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Inhibiting gene expression at transcription start sites in chromosomal DNA with antigene RNAs

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Transcription start sites are critical switches for converting recognition of chromosomal DNA into active synthesis of RNA. Their functional importance suggests that they may be ideal targets for regulating gene expression. Here, we report potent inhibition of gene expression by antigene RNAs (agRNAs) complementary to transcription start sites within human chromosomal DNA. Silencing does not require methylation of DNA and differs from all known mechanisms for inhibiting transcription. agRNAs overlap DNA sequences within the open complex formed by RNA polymerase, and silencing is acutely sensitive to single base shifts. agRNAs effectively silence both TATA-less and TATA-box-containing promoters. Transcription start sites occur within every gene, providing predictable targets for agRNAs. Potent inhibition of multiple genes suggests that agRNAs may represent a natural mechanism for controlling transcription, may complement siRNAs and miRNAs that target mRNA, and will be valuable agents for silencing gene expression.

Duplex RNAs complementary to mRNA inhibit translation in mammalian cells[1]. Duplex RNAs can also silence transcription of chromosomal DNA in plants[2,3] and yeast[4–6]. Two recent reports describe the use of duplex RNAs to induce DNA methylation and silence gene expression in mammalian cells[7,8], although a third report does not detect methylation[9] and a fourth study finds only low levels of methylation[10]. The methylation-induced silencing observed in mammalian cells was restricted to sequences within CpG islands (regions of DNA with a high proportion of the dinucleotide CpG), up to ten different synthetic RNAs were needed, and the potency of inhibition was less than that typically observed with standard mRNA-directed RNAs[7,8].

We hypothesized that DNA sequences essential for gene expression would be good targets for RNA-mediated recognition regardless of whether they were within CpG islands. When mammalian RNA polymerase binds to DNA at transcriptional start sites, it forms an open complex in which bases −9 to +2 are accessible to chemical agents that modify single-stranded DNA[11]. The partial single-stranded character of the open complex suggested that transcription start sites might be accessible to hybridization. The functional importance of start sites suggested that recognition by agRNAs would block gene transcription.

Here, we show that agRNAs targeting transcription start sites are potent inhibitors of gene expression. We did not detect methylation of DNA, and both TATA-less and TATA-box genes could be silenced. These results suggest a powerful ability for RNA to mediate recognition of chromosomal DNA in mammalian cells. The potency and generality of silencing are consistent with the suggestion that RNA-mediated recognition of chromosomal DNA may be a natural mechanism for regulating gene expression in human cells.

RESULTS

agRNAs inhibit gene expression

We designed agRNAs to be complementary to the upstream transcription start site for the human progesterone receptor (hPR) in T47D breast cancer cells[12–14]. hPR mediates the function of the hormone progesterone and is primarily expressed as two isoforms, B (hPR-B) and A (hPR-A). The promoter for hPR-B is upstream of the promoter for hPR-A (**Fig. 1**).

We designed agRNAs PR2, PR9 and PR24 to overlap different portions of the −9 to +2 region predicted to form the open complex within the promoter for hPR-B (**Table 1**). When added individually, these agRNAs reduced levels of hPR protein (**Fig. 1**) and RNA equivalently (**Fig. 2**), consistent with the hypothesis that mechanisms exist to allow RNA to mediate recognition of transcription start sites within chromosomal DNA and block gene expression. Out of 19 bases, PR9 has 10 bases complementary to hPR mRNA and PR24 has no complementarity to hPR mRNA[12], supporting the conclusion that the inhibition of gene expression we observed is mRNA-independent.

To determine the impact of target sequence on inhibition, we designed agRNAs complementary to sequences throughout the −49 to +17 region of the hPR-B promoter (**Table 1**). We observed a well-defined fingerprint of activity, with agRNAs PR7–PR10, PR13, PR24 and PR26 being the most potent inhibitors of hPR expression when assayed at 25 nM (**Table 1**, **Fig. 1c–e**). Each of these agRNAs partially overlaps the −9 to +2 open complex (**Fig. 1f**), supporting the

