

METHOD

Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing

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ABSTRACT

A large number of miRNAs have recently been discovered in plants and animals. Development of reverse genetic approaches that act to inhibit microRNA function would facilitate the study of this new class of noncoding RNA. Here we show that 2'-O-methyl oligoribonucleotides, but not 2'-deoxyoligonucleotides specifically inactivate the RNAi activity associated with miRNA-protein complexes in human cell extracts as well as in cultured human cells.

Keywords: RNA interference; miRNA; miRNP; posttranscriptional gene regulation; translational regulation; mRNA degradation

INTRODUCTION

RNA silencing is a fundamental mechanism of gene regulation that uses double-stranded RNA (dsRNA) derived 21– to 28 nucleotide (nt) small RNAs to guide mRNA degradation, control mRNA translation, or chromatin modification (for recent reviews, see Carrington and Ambros 2003; Dykxhoorn et al. 2003; Grewal and Moazed 2003; Pickford and Cogoni 2003). In the past 2 years, several hundred novel genes were identified in plants (Llave et al. 2002a; Mette et al. 2002; Park et al. 2002; Reinhart et al. 2002; Palatnik et al. 2003) and animals (Lee et al. 1993; Reinhart et al. 2000; Lagos-Quintana et al. 2001, 2002, 2003; Lau et al. 2001; Lee and Ambros 2001; Mourelatos et al. 2002; Ambros et al. 2003; Aravin et al. 2003; Brennecke et al. 2003; Dostie et al. 2003; Grad et al. 2003; Houbaviy et al. 2003; Lai et al. 2003; Lim et al. 2003a,b) that encode transcripts that contain short dsRNA hairpins. Defined 22-nt RNAs, referred to as microRNAs (miRNAs), are excised by dsRNA-specific endonucleases from the hairpin precursors (Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001; Lee et al. 2002, 2003). The miRNAs are incorporated into ribonucleoprotein particles (miRNPs; Caudy et al. 2002; Hutvagner and Zamore 2002; Lee et al. 2002, 2003; Mourelatos et al. 2002).

Plant miRNAs target mRNAs containing sequence segments with high complementarity for degradation (Llave et al. 2002b; Rhoades et al. 2002; Emery et al. 2003; Palatnik et al. 2003; Tang et al. 2003) or suppress translation of partially complementary mRNAs (Aukerman and Sakai 2003; Chen 2003). Animal miRNAs appear to act predominantly as translational repressors (Lee et al. 1993; Wightman et al. 1993; Moss et al. 1997; Olsen and Ambros 1999; Reinhart et al. 2000; Slack et al. 2000; Seggerson et al. 2002; Abrahante et al. 2003; Brennecke et al. 2003; Lin et al. 2003). However, these miRNAs can also guide RNA degradation if presented an artificial substrate with a high degree of complementarity (Hutvagner & Zamore 2002; Brennecke and Cohen 2003; Brennecke et al. 2003; Zeng et al. 2003). This indicates that animal miRNPs act like small interfering RNA (siRNA)-induced silencing complexes (RISCs). Cell-based reporter assays further support the idea that RISC and miRNPs have a similar composition and that their activity is determined by the degree of complementarity to the target mRNA (Doench et al. 2003; Saxena et al. 2003; Zeng et al. 2003). Considering that non-Watson-Crick base pairs are tolerated at certain positions with respect to RISC-mediated target degradation (Jackson et al. 2003; Saxena et al. 2003), it remains a possibility that some animal miRNAs may target mRNA degradation to a certain extent.

Investigation of miRNA expression combined with genetic and molecular studies in *Caenorhabditis elegans* and plants have identified cell lineage-specific miRNA expression patterns, which are required to control timing of development and tissue specification (for reviews, see Slack and Ruvkun 1997; Pasquinelli and Ruvkun 2002; Ambros

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Article and publication are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.5235104>.

2003; Carrington and Ambros 2003; Hake 2003). Recently, two miRNAs were identified that control cell death by regulating proapoptotic genes in *Drosophila melanogaster* (Brennecke et al. 2003; Xu et al. 2003). Deregulated expression of certain miRNAs has been linked to human proliferate diseases such as B-cell chronic lymphocytic leukemia (Calin et al. 2002; Lagos-Quintana et al. 2003) and colorectal neoplasia (Michael et al. 2003). Thus far, miRNA profiling was based on cloning of small RNAs, a technique that is limited by the availability of tissue (Pfeffer et al. 2003). The development of array technology for the systematic analysis of miRNA expression profiles may help to overcome these limitations (Krichevsky et al. 2003).

