Reports

Protein mediated miRNA detection and siRNA enrichment using p19

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p19 RNA binding protein from the Carnation Italian ringspot virus (CIRV) is an RNA-silencing suppressor that binds small interfering RNA (siRNA) with high affinity. We created a bifunctional p19 fusion protein with an N-terminal maltose binding protein (MBP), for protein purification, and a C-terminal chitin binding domain (CBD) to bind p19 to chitin magnetic beads. The fusion protein binds dsRNAs in the size range of 20–23 nucleotides, but does not bind ssRNA or dsDNA. Relative affinities of the p19 fusion protein for different-length RNA and DNA substrates were determined. Binding specificity of the p19 fusion protein for small dsRNA allows detection of miRNA:RNA probe duplexes. Using radioactive RNA probes, we were able to detect low levels of miRNAs in the sub-femtomole range and in the presence of a million-fold excess of total RNA. Detection is linear over three logs. Unlike most nucleic acid detection methods, p19 selects for RNA hybrids of correct length and structure. Rules for designing optimal RNA probes for p19 detection of miRNAs were determined by in vitro binding of 18 different dsRNA oligos to p19. These studies demonstrate the potential of p19 fusion protein to detect miRNAs and isolate endogenous siRNAs.

Introduction

MicroRNAs (miRNAs) are cleaved from RNA hairpins to generate dsRNAs 21–22 nucleotides long that associate with the RNA-induced silencing complex (RISC). microRNAs generally act by binding to a complementary region in the 3' untranslated region of mRNA to reduce protein expression. In plants, miRNA binding directs specific cleavage of the mRNA (1). Biological systems regulated by miRNAs include embryonic differentiation, hematopoiesis, cardiac hypertrophy, and a variety of cancers. Measuring miRNA levels in cancer is important in both diagnosis and prognosis of the disease (2).

To evaluate the role of miRNAs in gene expression, an accurate quantitative method of detection is needed. Existing methods using microarrays (3–5), single-molecule detection with lasers (6), or microsphere detection of miRNAs (7) often require expensive equipment. Other methods that use multiple enzymatic steps including RNA ligation, amplification, and gel electrophoresis (8–11) are susceptible to quantitative differences in detection. In this report, we describe

a novel and simplified method for miRNA detection that utilizes the small interfering RNA (siRNA) binding properties of the p19 protein from the Carnation Italian ringspot virus (CIRV), a plant tombusvirus. The protein's binding activity was discovered by its ability to block RNAi in plants (12–14). The dimeric p19 binds dsRNA in a size-dependent sequence-independent manner (15,16).

Hybridization of a miRNA-specific probe to a single-stranded target miRNA creates dsRNA that tightly binds the p19 fusion protein. The amino terminus of p19 was fused to maltose binding protein (MBP) to aid purification (17) and the carboxy terminus was fused to chitin binding domain (CBD) for tight binding to chitin magnetic beads (18). The recombinant fusion protein retains the ability to selectively bind siRNA. The p19 fusion protein did not bind ssRNA, rRNA, or dsDNA, and showed the greatest affinities for 19- and 20-nucleotide dsRNAs with blunt ends, as well as 21-nucleotide siRNA with 5' phosphates and 3' 2-nucleotide extensions. We describe a novel miRNA detection method which is based on selective binding of p19 fusion to siRNAs. The method is linear

over a three-log range and has a sensitivity of 0.1 fmoles. It is significantly faster and more sensitive than Northern blot analysis.

Materials and methods

In vitro RNA binding with p19 fusion

Size-specific binding of dsRNA to p19 fusion was measured using a gel mobility shift. Increasing amounts of MBP-p19-CBD fusion protein (0–3 µg) were bound to 90 ng of an equal mixture of 17-, 21-, and 25-nucleotide dsRNAs. The MBP-p19-CBD fusion protein was obtained from New England BioLabs (NEB; Ipswich, MA, USA). All dsRNAs contained 5' phosphates and 3' 2-nucleotide extensions. RNA was bound to the p19 fusion at room temperature (RT) for 2 h in 1× p19 binding buffer [20 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM TCEP [tris (2-carboxyethyl) phosphine], 100 mM NaCl, 0.02% Tween-20] with 10 units of murine RNase inhibitor (NEB) and 1× BSA (0.1 mg/mL) in a total volume of 20 µL. The reactions were mixed with 5 µL 5× loading buffer (15% Ficoll 400 and 5 mM EDTA) and analyzed by electrophoresis on 20% TBE acrylamide gel

