Small interfering RNA induced knockdown of green fluorescent protein using synthetic RNA molecules

RESEARCH

✍ José A Silva, Yamila Carpio, Elsa Rodríguez, Regla C Estrada, Mario P Estrada

[1]Center for Genetic Engineering and Biotechnology
Ave.31 e/158 y 190, Cubanacan, Playa, Aptdo. 6162,
CP 10 600, Ciudad de la Habana, Cuba
E-mail: jose.silva@cigb.edu.cu

ABSTRACT

Small interfering RNAs can be introduced into cells and cause silencing of specific genes. This can be used to study the effect of the gene on cellular function. The small interfering RNAs can be chemically prepared in an ordinary DNA/RNA synthesizer of a suitable quality for RNA interference. Here we report how these molecules are obtained at the Center for Genetic Engineering and Biotechnology (CIGB), Cuba, and their evaluation in the knowdown of green fluorescent protein in mammalian cells. In HEK293 cells, the eGFP expression was visualized at 24 and 48 h post-transfection under a fluorescence microscope. We observed that one RNA duplex (named RNA duplex B) silenced eGFP expression at 48 h post-transfection at a dose of 120 nM. No evident silencing effects were observed with the other RNA duplex (RNA duplex A). These results were corroborated by Northern blot analysis.

Key words: siRNA, green fluorescence protein, RNA synthesis

Biotecnología Aplicada 2007;24:49-52

RESUMEN

Silenciamiento de la proteína fluorescente verde inducido por ARN interferencia de tamaño pequeño mediante el empleo de moléculas de ARN sintéticas. Los ARNs de interferencia pequeños pueden ser introducidos en las células y provocar el silenciamiento de determinados genes. Este fenómeno de ARN interferencia puede ser utilizado en la determinación de la función de los genes en la célula. Los ARNs de interferencia de pequeño tamaño se pueden obtener por vía química, utilizando sintetizadores comunes de ADN/ARN con una calidad adecuada para mediar el fenómeno de ARN interferencia. En este trabajo presentamos la obtención de estas moléculas en el Centro de Ingeniería Genética y Biotecnología de Cuba y su evaluación en el silenciamiento de la proteína fluorescente verde en células de mamíferos. La expresión de eGFP fue visualizada a las 24 y 48 h después de la transfección en células HEK293 en un microscopio de fluorescencia. Se observó que el ARN doble cadena denominado B silenció la eGFP a las 48 h después de la transfección a una dosis de 120 nM. No se observó silenciamiento evidente de la expresión de la proteína con el ARN doble cadena denominado A Estos resultados fueron corroborados mediante análisis por Northem Blot.

Palabras claves: ARN interferencia de tamaño pequeño, proteína fluorescente verde, síntesis de ARN

*I*ntroduction

The introduction of double-stranded RNA (dsRNA) into a range of organisms induces both a potent and specific post-transcriptional gene silencing effect by directing degradation of homologous target RNAs. This form of gene suppression was first observed in *Caenorhabditis elegans* and termed RNA interference or RNAi [1]. RNAi has been observed in a wide range of organisms [2-5]. Biochemical analyses of the mechanism of RNAi have indicated that the gene silencing mediators are the 21-base pair small interfering RNAs (siRNAs) generated from longer dsRNA by the RNAse III-like enzyme Dicer [5]. These cleavage products are subsequently incorporated into the RNA-induced silencing complex (RISC) [5].

In mammalian cells, the use of long dsRNA has been restricted due to the proposed activation of an antiviral defense system that blocks protein translation leading to cell death [6]. Recently, this limitation to the application of RNAi in mammalian cells was overcome by the demonstration that chemically synthesized 21 base pair siRNAs, the effectors of RNAi, could be used in a wide range of human and mouse cell lines to induce gene silencing [7-12]. Delivery of chemically synthesized short interfering RNAs, mimicking Dicer cleavage substrates, results in the sequence-specific, robust silencing of the expression of the corresponding endogenous gene [7-12], thus bypassing the non-specific inhibitory mechanisms elicited by longer dsRNA in mammalian cells [6]. This approach for transiently controlling the expression of different target genes is rapidlybecoming the method of choice for determining gene function in mammalian cells [11, 12].

Here we present the protocols used to produce and evaluate siRNA-induced protein knockdown, specifically for knockdown enhanced green fluorescent protein (eGFP) in mammalian cells, with the siRNA oligonucleotides obtained by the DNA Synthesis Laboratory at the Center for Genetic Engineering and Biotechnology (CIGB), Cuba.

1. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 1998;391:806-81.

