

Effect of chemical modifications on modulation of gene expression by duplex antigene RNAs that are complementary to non-coding transcripts at gene promoters

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ABSTRACT

Antigene RNAs (agRNAs) are small RNA duplexes that target non-coding transcripts rather than mRNA and specifically suppress or activate gene expression in a sequence-dependent manner. For many applications *in vivo*, it is likely that agRNAs will require chemical modification. We have synthesized agRNAs that contain different classes of chemical modification and have tested their ability to modulate expression of the human progesterone receptor gene. We find that both silencing and activating agRNAs can retain activity after modification. Both guide and passenger strands can be modified and functional agRNAs can contain 2'F-RNA, 2'OMe-RNA, and locked nucleic acid substitutions, or combinations of multiple modifications. The mechanism of agRNA activity appears to be maintained after chemical modification: both native and modified agRNAs modulate recruitment of RNA polymerase II, have the same effect on promoter-derived antisense transcripts, and must be double-stranded. These data demonstrate that agRNA activity is compatible with a wide range of chemical modifications and may facilitate *in vivo* applications.

INTRODUCTION

The therapeutic potential of gene silencing by small interfering RNAs (siRNAs) is widely recognized (1). siRNA clinical candidates are commonly modified to

improve their stability, specificity and potency (2–4). Modified siRNAs have been shown to reduce off-target effects resulting from the miRNA pathway (5,6), the innate immune system (7,8) and loading of the wrong strand (6,9,10). In addition, chemically modified siRNAs have been used to deepen our understanding of the mechanism of RNAi (11–16) and improve siRNA delivery (17,18).

Antigene RNAs (agRNAs) are small duplex RNAs that target gene promoters. Like siRNAs, they are generally 19 bp with 2-nt overhangs at the 3'-ends. Depending on target sequence, cell type and basal level of expression, agRNAs can either silence (19–21) or activate (22–24) gene expression at the transcriptional level. Like gene silencing by siRNAs, modulation of gene expression by agRNAs involves argonaute (AGO) proteins (25,26). However, instead of targeting mRNA, agRNAs target non-coding RNA transcripts (ncRNAs) overlapping gene promoters (27,28). While agRNAs can target either sense or antisense non-coding transcripts, the agRNAs discussed in this study target an antisense transcript overlapping the promoter of the progesterone receptor (PR) gene (28). Thus the guide strand of these agRNA duplexes is the sense strand, in contrast to siRNAs, for which the guide strand is by definition antisense (i.e. complementary to the mRNA).

agRNAs that activate gene expression could be used to modulate expression of disease-associated genes whose upregulation might be beneficial. As with current gene silencing technology, however, it is likely that any development of agRNAs for *in vivo* use will require chemical modification to improve stability and biodistribution. In this study we examine the effects of modified

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nucleotides on the function of agRNAs. After examining over 120 duplexes, we identify several chemically modified compounds that retain partial or full activity.

MATERIALS AND METHODS

Double-stranded RNAs

RNAs were synthesized by Alnylam Pharmaceuticals or SIGMA Custom Products using standard protocols, or were purchased from Integrated DNA Technologies. The concentrations of single strands were determined by measuring their absorbance at 260 nm (A_{260}). Complementary single strands were combined in equimolar ratios and diluted to 20 μ M stock solutions in 2.5 \times phosphate-buffered saline. These duplexes were annealed by heating to 95°C and slowly cooled to room temperature in a heating block or thermal cycler. Duplex integrity was tested by a UV thermal melt experiment as described below. Duplexes were stored at -20°C .

Cell culture

T47D and MCF7 cells (American Type Culture Collection) were maintained in RPMI-1640 media (Sigma) supplemented with 10% (v/v) FBS, 0.5 \times MEM non-essential amino acids, 0.4 units ml^{-1} bovine insulin, 10 mM HEPES and 1 mM sodium pyruvate. Cells were cultured at 37°C and 5% (v/v) CO_2 .

Lipid-mediated transfection

Cells were plated at 110 000–160 000 cells per well in six-well plates (Costar) in RPMI. A higher number of cells was typically seeded for RNA analysis than for protein analysis. Transfections were performed after 1–2 days, when the cells had adhered and attained about 20–30% confluence. Lipofectamine RNAiMAX cationic lipid (1.1 μ l/well, Invitrogen) and RNA (31 pmol/well) were individually diluted in Opti-MEM (Invitrogen), then combined and allowed to incubate for 20 min (volume 250 μ l/well) before pipetting into cell-culture wells containing 1 ml fresh Opti-MEM (final volume 1.25 ml, 25 nM duplex RNA for all transfections except dose response experiments). The transfection solution was replaced with RPMI after 24 h, and media were changed again 2 days later. For dose response experiments, the ratio of lipid to RNA was kept constant, and the concentration of RNA was varied from 0 to 50 nM.

For competition experiments, the cells were passaged into new plates on Days 2 or 3 after transfection. The second transfection was carried out on Day 4 after the first transfection under identical conditions, and protein was harvested on Days 7 or 8. The negative control duplex Neg1 used in these experiments consisted of oligonucleotides 5'-UCAAGAAGCCAAGGAUAAUdTdT and 5'-A UUAUCCUUGGCUUCUUGAdTdT.

RNA harvest and quantitative real-time PCR

Total RNA from treated MCF7 or T47D cells was harvested 3 days after transfection. After washing with PBS, TRI reagent (1 ml, Sigma) was added to each well,

incubated for several minutes, mixed well and transferred to a 1.5-ml microcentrifuge tube. Chloroform (250 μ l) was added and the mixture was shaken vigorously for 1 min, then centrifuged at 12 500 rpm for 15 min. The clear aqueous layer was transferred to a new 1.5-ml microcentrifuge tube, avoiding any of the interphase or organic layer. Addition of cold isopropanol (500 μ l) followed by shaking, incubation at -20°C (at least 10 min), and centrifugation (12 500 rpm, 15 min, 4°C) gave a pellet, which was washed with 75% ethanol, dissolved in nuclease-free water (20 μ l), and quantitated by the A_{260} of an aliquot (2 μ l in 200 μ l TE buffer). Samples of 2 μ g RNA were removed and treated with DNase I (Worthington). After heat-inactivation of the DNase enzyme (75°C, 10 min), the samples were reverse transcribed using random hexamer primers with the High Capacity cDNA Archive kit (Applied Biosystems). Quantitative real-time PCR (qPCR) was used to measure RNA levels with 30–100 ng of cDNA (carried out in triplicate, and repeated for at least two independent transfections). For analysis of PR mRNA, we used a primer-probe assay (IDT) consisting of primers 5'-CTTA CCTGTGGGAGCTGTA-3' and 5'-GCACTTTCTAAG GCGACATG-3' as well as probe 5'-CTGCCCTTCCAT TGCCCTCTT-3'. For analysis of promoter-derived anti-sense transcripts, we used SYBRgreen qPCR with primers 5'-GGAGGAGGCGTTGTTAGAAA-3' and 5'-GAAGG GTCGGACTTCTGCT-3'. Data were normalized to levels of GAPDH mRNA as determined by a TaqMan Gene Expression Assay (Applied Biosystems).

Analysis of protein expression

Protein was generally harvested from treated MCF7 or T47D cells 5 days after transfection. The cells were washed with phosphate-buffered saline (PBS), trypsinized, transferred to 1.5-ml microcentrifuge tubes and centrifuged (3500 rpm, 15 min). The pellets were washed with cold PBS and gently lysed by addition of 50 μ l lysis buffer [50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5% Nonidet P40, 1 mM EDTA, 1 mM DTT]. Protease inhibitor cocktail set 1 (Calbiochem) was added to the lysis buffer just before use. After a freeze-thaw cycle to ensure complete lysis, the lysates were centrifuged to pellet insoluble cell debris and cell lysate solutions were transferred to new microtubes. Protein concentrations were calculated using the BCA assay (Thermo Scientific).

Western blots were performed on cell lysates (30 μ g protein per lane). Proteins were separated by SDS-PAGE using 7.5% Tris-HCl acrylamide gels, then transferred onto nitrocellulose membranes. After blocking the membrane (5% milk in PBS-T) for 1 h, the upper portion of the membrane was treated with PR monoclonal primary antibody (Cell Signaling, 1:1000 dilution in PBS-T, \sim 15 h) and the lower portion was treated with anti- β -actin (Sigma, 1:10 000 dilution, 1 h) as an internal control. Protein was visualized using HRP-conjugated secondary anti-mouse (Jackson Immunolabs, 1:5000 for PR and 1:20 000 for actin, 30–45 min) The membranes were then briefly treated with West Pico SuperSignal developing solution (Thermo Scientific) before exposing them to

film. All analogs were tested in at least two independent transfections, of which one representative western blot is shown.

Determination of melting temperature (T_m)

Annealed siRNA duplexes were diluted to 0.8 μ M in 0.1 M phosphate buffer, pH 7.4 (final volume 500 μ l) and transferred to quartz cuvettes. The solutions were topped with mineral oil (200 μ l) and heated from 18 to 99°C at 1°C/min while monitoring changes in A_{260} using a Cary 100 Bio spectrophotometer. Samples were then re-annealed in the spectrophotometer and the experiment was repeated several times. T_m values were calculated as the first derivatives of the plot of A_{260} vs temperature, and the results from three experiments were averaged.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed essentially as described (29). Two or three 150-mm dishes of T47D or MCF7 cells were reverse transfected with 25 nM of native agRNA, modified agRNA or luciferase control duplex siGL2 (30).

Media were changed on Day 2 and the cells harvested on Day 3. To crosslink and harvest nuclei, the cells were treated with 1% formaldehyde in PBS (10 ml) for 10 min, then the crosslinking reaction was quenched with glycine (0.125 M final concentration, at least 5 min). Cells were collected and nuclei were isolated by treatment with cold hypotonic lysis buffer (4 ml) containing 10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM MgCl₂ and 0.5% Nonidet P40. The pelleted nuclei were lysed with a buffer containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1) and 1× complete protease inhibitor cocktail (Roche). DNA was sonicated on ice to an average fragment size of ~500 bp. The lysate was then centrifuged to remove insoluble cell debris and the supernatant precleared with Protein G Plus/Protein A Agarose suspension (Calbiochem).