(Invitrogen, Carlsbad, CA, USA) for 2 h at 100 V in 1× TBE buffer followed by ethidium bromide staining.

siRNA isolation using p19 beads

The selectivity of siRNA binding to p19 chitin magnetic beads (NEB) in a large excess of total RNA was measured with 21-bp siRNA oligos

(Table 1, row 1). Up to 55 µg rat liver RNA and 10 ng 21-mer siRNA was incubated with 10 µL p19 beads suspended in 1× p19 binding buffer with RNase inhibitor and BSA (see “In vitro RNA binding with p19 fusion” section), and shaken at RT for 1–2 h in an Orbis shaker (MarketLab, Caledonia, MI, USA). Unbound RNA was removed by washing 6

times in 600 µL 1× p19 wash buffer (identical to 1× p19 binding buffer except without Tween-20 and TCEP). For each wash, the beads were shaken for 5 min at 37°C. The bound siRNA was eluted from beads in 20 µL 1× p19 elution buffer (20 mM Tris-HCl pH 7.0, 100 mM NaCl, 1 mM EDTA, and 0.5% SDS) by mixing on an Eppendorf thermo-

Table 1. Relative binding affinity of p19 fusion to different RNA and DNA substrates

	Name	Sequence	Structure	K_{rel}
1	21-mer ds siRNA	5' pCGUACGCGAAUACUUCGAUU 3' 3' UUGCAUGCGCCUUUGAAGCUp 5'		1
2	17-mer ds RNA	5' pCUCAACCAGCCACUGCU 3' 3' UAGAGUUGGUCGGUGACp 5'		106
3	25-mer ds RNA	5' pCGUACGCGAAUACUUCGAAUUGUU 3' 3' UUGCAUGCGCCUUUGAAGCUUACp 5'		230
4	21-mer ds RNA (w/o 5'p)	5' CGUACGCGAAUACUUCGAUU 3' 3' UUGCAUGCGCCUUUGAAGCU 5'		44
5	21-mer ds RNA (5'ppp)	5' pppCGUACGCGAAUACUUCGAUU 3' 3' UUGCAUGCGCCUUUGAAGCUppp 5'		8
6	19-mer blunt ds RNA	5' pCGUACGCGAAUACUUCGA 3' 3' GCAUGCGCCUUUGAAGCUp 5'		0.3
7	20-mer blunt ds RNA	5' pACGUACGCGAAUACUUCGA 3' 3' UGCAUGCGCCUUUGAAGCUp 5'		0.6
8	21-mer blunt ds RNA	5' pACGUACGCGAAUACUUCGAU 3' 3' UGCAUGCGCCUUUGAAGCUAp 5'		8.6
9	21-mer ds RNA/DNA	5' pCGUACGCGAAUACUUCGAUU 3' 3' TTGCATGCGCCTTATGAAGCTp 5'		61
10	21-mer ds DNA	5' pCGTACGCGAATCTTCGATT 3' 3' TTGCATGCGCCTTATGAAGCTp 5'		>1000
11	21-mer ds RNA-FAM	5' pCGUACGCGAAUACUUCGAUU 3' 3' UUGCAUGCGCCUAT(FAM)GAAGCUp 5'		1.3
12	21-mer ss RNA	5' pCGUACGCGAAUACUUCGAUU 3'		>1000
13	cel-let-7 ds microRNA	5' pUGAGGUAGUAGGUUGUUAUAGUU 3' 3' CCAUCCAUCUUUUUAAACGUAUCp 5'		20
14	gga-let-7 ds microRNA	5' pAGAGGUAGUGGGUUGCAUAGU 3' 3' UCU-UUCCGUCGUCCAACAUAU 5'		75
15	cel-mir-45 ds microRNA	5' pUGACUA---GAGACAAUUCAGCU 3' 3' AUACUGAUUGCUC-GUGUAGGUCp 5'		37
16	rRNA (rat liver ribosomal RNA)			>1000
17	tRNA (rat liver transfer RNA)			>1000