2. Sánchez A, Newmark PA. Double-stranded RNA specifically disrupts gene expression during planarian regeneration. Proceedings of the National Academy of Science USA, 1999;96:5049-54.

3.Kennerdell JR, Carthew RW. Use of dsRNA mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. Cell 1998; 95:1017-26.

4. Wianny F, Zernicka-Goetz M. Specific interference with gene function by double-stranded RNA in early mouse development. Nature Cell Biology 2000;2:70-5.

5. Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNAdirected nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature 2000; 404:293-6.

6. Clemens MJ. PKR-a protein kinase regulated by doublestranded RNA. Int J Biochem Cell Biol 1997; 29:945-9.

✍ Corresponding author

Materials and methods

Selection of siRNA sequences for targeting mRNAs

The target for the design of siRNA duplexes was the eGFP (GenBank accession number U57609). The design was made using BLOCK-iT™ RNAi Designer, Invitrogen. Two of the highest scoring siRNA duplexes were selected:

RNA duplex A

Sense: 5' CGGCAAGCUGACCCUGAAGdTdT 3' (position 119)

Antisense: 5' CUUCAGGGUCAGCUUGCCGdTdT

RNA duplex B

Sense: 5' GCCACAACGUCUAUAUCAUdTdT 3' (position 443)

Antisense: 5' ATGATATAGACGTTGTGGCdTdT 3'

Chemical synthesis

Chemical synthesis was performed on an ABI-394 synthesizer (Applied Biosystems) following standard procedures [13, 14]. We used protected phosphoramidites (proligo) with a ter-butylphenoxyacetyl radical at the amine exocyclic group and a ter-butyldimethylsilyl at the 2' hydroxyl position of the ribose [15].

The solid support was a controlled pore glass (CPG) functionalized with 2'-deoxythymidine (prime synthesis). Coupling time was 10 minutes in a 1.0 mmol synthesis scale.

For deprotection and cleavage from the solid support, the resin was incubated in NH_4OH/Ethanol (3:1) (V/V) at 55 ºC for 30 minutes, and after evaporating the supernatant, 0.4 mL of tetrabutylamonium fluoride (1 mol/L in tetrahydrofuran) were carefully added. This mixture was allowed to react 24 hours at room temperature [14].

The oligorribonucleotides were PAGE purified in denatured conditions. The gel used was 15% acrylamide and 7 mol/L urea. RNAse free water was used in the process. The recovery of the oligos from the gel was carried out incubating the previously crushed piece of gel with sterile water at 45 ºC for 3 hours [16].

Annealing of siRNAs to produce siRNA duplexes

The protocol below is based on a published procedure [17]. The dried RNA oligos were dissolved at a concentration of 1 µg/µL. Then each siRNA was diluted using sterile RNAse free water to a final concentration of 50 µM and 30 µL of each RNA solution and 15 µL of the annealing buffer (50 mM Tris pH 8.100 mM NaCl) were combined. The final concentration of the siRNA duplex was 20 µM. The solution was incubated at 90 °C for 1 min followed by an incubation for 1 h at 37 °C. Duplex RNA quality was checked onto 4% low melting point agarosa gels. The RNA bands were visualized under UV light after etidium bromide staining.

Cotransfection of CMV-eGFP reporter plasmid with siRNA duplexes

Human embryonic kidney (HEK 293) cells were grown in a 5% CO_2 humidified incubator at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Life technologies) supplemented with 10% fetal bovine serum (FBS) (Life technologies), 100 units/mL penicillin and 100 µg/mL streptomycin. The day (24 h) before plasmid /siRNA transfection, cells were trypsinized and a 24-well plate was seeded. Twenty four hours after seeding, a confluence of 70-80% was reached.

The transfection was duplicated using 8 uL of the CodeBreaker siRNA transfection reagent (Promega). The procedure followed was that recommended by the manufacturer. The experimental conditions were: 1 µg of pCMV-eGFP, 1 µg of pCMV-eGFP and RNA duplex at the final concentration of 60 nM and 120 nM. The negative control was 1 µg of pCMV-eGFP and a non-related RNA duplex against zebrafish somatostatin 14 synthetized with the T7 RiboMax Express RNAi system (Promega). The eGFP expression was visualized at 24 and 48 h post-transfection under a fluorescence microscope.

RNA isolation and Northen blot analysis

Total RNA was isolated with TriReagent (Sigma) according to the manufacturer's instructions. Northern blot was performed as previously described [18]. Briefly, RNA was separated under denaturing conditions in a MPOS/formaldehyde gel and after visualizing the RNA with UV light, a capillary blot was set up. The hybridization was done using eGFP ^{32}P as the probe.