An aliquot of the cleared lysate (100 μ l) was diluted to 1 ml in buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris pH 8.1, 167 mM NaCl and 1× complete protease inhibitor cocktail (Roche)]. Mouse anti-RNAPII IgG (Millipore, Catalogue #05-623, 3–6 μ g) or normal mouse IgG (Millipore Catalogue #12-371, 3–6 μ g) was added and the solution rotated at 4°C overnight. Protein G Plus / Protein A agarose suspension (30–60 μ l) was added to precipitate the antibody complexes and the beads were washed as described (29). Complexes were eluted using 2 × 250 μ l of NaHCO₃ (0.1 M) containing 1% SDS. NaCl was added (final concentration 200 mM) and the samples heated to 65°C for at least 2 h to reverse crosslinks. The samples were then treated with RNase A (1 μ l) at 37°C for 30 min, followed by Tris-HCl (pH 7.0, 20 μ l), EDTA (0.5 M, 10 μ l) and proteinase K (20 μ g) at 42°C for 45 min. DNA was isolated by phenol-chloroform extraction and ethanol precipitation. Pellets were diluted to 50 μ l in water and 5 μ l was used per qPCR reaction. qPCR primers used at the PR promoter were Fwd 5'-CCTAGAGGAGGAGGCGT TGT and Rev 5'-ATTGAGAATGCCACCCACA. Levels

of RNAPII at the GAPDH promoter were also measured with primers Fwd 5'-TACTAGCGGTTTTACGGGCG and Rev 5'-TCGAACAGGAGGAGCAGAGAGCGA and were used for normalization. Each sample was normalized to a corresponding 2% input sample.

Statistics, dose response profiles, and curve fitting

Error bars shown on qPCR graphs correspond to standard deviations from triplicate measurements of at least two independent transfections; they include the error from the qPCR measurement as well as from differences between the transfections. For Figures 2–7 we also examined whether RNA expression levels were significantly different from the mismatch control by conducting a two-tailed unpaired Student's *t*-test with equal variances, and for this test we considered only biological replicates (using averages of the triplicate qPCR measurements for each). Significance levels of *P*-values are: **P* < 0.05, ***P* < 0.01 and ****P* < 0.005.

Dose responses were fit to the model equation $y = 100[1 - x^m / (n^m + x^m)]$, where *y* is the percent of target (protein or mRNA) remaining, *x* is the concentration of agRNA in nanomolars. The regression was fit by optimizing *m* and *n* using SigmaPlot 11, and *n* was taken as the IC₅₀ value.

RESULTS AND DISCUSSION

Experimental design

We focused on the PR gene because it is a well-characterized target for agRNAs (20,23,25,28). In T47D breast cancer cells, which express a high level of PR, PR expression can be dramatically reduced by agRNA PR-9 that overlaps the start site from -9 to +10 (20,21). In MCF7 breast cancer cells, which express PR at a lower basal level, its expression can be increased by agRNA PR-11 targeting the region from -11 to +8 (23). PR-11 can also activate gene expression in T47D cells but activation becomes most apparent when the cells are grown under conditions that repress basal expression of PR (23). Whether activation or repression is observed depends primarily on the basal expression level of PR and the target sequence of the agRNA.

While PR-9 and PR-11 are benchmark agRNAs used for most experiments, we also examined other duplexes. For some modifications we tested analogs of silencing agRNA PR-26 (-26 to -8) or activating agRNA PR-22 (-22 to -4) to allow more general insights into gene modulation. To permit comparisons with an RNA that engages the post-transcriptional silencing pathway, we used modified analogs of PR2526, a siRNA that is complementary to PR mRNA from +2526 to +2544. As negative control we used MM4, a duplex containing four mismatches with respect to PR-9. Duplex and single-stranded oligomers were introduced into cells by standard transfection protocols using cationic lipid. Expression was analyzed by western analysis of PR protein and qPCR of PR mRNA.

We use descriptive compound identifiers (Table 1). Each type of modified duplex is assigned two uppercase letters.

The first letter describes the chemical modification of the passenger strand, while the second letter describes modification of the guide strand. A lowercase 'p' indicates that the 5'-end of the strand is phosphorylated. For example, a duplex labeled 'NpF' has a native passenger strand and a guide strand that is phosphorylated and fully modified with 2'-fluoro (2'-F-RNA) nucleotides. Passenger strands are listed above guide strands throughout.

Effects of 2'-F-RNA modification on gene silencing

The structure of 2'-F-RNA (Figure 1) is similar to that of RNA (31) but confers resistance to endonucleases (32,33). For siRNAs, partial modification with 2'-F RNA is tolerated throughout either strand of the duplex (34-36) and there have been reports of functional siRNAs that are extensively or completely modified with 2'-F-RNA (33,37,38). 2'-F-RNA has been shown to reduce the immunostimulatory activity of siRNAs (39,40).

We tested three types of 2'-F-RNA-modified strands: fully modified (F), modified at all of the pyrimidine units (Y), and modified at positions near the termini (Z) (Table 1 and Figure 2A). Relative to unmodified duplex PR-9, we observed reduced potency when either strand was replaced with a fully-fluorinated (PR-9 NpF, PR-9 pFN) or pyrimidine-fluorinated strand (PR-9 NpY, PR-9 pYN) (Figure 2B). siRNA PR2526, by contrast, retained most silencing activity after total or partial modification of its passenger strand (PR2526 pFN, PR2526 pYN) but activity was lost after modification of the guide strand (PR2526 NpF, PR2526 NpY) (Figure 2B). Duplexes based on either PR-9 or PR2526 containing heavy modification in both strands were largely inactive (Supplementary Figure S1).

Given the loss of activity after aggressive modification of PR-9, we tested more limited terminal substitutions. A

PR-9 analog containing minimal modification of the guide strand was effective when paired with an unmodified passenger strand (PR-9 NZ) but not when the passenger strand was heavily modified (PR-9 YZ and PR-9 FZ) (Figure 2C). Analogs of PR2526 containing a minimally modified guide strand (PR2526 NZ, PR2526 YZ, PR2526 FZ) were active regardless of the extent of modification of the passenger strand (Figure 2C).

We also examined the effect of 2'-F-RNA modification on the activity of silencing agRNA PR-26 in an effort to determine how modification effects might vary depending on the sequence of the agRNA. When the pyrimidines of either or both strands of PR-26 were replaced with 2'-F-RNA (PR-26 YN, PR-26 NY, PR-26 YY), most silencing activity was retained (Figure 2D). Taken together, data from PR-9 and PR-26 demonstrate that 2'-F-RNA substitutions are compatible with the mechanism for agRNA activity but that the activity of individual patterns of modification can vary between duplexes.

Effect of 2'-F-RNA modification on gene activation

We introduced 2'-F-RNA into activating agRNA PR-11 (Figure 3A) and observed that the modification was broadly tolerated. Modification of the pyrimidines of the passenger strand (PR-11 pYN), total modification of the passenger strand (PR-11 pFN), or heavy modification of the guide strand (PR-11 NpF and PR11 NpY) all led to gene activation, albeit at levels below those of the unmodified PR-11 duplex (Figure 3B). Combining the unmodified or heavily modified passenger strands with a minimally modified guide strand showed a loss of potency as the degree of modification increased (Figure 3C). Nevertheless, all of these duplexes retained significant activity (2-fold to 6-fold at the RNA level). PR-11 analogs with both strands heavily modified were inactive (Supplementary Figure S2).

To assess the generality of 2'-F-RNA modification, we also tested a second activating agRNA, PR-22. For PR-22, 2'-F-RNA was well-tolerated in the passenger strand and duplexes PR-22 FN and PR-22 YN were able to activate gene expression at both RNA and protein levels without loss of potency (Figure 3D). The guide strand of PR-22 was more sensitive to 2'-F-RNA modification, with lower levels of gene activation by agRNAs PR-22 NY and PR-22 YY. Taken together with results from PR-11, these results show that 2'-F-RNA substitutions can be well tolerated by activating agRNAs and that chemical modifications on the passenger strand tend to be better tolerated.

Table 1. Descriptive strand names used throughout this work

Abbreviation	Strand chemistry
N	Native RNA strand
F	2'-F-RNA, fully modified
Y	2'-F-RNA, all pyrimidines
Z	2'-F-RNA, contains three units near termini
P	2'-OMe-RNA, all pyrimidines
J	2'-OMe-RNA, two units at the 5'-end
L	LNA, contains four units distributed evenly
L3	LNA, contains two units toward the 3'-end
L5	LNA, contains two units toward the 5'-end
p	A lower case 'p' preceding any strand abbreviation indicates a 5'-phosphate

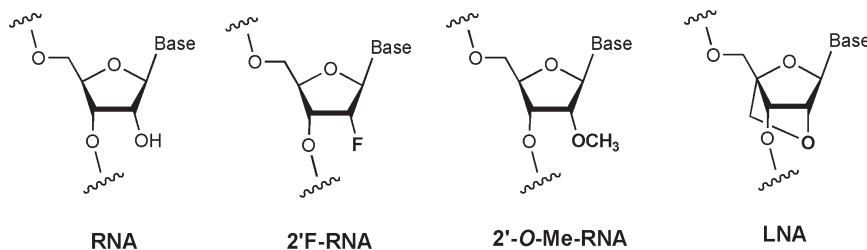


Figure 1. Chemical modifications used in this study.