The relative binding affinities were determined by competitive gel shift. Unlabeled RNA or DNA were added in increasing amounts to compete with a radioactive 21-nucleotide siRNA (row 1) for binding to p19 fusion protein. Each experiment included a control which consisted of cold 21-nucleotide siRNA competing with the same radioactive labeled siRNA for p19 binding. The amount of cold siRNA that gave a 50% reduction in binding of the radioactive siRNA was given a value of 1 (K_{rel}). The K_{rel} for other oligos was calculated by comparing the oligo concentration to this standard. Oligonucleoties with a lower affinity have a higher K_{rel} . The name, nucleotide sequence, structure and the relative binding to MBP-p19-CBD fusion for each tested substrate (rows 2–17) are listed. The guide strand of each miRNA is shown in red (rows 13–15). The DNA strand for the RNA:DNA hybrid in row 9 is colored blue.

Figure 1. Size-specific binding of siRNAs and siRNA isolation using MBP-p19-CBD chitin magnetic beads. (A) A mixture of three dsRNAs of 17, 21, and 25 nucleotides (30 ng each) was incubated with 0–3 μ g MBP-p19-CBD fusion protein in 20 μ L 1 $\times$ p19 binding buffer, 1 $\times$ BSA (NEB), and 10 units of murine RNase inhibitor. The reactions were incubated at RT for 2 h, followed by PAGE and ethidium bromide staining. The siRNA markers are shown at the right of the figure. (B) siRNA isolation by p19 chitin magnetic beads. Ten microliters of p19 beads were incubated with 27.5 μ g rat liver total RNA and 5 ng 21-nucleotide siRNA (a quantitative standard); Lane 1, 5 ng of 21-nucleotide siRNA (a quantitative standard); Lane 2, unbound RNA; Lane 3, first wash (20 out of 600 μ L); Lane 4, sixth wash (20 out of 600 μ L); Lane 5, eluate; Lane 6, siRNA size marker.

mixer (New Brunswick Scientific, Edison, NJ, USA) for 20 min at 37°C. The eluted RNA was analyzed by electrophoresis on a 20% acrylamide gel in 1 $\times$ TBE buffer.

Gel mobility shift competition assay

A synthetic 21-nucleotide ssRNA oligonucleotide (5'-CGUACGCGGAAUACUUCGAUU-3') was radiolabeled with [γ - 32 P]-ATP (Perkin Elmer Life and Analytical Sciences, Waltham, MA, USA) by T4 polynucleotide kinase (NEB) using the manufacturer's protocol. The labeled strand was hybridized to the phosphorylated complementary ssRNA strand (5'-pUGCAAGUAUCCGCGUACGUU-3') in 30 μ L 1 $\times$ T4 polynucleotide kinase buffer (NEB) by heating to 90°C for 3 min and then slowly cooled to RT. The 32 P-labeled siRNA duplex was separated from unincorporated [γ - 32 P]-ATP using a Centri-Sep spin column (Princeton Separations, Inc, Adelphia, NJ, USA). Unlabeled double stranded competitors were made by annealing complementary oligos in equal molar ratios using the above protocol.

Competitive binding studies were performed by mixing increasing amounts (0–1280 ng) of various kinds of unlabeled nucleic acids with 1 ng radioactively labeled siRNA followed by the addition of 16 ng p19 fusion protein. The binding reaction was in 1 $\times$ p19 binding buffer containing RNase inhibitor at RT for 1 h. The reaction mixture was analyzed by gel electrophoresis using a 6% TBE DNA retardation gel (Invitrogen) in 0.5 $\times$ TBE buffer for one hour at 100 V. The gels were dried before exposure to Hyperfilm (GE Healthcare, Piscataway, NJ, USA) and band intensities determined with the Quantity One program (Bio-Rad, Hercules, CA, USA). Each series of experiments included a control where different amounts of unlabeled 21-nucleotide siRNA competed for p19 binding with radioactive 21-nucleotide siRNA. The amount of 21-nucleotide siRNA that gave a 50% reduction in binding of the labeled RNA to p19 was arbitrarily given a value of 1. If another nucleic acid required a 10-fold greater amount for a 50% reduction in binding of labeled 21-nucleotide siRNA to p19, it was given a value of 10. These

values are reported as K_{rel} or relative binding constants.