Results and discussion

The analysis of siRNA function in Drosophila embryos indicated that siRNA duplexes composed of 21-nt sense and 21-nt antisense strands, paired in a way giving 2-nt 3' overhangs, are the most efficient triggers of sequence specific mRNA degradation [19]. In general, twenty-one-nucleotide RNAs are not too difficult to synthesize. The selection of target regions with uridine residues in the 2-nt overhangs is preferred. In the present study these uridine residues were replaced by 2' deoxythymidine because it has been shown that this replacement does not affect siRNA activity. In addition, this modification significantly reduces the costs of RNA synthesis and may also enhance the nuclease resistance of siRNA duplexes when applied to mammalian cells [12, 20].

The siRNA molecules are preferably chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. We used 10 minutes for coupling, as recommended by the manufacturer, and the average yield was of 98%, which is appropriate for this type of synthesis [16]. In RNA synthesis coupling time is longer than DNA synthesis. This is due to the presence of a protecting group at 2' OH positions in the RNA, which produces steric hindrance when the nucleosidic phosphoramidite approaches the phosphorus of position 3'.

The common problems of decomposition during the deprotection process were reduced because of the use of more basic labile ter-butylphenoxyacetyl protected monomers [15]. This protecting group enabled the deprotection step, incubating the resin in NH_4OH/Ethanol (3:1) (V/V) at 55 ºC, to take place in 30 minutes, instead of the 12-16 hours neeeded in standard procedures [16].

7. Elbashir SM, Harborth J, Weber K, Tuschl T. Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods 2002;26:199-213.

8. Paddison PJ, Hannon GJ: RNA interference: the new somatic cell genetics? Cancer Cell 2002;2:17-23.

9. Paddison PJ, Caudy AA, Hannon GJ. Stable suppression of gene expression by RNAi in mammalian cells. Proc Natl Acad Sci U S A, 2002;99:1443-48.

10. McManus MT, Sharp PA. Gene silencing in mammals by small interfering RNAs. Nat Rev Genet 2002;3:737-47.

11. Bannan M, Puri N. The ins and outs of RNAi in mammalian cells. Current Pharmaceutical biotechnology 2004;5: 441-50.

12. Ui-Tei K, Naito Y, Saigo K. Essential notes regarding the design of functional siRNAs for efficiente mammalian RNAI. J Biomed Biotechnol 2006;65052:1-8.

13. Usman N, Ogilvie KK, Jiang MY, Cedergren RJ. The automated chemical synthesis of long oligoribonucleotides using 2'-O-silylated ribonucleoside 3'-O-phosphoramidites on a controlled pore glass support: synthesis of a 43- nucleotide sequence similar to the 3'-half molecule of an *Escherichia coli* formylmethionine tRNA. Journal of the American Chemical Society 1987;109:7845-54.

14. Scaringe SA, Francklyn C, Usman N. Chemical synthesis of biologically active oligoribonucleotides using ®-cyanoethyl protected ribonucleoside phosphoramidites. Nucleic Acid Research 1990;18:5433-41.

15. Sinha ND, Davis P, Usman N, Pérez J, Hodge R, Kremsky J, Casale R. Labile exocyclic amine protection of nucleosides in DNA, RNA and oligonucleotide analog synthesis facilitating N-deacylation, minimizing depurination and chain degradation. Biochimie 1993;75:13-23.

16. Ekstein F. Oligonucleotides and analogues.Second ed., Oxford University Press, Oxford UK, 1991.

17. Elbashir MS, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide mediate RNA interference in culture mammalian cells. Nature 2001; 411:494-8.

18. Alwine JC, Kemp DJ, Parker, BA, Reiser J, Renart J, Stark, GR, Wahl GM. Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzyloxymethyl paper. Methods Enzymology 1979;68:220.

19. Aravin AA, Lagos-Quintana M, Yalcin A, Zavolan M, Marks D, Snyder B *et al*. The small RNA profile during *Drosophila melanogaster* development. Developmental Cell 2003;5:337-50.

20. Elbashir MS, Harborth J, Weber K, Tuschl T. Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods 2002; 26:199-213.