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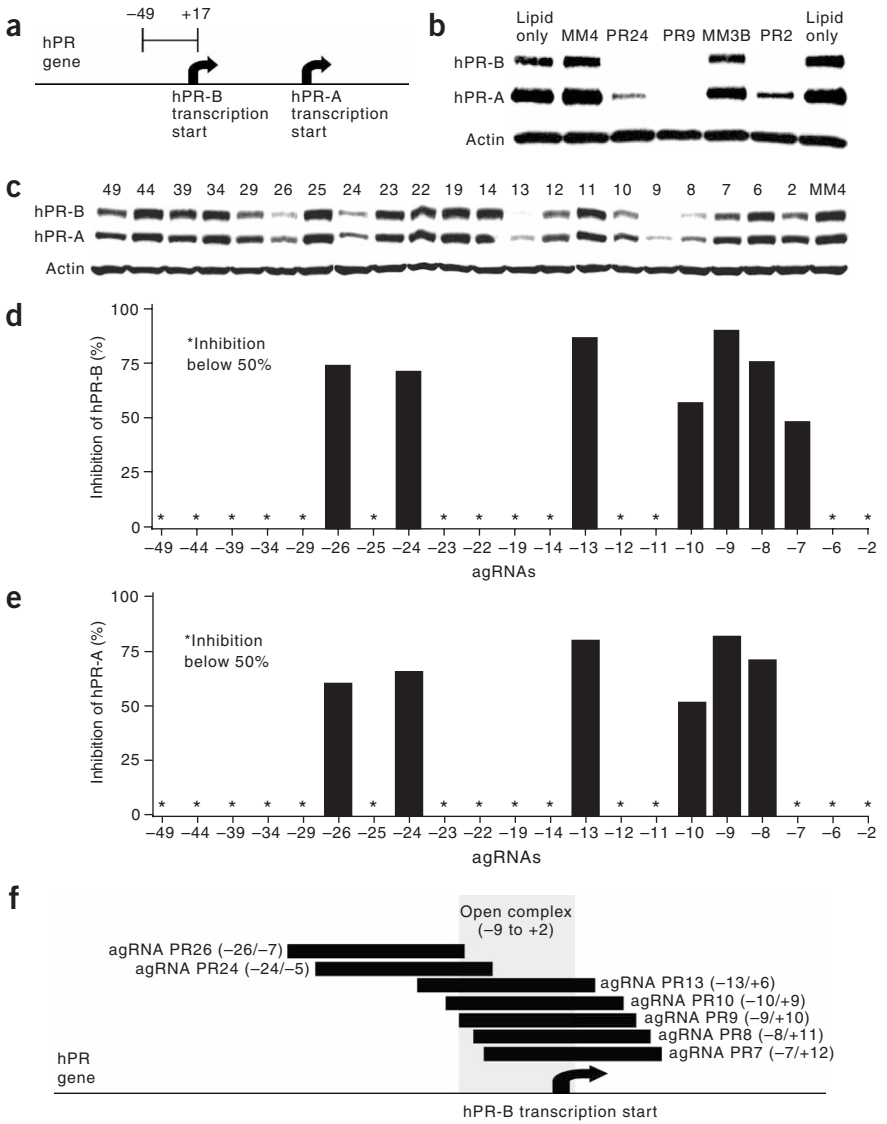

Figure 1 Inhibition of gene expression by agRNAs complementary to the transcription start site of hPR-B. (**a**) −49 to +17 target region for agRNAs. (**b**) Western analysis: inhibition of hPR expression by PR24, PR9 and PR2. All agRNAs were present at 25 nM. Active agRNAs are defined as any agRNA that yielded ≥50% inhibition of either hPR-B or hPR-A. (**c**) Western analysis showing inhibition of hPR-B and hPR-A by active agRNAs. (**d**,**e**) Quantification of inhibition for hPR-B (**d**) and hPR-A (**e**) expression by active agRNAs (duplexes are described in **Table 1**). (**f**) Location of active agRNAs relative to the proposed location of the open complex.

hypothesis that the open complex or transcription start site creates a susceptible chromosomal target for RNA-mediated recognition.

