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Simple and efficient DNA vector-based RNAi systems in mammalian cells

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Abstract

We have constructed four different RNA polymerase III (Pol III)-based expression vectors, containing H1 or U6 promoters from human and mouse, which enable the endogenous production of small RNA transcripts for gene silencing applications. In addition, to facilitate the selection of recombinant clones, we have further improved these vectors by constructing a stuffer of puromycin resistance gene (Puro^r) between *Cla*I and *Hin*dIII sites, which makes the preparation of vectors easy for rapid and efficient cloning of targeting sequences. A comparative analysis of the silencing efficiency between shRNA, sense-RNA, antisense-RNA, and siRNA showed that both the shRNA and siRNA, but not the sense-RNA and antisense-RNA, dramatically inhibit the targeting gene firefly luciferase activity in mammalian cells. However, there were no significant differences in the inhibition of firefly luciferase expression by shRNA and siRNA expressed from these DNA vectors. In summary, these improved DNA vector-based RNAi systems should provide a simple, convenient, and efficient cloning strategy for studying gene functions in mammalian cells.

Keywords: RNA polymerase III promoter; H1 promoter; U6 promoter; shRNA; Antisense-RNA; Sense-RNA; siRNA; RNA interference

RNA interference (RNAi) is a process of posttranscriptional gene silencing by which double-stranded RNA (dsRNA) induces sequence-specific degradation of homologous gene transcripts [1,2]. RNAi is triggered by exposing cells to dsRNAs either via exogenous delivery or endogenous expression. The long dsRNA molecules are first processed into 21- to 23-nucleotide (nt) small interfering RNA duplexes (siRNAs) with symmetrical 2-nt 3′ overhanging ends by the action of an endogenous dsRNA-specific endonuclease, Dicer, a member of the RNase III family [3–6]. Subsequently, the siRNA products are effectively incorporated into the RNA-induced silencing complex (RISC), which is then guided to catalyze the enzymatic cleavage of complementary mRNA at the site where the antisense siRNA strand is bound [7,8].

RNAi is evolutionarily conserved to each of the eukaryotic lineages. It appears to have a primary function as a cellular defense mechanism against viral infection [9,10] and transposable element-induced genomic instability [11–13]. In addition, it also appears to be involved in the regulation of cellular genes important for metazoan development [14,15]. RNAi has become a powerful and widely used approach for the analysis of gene function in a variety of organisms, including plants and animals. In plants and invertebrates, introduction of dsRNA into the cells leads to destruction of endogenous mRNA that is homologous to the dsRNA. In mammals, however, long dsRNAs (>30 nt in length) activate a

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dsRNA-dependent protein kinase (PKR), which subsequently phosphorylates and inactivates the eukaryotic initiation factor 2α (eIF2α) subunit resulting in general inhibition of cellular protein synthesis, as well as, the 2′,5′-oligoadenylate synthetase (OAS), which in turn activates RNaseL causing a non-specific degradation of cellular mRNA [16–20]. However, by using short synthetic 21-nt siRNAs with 2-nt 3′ overhangs allowed for sequence-specific gene silencing yet avoided the non-selective cytotoxic effects of long dsRNAs in mammalian cells [21,22].

In mammalian systems including in vitro and in vivo, the silencing effect induced by exogenous delivery of synthetic siRNAs is transient and reactivation of the target gene normally occurs after a few days [21,22]. In addition, the efficacy of siRNAs is dependent on the specificity of the target sites within a gene. In order to obtain effective siRNAs, it is necessary to design, synthesize, and screen many different siRNAs, which are expensive due to the cost of chemical synthesis of RNA oligonucleotides [23–26]. To overcome these limitations, DNA vector-based RNAi systems driven by RNA polymerase III (Pol III) promoters have been developed to express transcripts that can be converted into siRNAs in mammalian cells [27–31]. RNA Pol III promoters, especially H1 and U6 from human and mouse, have been used most frequently, since they have a well-defined start site of transcription and a simple effective termination signal consisting of only five or six consecutive thymidine residues (Ts), and therefore they are suitable for the synthesis of small RNA transcripts. Moreover, they can efficiently transcribe small RNA transcripts lacking both the 5′ cap and 3′ polyadenosine [poly(A)] tail [32,33].