As showed in Figure 1, siRNA duplex was successfully formed after annealing for 1 min at 90 ºC and incubation at 37 ºC for 1h. It has been demonstrated that siRNA triggers specific gene silencing in HEK293 cells [12, 20]. After the transfection assays, we observed that the RNA duplex B blocked eGFP expression (Figure 2). Silencing in terms of the number of green cells and fluorescence intensity was observed at the highest siRNA dose (120 nM) 48 h after transfection. Visible silencing effects were noticed using 60 nM siRNA but quantification of fluorescence by flow cytometry would be needed to further determine whether there was a reduction of cell fluorescence (data not shown). Similar results were obtained by others [21] when using 60 nM of a synthetic eGFP siRNA at 48 h post-transfection. These dose effectiveness discrepancies could be due to the use of different transfection reagents. We did not obtain evident silencing effects with RNA duplex A (data not shown). Northern blot analysis (Figure 3) shows no visible reduction of the eGFP mRNA levels in cells co-transfected with pCMVeGFP/RNA duplex A and pCMV-eGFP/RNA duplex B at 60 nM when compared with pCMV-eGFP transfected cells. The determination of eGFP target mRNA levels indicates that the RNA duplex B reduced target mRNA at 120 nM.

In general, RNAi activity induced in mammalian cells is highly dependent on the particular sequence of the siRNA used [22, 23]. The selection of the target region is currently a trial and error process, but with a likelihood of 80-90% success given a large enough random selection of target genes [20]. It is therefore advisable to synthesize several siRNA duplexes and also to control the specificity of the knockdown experiments. Furthermore, a non-specific siRNA duplex may be needed as a control, preferably, a siRNA duplex which is targeting a gene absent from the selected model organism [20], for which reason we selected a siRNA target zebrafish somatostatin 14. In the design of a highly functional siRNAs for mammalian RNAi, suitable sequence conditions or good algorithms for selection of highly functional siRNAs and good computer software suitable for genome-wide short-sequence homology search to minimize the off-target effect are indispensable. Many websites are available for functional siRNA. These websites may incorporate one or a few algorithms for functional siRNA selection previously determined through biological validation data. considerable mammalian RNAi data are now available so that, in certain websites, the original algorithms may have been replaced by modifications that are more