Gene silencing with 2'-F-RNA in T47D cells

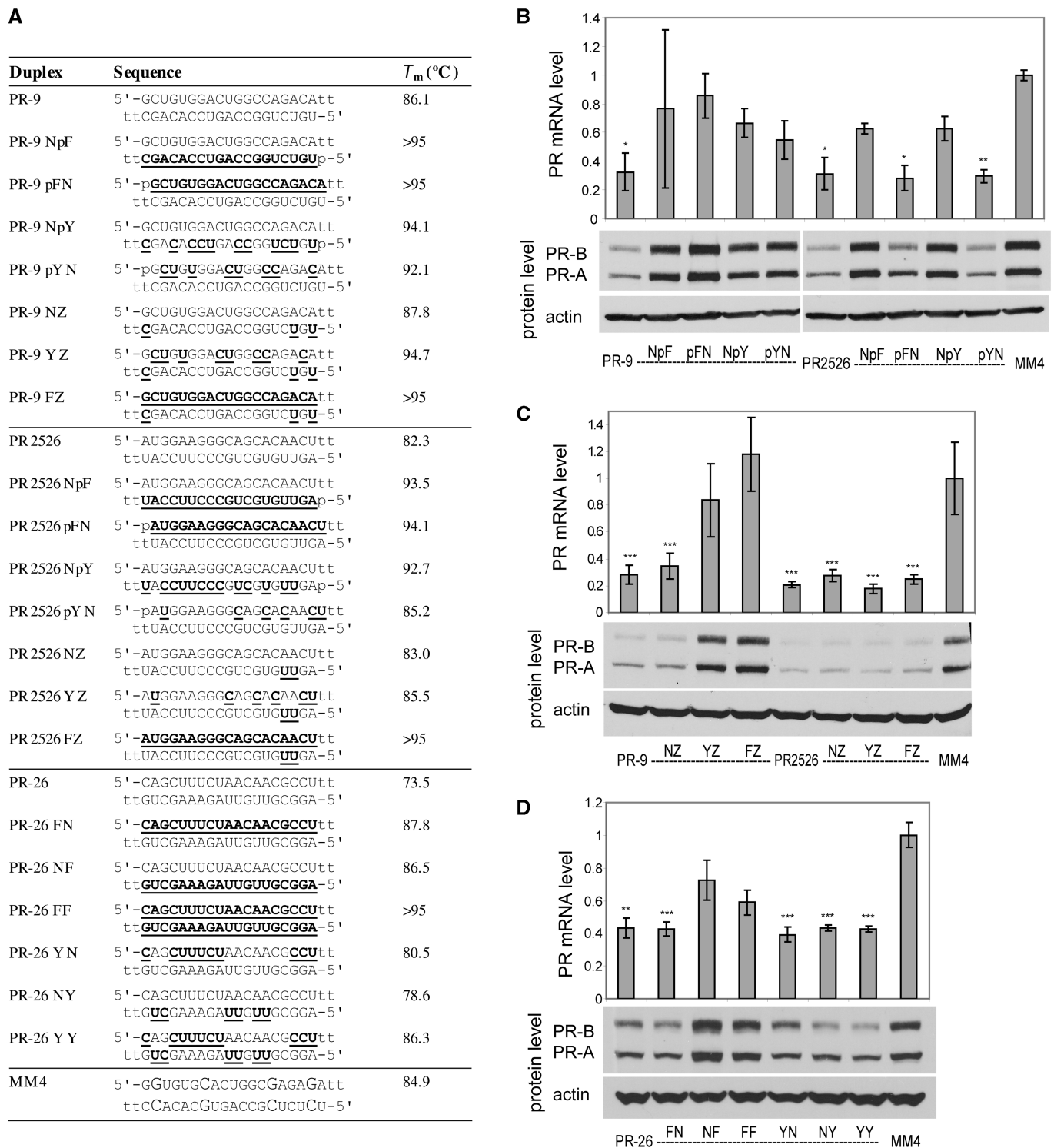


Figure 2. PR gene expression can be silenced by agRNAs containing 2'-F-RNA. (A) Duplex sequences: passenger strands are listed on top in all cases; for siRNA PR2526 this is the sense strand, but for agRNAs PR-9 and PR-26 this is the antisense strand. Modification codes: dna, RNA, **2'-F-RNA**; p = phosphate. (B) PR-9 analogs and controls containing one strand heavily or fully modified with 2'-F-RNA. (C) PR-9 analogs and controls containing a minimally 2'-F-RNA-modified guide strand. (D) PR-26 analogs containing one or both strands heavily or fully modified with 2'-F-RNA. All data are normalized to mismatch control MM4. RNA levels are the average (\pm SD) from at least two independent transfections. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$. Protein levels were also confirmed by at least two independent transfections, from which a typical western blot is shown.

Gene activation with 2'F-RNA in MCF7 cells

A

Duplex	Sequence	T_m (°C)
PR-11	5' -UGUGGACUGGCCAGACAGCtt ttACACCUGACCGGUCUGUCG-5'	85.2
PR-11 NpF	5' -UGUGGACUGGCCAGACAGCtt ttACACCUGACCGGUCUGUCGp-5'	>95
PR-11 pFN	5' -pUGUGGACUGGCCAGACAGCtt ttACACCUGACCGGUCUGUCG-5'	>95
PR-11 NpY	5' -UGUGGACUGGCCAGACAGCtt ttACACCUUGACCGGUCUCGp-5'	95.3
PR-11 pYN	5' -pUGUGGACUGGCCAGACAGCtt ttACACCUGACCGGUCUGUCG-5'	91.2
PR-11 NZ	5' -UGUGGACUGGCCAGACAGCtt ttACACCUUGACCGGUCUCG-5'	89.1
PR-11 YZ	5' -UGUGGACUGGCCAGACAGCtt ttACACCUUGACCGGUCUCG-5'	94.6
PR-11 FZ	5' -UGUGGACUGGCCAGACAGCtt ttACACCUUGACCGGUCUCG-5'	>95
PR-22	5' -CAGACAGCUUUCUAAACAActt ttGUCUGUCGAAAGAUGUUG-5'	70.0
PR-22 FN	5' -CAGACAGCUUUCUAAACAActt ttGUCUGUCGAAAGAUGUUG-5'	81.9
PR-22 NF	5' -CAGACAGCUUUCUAAACAActt ttGUCUGUCGAAAGAUGUUG-5'	81.2
PR-22 FF	5' -CAGACAGCUUUCUAAACAActt ttGUCUGUCGAAAGAUGUUG-5'	>95
PR-22 YN	5' -CAGACAGCUUUCUAAACAActt ttGUCUGUCGAAAGAUGUUG-5'	76.0
PR-22 NY	5' -CAGACAGCUUUCUAAACAActt ttGUCUGUCGAAAGAUGUUG-5'	76.0
PR-22 YY	5' -CAGACAGCUUUCUAAACAActt ttGUCUGUCGAAAGAUGUUG-5'	83.9
MM4	5' -GGUGUGCACUGGCAGAGAtt ttCCACACGUGACCGCUCUCU-5'	84.9

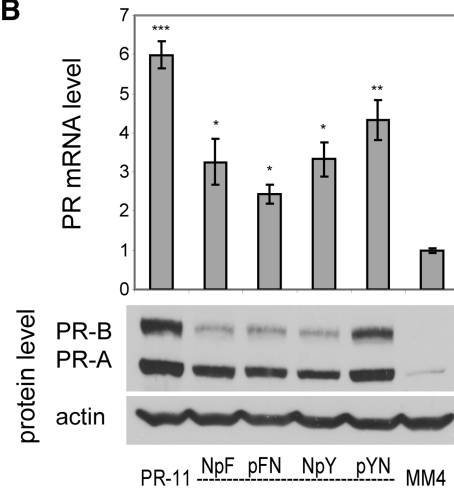
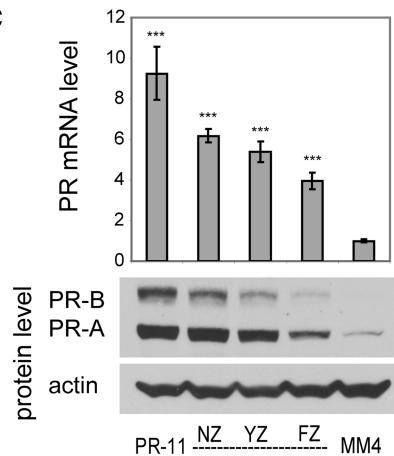
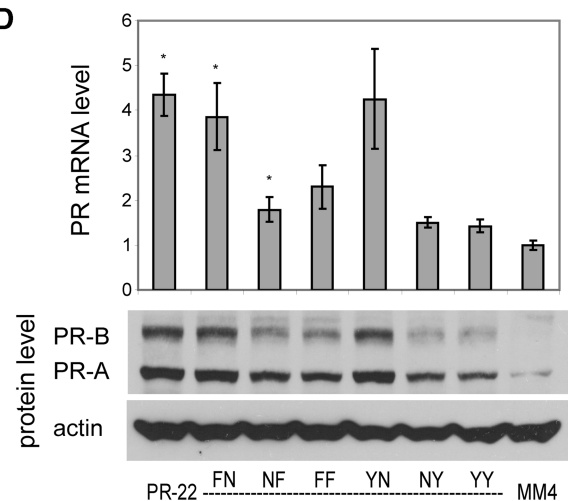
B**C****D**

Figure 3. PR gene expression can be activated by agRNAs containing 2'F-RNA. (A) Duplex sequences: passenger strands are listed on top in all cases; for agRNAs PR-11 and PR-22 this is the antisense strand. Modification codes: dna, RNA, **2'F-RNA**; p = phosphate. (B) PR-11 analogs containing one strand heavily or fully modified with 2'F-RNA. (C) PR-11 analogs containing a minimally 2'F-RNA-modified guide strand. (D) PR-22 analogs containing one or both strands heavily or fully modified with 2'F-RNA. All data are normalized to mismatch control MM4. RNA levels are the average (\pm SD) from at least two independent transfections. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$. Protein levels were also confirmed by at least two independent transfections, from which a typical western blot is shown.

Effect of 2'-O-methyl-RNA on gene silencing

2'-O-Methyl-RNA (2'OMe) nucleotides (Figure 1) improve the thermal and nuclease stability of siRNA duplexes (41,42). Inclusion of 2'OMe-RNA units also reduces off-target effects mediated either through the miRNA pathway (5) or the innate immune system (43–45). We tested duplex RNAs containing two 2'OMe ribonucleotides at the 5'-terminus of each strand (J) or at all pyrimidine nucleotides (P) (Figure 4A). Duplexes PR-9 JJ, containing 2'OMe nucleotides at both 5'-termini, and PR-9 PN, containing 2'OMe modification at the pyrimidine nucleotides of the passenger strand, were both effective gene silencing agents (Figure 4B). Duplexes containing pyrimidine modifications on the guide strand (PR-9 NP, PR-9 PP) were inactive, consistent with previous observations that 2'OMe modification is often better tolerated in the passenger strand (41). Variants of PR2526 (PR2526 PN, PR2526 NP and PR2526 PP) had activities similar to the PR-9 variants (Figure 4B).

Gene silencing with 2'OMe-RNA in T47D cells

Duplex	Sequence	T_m (°C)
PR-9	5' -GCUGUGGACUGGCCAGACAtt ttCGACACCUGACCGGUCUGU-5'	86.1
PR-9 JJ	5' - gc UGUGGACUGGCCAGACAtt ttCGACACCUGACCGGUCU gu -5'	88.5
PR-9 PN	5' - GcuGu GGAcuGGccAGAcAtt ttCGACACCUGACCGGUCUGU-5'	89.2
PR-9 NP	5' -GCUGUGGACUGGCCAGACAtt tt cgAc accuGaccGGuc <u>gu</u> -5'	90.0
PR-9 PP	5' - GcuGu GGAcuGGccAGAcAtt tt cgAc accuGaccGGuc <u>gu</u> -5'	91.4
PR2526	5' -AUGGAAGGGCAGCACAAcUtt ttUACCUUCCCGUCGUGUUGA-5'	82.3
PR 2526 JJ	5' - au GGAAAGGGCAGCACAAcUtt ttUACCUUCCCGUCGUGU ga -5'	81.9
PR 2526 PN	5' - Au GGAAAGGGcAGcAAcUtt ttUACCUUCCCGUCGUGUUGA-5'	82.7
PR 2526 NP	5' -AUGGAAGGGCAGCACAAcUtt tt uaccuucc GucGu <u>guu</u> GA-5'	86.1
PR 2526 PP	5' - Au GGAAAGGGcAGcAAcUtt tt uaccuucc GucGu <u>guu</u> GA-5'	87.0
PR-26	5' -CAGCUUUCUAACAACGCCUtt ttGUCGAAAGA <u>uuuu</u> GCGGA-5'	73.5
PR-26 PN	5' - cagcuuuu uAACAACGCCUtt ttGUCGAAAGA <u>uuuu</u> GCGGA-5'	77.1
PR-26 NP	5' -CAGCUUUCUAACAACGCCUtt ttG uc GAAAGA <u>uuuu</u> GCGGA-5'	76.5
PR-26 PP	5' - cagcuuuu uAACAACGCCUtt ttG uc GAAAGA <u>uuuu</u> GCGGA-5'	80.1
MM4	5' -GGUGUGCACUGGCAGAGAtt ttCCACACGUGACCGCUCUCU-5'	84.9

We also examined the effects of 2'OMe modification of a second silencing agRNA, PR-26, and identified analogs that inhibited PR expression (Figure 4C). However, for PR-26, modification of the guide strand in PR-26 NP did not prevent gene silencing. These data demonstrate that agRNA-mediated gene silencing can tolerate the introduction of 2'OMe-RNA modifications. The influence of a specific pattern of modification, however, depends on the identity of the parent sequence and can vary between agRNAs.