There are mainly two strategies in producing active siRNAs by DNA vector-based RNAi systems in mammalian cells [34]. The short hairpin RNA (shRNA) strategy uses a single promoter followed by the sense, a loop, and the antisense sequences [29,31,35]. The dual promoters' strategy uses tandem promoters that drive the expression of sense and antisense strands from separated transcriptional units [36]. To develop suitable and effective DNA vectors for simple and rapid cloning of targeting sequences, we have constructed four different expression vectors, containing the widely used RNA Pol III H1 and U6 promoters from human and mouse, for efficient expression of small RNA transcripts in mammalian cells. The expression cassettes are designed in which small RNA coding sequences are inserted between unique *Cla*I and *Hin*dIII sites. However, one big obstacle for these DNA vector-based RNAi systems is that it takes a lot of time and trouble to make the DNA constructs. To facilitate the cloning of targeting sequences into these expression cassettes, we have improved upon these DNA vectors by constructing a stuffer of puromycin resistance gene (Puro^r) between *Cla*I and *Hin*dIII sites. Moreover, systematic comparison of the knockdown efficiency among these expression vectors remains to be elucidated. These promoters were used to drive the transcription of shRNA, sense-, antisense-RNA, and siRNA molecules for targeting directed against firefly luciferase reporter gene in mammalian cells. The silencing efficiency of different promoters and transcripts was compared.

Materials and methods

Cell culture. Human neuroblastoma cell line SK-N-SH, mouse embryo fibroblast NIH3T3, and baby hamster kidney fibroblast BHK were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) supplemented with heat-inactivated 10% fetal calf serum (FCS; Biological Industries, Kibbutz Beit Haemek, Israel) and 1% antibiotic/antimycotic solution (Gibco-BRL) at 37 °C in a humidified incubator with 5% CO_2. The cell line was routinely split two to three times a week after trypsinization.

Transfection and luciferase assay. Twenty-four hours before transfection, cells were trypsinized and seeded in 6-well culture plates at 1×10^5 cells per well. The cells were transiently transfected with 0.5 μg of the target vectors and 1.5 μg of each of the various shRNA, siRNA, antisense RNA, and sense RNA expression vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were collected at 60 h later and aliquots of the cell lysates containing equal amounts of protein were analyzed by Dual-luciferase Reporter Assay System (Promega, Madison, WI, USA) as recommended by the manufacturer or Western blotting. The total protein in the cell lysates was determined using the BCA assay (Pierce, Rockford, IL, USA).

Construction of dual-luciferase reporter plasmid pCMV-FL/RL and pCMV-p53/EGFP expression vector. Plasmid vectors were constructed by using standard molecular cloning techniques. The dual-luciferase reporter plasmid pCMV-FL/RL (Fig. 2A), containing firefly (*Photinus pyralis*) luciferase (*luc*+) and *Renilla* (*Renilla reiformis*) luciferase (R*luc*) genes, was constructed by inserting the *luc*+ gene from pGL3-Basic (Promega) and the R*luc* gene from pRL-TK (Promega) into the pCMVβ (BD Biosciences Clontech, Palo Alto, CA, USA) vector to generate pCMV-FL and pCMV-RL, respectively, and then the R*luc* gene expression cassette from pCMV-RL was cloned into the pCMV-FL vector. The pCMV-p53/EGFP expression vector (Fig. 2A), containing tumor suppressor protein p53 (*Tp53*) and enhanced green fluorescent protein (EGFP) genes, was generated by inserting the *Tp53* gene from pRev-TRE-p53 (kindly provided by Ming-Derg Lai, Department of Biochemistry, National Cheng Kung University, Tainan, Taiwan) and EGFP gene from pEGFP-N1 (BD Biosciences Clontech) into the pCMVβ vector to create pCMV-p53 and pCMV-EGFP, respectively, and then the *Tp53* gene expression cassette from pCMV-p53 was cloned into the pCMV-EGFP vector.

Construction of Pol III promoter expression vectors. The RNA Pol III promoters, H1 and U6 from human and mouse, were PCR-amplified using synthetic oligonucleotides and cloned into an *Eco*RI/*Hin*dIII restriction site of a pGEM-7Zf(+) vector (Promega) or an *Eco*RI/*Cla*I restriction site of a pGEM-7Zf(+)-derived vector. Oligonucleotides were purchased from commercial suppliers. Oligonucleotides used for the amplification of H1 and U6 promoters from human and mouse were: HsU6-S: 5′-GGAATTCAAGGTCGGGCAGGAAGAGG-3′ and HsU6-AS: 5′-CCCAAGCTTCCATCGATGTTTCGTCCTTTCCACAAGATAT-3′; HsH1-S(T7): 5′-TAATACGACTCACTATAGGG-3′ and HsH1-AS : 5′-CCATCGATAAAGAGTGGTCTCATACAG-3′; MmU6-S: 5′-GGAATTCATCCGACGCCGCCATCTCTAGG-3′ and MmU6-AS: 5′-CCATCGATCAAGGCTTTTCTCCAAGGGATA-3′; and MmH1-S: 5′-GGAATTCCGCTCTTGAAGGACGACGTCATC-3′ and MmH1-AS: 5′-CCATCGATAGGGTGTAGACCGGCCGCCAC-3′. The resulting plasmids were designated as