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Biochemical and Biophysical Research Communications 330 (2005) 53–59
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Simple and efficient DNA vector-based RNAi systems in mammalian cells

Meng-Tsai Wu [a], Ren-Huang Wu [a], Chuan-Fu Hung [a], Tsung-Lin Cheng [b], Wen-Hui Tsai [c,d], Wen-Tsan Chang [a,b,*]

[a] Department of Biochemistry, National Cheng Kung University Medical College, Tainan 701, Taiwan, ROC
[b] Institute of Basic Medical Sciences, National Cheng Kung University Medical College, Tainan 701, Taiwan, ROC
[c] Institute of Clinical Medicine, National Cheng Kung University Medical College, Tainan 701, Taiwan, ROC
[d] Department of Pediatrics, Chi Mei Foundation Medical Center, Tainan 701, Taiwan, ROC

Received 17 February 2005

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.
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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

* Corresponding author. Fax: +886 6 2741694.
E-mail address: wtchang@mail.ncku.edu.tw (W.-T. Chang).

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doi:10.1016/j.bbrc.2005.02.129

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

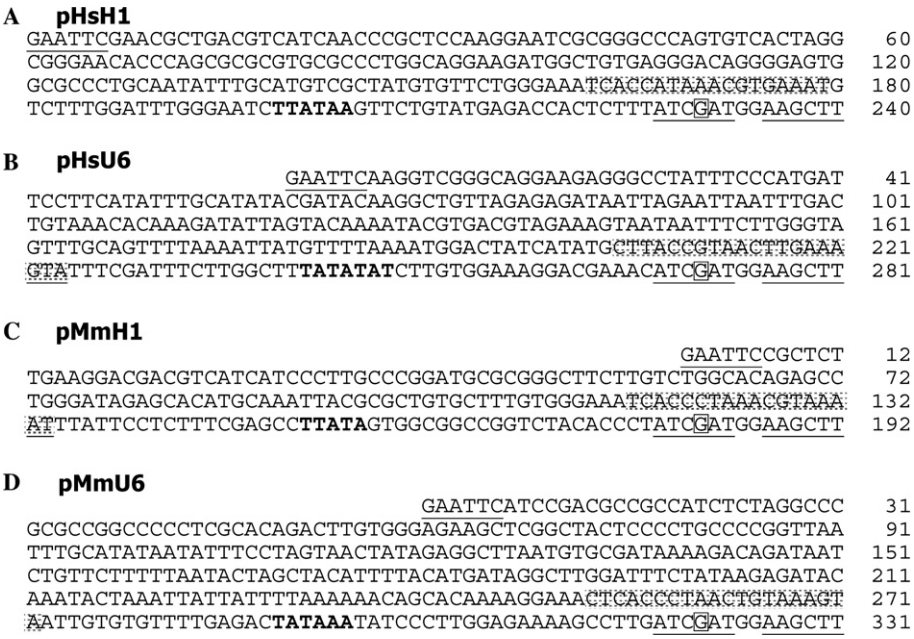

Fig. 1. Sequence analysis of RNA Pol III promoters. The human H1 (A) and U6 (B), and mouse H1 (C) and U6 (D) promoters were isolated from human and mouse genomic DNAs by PCR amplification. The proximal sequence element (PSE) is shaded in gray, TATA box is in bold, restriction sites of *Eco*RI (GAATTC), *Cla*I (ATCGAT), and *Hin*dIII (AAGCTT) are underlined, and G is the transcription initiation site (+1).

pHsH1, pHsU6, pMmH1, and pMmU6 (Fig. 1). To construct the improved pHsH1puro, pHsU6puro, pMmH1puro, and pMmU6puro vectors (Fig. 2B), the *Cla*I/*Hin*dIII-Puro^r gene fragment from pMSCVpuro vector (BD Biosciences Clontech) was inserted into the *Cla*I/*Hin*dIII-digested pHsH1, pHsU6, pMmH1, and pMmU6 vectors.