Effect of 2'-O-methyl-RNA on gene activation

We introduced 2'-O-methyl modifications into two different activating agRNAs, PR-11 and PR-22 (Figure 5A). Substitution of 2'OMe units into PR-11 reduced activation from 8-fold to 2–3-fold (Figure 5B). PR-11 PP with two heavily modified strands were the least activating. By contrast, activation by modified PR-22 duplexes was relatively stable at 4- to 6-fold regardless of modification

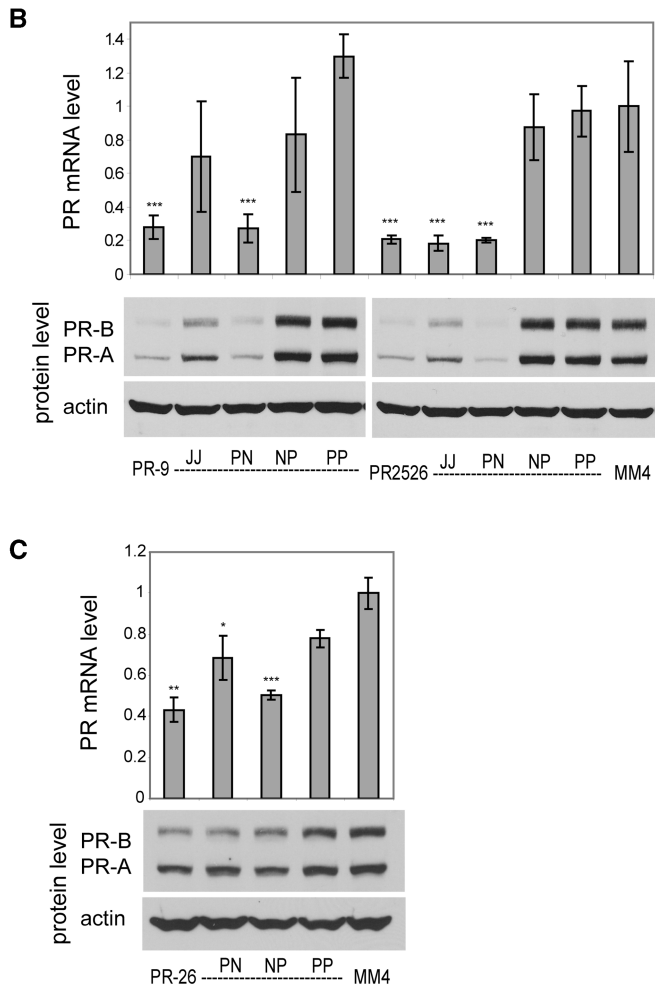


Figure 4. PR gene expression can be silenced by agRNAs containing 2'OMe-RNA. (A) Duplex sequences: passenger strands are listed on top in all cases; for siRNA PR2526 this is the sense strand, but for agRNAs PR-9 and PR-26 this is the antisense strand. Modification codes: dna, RNA, 2'OMe-RNA. (B) PR-9 analogs and controls containing 2'OMe-RNA. (C) PR-26 analogs containing 2'OMe-RNA. All data are normalized to mismatch control MM4. RNA levels are the average (\pm SD) from at least two independent transfections. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$. Protein levels were also confirmed by at least two independent transfections, from which a typical western blot is shown.

Gene activation with 2'OMe-RNA in MCF7 cells

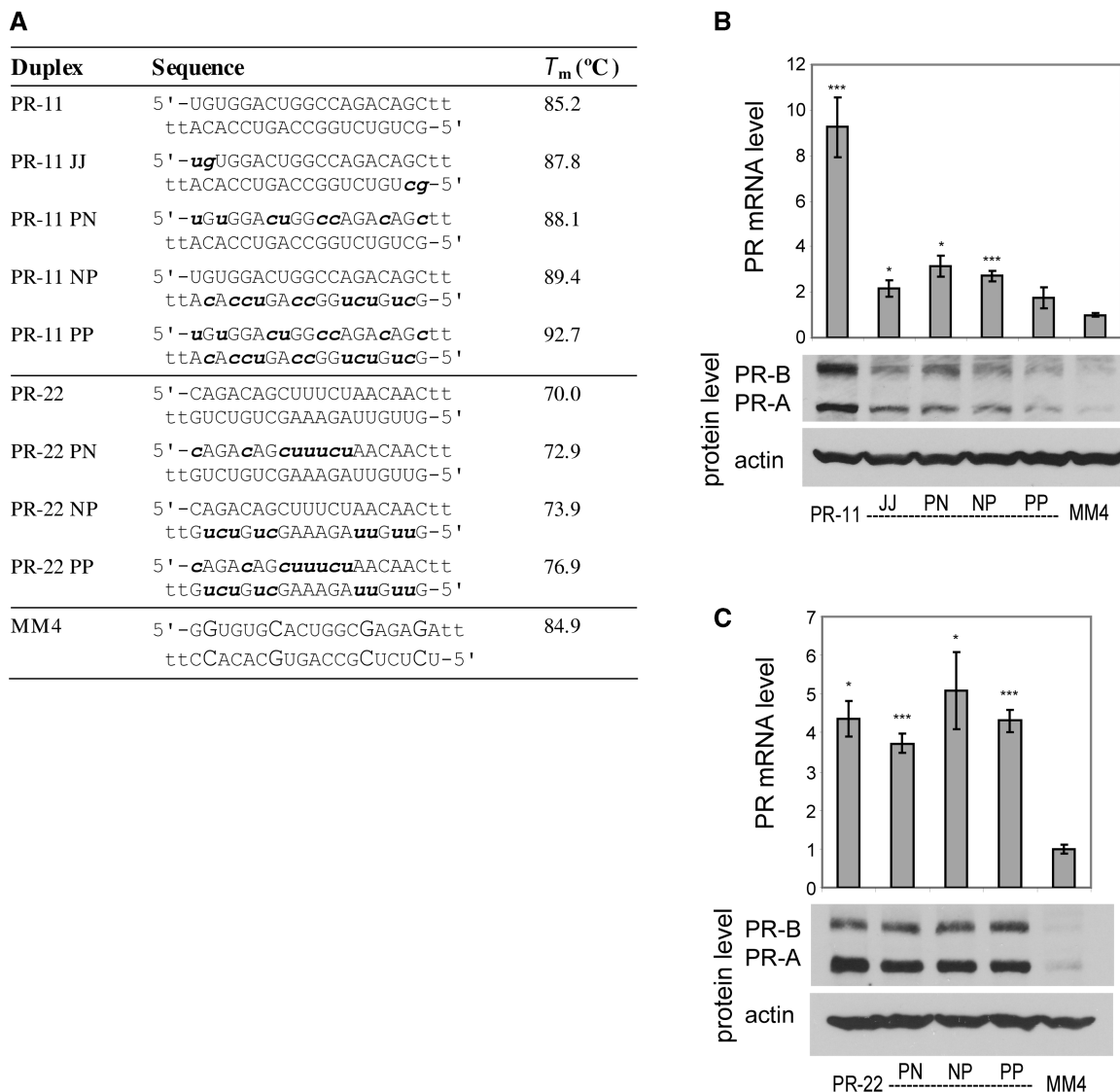


Figure 5. PR gene expression can be activated by agRNAs containing 2'OMe-RNA. (A) Duplex sequences: passenger strands are listed on top in all cases; for agRNAs PR-11 and PR-22 this is the antisense strand. Modification codes: dna, RNA, 2'Ome-rna. (B) PR-11 analogs containing 2'OMe-RNA. (C) PR-22 analogs containing 2'OMe-RNA. All data are normalized to mismatch control MM4. RNA levels are the average (\pm SD) from at least two independent transfections. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$. Protein levels were also confirmed by at least two independent transfections, from which a typical western blot is shown.

(Figure 5C). These data suggest that, as observed above for gene silencing, the impact of 2'OMe modifications on gene activation will vary depending on the parent sequence.

Effect of LNA on gene silencing

Locked nucleic acid (LNA) (Figure 1) is a rigid nucleotide analog containing a constrained, bicyclic sugar. Introduction of locked nucleotides into DNA or RNA oligomers leads to substantially enhanced affinities for complementary sequences (46–49) and enhanced resistance to digestion by nucleases (49–51). Several studies have also shown that the introduction of LNA nucleotides

can improve the properties of siRNAs (9,35,51–53). LNA has been shown to improve loading of the desired strand (51), increase nuclease resistance (51) and lower immune stimulation (53).

We tested a series of duplexes containing four LNA nucleotides evenly distributed across either the passenger or guide strand (Figure 6A). PR-9 LN and PR-9 NL both retained the ability to silence gene expression, albeit to a lesser extent than PR-9 (Figure 6B). Significant silencing activity was maintained regardless of whether the sense or antisense strand was modified.