Understanding the biological function of miRNAs requires knowledge of their mRNA targets. Bioinformatic approaches have been used to predict mRNA targets, among which transcription factors and proapoptotic genes were prominent candidates (Rhoades et al. 2002; Enright et al. 2003; Stark et al. 2003). Processes such as *Notch* signaling, cell proliferation, morphogenesis, and axon guidance appear to be controlled by miRNA genes.

To validate the predicted miRNA targets, new and rapid methods for sequence-specific inactivation of miRNPs are needed. We adapted an antisense-based strategy to block miRNA function in cell extracts as well as cultured mammalian cells. The use of nuclease-resistant 2'-O-methyl oligoribonucleotides allowed us to sequence-specifically inactivate miRNPs in HeLa cell extracts. Transfection of 2'-O-methyl oligoribonucleotides, but not of antisense deoxyoligonucleotides into cultured mammalian cells efficiently blocked the RISC-associated miRNP activity. This demonstrates that 2'-O-methyl oligoribonucleotides are useful inhibitors of miRNAs that will be valuable reagents for the study of miRNA function.

RESULTS AND DISCUSSION

To assess the ability of modified oligonucleotides to specifically interfere with miRNA function, we used our previously described mammalian biochemical system developed for assaying RISC activity (Martinez et al. 2002). Zamore and colleagues (Hutvagner and Zamore 2002) showed that crude cytoplasmic cell lysates and eIF2C2 immunoprecipitates prepared from these lysates contain let-7 RNPs that specifically cleave let-7-complementary target RNAs. We previously reported that in HeLa cells, numerous miRNAs are expressed including several let-7 miRNA variants (Lagos-Quintana et al. 2001). To assess if other HeLa cell miRNAs are also engaged in RISC-like miRNPs we examined the cleavage of a ³²P-cap-labeled substrate RNA with a complementary site to the highly expressed miR-21 (Lagos-Quintana et al. 2001; Mourelatos et al. 2002). Sequence-specific target RNA degradation was readily observed and appeared to be approximately two- to fivefold more effective than cleavage of a similar let-7 target RNA (Fig. 1A, lane

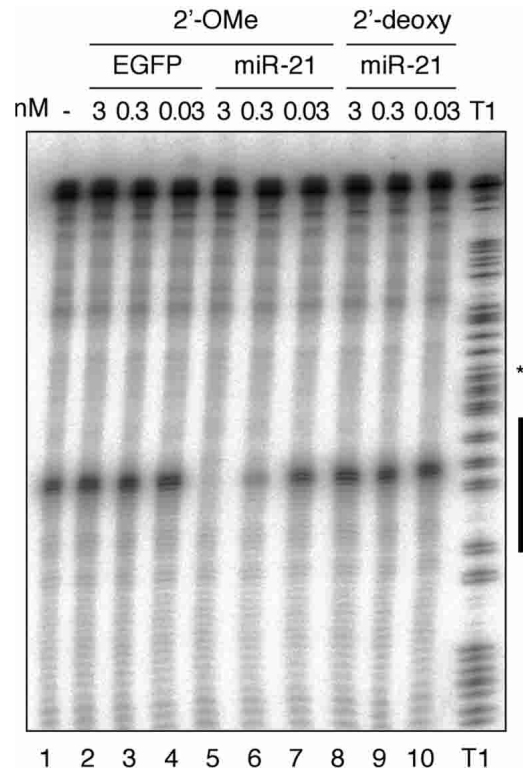


FIGURE 1. Antisense 2'-O-methyl oligoribonucleotide specifically inhibit miR-21 guided cleavage activity in HeLa cell S100 cytoplasmic extracts. The black bar to the left of the RNase T1 ladder represents the region of the target RNA complementary to miR-21. Oligonucleotides complementary to miR-21 were preincubated in S100 extracts prior to the addition of ³²P-cap-labeled cleavage substrate. Cleavage bands and T1 hydrolysis bands appear as doublets after a 1-nt slipping of the T7 RNA polymerase near the middle of the transcript indicated by the asterisk.

1; data not shown). We therefore decided to interfere with miR-21 guided target RNA cleavage.

