin A/C gene expression (19). Since hairpin structure siRNA with a 9-mer spacer is reported to show the most potent gene silencing effect using an expression vector with different promoters, indicating that the size of the loop is important in siRNA activity (20), we investigated a DNA vector producing 9-mer spacer hairpin siRNA under the hU6 promoter. Here we report that transfection of the hU6 promoter-

driven 9-mer spacer hairpin siRNA effectively suppresses exogenous genes and enhanced green fluorescence protein (EGFP) expression in HEK 293T cells.

Human U6 promoter (GenBank accession #M14486, gene sequence 64–355) was amplified from normal human genomic DNA (Clontech, Palo Alto, CA, USA) by the polymerase chain reaction (PCR), cloned into pUC19 (Invitrogen, Carlsbad, CA, USA) plasmid (pUC-hU6), and sequenced (17). As shown in Figure 1a, we constructed a hU6 promoter-driven siRNA vector to suppress EGFP-gene expression (pUC-hU6-siGFP) by inserting an siRNA sequence for EGFP (siGFP), which consisted of two 21-nt complementary sequences placed in opposition to each other with the insertion of a 9-mer spacer sequence and the attachment of 5 Ts at the 3'-end of the sequence downstream of the hU6 promoter. Transcription of RNA-pol III begins after the hU6 promoter and is stopped by the insertion of 5 Ts. The outcome should be the formation of hairpin dsRNA with a 9-mer spacer, with a 5 Us overhang at the 3'-end and 27-mer of the hU6 small nuclear RNA sequence at the 5'-end. The hairpin siRNA with the 27-mer of hU6 small nuclear RNA sequence at the 5'-end is reported to be more abundant than siRNA without this extra sequence (19).

To assess hU6 promoter activity in HEK 293T cells, we used pUC-hU6-extra, which is a plasmid containing an extra sequence (ggc aca caa cac tat ttt) instead of the siGFP downstream of the hU6 promoter (Fig. 1b). We found that HEK 293T transfected with pUC-hU6-random expressed a high level of the extra sequence using reverse transcription-PCR (RT-PCR), while HEK 293T cells without transfection did not show any

Fig. 1. Construction of the human U6 (hU6) promoter-driven plasmid vector for siRNA and measurement of hU6 promoter activity in HEK 293 cells. a: A schematic illustration of the hU6 promoter construct for hairpin siRNA. The sequence of the hU6 promoter was amplified by PCR from human genomic DNA with sense (5'-TCTTTGGAATTCAAGGTCGGGCAGGAA-GAGGGCCTA-3') and antisense (5'-AAAAATTCTAGATGTAAAAATAGTGTGTGTGCCTAGTATATGTGCTGCCGAA-GCGAGCAC-3') primers (17). The amplified DNA fragment was digested with *EcoRI* and *XbaI* and cloned into the *EcoRI*–*XbaI* site of pUC 19 (pUC-hU6-extra). A siRNA consisting of 21-mer sense and antisense sequences of EGFP (106–127) with a 9-mer insert and 5 Ts was synthesized with sense 5'-CGC GGATCCGGCGATGCCACCTACGGCAAGTTCAAGAGA-3' and antisense 5'-GCTCTAGAAAAAAGGCGATGCCACCTA CCGCAAGTCTCTTGAAC-3' primers using PCR. The DNA fragment was digested with *BamHI* and *XbaI* and then cloned into the *BamHI*–*XbaI* site of pUC-hU6-extra. b: Activity of hU6

promoter in the HEK 293 cell was measured by RT-PCR using 1 μ g of total RNA extracted from the cells with a primer set (5'-GTGCTCGC TTCGGCAGCACAT-3', 5'-AATAGTGTGTGTGCCTAGGA-3'). HEK 293 cells transfected with pUC-hU6-extra expressed the reference sequence (lane 2), while the cells transfected with null vector did not show any PCR product on the gel (lane 1).