The high binding affinity of LNA has been used to direct loading of the appropriate strand into AGO and bias strand usage (51). We tested a series of

Gene silencing with LNA in T47D cells

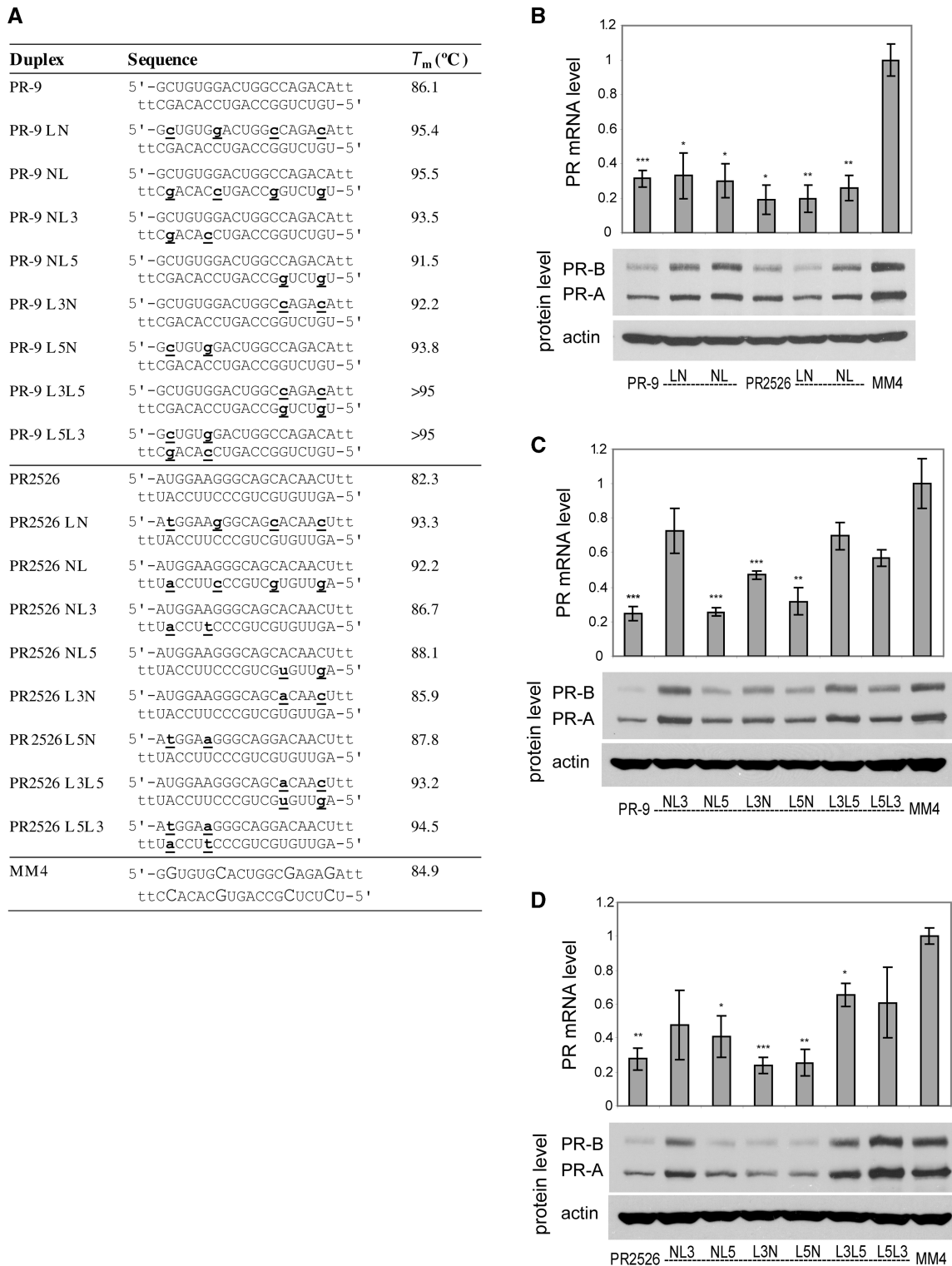


Figure 6. PR gene expression can be silenced by agRNAs containing LNA. (A) Duplex sequences: passenger strands are listed on top in all cases; for siRNA PR2526 this is the sense strand, but for agRNA PR-9 this is the antisense strand. LNA sequences contain 5-methylcytosine instead of cytosine. Modification codes: dna, RNA, lna. (B) PR-9 analogs and controls containing four evenly distributed LNA units. (C) PR-9 analogs containing LNA at strand ends. (D) PR2526 analogs containing LNA at strand ends. All data are normalized to mismatch control MM4. RNA levels are the average (\pm SD) from at least two independent transfections. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$. Protein levels were also confirmed by at least two independent transfections, from which a typical western blot is shown.

thermodynamically biased LNA analogs, to explore whether their activities would differ (Figure 6C). Strand L3 contains LNA at the second and sixth positions from its 3'-end, while strand L5 contains LNA at the second and sixth positions from its 5'-end. Modification of the 3'-end of the guide strand (PR-9 NL3), reduced activity of PR-9 (Figure 6C). All other RNA duplexes containing two LNA substitutions on one strand (PR-9 NL5, PR-9 L3N, PR-9 L5N) were active regardless of which strand was modified or the location of the modification. Some activity was lost when both strands were modified with LNA (PR-9 L3L5, PR-9 L5L3). Variants of PR2526 containing LNA bases yield results that were analogous to LNA-substituted variants of PR-9 (Figure 6B and D).

These results demonstrate that LNA modifications are compatible with gene silencing by agRNAs, but show no

evidence for biased strand loading in the context of the PR-9 or PR2526 sequences. The reduction in activity upon modification of the 3'-end of the guide strand ('NL3') is consistent with previous observations that LNA modification of the sixth nucleotide from the 3'-end of this strand leads to reduced potency (51). Results were similar for each pattern of modification regardless of whether agRNA PR-9 or siRNA PR2526 was the parent duplex, emphasizing that the response of agRNAs and siRNAs to chemical modification can be similar.

Effect of LNA on gene activation

We tested LNA-modified analogs of PR-11 for gene activation in MCF7 cells (Figure 7A). For activating duplex PR-11, we observed activation (2- to 3-fold versus 6-fold

Gene activation with LNA in MCF7 cells

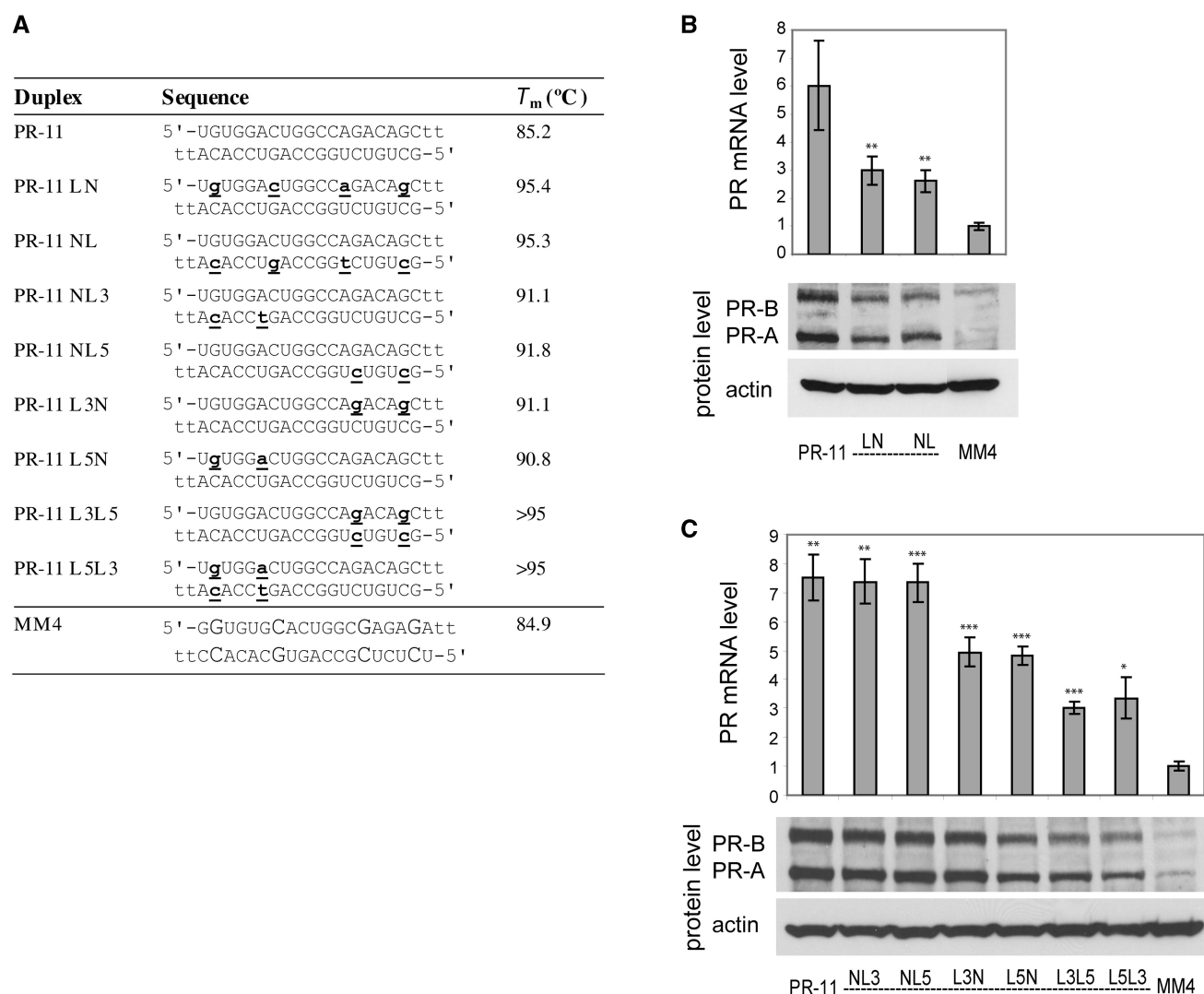


Figure 7. PR gene expression can be activated by agRNAs containing LNA. (A) Duplex sequences: passenger strands are listed on top in all cases; for agRNA PR-11 this is the antisense strand. LNA sequences contain 5-methylcytosine instead of cytosine. Modification codes: dna, RNA, **l**na. (B) PR-11 analogs containing four evenly distributed LNA units. (C) PR-11 analogs containing LNA at strand ends. All data are normalized to mismatch control MM4. RNA levels are the average (\pm SD) from at least two independent transfections. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$. Protein levels were also confirmed by at least two independent transfections, from which a typical western blot is shown.

for the unmodified duplex) after modification of either strand with four LNA nucleotides (PR-11 LN, PR-11 NL) (Figure 7B). The potency was reduced to the same extent whether the guide or passenger strand was modified. For activation by PR-11, all of the thermodynamically biased LNA analogs maintained significant activity (Figure 7C). Modification of either end of the guide strand (PR-11 NL3, PR-11 NL5) gave activity indistinguishable from that of the native PR-11. Passenger strand modification (PR-11 L3N, PR-11 L5N) gave a slight reduction in activation, and modification of both strands (PR-11 L3L5, PR-11 L5L3) led to a further reduction in activation. Nonetheless, even these duplexes modified in both strands gave >3-fold activation at the RNA level and clear activation at the protein level. Together with results of 2'F and 2'OMe substitution, these data suggest that agRNA-mediated gene activation is broadly tolerant of chemical modification.

Effect of combining modifications on gene silencing

Many examples exist of potent and stable siRNAs containing multiple types of chemical modifications (38,54–56). In some cases, combination of multiple modifications led to potency and stability unattainable by the individual modifications alone (54,56). We set out to explore whether combination agRNA duplexes (Figure 8A) would yield better gene inhibition or

activation in their respective cell lines. Even if existing potency is only maintained, the combination of different chemistries could afford better stability and other desirable drug properties.