Relative binding of dsRNAs to p19 beads

The optimal RNA probe size was determined by binding 18 different length dsRNAs to p19 beads. Thirty nanograms of each dsRNA was incubated with 10 μ L p19 beads resuspended in 1 $\times$ p19 binding buffer, RNase inhibitor and BSA followed by washing and elution as described previously in siRNA isolation using p19 beads.

After ethidium bromide staining, the amount of dsRNA eluted from the beads was compared with the total dsRNA added. Band intensities were determined with Quantity One and the percent recovery calculated.

Detection of miR-122a with p19 beads

The p19 magnetic beads were used to measure the amount of miR-122a in rat liver RNA (Molecular Research Center, Cincinnati, OH, USA). A miR-122a-specific RNA probe, 5'-AACACCAUUGU-CACACUCCAUA-3', complementary to miR-122a, was end-labeled with 6000 Ci/mM [γ - 32 P]-ATP using T4 polynucleotide kinase (NEB). Different amounts of total rat liver RNA (5 and 10 μ g) were hybridized with 1 ng (500,000 cpm) 32 P-labeled miR-122a probe at 75° for 5 min and 52° for 7 h in a programmed PCR machine in 1 $\times$ p19 binding buffer in a total volume of 10 μ L. The hybridization mixture was then bound to 10 μ L p19 beads, washed, and eluted as described in siRNA isolation using p19 beads (see "Materials and methods" section). A 1/50 aliquot of the eluted miR-122a:RNA probe duplex—which represents 100 ng and 200 ng of rat liver RNA—was analyzed by electrophoresis on a 20% TBE gel. The gel was dried and exposed to an Imaging Screen K (Bio-Rad). The amount of radioactivity in the hybrid was determined by a scan of the screen using a Typhoon 9400 scanner with Image Quant TL software (GE Healthcare). A standard curve for miR-122a detection was generated by hybridizing a 1-ng aliquot of the same radioactive miR-122a probe with different amounts (20.1–0.1 fmoles) of synthetic miR-122a (5'-pUGGAGUGUGA-CAAUGGUGUUUG-3') in the presence of 10 μ g yeast total RNA (Invitrogen). The same p19 binding protocol was used for the standard curve and rat liver RNA samples. All assays were done in triplicate.

Northern blot analysis for detection of miR-153

RNA samples were prepared in 2 $\times$ TBE-urea sample loading buffer and loaded into lanes of a 15% TBE urea gel (Invitrogen). Each gel lane contained either 4 μ g yeast RNA (Invit-

Figure 2. Quantitative detection of miR-122a in rat liver RNA. A standard curve for miR-122a detection was created with defined amounts of an RNA oligo hybridized to a constant amount of complementary radioactive RNA probe. (A) The miRNA:probe hybrids were isolated with p19 beads and analyzed with a non-denaturing gel. The background of ssRNA probe, seen on this gel, was also observed with chitin magnetic beads without p19 (data not shown). (B) A standard curve was created by plotting the quantitative values for the dsRNA hybrids, from Supplementary Table S1, against the amount of synthetic miR-122a (in fmoles). (C) The amount of miR-122a in 100 ng and 200 ng rat liver RNA was determined with the identical p19 protocol and the same labeled RNA probe used for the standard curve (Supplementary Table S2). The assays were done in triplicate.