2'-O-Methyl modification is frequently used to protect oligoribonucleotides from degradation in cell extracts or cultured cells (reviewed in Lamond and Sproat 1993; Verma and Eckstein 1998). 2'-O-Methyl oligoribonucleotides are also well known for their rapid and very stable hybridization to single-stranded RNA (Cummins et al. 1995; Majlessi et al. 1998). We chemically synthesized a 24-nt 2'-O-methyl oligoribonucleotide that contained a 3' C7 aminolinker and was complementary to the longest form of the miR-21. The aminolinker was introduced to enable postsynthetic conjugation of nonnucleotidic residues such as biotin.

Increasing concentrations of anti-miR-21 2'-O-methyl oligoribonucleotide and a control 2'-O-methyl oligoribonucleotide cognate to an EGFP sequence were added to the S100 extract 20 min prior to the addition of ³²P-cap-labeled substrate. We determined the concentration of miR-21 in the S100 extract by quantitative Northern blotting to be 50 pM (Lim et al. 2003b). The control EGFP oligonucleotide did not interfere with miR-21 cleavage even at the highest

applied concentration (Fig. 1A, lanes 2,3). In contrast, the activity of miR-21 was completely blocked at a concentration of only 3 nM (Fig. 1A, lane 5), and a concentration of 0.3 nM showed a substantial 60–70% reduction of cleavage activity (Fig. 1, lane 6). At a concentration of 0.03 nM, the cleavage activity of miR-21 was not affected when compared to the lysate alone (Fig. 1, lanes 1,7). It was previously reported that antisense 2'-deoxyoligonucleotides were able to sequence-specifically inactivate miRNPs in microinjected *D. melanogaster* embryos (Boutla et al. 2003). However, at concentrations identical to those of 2'-O-methyl oligoribonucleotides, we could not detect blockage of miR-21 induced cleavage (Fig. 1A, lanes 8–10). The 2'-deoxynucleotides used in this study were protected against 3'-exonucleases by the addition of three 2'-O-methyl ribonucleotide residues.

To assess the ability of 2'-O-methyl oligoribonucleotides for interfering with RISC in living cells, we first examined if siRNA duplex initiated RISC was blocked by 2'-O-methyl oligoribonucleotides complementary to the guide siRNA strand. Control or inhibitor oligonucleotides were cotransfected in equimolar amounts with preannealed siRNA duplex targeting EGFP into HeLa S3 cells stably expressing EGFP (Fig. 2A). The 2'-O-methyl oligoribonucleotide complementary to the guide siRNA strand specifically blocked duplex siRNA function (Fig. 2A, lane 5) whereas a 2'-O-methyl oligoribonucleotide of the reversed sequence did not interfere with silencing or compromise the viability of the treated cells (Fig. 2A, lane 3). Cotransfection of the reverse 2'-O-methyl oligoribonucleotide with nonspecific, luciferase-targeting siRNA had also no effect on GFP expression (data not shown). A 2'-deoxyoligonucleotide complementary to the guide EGFP siRNA was unable to block EGFP RISC activity (Fig. 2A, lane 4). Inhibition of EGFP RISC and recovery of EGFP fluorescence was also observed when the inhibitory 2'-O-methyl oligoribonucleotide was transfected 24 h after the initial introduction of the EGFP-targeting siRNA duplex (Fig. 2B).

To monitor the activity of miR-21 in HeLa cells, we constructed reporter plasmids that express EGFP mRNA that contains in its 3' UTR a 22-nt sequence complementary to miR-21 (pEGFP-S-21) or in sense orientation to miR-21