Construction of shRNA, sense-, antisense-RNA, and siRNA expression vectors. A general strategy for constructing shRNA, sense-RNA, and antisense-RNA expression systems involved ligating an annealed oligonucleotide duplex into *Cla*I/*Hin*dIII restriction site of an expression vector. Oligonucleotides for shRNA, sense-RNA, and antisense-RNA syntheses targeting the firefly luciferase and tumor suppressor protein p53 genes were purchased from commercial suppliers. (i) Oligonucleotides for pSUPER-expressed *luc*+ shRNA as published previously [21]: forward, 5′-CTTACGCTGAGTACTTCGAttcaagagaTCGAAGTACTCAGCGTAAGT TTTTTGGAAA-3′; reverse, 5′-CTTACGCTGAGTACTTCGAtctcttgaaTCGAAGTACTCAGCGTAAG-3′. (ii) Oligonucleotides for pHsH1-, pHsU6-, pMmH1-, and pMmU6-expressed *luc*+ shRNA: forward, 5′-CGACATCACTTACGCTGAGTACTTCGAttcaagagaTCGAAGTACTCAGCGTAAGTGATGTCTTTTTTG GAAA-3′; reverse, 5′-AGCTTTTCCAAAAAAGACATCACTTACGCTGAGTACTTCGAtctcttgaaTCGAAGTACTCAGCGTAAGTGATGT-3′. (iii) Oligonucleotides for pHsH1-, pHsU6-, pMmH1-, and pMmU6-expressed *luc*+ sense-RNA: forward, 5′-CGACATCACTTACGCTGAGTACTTCGATTTTTTGGAAA-3′; reverse, 5′-AGCTTTTAAAAAATCGAAGTACTCAGCGTAAGTGATGT-3′. (iv) Oligonucleotides for pHsH1-, pHsU6-, pMmH1-, and pMmU6-expressed *luc*+ antisense-RNA: forward, 5′-CGGTCGAAGTACTCAGCGTAAGTGATGTCTTTTTA-3′; reverse, 5′-AGCTTAAAAAAGACATCACTTACGCTGAGTACTTCGAC-3′. (v) Oligonucleotides for pHsH1-, pHsU6-, pMmH1-, and pMmU6-expressed *Tp53* shRNA: forward, 5′-CGACTCCAGTGGTAATCTACttcaagagaGTAGATTACCACTGGAGTCTTTTTTGGAAA-3′; reverse, 5′-AGCTTTTCCAAAAAAGACTCCAGTGGTAATCTACtctcttgaaGTAGATTACCACTGGAGT-3′. The underlined sequences are the targeting sequences on the firefly luciferase or p53 gene. To construct the DNA vector-expressed siRNA system, the antisense-RNA expression cassettes were inserted into the sense-RNA expression vectors.

Western blot analysis of p53 and EGFP. Cells were harvested 60 h after transfection and lysed in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 2 mM EDTA, and 1% NP-40) containing protease inhibitors (Roche, Mannheim, Germany). Total protein extracts (30 μg per lane) were resolved on a 10% SDS–polyacrylamide gel, transferred onto an Immobilon-P membrane (Millipore, Billerica, MA, USA), and incubated with anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-GFP monoclonal antibody (Santa Cruz Biotechnology), followed by incubation with horseradish-peroxidase-conjugated anti-mouse IgG secondary antibody (Santa Cruz Biotechnology). The bands were visualized by using the enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Results and discussion

Construction of improved DNA vector-based RNAi systems

The active, in vivo identified or in vitro synthesized siRNA has been shown to be a small 21- to 23-nt RNA duplex with symmetrical 2-nt 3′ overhangs [37,38]. The input RNA molecule can be either in the form of a long dsRNA or a hairpin dsRNA [39,40], both being subsequently processed into siRNA by Dicer endonuclease [3–6]. However, the long dsRNAs provoke a strong cytotoxic response through the activation of PKR and OAS in mammalian cells [16–20]. This non-specific cytotoxic effect can be overcome by directly applying synthetic siRNAs, pools of siRNAs, or DNA vector-expressed small RNA transcripts, including shRNAs and siRNAs.