Our criteria for choosing combinations was to select modified strands from PR-9 and PR-11 analogs showing the highest activity and recombine them in new ways. For PR-9 analogs, this led to the identification of several potent new duplexes (Figure 8B). Duplexes including PR-9 L5L5, PR-9 JL, PR-9 JZ and PR-9 LZ retained substantial activity. Duplexes PR-9 LL5 and PR-9 PpJ lost most activity. The effects appear complex and cannot simply be characterized by considering additive effects from the individual modifications. Nonetheless, it appears that terminal 2'F-RNA modification (Z) on the guide strand and LNA modification at the 5'-end of the passenger strand (L5) are generally well-tolerated, whereas 2'OMe modification of all pyrimidines (P) on the passenger strand tends to disrupt the activity.

It is noteworthy that two of the most active combination duplexes also had T_m values that were too high for us to measure (PR-9 L5L5 and PR-9 JL). It is sometimes assumed that high T_m values prevent the strand separation required for siRNA/agRNA activity, but our findings confirm that the relationship between T_m and activity is more complex. This result is consistent with our previous observation that the limited number of agRNA duplexes

Gene silencing with combination duplexes in T47D cells

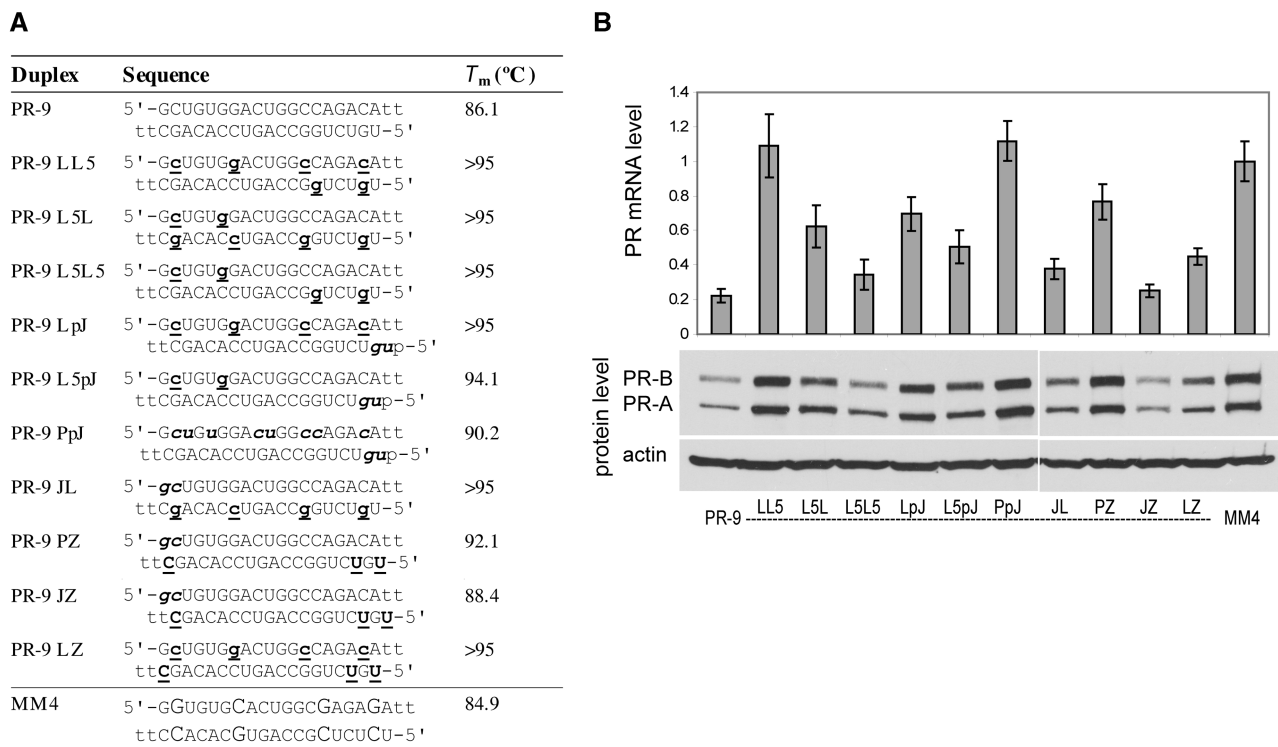


Figure 8. PR gene expression can be silenced by agRNAs containing combinations of modifications. (A) Duplex sequences; passenger strands are listed on top in all cases; for agRNA PR-9 this is the antisense strand. LNA sequences contain 5-methylcytosine instead of cytosine. Modification codes: dna, RNA, 2'F-RNA, 2'OMe-rna, lna; p = phosphate. (B) PR-9 analogs containing combinations of previously identified active strands. All data are normalized to mismatch control MM4. RNA levels are the average (\pm SD) from at least two independent transfections. Protein levels were also confirmed by at least two independent transfections, from which a typical western blot is shown.

tested to date do not rigidly adhere to published guidelines for predicting the activity of duplex RNAs (23).

We combined modified strands from active PR-11 analogs to give a new series of activating agRNAs (Figure 9A). All the combination duplexes based on PR-11 lost significant activity with respect to the unmodified control. Duplexes containing LNA in the passenger strand and 2'F-RNA at the termini of the guide strand (PR-11 LZ and PR-11 L5Z) were the best of this series, but only showed 2- to 3-fold activation at the RNA level (Figure 9B).

No evidence that single-strands mediate agRNA activity

There have been reports that single-stranded antisense RNA can lead to inhibition via the RNAi pathway (12,57,58). In addition, previous work from our lab (59–61) and others (62–65) has shown that single-stranded oligonucleotides including peptide nucleic acid (PNA) and LNA–DNA chimeras were able to bind chromosomal DNA and repress gene expression at the transcriptional level.

To explore whether the single-stranded RNAs contained within agRNAs can mediate such effects, we transfected T47D and MCF7 cells with various native and modified single strands from functional silencing or activating agRNA duplexes (Figure 10A). Transfections were carried out under the same conditions and at the same concentration (25 nM) as duplex RNAs. Results from both qPCR and western blots demonstrate that single-stranded agRNA, when transfected alone, had no

effect on silencing or activation of PR gene expression (Figure 10B and C). These data demonstrate that potent modulation of gene expression by anti-PR agRNAs requires the presence of intact duplex RNA regardless of chemical modification. Our data indicate that gene silencing or activation by the agRNA in our study is not due (i) to slight excess of one strand, (ii) to degradation of one strand (prior to incorporation into AGO), or (iii) to single-strand-mediated off-target effects. The requirement for RNA duplexes also provides further evidence that both native and chemically modified agRNAs make use of the RNAi machinery.

Concentration dependence of modified duplexes

To understand the effect of chemical modification on the potency of agRNAs we examined the dependence of gene silencing or activation on the concentration of selected RNAs. We observed that potencies of gene silencing by modified agRNAs PR-9 L5N and PR-9 NZ were similar to parent agRNA PR-9 (Figure 11A, see also Supplementary Figures S3 and S4). Likewise, potencies of gene activation by PR-11 pYN and PR-11 pFN were similar to gene activation by parent agRNA PR-11 (Figure 11B). Thus it is possible to achieve potent, dose-dependent gene modulation by both activating and inhibitory chemically modified agRNAs. For silencing agRNAs based on PR-9, we calculated IC₅₀ values for the native duplex and several modified analogs (Table 2) (Supplementary Figures S3 and S4). At both the protein and RNA levels, some of the modified analogs were of

Gene activation with combination duplexes in MCF7 cells

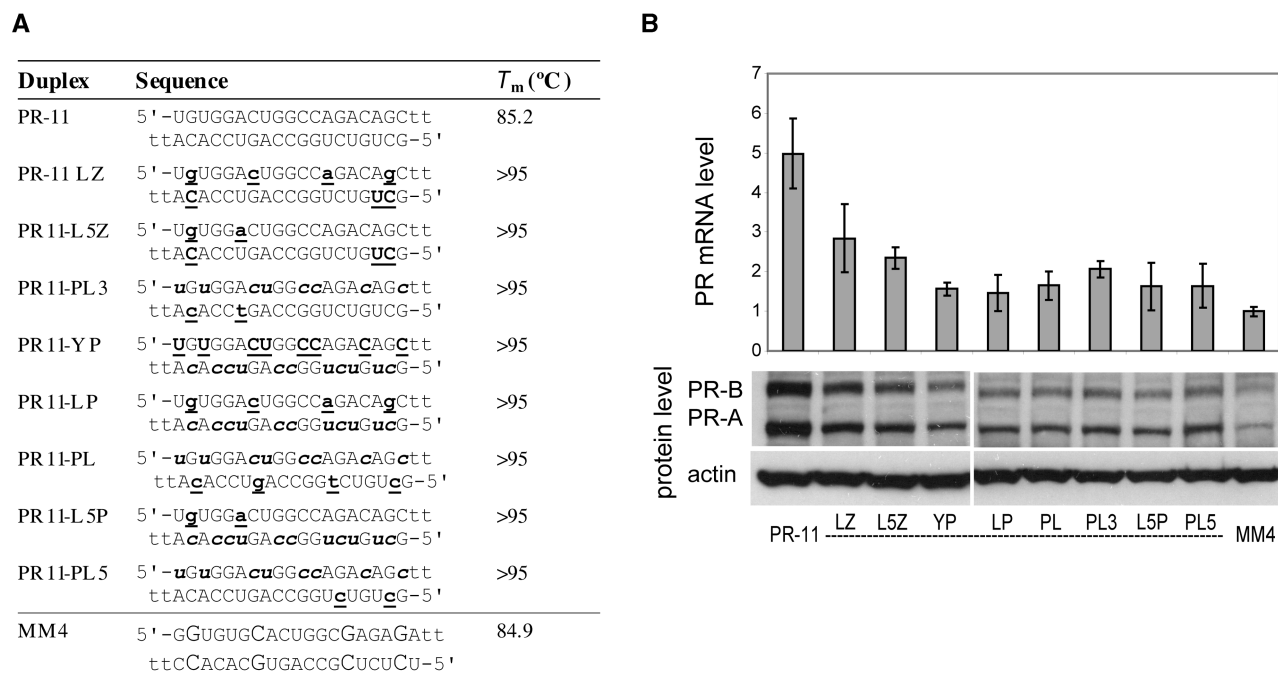


Figure 9. PR gene expression can be activated by agRNAs containing combinations of modifications. (A) Duplex sequences: passenger strands are listed on top in all cases; for agRNA PR-11 this is the antisense strand. LNA sequences contain 5-methylcytosine instead of cytosine. Modification codes: dna, RNA, 2'F-RNA, 2'F-RNA, 2'F-RNA, LNA. (B) PR-11 analogs containing combinations of previously identified active strands. All data are normalized to mismatch control MM4. RNA levels are the average (\pm SD) from at least two independent transfections. Protein levels were also confirmed by at least two independent transfections, from which a typical western blot is shown.