Fig. 2. Typical fluorescent microscopic pictures of HEK 293 48 h after a transient transfection performed with Lipofectamine™ 2000 (Invitrogen) on subconfluent HEK 293 according to the manufacturer’s protocol in a 24-well culture plate. a: HEK 293 cells transfected with 400 ng of pcDNA 3.1 as a negative control for the fluorescent signal. b: HEK 293 cells with 400 ng of EGFP expression vector (pEGFP-C1) as a positive control. c: HEK 293 cells cotransfected with 400 ng of pEGFP-C1 and 2 μg of pUC-hU6-extra as a control for siRNA. d: HEK 293 cells cotransfected with 400 ng of pEGFP-C1 and 2 μg of pUC-hU6-siGFP. EGFP protein expression in HEK 293 cells was dramatically suppressed by cotransfection with pUC-hU6-siGFP.

PCR product on the gel. This result indicates that the hU6 promoter has a high efficiency for transcription of a small gene sequence such as siRNA in HEK 293T cells.

We investigated gene silencing by siGFP transcribed from pUC-hU6-siGFP in HEK 293T cells transfected with pEGFP-C1, which is a mammalian EGFP expression plasmid (Clontech). Protein expression levels of EGFP were assessed by green fluorescent signals under a microscope, and the gene expression level of EGFP was assessed by gel electrophoresis analysis after RT-PCR of an RNA sample was isolated from HEK 293T cells. HEK 293T cells transfected with pEGFP-C1 expressed EGFP protein (Fig. 2b) and genes (Fig. 3, lane 2), with the gene expression ratio between EGFP and β-actin ($EGFP/\beta actin = 1.14 \pm 0.141$ (mean ± S.D., n = 3), while cells transfected with pcDNA 3.1 (Clontech) did not show any fluorescent signals (Fig. 2a) or any bands on the gel (Fig. 3, lane 1, $EGFP/\beta actin = 0.07 \pm 0.008$). Co-transfection of pUC-hU6-siGFP almost shut down protein (Fig. 2d) and gene (Fig. 3, lane 4, $EGFP/\beta actin = 0.16 \pm 0.026$) expression of EGFP, but co-transfection of pUC-hU6-random failed to alter protein (Fig. 2c) and gene (Fig. 3, lane 3, $EGFP/\beta actin = 1.21 \pm 0.107$) expression of EGFP in HEK 293T cells transfected with pEGFP-C1. These results indicate that siRNAs, which have hairpin struc-

Fig. 3. Effects of pUC-hU6-siGFP on EGFP gene expression levels in HEK 293 cells. Transient transfection performed with Lipofectamine™ 2000 on subconfluent HEK 293 in a 6-well culture plate. After 48 h of transfection, total RNA was extracted from the HEK 293 cells and 1 μg of the RNA was reverse-transcribed and amplified using the SuperScript™ ONE-STEP™ RT-PCR System (Gibco, Carlsbad, CA, USA) with EGFP-specific oligo sense 5'-CAAGGACGACGGCAACTACAAGACC-3' and antisense 5'-GCG GACTGGGTGCTCAGGTAGTGGT-3' primers. Amplification of β-actin was used as an internal control (sense primer: 5'-GACAGGAT GCAGAAGGAGAT-3', antisense primer: 5'-TTGCTGATCCACA TCTGCTG-3'). A sample from HEK 293 cells was transfected with 2 μg of pcDNA 3.1 as a negative control (lane 1). A sample from HEK 293 cells was transfected with 2 μg of pEGFP-C1 alone as a positive control (lane 2). A sample from HEK 293 cells was cotransfected with 2 μg of pEGFP-C1 and 10 μg of pUC-hU6-extra (lane 3). A sample from HEK 293 cells was cotransfected with 2 μg of pEGFP-C1 and 10 μg of pUC-hU6-siGFP (lane 4). EGFP gene expression in HEK 293 cells was dramatically suppressed by cotransfection with pUC-hU6-siGFP.

tures with a 9 nt insert and a 5' U3' overhang, transcribed from a hU6 promoter-driven plasmid in human cells are able to knock down target gene expression.

We are able to efficiently shut down exogenous target protein expression in human cells by gene silencing technology using 21 nt hairpin siRNA. Since this siRNA is transcribed from the siRNA expression plasmid vector under the hU6 promoter that requires only a simple DNA vector construction, it will be helpful to investigate RNAi for a variety of genes that encode physiologically significant proteins. Although selectivity of RNAi may vary by target sequence and should be checked in each individual case, since stable transfection of siRNA expression plasmid is expected to permanently knock down the target gene, our RNAi system will be useful to investigate the function of human cells.

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