To synthesize a defined small RNA transcript displaying features close to above requirements, we used

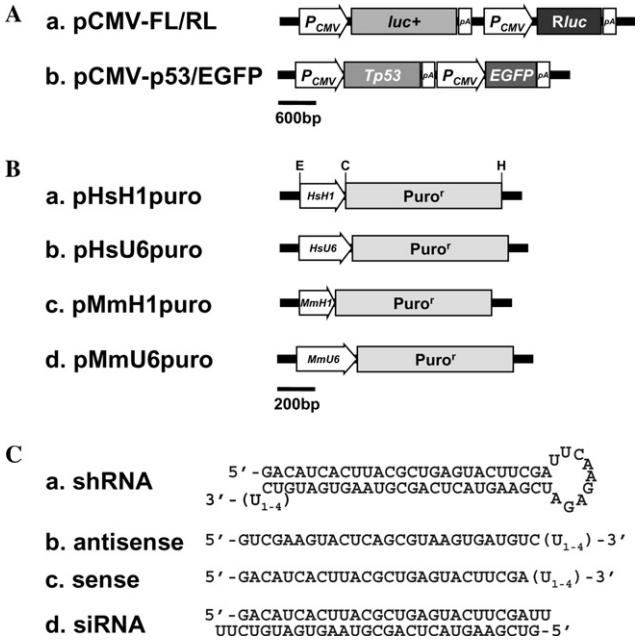

Fig. 2. RNAi target vectors and DNA vector-based RNAi systems. (A) Constructs of RNAi target vectors. The dual-luciferase reporter plasmid pCMV-FL/RL (a) contained the firefly luciferase (*luc*+) and *Renilla* luciferase (R*luc*) expression cassettes, and pCMV-p53/EGFP (b) contained the tumor suppressor protein p53 (*Tp53*) and enhanced green fluorescent protein (*EGFP*) expression cassettes. The R*luc* and *EGFP* expression cassettes serve as reference protein expression systems for the RNAi target vectors, pCMV-FL/RL and pCMV-p53/EGFP, respectively. (B) Constructs of the improved DNA vector-based RNAi systems. The puromycin resistance gene (Puro^r) was cloned between *Cla*I and *Hin*dIII sites of the vectors as a stuffer that was convenient for the construction of the RNAi target sequence. E, C, and H are the *Eco*RI, *Cla*I, and *Hin*dIII restriction enzyme sites. (C) Small RNA transcripts expressed from Pol III promoter-based RNAi systems. The predicted shRNA (a), antisense-RNA (b), sense-RNA (c), and siRNA (d) variants of the firefly luciferase *luc*+ transcribed from pHsH1, pHsU6, pMmH1, and pMmU6 vectors.

the RNA Pol III promoters that transcribe the RNA from a defined start site (+1) and terminate at a run of 5–6 Ts, making it possible to design RNA with defined sizes. We have cloned the widely used RNA Pol III promoters including H1 and U6 from human and mouse by PCR amplification. The PCR products were cloned into an *Eco*RI/*Hin*dIII restriction site in a pGEM-7Zf(+) vector (Promega) or an *Eco*RI/*Cla*I restriction site in a pGEM-7Zf(+)-derived vector and the resultants were designated as pHsH1, pHsU6, pMmH1, and pMmU6 (Fig. 1). To make the construction simple and convenient, these vectors all contained the unique cloning sites, *Cla*I and *Hin*dIII, for construction of RNAi target sequences. Since U6 promoter transcribes preferentially from a "G" nucleotide at the +1 position, whereas the H1 promoter is less strict. We designed particularly that RNA transcripts start with a G-nt in the vectors, where it locates within the *Cla*I restriction site. Nucleotide sequence of the PCR products was analyzed by DNA sequencing and verified by a BLAST search of the non-redundant NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/).