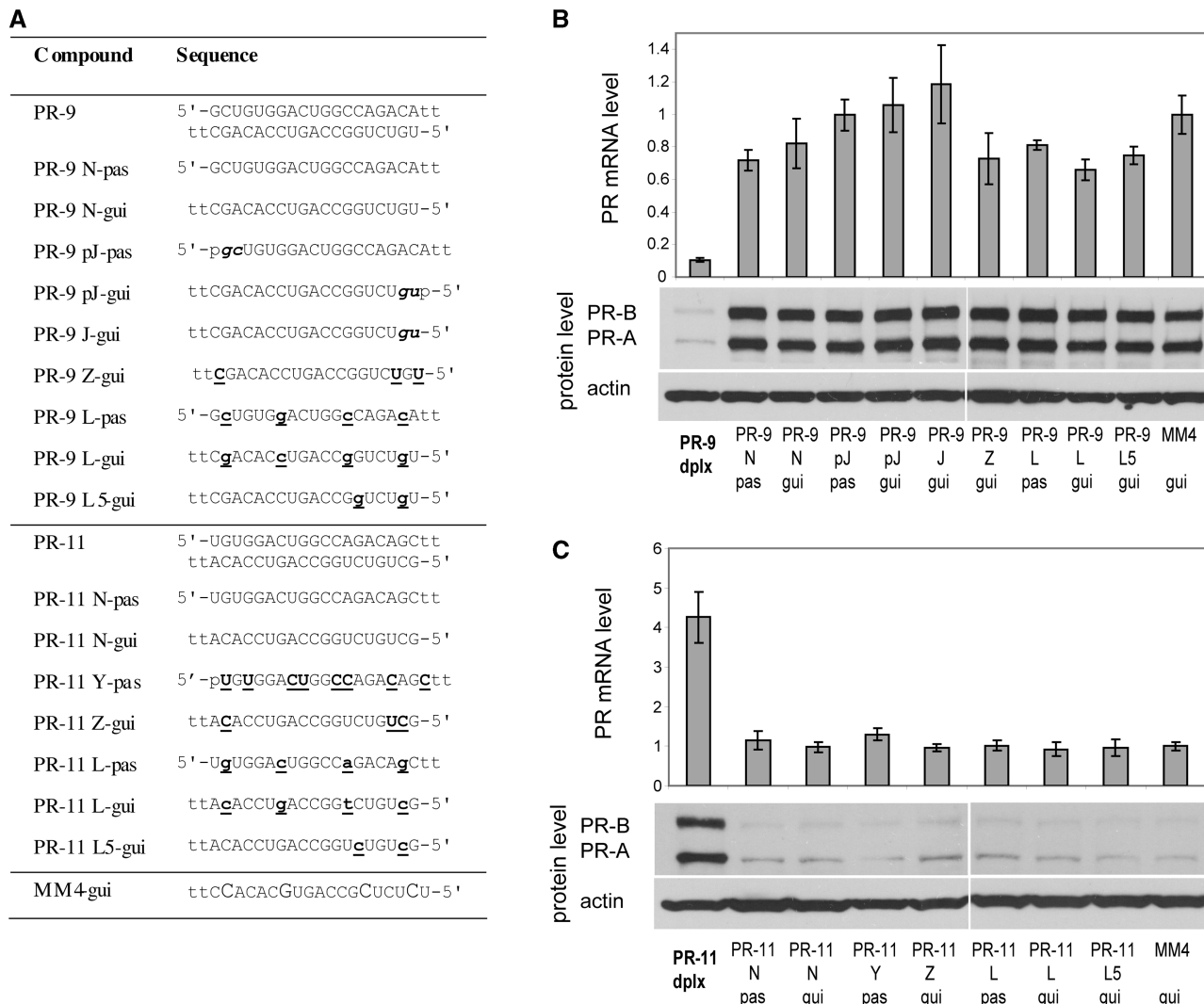


Figure 10. Single strands of native or modified agRNAs are inactive. (A) Sequences: passenger strands are given 5'-3', guide strands are given 3'-5' as shown. For these agRNAs the passenger strand is the antisense strand. Modification codes: gna, RNA, **2'F-RNA**, **2'Ome-rna**, **lna**; p = phosphate. (B) PR-9 duplex and native or modified single-strands. (C) PR-11 duplex and native or modified single-strands. All data are normalized to the guide strand of mismatch control MM4. RNA levels are the average (\pm SD) from at least two independent transfections. Protein levels were also confirmed by at least two independent transfections, from which a typical western blot is shown.

comparable potency to PR-9 itself (e.g. PR-9 NL). Others, while still active, showed reduced potency (e.g. PR-9 NZ).

Modified duplexes affect recruitment of RNA polymerase II

We have previously used ChIP of RNA polymerase II (RNAPII) [see the supporting information of ref. (28)] and nuclear run-on assays (25) to show that both silencing and activating agRNAs affect PR transcription. To investigate whether chemically modified agRNAs operate through the same mechanism as their native counterparts, we carried out RNAPII ChIP using two active modified duplexes (Figure 12). In T47D cells, treatment with either PR-9 or its modified analog PR-9 PN led to reduced occupancy of RNAPII at the PR promoter relative to cells treated with the luciferase control duplex siGL2 (Figure 12A). In MCF7 cells, treatment with either PR-11 or the modified analog PR-11 pYN increased

levels of RNAPII at the PR promoter (Figure 12B). The finding that native and modified duplexes behave similarly supports the hypothesis that they act through similar mechanisms.

Native and modified duplexes have the same effect on antisense transcript levels

We have previously observed that both activating and inhibitory agRNAs bind to an antisense transcript that overlaps the PR promoter (28). This transcript originates within PR mRNA and extends 70 000-bp upstream. It is spliced, and the spliced product contains target sequences for all the agRNAs used in this study. When biotinylated agRNAs are transfected into cells they bind the antisense transcript. RNA immunoprecipitation shows that both activating and silencing agRNAs recruit AGO to the antisense transcript. The antisense transcript, therefore,

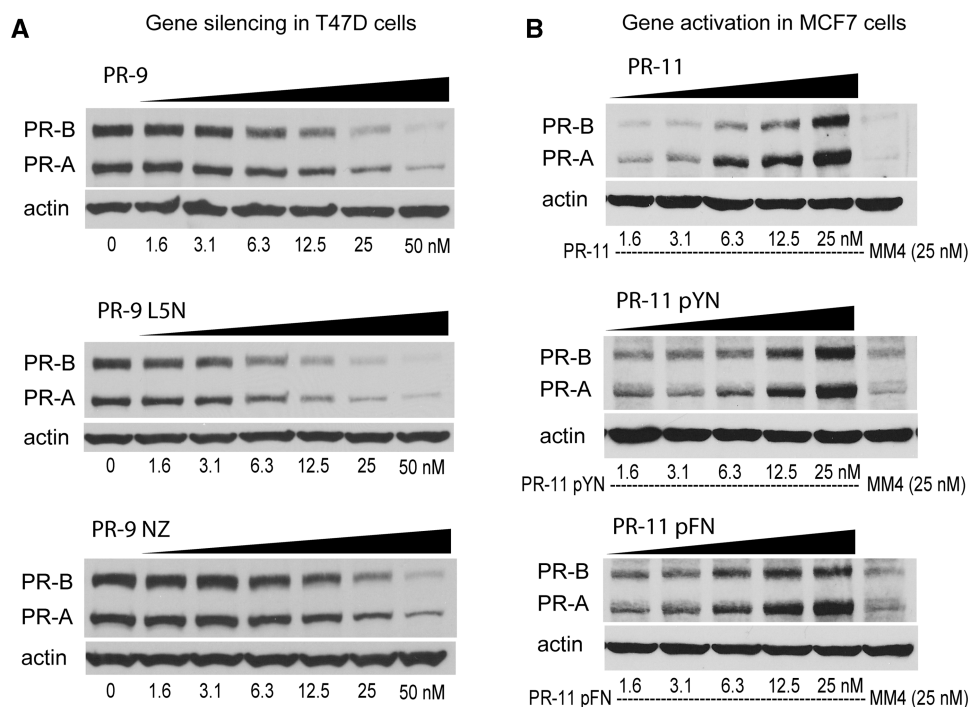


Figure 11. Sample dose response experiments for both (A) silencing and (B) activation. More dose response experiments for PR-9 analogs are shown in Supplementary Figures S3 and S4.

Table 2. IC₅₀ values for PR-9 and modified analogs in T47D cells^a

Silencing agRNA	Type of chemistry	IC ₅₀ (RNA) (nM)	IC ₅₀ (protein) (nM)
PR9	native	5.6	12.2
PR9 NZ	2'F	18.6	19.6
PR9 JpJ	2'OMe	9.4	10.2
PR9 PN	2'OMe	12.9	12.6
PR9 NL	LNA	6.8	13.2
PR9 L5N	LNA	13.3	7.4

^aPlots used to derive these data are shown in Supplementary Figures S3 and S4.

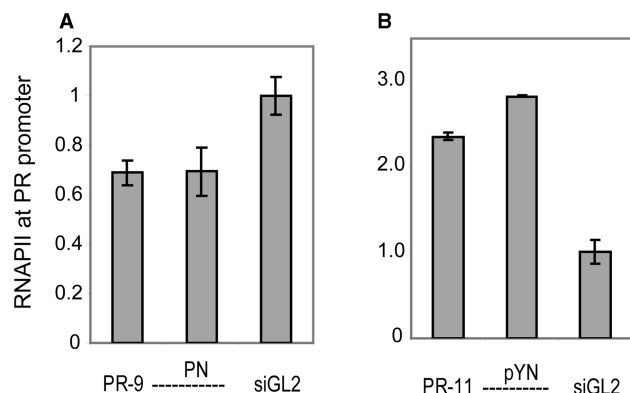


Figure 12. ChIP of RNA Polymerase II (RNAPII) shows that both native and modified agRNAs operate at the transcriptional level. (A) Treatment of T47D cells with PR-9 or PR-9 PN leads to reduced occupancy of RNAPII at the PR promoter relative to cells treated with control duplex siGL2. (B) Treatment of MCF7 cells with PR-11 or PR-11 pYN leads to increased occupancy of RNAPII at the PR promoter relative to cells treated with control duplex siGL2.

appears to play a central role in the mechanism of agRNAs.

We explored the effect of chemically modified agRNAs on levels of the promoter-associated antisense transcript. We previously observed that treatment of T47D cells with silencing agRNA PR-9 led to a reduction of antisense transcript levels, while treatment of MCF7 cells with activating agRNA PR-11 led to no significant change in antisense transcript levels (28). We repeated these experiments using both the native duplexes and several of the most active modified analogs. We monitored expression of PR mRNA and the antisense transcripts that span the agRNA target region using qPCR.