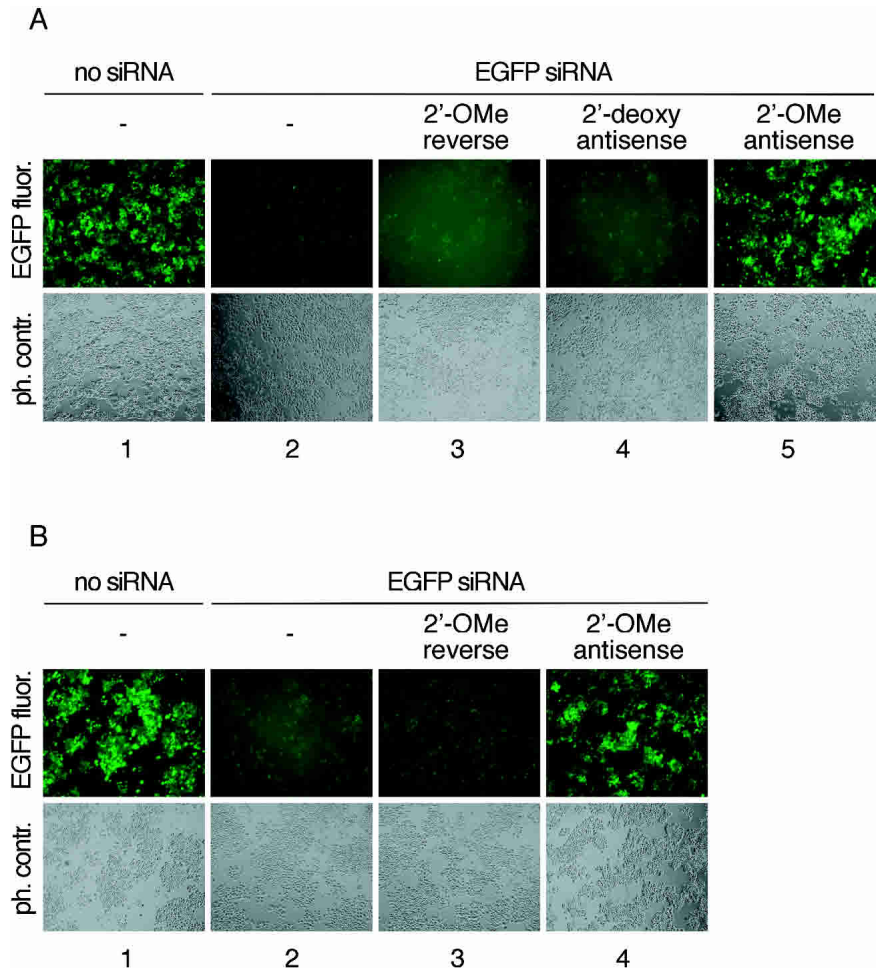


FIGURE 2. Antisense 2'-O-methyl oligoribonucleotides block EGFP-targeting RISC in EGFP stably expressing HeLa cells. (A) EGFP expressing HeLa cells were cotransfected with EGFP siRNA duplex and the indicated single-stranded oligonucleotides. EGFP fluorescence was recorded 48 h past transfection. The antisense 2'-O-methyl oligoribonucleotide is complementary to the guide siRNA strand. The reversed sequence (not the reverse complement) of antisense 2'-O-methyl oligoribonucleotide was used as control. The guide siRNA is complementary to a segment of the EGFP mRNA. The *top* row shows fluorescence microscopy images recording the EGFP fluorescence; the *bottom* row shows phase contrast images. (B) EGFP expressing HeLa cells were first transfected with EGFP siRNA duplex followed by transfection of and the indicated single-stranded 2'-O-methyl oligoribonucleotides after 24 h.

(p-EGFP-A-21). Endogenous miRNAs have previously been shown to act like siRNAs by cleaving reporter mRNAs carrying sequences perfectly complementary to miRNA (Zeng et al. 2002, 2003; Brennecke et al. 2003; Stark et al. 2003). To monitor transfection efficiency and specific interference with the EGFP indicator plasmids, the far-red fluorescent protein encoding plasmid pHcRed-C1 was cotransfected. Expression of EGFP was observed in HeLa cells transfected with pEGFP and pEGFP-A-21 (Fig. 3, rows 1,2), but not from those transfected with pEGFP-S-21 (Fig. 3, row 3). However, expression of EGFP from pEGFP-S-21 was restored upon cotransfection with anti miR-21 2'-O-methyl oligoribonucleotide (Fig. 3, row 4). Consistent with our previous observation, the 2'-deoxy anti miR-21 oligo-