Although siRNA has a great potential to silence genes in a sequence-specific manner, the efficiency of siRNA is dependent on the identification of specific target sites, because not all sequences are effective in RNAi-mediated gene silencing. Specifically selected target sequences can be easily cloned into an expression cassette, providing an optimal system for testing endogenous expression and activity of siRNAs. However, one big obstacle for DNA vector-based RNAi systems is that it takes a lot of time and effort to make the DNA constructs. To enhance the convenience of constructing a DNA vector-based RNAi system and facilitate the selection of recombinant clones, we have further improved these systems by inserting a stuffer of puromycin resistance gene (Puro^r) between *Cla*I and *Hin*dIII sites, which makes the preparation of vectors simple and easy by just removing the stuffer of Puro^r DNA fragment with *Cla*I and *Hin*dIII double digestion. The improved vectors were named as pHsH1puro, pHsU6puro, pMmH1puro, and pMmU6puro (Fig. 2B).

The sensitive luciferase assay combined with the DNA vector-based RNAi system provides a reliable readout even at low transfection efficiencies. Dual-luciferase expression plasmid pCMV-FL/RL, encoding firefly (*P. pyralis*) luciferase and *Renilla* (*R. reiformis*) luciferase, was used to test the efficiency of gene silencing induced by the DNA vector-expressed small RNA transcripts. The target mRNA sequence was 19 nt in length from the luciferase gene, which has been shown to be an effective target for RNAi [21]. In addition, it has been reported that when the sequence for RNAi is effective, the knockdown efficiency increases with the increasing siRNA length [7,41]. We expanded the sequence to 26 nt due to the requirement of a G-nt at the +1 position and designed the sequence for shRNA transcripts accordingly (Fig. 2C). The shRNA expression cassette contained 19- or 26-nt sequences (sense) from the target mRNA followed by a short 9-nt spacer, the reverse complement of the sense sequence (antisense), and 6 Ts as a termination signal. The *Cla*I site at 5′ end and the *Hin*dIII site at the 3′ end corresponded to the same restriction sites at both ends of the Puro^r stuffer.

Comparison of RNAi efficiency induced by shRNA, antisense-RNA, sense-RNA, and siRNA

To determine the knockdown efficiency of the 19-nt siRNA expressed from pSUPER and 26-nt siRNAs expressed from pHsH1 on firefly luciferase expression, human neuroblastoma cell line SK-N-SH cells were cotransfected with dual-luciferase expression plasmid pCMV-FL/RL (Fig. 2A) and both the shRNA expression constructs pHsH1-shluc+ and pSUPER-shluc+.

Following 60 h incubation period, cells were lysed, and firefly and *Renilla* luciferase activities were measured by Dual-luciferase Reporter Assay System (Promega). In pHsH1-shluc+ system, the shRNA with 26 nt exhibited a silencing efficiency of more than 90%, whereas the pSUPER-shluc+ system with 19 nt had a knockdown efficiency of nearly 60% (data not shown). These results indicated that the isolated human H1 promoter is effective in mediating shRNA expression and the shRNA with 26 nt is more effective than 19 nt.

We then compared the silencing efficiency between shRNA, sense, antisense-RNA, and siRNA transcripts expressed from pHsH1. The target sequence is 26 nt for the firefly luciferase mRNA. The levels of firefly and *Renilla* luciferase activities were determined by Dual-luciferase Reporter Assay System at 60 h after cotransfection of pCMV-FL/RL and pHsH1 carrying various small RNA coding sequences. As the results shown in Fig. 3A, both the shRNA and siRNA exhibited high inhibition effects with a knockdown efficiency of more than 80%, whereas the sense- and antisense-RNA transcripts had no effect on firefly luciferase expression (Fig. 3A). We examined further the silencing effect among shRNA, sense, antisense-RNA, and siRNA transcripts expressed from the other pHsU6, pMmH1, and pMmU6 vectors. Consistent with the results obtained from the pHsH1 system, both the shRNA and siRNA expressed from pHsU6, pMmH1, and pMmU6 also displayed strong silencing effect on firefly luciferase expression with similar inhibiting efficiency. The shRNA system was slightly better than siRNA system, with a knockdown efficiency of more than 90% (Fig. 3). However, the antisense and sense transcripts were ineffective in all of the RNA Pol III promoter systems.