Native and modified duplexes gave comparable results. PR-9 and modified PR analogs reduced expression of both PR mRNA and the promoter-derived antisense transcript similarly (Figure 13A). Conversely, PR-11 and modified analogs increased expression of PR mRNA, but caused no significant change in antisense transcript levels (Figure 13B). The finding that native and modified duplexes have the same effect on levels of the antisense transcript supports the conclusion that they act through similar mechanisms.

Inactive modified duplexes do not compete for target sites

It is interesting to speculate as to why some patterns of modification cause reduced activity. Inactive agRNAs could be failing at any of a number of steps: (i) loading into the RNA-induced silencing complex (RISC), (ii) cleavage or dissociation of the passenger strand, (iii) import into the nucleus, (iv) binding of the RNA target, or (v) execution of the effect (activation or silencing). We previously observed that inactive native duplexes such as

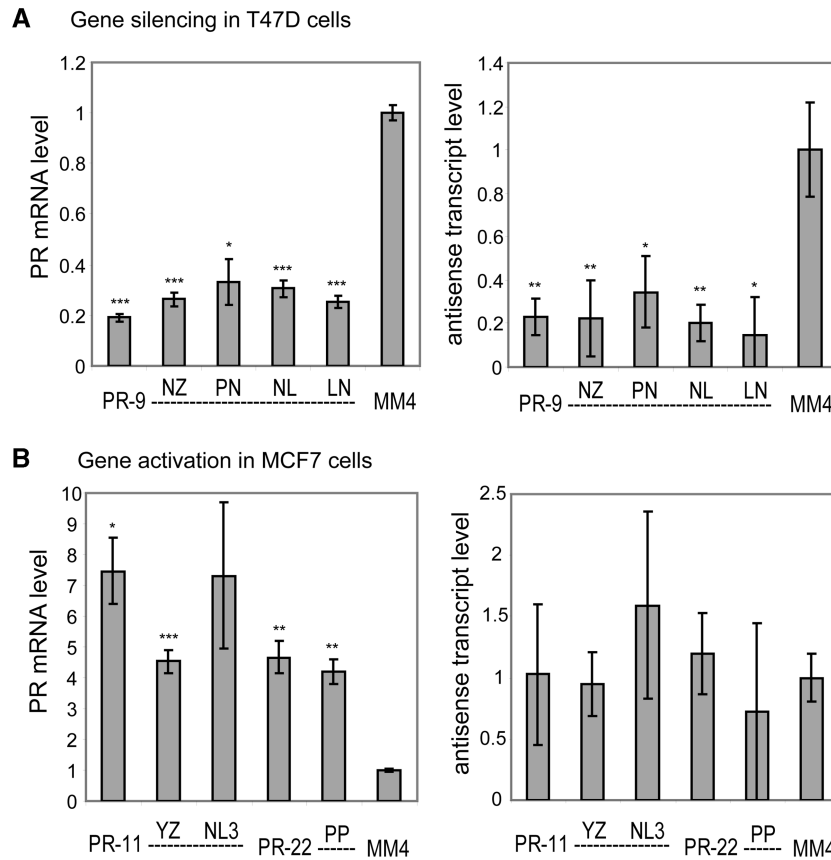


Figure 13. Native and modified duplexes have the same effect on antisense transcript levels. **(A)** Treatment of T47D cells with PR-9 and modified duplexes leads to a significant downregulation of both PR mRNA (left) and the promoter-associated antisense transcript (right). **(B)** Treatment of MCF7 cells with PR-11 and modified duplexes leads to a significant upregulation of PR mRNA (left) but no significant change in levels of the promoter-associated antisense transcript (right). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

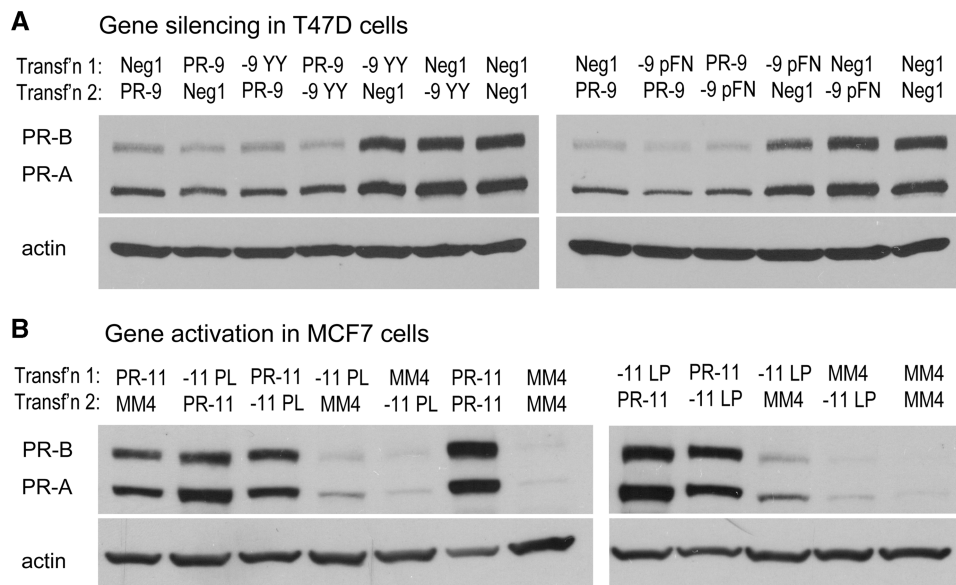


Figure 14. Inactive modified duplexes do not compete for target sites. Western analysis showing no competition between native agRNAs and inactive modified duplexes applied to cells in two transfections 4 days apart. **(A)** Native PR-9 silences gene expression equally effectively whether it is transfected before or after an inactive chemically modified duplex (or negative control). **(B)** Native PR-11 activates gene expression equally effectively whether it is transfected before or after an inactive chemically modified duplex.

PR-8 (targeting -8 to +11) and PR-12 (-12 to +7) could compete for target sites with the active duplex PR-11 (23). Thus, treating MCF7 cells with inactive duplex PR-12 prevented gene activation by PR-11 in a subsequent transfection several days later. On the other hand, initial treatment with PR-11 followed later by PR-12 gave activation comparable to PR-11 alone (23). This experiment showed that the order of addition is crucial and the duplexes compete for the same target sites. Duplexes PR-8 and PR-12 failed at step (v) above—they were loaded into RISC and even bound the correct RNA target, but failed to execute an agRNA effect. Therefore, since shifting the duplex–target interaction by 1–3 bp is sufficient to eliminate activity, the geometry of interaction at the promoter may be crucial for induction of gene activation.

To test where inactive chemically modified agRNAs might be deficient we carried out a similar experiment using two inactive modified duplexes each for silencing and activation. In T47D cells we used the inactive duplexes PR-9 YY and PR-9 pFN. In both cases, levels of PR expression were identical whether the inactive duplexes were transfected before or after the active duplex PR-9 (Figure 14A). Similarly, treatment of MCF7 cells with PR-11 gave gene activation regardless of whether the duplexes were transfected before or after inactive modified duplexes PR-11 PL and PR-11 LP (Figure 14B). These data suggest that the modified duplexes did not compete for target sites with native duplexes. Since the inactive modified duplexes are not binding the target site, they must be failing at one of the first four steps in the process: RISC loading, passenger strand removal, nuclear import or target binding. Furthermore, some of the inactive modified duplexes contain native guide strands, thus it seems clear that they are failing at one of the first two steps—RISC loading or passenger strand removal—since after this point they would be identical with native duplexes. Therefore in most cases, the challenges causing modified agRNAs to fail are the same as those faced by chemically modified siRNAs that are complementary to mRNA.

agRNAs and chemical modifications

Double stranded RNAs that are complementary to mRNA are currently being tested in clinical trials (1–4). Given the success of siRNAs in the laboratory and progress in the clinic, why would agRNAs enhance the potential for *in vivo* application of double-stranded RNA? One reason is that activating gene expression would open up a wider range of therapeutic targets and may provide new opportunities for development. For example, if more of a protein product is needed to treat a disease, upregulating expression using agRNAs might prove advantageous, especially if the protein's site of action is intracellular and cannot be accessed by systemically administered protein. Even in the case of gene silencing, where existing siRNA technology often gives superb results, it is possible that silencing at the transcriptional level may be advantageous for some targets. It is possible to envision that agRNAs may be more potent in

some cases, produce longer lasting effects, or produce different modulation of isoform expression. Finally, understanding the effect of modifications will also be important for future studies of agRNA function in cultured cells. Potential applications for promoter-targeted duplex RNAs have been summarized in a recent review (66).

Our data have several implications for the mechanism of chemically modified agRNAs: (i) As with any structure–function analysis, the most important finding is that some designs yield active compounds whereas others do not; (ii) Two strands are necessary for efficient action of agRNAs, we observe no silencing or activation by single strands at the highest concentrations used in this study; (iii) Activation and silencing show a regular, predictable dose dependence; (iv) As with siRNAs, optimal modifications need to be determined empirically for each sequence; (v) As with siRNAs, both guide and passenger strands can be modified, but modifications are generally better tolerated on the passenger strand; (vi) IC₅₀ values and maximal efficacies of modified and unmodified agRNAs are similar, consistent with the expectation that they operate by a common mechanism; (vii) Both native and modified duplexes affect recruitment of RNAPII, also consistent with a common mechanism; (viii) Addition of modified and unmodified agRNAs leads to almost identical changes in antisense transcript levels, again consistent with a shared mechanism; and (ix) Inactive duplexes do not compete with active duplexes, suggesting that the inactive duplexes fail at a step prior to association with the antisense transcript.

The finding that agRNAs containing 2'-F-RNA, 2'-OMe-RNA, or LNA retain the ability to silence or activate gene expression is significant because it demonstrates that agRNAs tolerate the introduction of modifications known to be powerful tools for improving *in vivo* properties. For duplex RNAs that are complementary to mRNA, it is commonly observed that different sequences tolerate different chemical modifications or different patterns of the same chemical modification. The interplay of chemical modifications with each particular sequence is the ultimate determinant of duplex potency and duplexes with optimal properties must be determined empirically. No universal rules of siRNA modification have been developed because our understanding of the nature of this interplay is not yet sufficient.

Our data on agRNAs suggest the same conclusion. We have tested two different silencing agRNAs, PR-9 and PR-26, and two different activating agRNAs, PR-11 and PR-22. While both tolerate a wide range of modifications, the relative potencies of analogous modified duplexes differ. Like siRNAs, we conclude that empirical testing will be needed to identify agRNAs with the best balance of properties. While empirical testing may be necessary, we found that a wide range of modifications were compatible with gene silencing or activation by an agRNA-mediated mechanism and we predict that it will not be difficult to develop active chemically modified agRNAs for genes of interest where a parent unmodified agRNA has already been identified.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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