rogen) or 4 µg RNA isolated from the microfilariae of the parasitic nematode *Brugia malayi* (TRS Labs, Athens, GA, USA). Positive controls contained 4 µg yeast RNA and 100, 50, or 10 pg miR-153 RNA oligo. Following electrophoresis, the RNA was electroblotted onto Genescreen Plus membrane (Perkin Elmer Life and Analytical Sciences) using a Semi-Dry Electro blotter (Continental Lab Products, San Diego, CA, USA) in 1× TBE, as described by the manufacturer, then crosslinked to the membrane (Spectrolinker XL-1000 UV crosslinker; Spectronics Corporation, Westbury, NY, USA) at 120 mJ/cm². After prehybridization, the membrane was hybridized overnight at 37°C in Ultrahyb-Oligo hybridization buffer (Invitrogen) with ³²P-labeled oligonucleotide probes (2 × 10⁶ cpm/mL hybridization solution).

After hybridization, the blot was washed twice at 42°C for 30 min each in 2× SSC, 0.1% SDS. The 19-nucleotide oligonucleotide probe, 5'-CACUUUUGUGACUAUGCAA-3', complementary to miR-153, 5'-pUUGCAUAGUCACAAAAGUGAU-3', was labeled with [γ-³²P]-ATP (10mCi/mL, 6000 Ci/mmol; Perkin Elmer) and T4 polynucleotide kinase (NEB). The blot was exposed to Hyperfilm MP (GE Healthcare) for 3 days at -70°C using an intensifying screen.

Results and discussion

Modeling of dsRNA binding to MBP-p19-CBD fusion protein

To assist purification and retention of p19, two separate affinity tags were used: an amino-terminal fusion to MBP and a

carboxy-terminal fusion to CBD (Supplementary Figure 1A). The MBP moiety allows single column purification of the 70-kDa p19 fusion protein using amylose resin, while the CBD moiety allows the purified p19 fusion protein to tightly bind chitin magnetic beads. The locations of the two affinity tags fused to p19 were modeled using the structure of p19 bound to siRNA (16). The p19 binds to the sugar phosphate backbone of siRNA on one side of the helix (Supplementary Figure 1B). When viewed from the side (Supplementary Figure 1C), the CBDs are located away from the RNA helix. This model suggests that the spatially separated MBPs and CBDs will not interfere with siRNA binding to p19.

MBP-p19-CBD fusion preferentially binds 21 nucleotide siRNAs

The length of a dsRNA has a significant effect on p19 binding (16). We demonstrated the binding specificity of the p19 fusion protein with a gel shift assay using 30 ng for each of three dsRNAs (17-, 21- and 25-nucleotide). The RNAs have a 5' phosphates and 3' 2-nucleotide extensions. Figure 1A shows that the p19 fusion preferentially binds 21-nucleotide dsRNA compared with 17- and 25-nucleotide dsRNAs. Efficient binding of 30 ng 21-nucleotide dsRNA occurs with 500 ng of the p19 fusion which is a 1.5–2 fold molar excess or 15–20× weight excess of the p19 fusion protein. Binding of 17-nucleotide dsRNA or 25-nucleotide dsRNA required four to six times more p19 fusion than required for 21-nucleotide binding (Figure 1A). These results were consistent with the observations by others (12,16). The binding affinity of the p19 fusion protein for a 21-nucleotide dsRNA is in the low nanomolar range (data not shown). These studies demonstrate that the MBP-p19-CBD fusion protein retains the binding properties of CIRV p19.

Relative binding affinities of the p19 fusion to various RNAs and DNAs

The binding of p19 fusion to different RNA and DNA oligonucleotides was measured by a competitive gel shift assay. As shown in Supplementary Figure S2, dsDNA, ssRNA, or ribosomal RNA did not compete for binding with radioactive 21-nucleotide siRNA to p19. As a standard, the amount of unlabeled 21-nucleotide siRNA that reduced binding of the labeled 21-nucleotide siRNA by 50% is arbitrarily given a value of one for relative binding, K_{rel} (Supplementary Figure S2A, and Table 1, row 1).