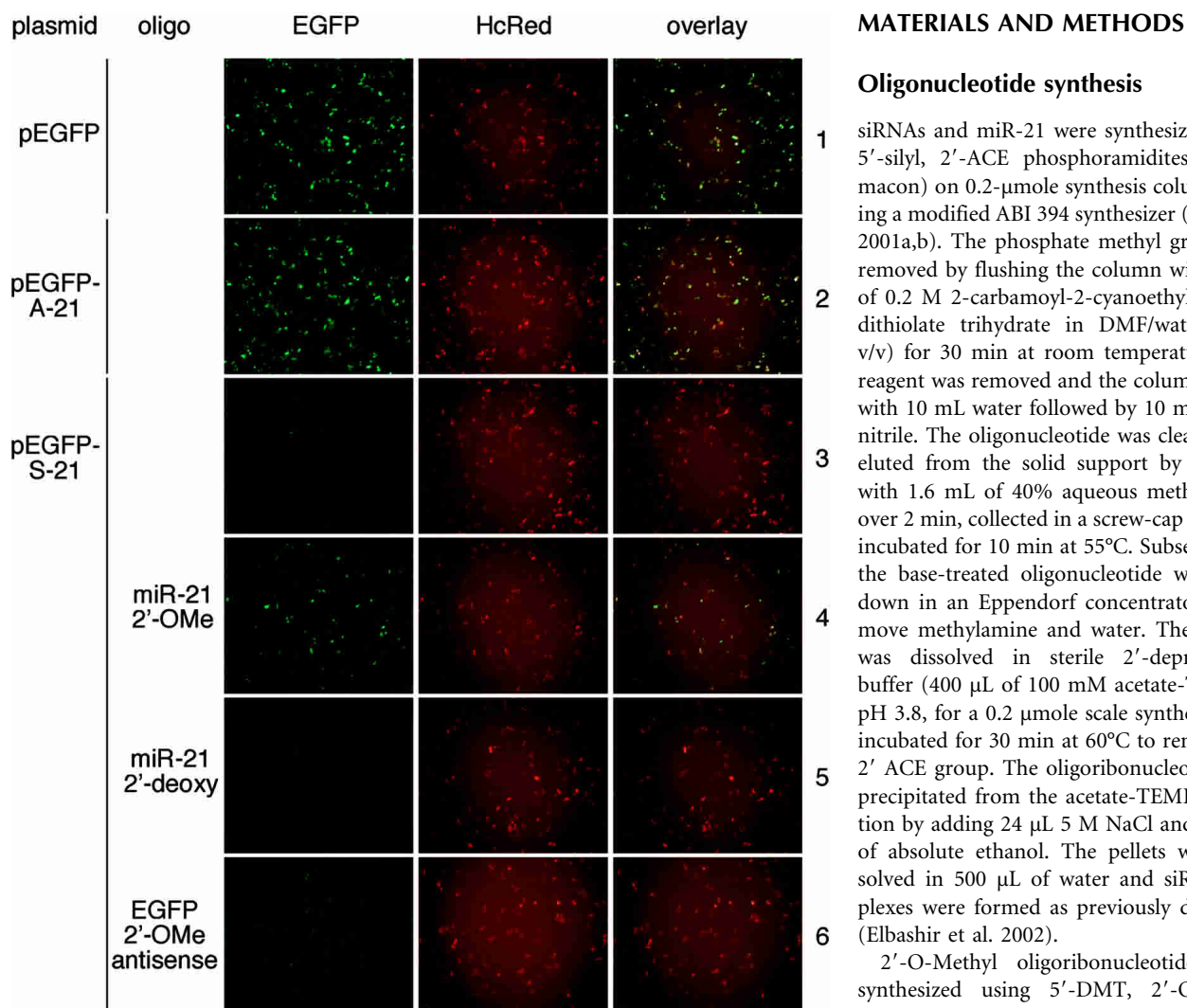


FIGURE 3. Antisense 2'-O-methyl oligoribonucleotides interfere with endogenous miR-21 RNP cleavage in HeLa cells. HeLa cells were transfected with pHcRed and pEGFP or its derivatives, with or without inhibitory or control oligonucleotides. EGFP and HcRed protein fluorescence were excited and recorded individually by fluorescence microscopy 24 h after transfection. Coexpression of cotransfected reporter plasmids was documented by superimposing of the fluorescence images in the *right* panel.

nucleotide showed no effect (Fig. 3, row 5). Similarly, cotransfection of the EGFP 2'-O-methyl oligoribonucleotide in sense orientation with respect to the EGFP mRNA (or antisense to EGFP guide siRNA) had no effect (Fig. 3, row 6).