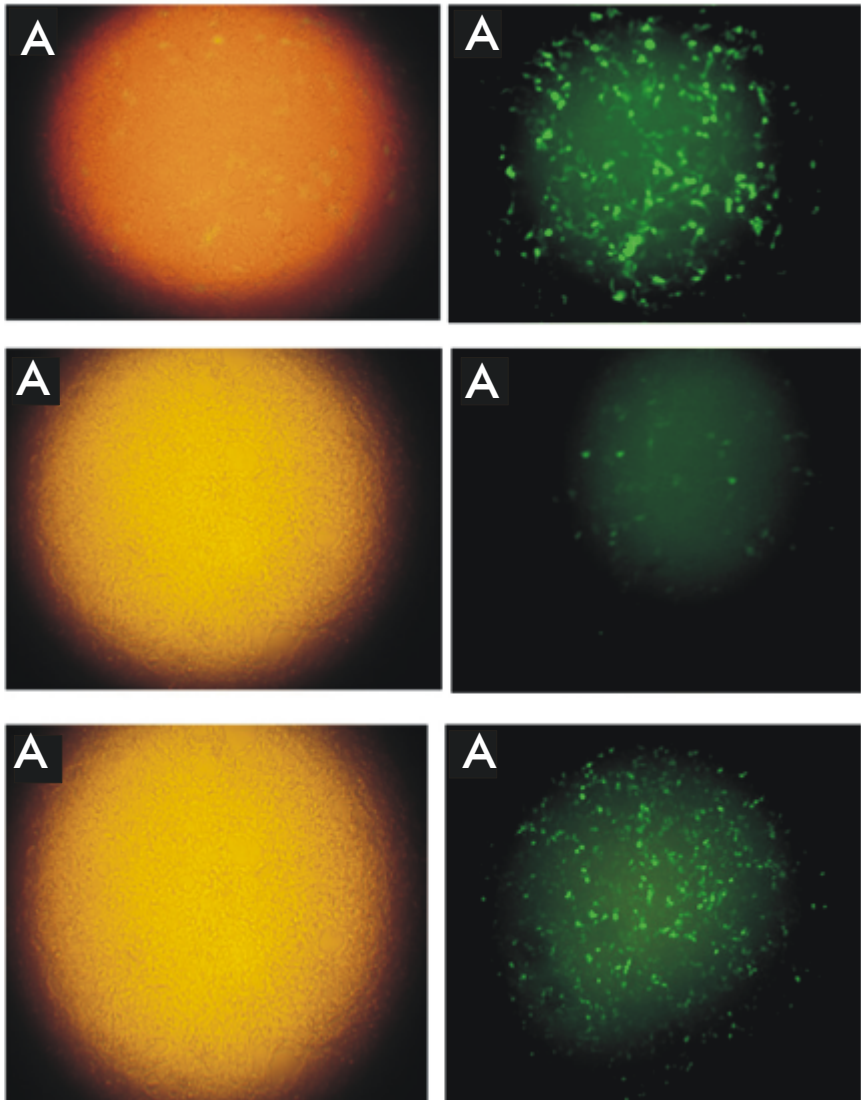

Figure 2. Fluorescence microscopy of the HEK293 cells transfected with CMV-eGFP reporter plasmid and siRNA duplexes: A, B. pCMV-eGFP; C, D. pCMV-eGFP and RNA duplex B 120 nM; E, F. pCMV-eGFP with RNA duplex against zebrafish SS14 120 nM. A, C, E: light field; B, D, F: dark field.

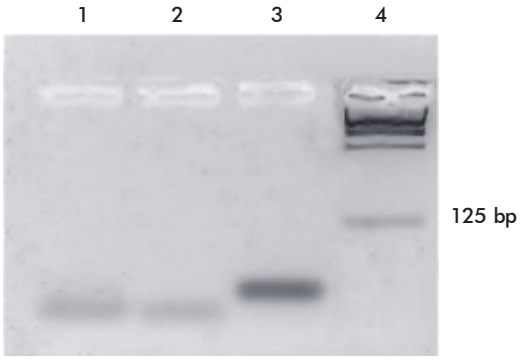

Figure 1. Duplex RNA quality was checked onto 4% low melting point agarose gels. The RNA bands were visualized under UV light after etidium bromide staining: 1. Single stranded sense RNA A, 2. Single stranded antisense RNA A, 3. Annealed siRNA A, 4. DNA from phage λ digested with endonuclease Hind III was loaded as a marker.

21. Tran N, Cairns MJ, Dawes IW, Arndt GM. Expressing functional siRNAs in mammalian cells using convergent transcription. BMC Biotechnology 2003; 3:21.

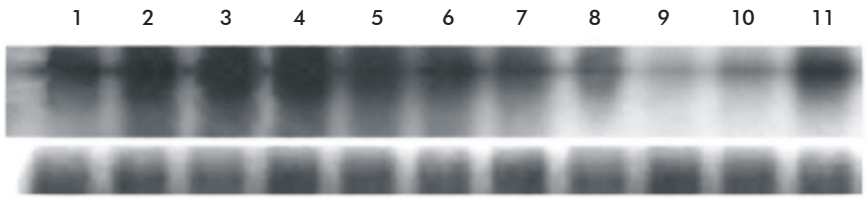

Figure 3. Northern blot analysis of eGFP expression in HEK293 cells co-transfected with CMV-eGFP reporter plasmid and siRNA duplexes: 1,2 pCMV-GFP; 3,4 pCMV-GFP and RNA duplex A 60 nM; 5,6 pCMV-GFP and RNA duplex A 120 nM; 7,8 pCMV-GFP and RNA duplex B 60 nM; 9,10 pCMV-GFP and RNA duplex B 120 nM, 11 CMV-GFP plus RNA duplex SS14 pez zebra 120 nM. Upper panel: eGFP mRNA. Lower panel: Ribosomal 28S subunit as the control for loading.

effective yet do not appear in scientific journals, thus hindering the evaluation of the individual website [12]. In this case, we selected BLOCK-iT RNAi Designer (https://rnaidesigner.invitrogen.com/ Invi trogen), which is one of the most popular websites.

SiRNA-mediated RNAi activity may also vary depending on other parameters such as siRNA concentration, duration of siRNA exposure, target mRNA concentration and secondary structure within cells [22, 24].

Received in september, 2006. Accepted for publication in january, 2007.

Small interfering RNAs have become powerful molecules for triggering gene-specific silencing. The present study shows specific eGFP gene silencing in mammalian cells through RNAi using RNA oligos synthesized by the DNA Synthesis Laboratory at the Center for Genetic Engineering and Biotechnology (CIGB), Cuba. The successful synthesis of these molecules at CIGB provides a novel tool for the genome wide analysis of gene function and may become very useful in several therapeutic applications.

22. Ui-Tei K, Naito Y, Takahashi F, *et al*. Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acids Research 2004;32(3):936-48.

23. Holen T, Amarzguioui M, Wiiger MT, Babaie E, Prydz H. Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. Nucleic Acids Research 2002; 30(8):1757-66.

24. Omi K, Tokunaga K, Hohjoh H. Long-lasting RNAi activity in mammalian neurons. FEBS Letters 2004;558(1-3):89-95.