The p19 fusion bound dsRNA in a size-dependent manner (Table 1, rows 1–3). The three phosphorylated dsRNAs with the greatest affinities are: blunt 19-bp dsRNA > blunt 20-bp dsRNA > siRNA (19-bp dsRNA core with 3' 2-nucleotide extensions) shown

Figure 3. Binding specificity of p19 beads to dsRNA. The top blue lines represent miRNAs and the bottom red lines indicate the probe strands. The amount of dsRNA bound and eluted from p19 beads was determined by a scan of the gel (as described in the "Materials and methods" section). A [+] indicates that >85% of the starting RNA was eluted, [+/-] indicates that 15–85% of the starting RNA was eluted, and [-] denotes that <15% of the RNA was eluted. The number below each RNA duplex indicates the number of base-paired nucleotides. The vertical lines at the ends of the RNA strands represent unpaired nucleotides.

in Table 1, rows 6, 7, and 1. It is interesting that the p19 fusion protein has greater affinity for the blunt 19-bp dsRNA than 21-nucleotide siRNA. A similar result was

also reported by Vargason (16), except p19 rather than p19 fusion was used in their binding assay. The 21-nucleotide siRNA has greater binding affinity than the 21-bp blunt

dsRNA (Table 1, rows 1 and 8). Removal of the 5' phosphate from the 21-nucleotide siRNA reduces binding affinity by >40-fold (Table 1, row 4). This suggests that the 5' phosphate and a 19- or 20-bp dsRNA core are critical for tight binding to the p19 fusion. The addition of a 3' 2-nucleotide extension to a 19-bp dsRNA core (as in the 21-nucleotide siRNA) does not enhance binding.

Increasing or decreasing the length by 4 bp (25- or 17-nucleotide dsRNAs) significantly reduces binding affinity to the p19 fusion, by 230- and 106-fold, respectively (Table 1, rows 3, 2). These relative affinities were determined when the oligo concentration competing for 32 P-labeled 21-nucleotide siRNA binding, is in large excess compared with the concentration of the p19 fusion. When p19 was in excess, only a 6- to 10-fold molar excess of 17- or 25-nucleotide dsRNAs was needed for binding (Figure 1A). Changes in length of a few nucleotides (Table 1, rows 2, 3) have a greater effect on binding than mismatched base pairs in a 21- to 22-nucleotide dsRNA (Table 1, rows 13–15).

Since assays for siRNA enrichment and miRNA detection take place in a very large excess of cytoplasmic RNA, we tested if rRNA or tRNA could compete with siRNA for p19 fusion binding. No reduction in binding was observed with a 1000-fold excess of rat liver

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Figure 4. Comparison of p19 beads to Northern blot analysis for miRNA-153 detection. (A) Northern blot analysis of miR-153 in *B. malayi* mf. The first three lanes contain 4 μ g yeast RNA mixed with 100, 50, or 10 pg of *B. malayi* miR-153 oligo. The yeast RNA lane contains 4 μ g yeast RNA only (negative control) and the mf RNA lane contains 4 μ g *B. malayi* mf RNA. (B) Detection of miR-153 from *B. malayi* mf RNA using p19. The same labeled RNA probe used for the Northern blot analysis (A) was used for p19 detection (B). The probe was hybridized to 4 μ g *B. malayi* mf RNA followed by p19 binding, washing, and elution. The ssRNA (miR-153 probe) and dsRNA (miR-153:probe hybrid) were included in the non-denaturing gel as size markers. The mf RNA lane contained the miR-153:RNA probe hybrid eluted from the p19 beads. The yeast RNA lane represents a negative control where 4 μ g yeast RNA was hybridized to the miR-153 probe followed by p19 binding, washing and elution.

rRNA (Supplementary Figure S2C and Table 1, row 16), tRNA (Table 1, row 17), or ssRNA (Supplementary Figure S2D and Table 1, row 12). These studies demonstrated the specificity of p19 for short dsRNA. A report using endogenously expressed p19 in embryonic stem cells found short (19–22 nucleotides) fragments of rRNA were immunoprecipitated with antibodies to tagged p19. They did not report any bound 21-nucleotide siRNAs (19). Our results showed that if 21-nucleotide siRNA was present, rRNAs did not compete for p19 binding. The gel shift assay used in this study is likely more stringent for RNA binding to p19 than the immunoprecipitation assays used in the stem cell studies.