The knockdown efficiency between human H1 and U6, and mouse H1 and U6 promoter systems has not been compared at the same time before. We cotransfected pCMV-FL/RL and shRNA or siRNA expression constructs driven by the four different RNA Pol III promoters into SK-H-SH, NIH3T3, and BHK cells. The levels of firefly and *Renilla* luciferase activities were assayed by Dual-luciferase Reporter Assay System at 60 h after cotransfection. The firefly luciferase activity was reduced by more than 80% with little difference between these promoters in all three different cell types (Fig. 4). In addition, the shRNA systems showed slightly better effects on the inhibition of firefly luciferase expression than siRNA systems. To further test the inhibition effect of these shRNA systems, we cotransfected pCMV-p53/EGFP and shRNA expression constructs driven by these four different RNA Pol III promoters into BHK cells. The levels of p53 and EGFP protein production were determined by Western blot analysis at 60 h after transfection. As the results shown in Fig. 5, all of the shRNAs expressed from pHsH1, pHsU6, pMmH1, and pMmU6 vectors exhibited a complete inhibition of the pCMV-p53/EGFP-induced p53 protein production, but control vectors displayed no inhibition effects on p53 protein production. These results indicate that all of the four promoters can efficiently drive shRNA and siRNA expression, and effectively mediate gene inhibition.

In summary, our results clearly demonstrated that silencing effects were improved by extending the length

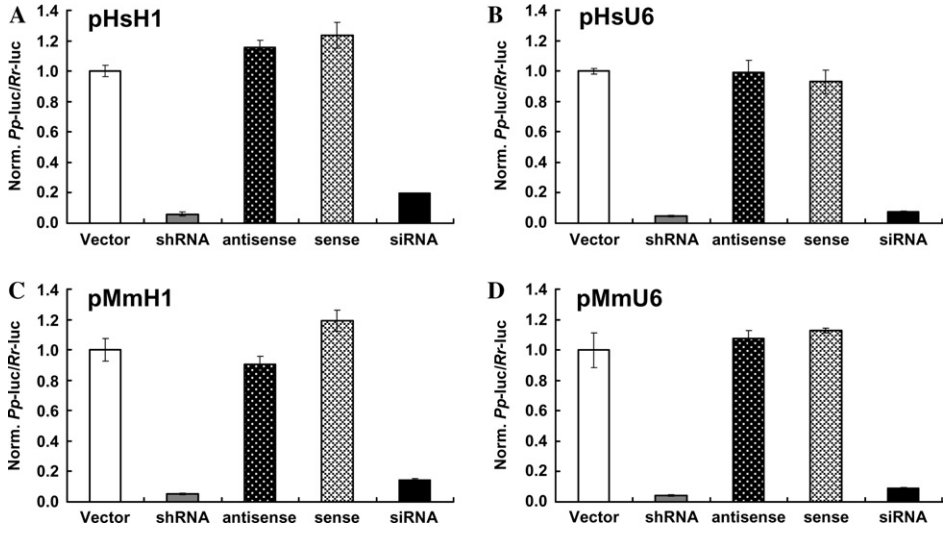

Fig. 3. Efficiency of gene silencing induced by different small RNA transcripts. Inhibition effects of shRNAs, antisense-RNAs, sense-RNAs, and siRNAs expressed from different RNA Pol III promoters, including pHsH1 (A), pHsU6 (B), pMmH1 (C), and pMmU6 (D) vectors, on pCMV-FL/RL-induced firefly luciferase expression. SK-N-SH cells were cotransfected with 0.5 μg of dual-luciferase reporter plasmid pCMV-FL/RL and 1.5 μg DNA vector-based RNAi constructs as indicated by Lipofectamine 2000. At 60 h posttransfection, the expression levels of firefly and *Renilla* luciferases in the total protein extracts were measured by Dual-luciferase Reporter Assay System. The firefly luciferase/*Renilla* luciferase (*Pp-luc Rr-luc*) ratio was normalized and calculated against the control vector (A, pHsH1; B, pHsU6; C, pMmH1; and D, pMmU6). The plotted data were averaged from three independent experiments and the bars indicate standard deviation.