In summary, we have demonstrated that RISC and miRNP complexes can be effectively and sequence-specifically inhibited with 2'-O-methyl oligoribonucleotides antisense to the guide strand positioned in the RNA silencing complex. This technique will be essential to assess the function of miRNA genes expressed in cultured cells. It is also conceivable that 2'-O-methyl oligoribonucleotides or other antisense reagents could be valuable for therapeutic intervention to control miRNA activity in disease states.

MATERIALS AND METHODS

Oligonucleotide synthesis

1 siRNAs and miR-21 were synthesized using 5'-silyl, 2'-ACE phosphoramidites (Dharmacon) on 0.2- μ mole synthesis columns using a modified ABI 394 synthesizer (Scaringe 2001a,b). The phosphate methyl group was removed by flushing the column with 2 mL of 0.2 M 2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate in DMF/water (98:2 v/v) for 30 min at room temperature. The reagent was removed and the column rinsed with 10 mL water followed by 10 mL acetonitrile. The oligonucleotide was cleaved and eluted from the solid support by flushing with 1.6 mL of 40% aqueous methylamine over 2 min, collected in a screw-cap vial, and incubated for 10 min at 55°C. Subsequently, the base-treated oligonucleotide was dried down in an Eppendorf concentrator to remove methylamine and water. The residue was dissolved in sterile 2'-deprotection buffer (400 μ L of 100 mM acetate-TEMED, pH 3.8, for a 0.2 μ mole scale synthesis) and incubated for 30 min at 60°C to remove the 2' ACE group. The oligoribonucleotide was precipitated from the acetate-TEMED solution by adding 24 μ L 5 M NaCl and 1.2 mL of absolute ethanol. The pellets were dissolved in 500 μ L of water and siRNA duplexes were formed as previously described (Elbashir et al. 2002).

2'-O-Methyl oligoribonucleotides were synthesized using 5'-DMT, 2'-O-methyl phosphoramidites (Proligo) on 1- μ mole synthesis columns loaded with 3'-aminomodifier (TFA) C7 Icaa control pore glass support (Chemgenes). The aminolinker was added to also use the oligonucleotides for conjugation to amino group reactive reagents, such as biotin succinimidyl esters. The synthesis products were deprotected for 16 h at 55°C in 30% aqueous ammonia and then precipitated by the addition of 12 mL absolute 1-butanol. The full-length product was then gel-purified using a denaturing 20% polyacrylamide gel. 2'-Deoxyoligonucleotides were prepared using 0.2- μ mole scale synthesis and standard DNA synthesis reagents (Proligo).

The sequences of the 2'-O-methyl oligoribonucleotides were 5'-GUCAACAUCAGUCUGUAAGCUAL (L, 3' aminolinker) for 2'-OMe miR-21, 5'-AAGGCAAGCUGACCCUGAAGUL for EGFP 2'-OMe antisense, 5'-UGAAGUCCCAGUCGAACGGAAL for EGFP 2'-OMe reverse; the sequence of chimeric 2'-OMe/DNA oligonucleotides was 5'-GTCAACATCAGTCTGATAAGCTAGC GL for 2'-deoxy miR-21 (underlined, 2'-OMe residues) and 5'-AAGGCAAGCTGACCCTGAAGTGCCGL for EGFP 2'-deoxy antisense; the sequences of the EGFP siRNAs were GGCAAGCUGACCUGAAGUUT for sense and 5'-pACUUCAGGGUCAGCUU

GCCUT for antisense (p, 5'-monophosphate; T, 2'-deoxythymidine).

The miR-21 cleavage substrate was prepared by PCR-based extension of the partially complementary synthetic DNA oligonucleotides 5'-GAACAATTGCTTTTACAGATGCACATATCGA GGTGAACATCACGTACGTCAACATCAGTCTGATAAGCTATC GGTGGCAGAAGCTAT and 5'-GGCATAAAGAATTGAAGA GAGTTTTCACTGCATACGACGATTCTGTGATTTGTATTTCAG CCCATATCGTTTCATAGCTTCTGCCAACCGA. The extended dsDNA was then used as template for a new PCR with primers 5'-TAATACGACTCACTATAGAACAATTGCTTTTACAG and 5'-ATTTAGGTGACACTATAGGCATAAAGAATTGAAGA to introduce the T7 and SP6 promoter sequences for in vitro transcription. The PCR product was ligated into pCR2.1-TOPO (Invitrogen). Plasmids isolated from sequence-verified clones were used as templates for PCR to produce sufficient template for run-off in vitro transcription reactions using phage RNA polymerases (Elbashir et al. 2001). ³²P-Cap-labeling was performed as reported (Martinez et al. 2002).