Structural studies show that p19 does not contact nucleotides that are 6–8 bases from the 5' end of siRNA (16). When one strand of siRNA was labeled with dT FAM nucleotide seven bases from the 5' end, it had similar binding affinity for p19 fusion as unmodified siRNA (Table 1, rows 11 and 1). An siRNA with an internal biotinylated nucleotide can also bind p19 and streptavidin (data not shown). The ability of p19 fusion to bind dsRNAs with modified nucleotides expands the scope of miRNA detection methods.

The p19 fusion did not bind 21-nucleotide dsDNA (Supplementary Figure S2B and Table 1, row 10) but did bind a 21-nucleotide RNA:DNA hybrid with 60-fold lower affinity than 21-nucleotide siRNA (Table 1, rows 9 and 1). This difference in binding may be caused by their structures. dsRNA and RNA:DNA hybrids are A-form helices which are wider and approximately 25% shorter than the B-form helix of dsDNA (20).

Since the discovery that 5' triphosphorylated secondary siRNAs are the majority

of endogenous siRNAs in *C. elegans* (21), we tested if the extent of 5' phosphorylation could influence dsRNA binding to p19 fusion. The 21-nucleotide siRNA with 5' triphosphates bound with only an 8-fold lower affinity than 21-nucleotide siRNA with 5' monophosphates (Table 1, rows 5 and 1), suggesting the p19 fusion can be used to isolate both types of endogenous siRNAs.

The binding of p19 fusion to dsRNAs with mismatched base pairs was tested. In vivo, mismatched double-stranded miRNAs are generated by cleavage of endogenous hairpins by Drosha then Dicer. Synthetic dsRNA oligos for *C. elegans* let-7, *Gallus gallus* let-7, and *C. elegans* miR-45, which have four or five mismatched base pairs, bound to the p19 fusion with a 20- to 75-fold reduced affinity (Table 1, rows 13–15) compared with 21-nucleotide siRNA (Table 1, row 1). The lower affinity binding may relate to the distortion of the A-form RNA helix. It is unlikely these mismatched RNAs would be detected using p19 beads because an RNA:DNA hybrid with the same K_{rel} of 60 as the mismatched dsRNAs was not detected with p19 beads (data not shown).

Application of p19 fusion protein for siRNA isolation

Based on the bifunctional binding property of the p19 fusion, an siRNA enrichment assay was developed. The p19 fusion protein was bound to chitin magnetic beads, which have a very high affinity for the CBD. Binding capacity is 300–500 μ g of CBD fusion protein per 1 mL beads (18). To determine if total cellular RNA would interfere with siRNA binding, 30 μ g rat liver RNA and 10 ng 21-nucleotide siRNA were mixed and

incubated with p19 beads. After extensive washing, siRNA bound to the p19 beads was eluted and analyzed on a 20% TBE gel (Figure 1B). Even with a 5000-fold excess of total RNA, about 50% of input siRNA was recovered with a low background of cytoplasmic RNA. Based on this selective binding, we developed a novel miRNA detection assay using p19 beads to bind a miRNA:³²P-RNA probe duplex.

Rules for probe design in p19 detection of miRNAs

Knowing the binding specificities of the p19 fusion to different oligos is important in designing miRNA-specific probes for the p19 chitin magnetic beads. To determine optimum size and structure of miRNA/RNA probe hybrids for miRNA detection, 18 different dsRNA oligos were tested for p19 binding. This semiquantitative assay used the nonradioactive protocol for siRNA isolation (see “Materials and methods” section). The percentage of bound RNA was calculated by dividing eluted RNA by total RNA added to the reaction. The results show that probe length is more important than miRNA length for binding (Figure 3). For example, a 19-nucleotide RNA probe that forms a blunt end when hybridized to miRNAs 19–25 nucleotides long effectively binds p19 beads. The best size for an RNA probe is 19 or 20 nucleotides. Shorter (17–18 nucleotides) or longer (21–23 nucleotides) RNA probes form duplexes that bind poorly, if at all, to p19. The best probe for p19 binding (*i*) is 19 to 20 nucleotides long, (*ii*) forms a blunt end when hybridized to target RNA, and (*iii*) has a 5' phosphate (Figure 3). These probes can detect miRNAs 19–25 nucleotides in length. Calabrese and Sharp have reported that dsRNA with a single 12-nucleotide extension can bind p19 (19). The possibility of p19 beads binding longer RNAs is being investigated.