Potent inhibition by clusters of active agRNAs (PR7–PR10, PR12, PR13, PR24 and PR26) is especially striking, because these clusters bracket inactive RNAs PR11 and PR25 (**Table 1**, **Fig. 1c–e**). PR10–PR12 and PR24–PR26 have similar melting temperature values, suggesting that thermal stability (**Table 1**) is not the cause of the dramatic variation in efficacy. agRNAs PR11 and PR25 were re-synthesized and re-tested with the same negative outcome, suggesting that their inactivity is not caused by trivial errors during synthesis.

This sharp fingerprint for activity indicates that the mechanism for recognition of DNA by agRNAs is sensitive to small differences in sequence and structure at target sites. Discrimination between potent and nonpotent agRNAs might be at the level of protein recognition and processing or might occur during initiation of hybridization to the nucleic acid target. Cotransfection of less active agRNAs in combination (PR2, PR6 and PR7 or PR29, PR39 and PR49) modestly increased levels of inhibition over those achieved by the individual agRNAs (**Supplementary Fig. 1** online).

Inhibition by agRNAs was potent. A dose-response profile for inhibition of hPR expression by agRNA PR9 indicated a half-maximal inhibitory concentration (IC_{50}) value of ~2.5 nM (**Fig. 2a**). This IC_{50} value was better than those obtained with an anti-hPR-B duplex RNA that targets mRNA (12 nM), an antisense peptide nucleic acid (PNA) that targets mRNA (12 nM), or antigene PNAs (25 nM) (**Supplementary Fig. 2** online). Inhibition in the low nanomolar range indicated that agRNAs are an efficient strategy for blocking gene transcription. We also observed efficient inhibition of hPR expression in T47D cells treated with estrogen (**Supplementary Fig. 3** online), conditions known to lead to upregulation of hPR levels[15,16].

Inhibition by agRNAs was sequence-specific. Mismatch-containing agRNAs MM2, MM3A, MM4 (mismatches spaced throughout) and MM3B (three mismatches grouped within the portion of the RNA that is not complementary to mRNA) did not inhibit hPR expression (**Table 1**, **Fig. 1b,c**, **Fig. 2b,c**). The inability of MM3B to inhibit transcription suggests that partial complementarity to the 5′-terminus of mRNA is not sufficient for silencing. DNA duplexes analogous to agRNA PR9 did not inhibit expression (**Table 1**, **Fig. 2b**), indicating that recognition must be mediated by RNA. The failure of DNA duplexes to inhibit gene expression also offered strong evidence that the mechanism of inhibition does not involve binding to transcription factors.

agRNAs inhibited expression of both hPR-B and hPR-A even though the transcription start of hPR-A is approximately 760 bases downstream of the target site (**Fig. 1a**). We have also observed this result using three other knockdown strategies (antisense PNAs, antigene PNAs (agPNAs) and anti-mRNA duplex RNAs) that target hPR-B, confirming that inhibition of hPR-A expression is a natural consequence of efficient sequence-specific inhibition of hPR-B (**Supplementary Fig. 2**, ref. 17). agRNAs and agPNAs[17] yielded a similar, nearly linear linkage between expression of hPR-B and hPR-A. By contrast, standard siRNA duplexes that target mRNA produced a different, almost exponential linkage profile[17] (**Fig. 3**). The similar inhibition of hPR-B and hPR-A by agRNAs and agPNAs is evidence that both approaches involve recognition of chromosomal DNA rather than RNA and that the mechanism of inhibition differs from that used by standard siRNAs that target mRNA.