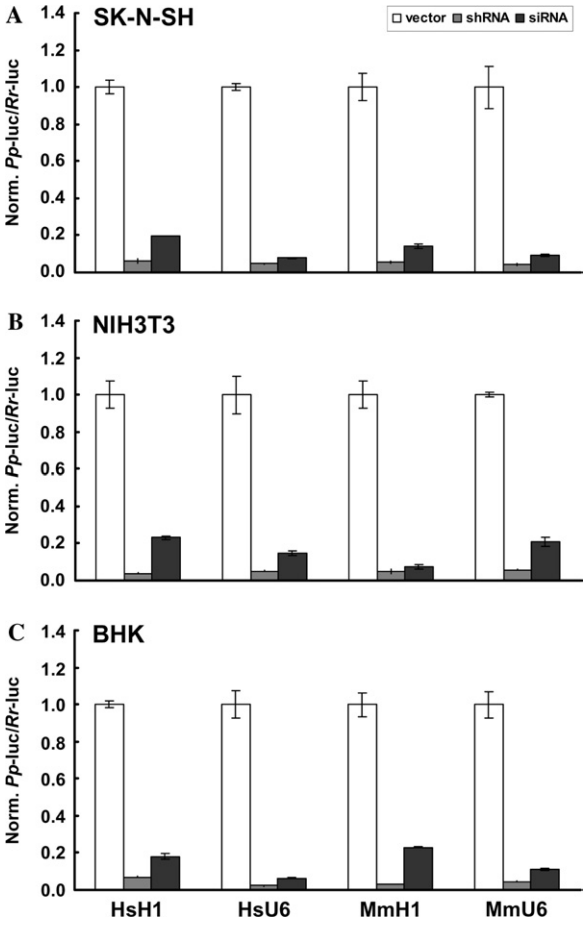

Fig. 4. Comparison of gene silencing induced by shRNA and siRNA expressed from different RNA Pol III promoters. Inhibition effects of shRNAs and siRNAs expressed from different RNA Pol III promoters, including pHsH1, pHsU6, pMmH1, and pMmU6 vectors as indicated, on pCMV-FL/RL-induced firefly luciferase expression in SK-N-SH (A), NIH3T3 (B), and BHK (C) cells. The experimental procedures were performed exactly as Fig. 3. The plotted data were averaged from three independent experiments and the bars indicate standard deviation.

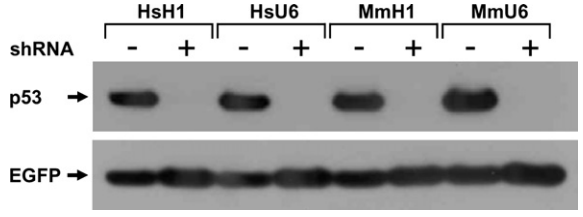

Fig. 5. Inhibition effects of shRNAs expressed from different RNA Pol III promoters on tumor suppressor protein p53 expression. BHK cells were cotransfected with 0.5 μg pCMV-p53/EGFP and 1.5 μg DNA vector-based RNAi constructs as indicated by Lipofectamine 2000. At 60 h posttransfection, the expression levels of p53 and EGFP in the total protein extracts were analyzed by Western blotting. The levels of EGFP serve as reference protein for transfection efficiency.

of siRNA sequence, and that both the shRNAs and siRNAs expressed from these DNA vector-based RNAi systems were effective in inhibiting the homologous RNA transcripts in cultured mammalian cells. Since RNAi has evolved as a powerful strategy for reverse functional genomics in various organisms and with tremendous therapeutic potentials in mammals, our systems should provide a simple and rapid cloning tool for expression of the small RNA transcripts, which can effectively induce RNAi-mediated gene silencing in mammalian cells. Moreover, the construction of our DNA vector-based RNAi systems is also cost and time saving in comparison to previous methods.

Acknowledgment

This work was supported by grants from the National Science Council of Taiwan, ROC (to Wen-Tsan Chang).

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