Plasmids

Plasmids pEGFP-S-21 and pEGFP-A-21 were generated by T4 DNA ligation of preannealed oligodeoxynucleotides 5'-GGCCT CAACATCAGTCTGATAAGCTAGGTACCT and 5'-GGCCAGGT ACCTAGCTTATCAGACTGATGTTGA into NotI digested pEGFP-N-1 (Clontech). The plasmid pHcRed-C1 was from Clontech.

HeLa extracts and miR-21 quantification

HeLa cell extracts were prepared as described (Dignam et al. 1983); 5×10^9 cells from HeLa suspension cultures were collected by centrifugation and washed with PBS (pH 7.4). The cell pellet (approx. 15 mL) was resuspended in two times of its volume with 10 mM KCl/1.5 mM MgCl₂/0.5 mM dithiothreitol/10mM HEPES-KOH (pH 7.9) and homogenized by douncing. The nuclei were then removed by centrifugation of the cell lysate at $1000 \times g$ for 10 min. The supernatant was spun in an ultracentrifuge for 1 h at $105,000 \times g$ to obtain the cytoplasmic S100 extract. The concentration of KCl of the S100 extract was subsequently raised to 100 mM by the addition of 1 M KCl. The extract was then supplemented with 10% glycerol and frozen in liquid nitrogen.

Two hundred eighty micrograms of total RNA were isolated from 1 mL of S100 extract using the acidic guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi 1987). A calibration curve for miR-21 Northern signals was produced by loading increasing amounts (10 to 30,000 pg) of synthetically made miR-21 (Lim et al. 2003b). Northern blot analysis was performed as described using 30 µg of total RNA per well (Lagos-Quintana et al. 2001).

In vitro miRNA cleavage and inhibition assay

2'-O-Methyl oligoribonucleotides or 2'-deoxyoligonucleotides were preincubated with HeLa S100 at 30°C for 20 min prior to the addition of the cap-labeled miR-21 target RNA. The concentration of the reaction components were 5 nM target RNA, 1 mM ATP, 0.2 mM GTP, 10 U/mL RNasin (Promega) and 50% HeLa S100

extract in a final reaction volume of 25 µL. The reaction time was 1.5 h at 30°C. The reaction was stopped by addition of 200 µL of 300 mM NaCl/25 mM EDTA/20% w/v SDS/200 mM Tris HCl (pH 7.5). Subsequently, proteinase K was added to a final concentration of 0.6 mg/mL and the sample was incubated for 15 min at 65°C. After phenol/chloroform extraction, the RNA was ethanol-precipitated and separated on a 6% denaturing polyacrylamide gel. Radioactivity was detected by phosphorimaging.

Cell culture and transfection

HeLa S3 and HeLa S3/GFP were grown in 5% CO₂ at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin, and 100 µg/mL streptomycin. One day before transfection, 10^5 cells were plated in 500 µL DMEM containing 10% FBS per well of a 24-well plate. Plasmid and plasmid/oligonucleotide transfection was carried out with Lipofectamine2000 (Invitrogen). pEGFP (0.2 µg) or its derivatives were cotransfected with 0.3 µg pHcRed with or without 10 pmole of 2'-O-methyl oligoribonucleotide or 10 pmole of 2'-deoxyoligonucleotide per well. For siRNA transfection, 20 pmole of siRNA duplex and 60 pmole of 2'-O-methyl oligoribonucleotide or 60 pmole of 2'-deoxyoligonucleotide were used per well. Fluorescent cell images were recorded on a Zeiss Axiovert 200 inverted fluorescence microscope (Plan-Apochromat 10×/0.45) equipped with Chroma Technology Corp. filter sets 41001 (EGFP) and 41002c (HcRed) and AxioVision 3.1 software.

ACKNOWLEDGMENTS

We thank P.Y. (Jeff) Chen and Jutta Meyer for expert technical assistance, R. Lührmann (MPIBPC, Göttingen, Germany) for HeLa cell cytoplasmic extracts, and J. Martinez for discussion. G.M. was supported by fellowship ATLF-219-2003 of the European Molecular Biology Organization. This work was also supported by NIH Grant R01 GM068476-01.

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Received November 18, 2003; accepted December 11, 2003.

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