miRNA-122a detection using p19 beads

A very sensitive and reproducible assay for miRNA detection was developed using p19 chitin magnetic beads. To test this method, the amount of endogenous miR-122a in rat liver RNA was measured. A ³²P-labeled ssRNA probe, complementary to the microRNA, was hybridized to the RNA samples and the dsRNA isolated using p19 beads. The eluted RNA samples were run on a nondenaturing polyacrylamide gel to separate dsRNA from ssRNA and the radioactivity in the dsRNA bands was measured by phosphor imaging.

A standard curve was first generated with known amounts of synthetic miR-122a RNA oligo mixed with an excess of yeast RNA (Figure 2, A and B; Supplementary Table S1). This assay is very sensitive; even at 0.1 fmoles, the signal was still 20 times the background

(compare 0.1 fmoles with 0 fmoles miR-122a in Supplementary Table S1). The remaining ssRNA probe represents only a few femtomoles, which is <1% of the total probe used in the assay.

The amount of miR-122a in triplicate samples of 100 and 200 ng total rat liver RNA was then determined (Figure 2C and Supplementary Table S2). Based on the standard curve, there was 67.9 pg (9.1 fmoles) and 59.1 pg (8.0 fmoles) per 1 µg rat liver total RNA for the 100- and 200-ng samples, respectively. The average value of 64 pg miR-122a per 1 µg rat liver total RNA from this assay was in agreement with the value of 58 pg/µg reported for miR-122a from mouse liver RNA (22).

Comparison of p19 beads to Northern blot for detection of miR-153 from *Brugia malayi*

A comparison was made between p19 beads and Northern blot analysis for detection of a very low abundance miRNA, miR-153. Deep sequencing of *Brugia malayi* small RNAs from the microfilariae (mf) identified 500 reads for miR-153 compared with 146,000 reads for miR-71, out of a total of 3 million reads (data not shown). For an equal comparison between the two methods, the same amount of *B. malayi* mf RNA (4 µg), the same radioactive probe, and the same time of exposure to the film were used. Under these conditions, miR-153 was not detected by Northern blot analysis (Figure 4A), but p19 beads generated a very strong signal (Figure 4B). Internal controls for the Northern blot using a miR-153 oligo detected 10–50 pg RNA.

The p19 protein from the CIRV plant tombusvirus has been selected by evolution to tightly and selectively bind small dsRNAs which contain a 19-bp double-stranded region. We have utilized these properties to design assays for siRNA enrichment and miRNA detection, using a bifunctional recombinant fusion protein. The double-tagged p19 fusion can be used as a general miRNA detection method because binding is sequence-independent. The best RNA probes for miRNA detection are 19–20 nucleotides long, have a 5' phosphate, and form one blunt end when hybridized to miRNAs. These probes can detect miRNAs of 19–25 nucleotides. The length of the dsRNA core (19–20 bp) is more important for p19 binding than the length of the miRNA targets.

Detection of miR-122a in liver RNA demonstrated that the p19 method was very sensitive, quantitative, and reproducible. It detected 100 amoles RNA with a standard deviation of ~25% of the total signal. The high avidity and specificity of the p19 beads allow the detection of miRNA

in a millionfold excess of total RNA. This detection method is much faster and more sensitive than Northern blot analysis, enabling the detection of rare miRNAs, like miR-153, in the sub-femtomole range.

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Competing interests

J.J., C.B.P., and L.A.M. are employees of New England BioLabs, a company that sells p19 and other proteins for RNA and DNA research. M.C. was a master's student from the Ecole Supérieure de Biotechnologie Strasbourg program in France, whose research on the use of p19 fusion for miRNA detection was performed at New England BioLabs.

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