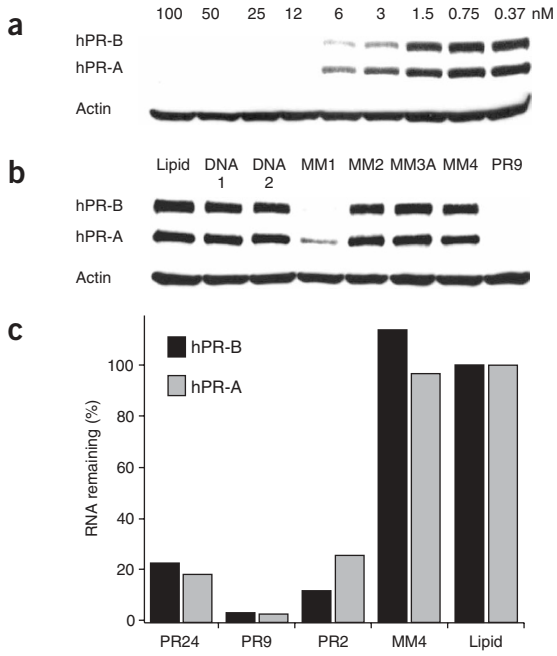

Figure 2 Inhibition of gene expression by agRNAs is potent and selective and is observed at the level of RNA. (**a**) Dose-dependent inhibition of hPR expression by PR9. (**b**) Effect of introducing mismatches or DNA bases on inhibition of hPR by analogs of PR9. (**c**) Quantitative PCR measurement of hPR mRNA in cells treated with PR24, PR9 and PR2. MM4, duplex RNA containing four mismatches spaced throughout its sequence. MM3B, duplex RNA containing three mismatches clustered within the bases targeted upstream of the hPR-B start site. Lipid only, Oligofectamine added alone, no RNA added. All duplex RNAs or DNAs were present at 100 nM unless otherwise noted.

To determine the generality of silencing by agRNAs, we designed agRNAs complementary to transcription start sites for major vault protein (MVP)[18], androgen receptor (AR)[19] and cyclooxygenase-2 (COX-2, ref. 20). Like hPR, MVP and AR have TATA-less promoters. The promoter for COX-2 contains a TATA box. We designed agRNAs (**Table 1**) complementary to the –14 to +13 regions of these three genes and observed inhibition of expression of MVP, AR and COX-2 (**Fig. 4**). Seven agRNAs were targeted to AR, and these produced a sharply defined activity fingerprint. Duplex RNAs that lacked complementarity to MVP, AR or COX-2 were not inhibitors (**Fig. 4a–c**). agRNA PR9 did not inhibit expression of MVP, AR or COX-2 (data not shown). These results indicate that the mechanism of agRNA silencing is general for both TATA-less and TATA-box promoters and is likely inclusive of most genes.

Inhibition of expression does not involve methylation of DNA

As noted earlier, three previous studies had suggested that short RNAs could induce methylation of chromosomal DNA[7,8,10]. We used multiple methods to examine whether methylation might be associated with the silencing that we observed. We treated cells with agRNAs and used sodium bisulphite sequencing to examine the regions surrounding the AR and hPR-B transcription start sites. Between 5 and 15 cloned PCR products were sequenced for each sample of sodium bisulfite–treated DNA. No methylation was observed by sequencing (**Supplementary Figs. 4** and **5** online) or by methylation-specific PCR (**Fig. 5a,b**). Silencing DNA methyltransferase-1 (DNMT1), an enzyme responsible for methylating DNA, had no effect on inhibition of hPR expression by PR9 (**Fig. 5c**). Addition of 5-aza-2′-deoxycytidine (5-aza-dC), an inhibitor of DNA methylation, to cells also had no effect on inhibition of hPR by agRNAs PR9, PR24 and PR49 (**Fig. 5d**).

Methylation would be predicted to yield prolonged silencing of expression. We observed, however, that inhibition of AR expression by agRNA AR9 peaked at five days after transfection and had fully recovered by day 10 (**Fig. 4d**). This transient silencing provides additional support for the conclusion that the mechanism of gene inhibition by agRNAs is independent of methylation and relies on reversible RNA-mediated recognition of target DNA sequences.

According to published standards for identification, there are no CpG islands within 1 kB of the transcription start site of MVP. hPR has a CpG island immediately downstream of its transcription start site[21], and AR[22] and COX-2 (ref. 23) have CpG islands that include their transcription start sites. Thus, agRNAs yield silencing, but not methylation, in four genes with varying potentials for promoter methylation.

DISCUSSION

The ability of RNA to mediate methylation-independent recognition of chromosomal DNA has not been reported previously in any organism. The simplest explanation for our results is that proteins can promote the recognition of DNA sequences without also causing methylation or requiring the presence of a CpG dinucleotide or a CpG island.

When agRNAs mediate recognition at transcription start sites, these critical switch regions are blocked and RNA synthesis cannot occur. This blockade occurs regardless of whether the promoter contains a TATA box. Although we have focused on transcription start sites, it is possible that many other important sites within chromosomal DNA may also be susceptible to RNA-mediated recognition. A steric

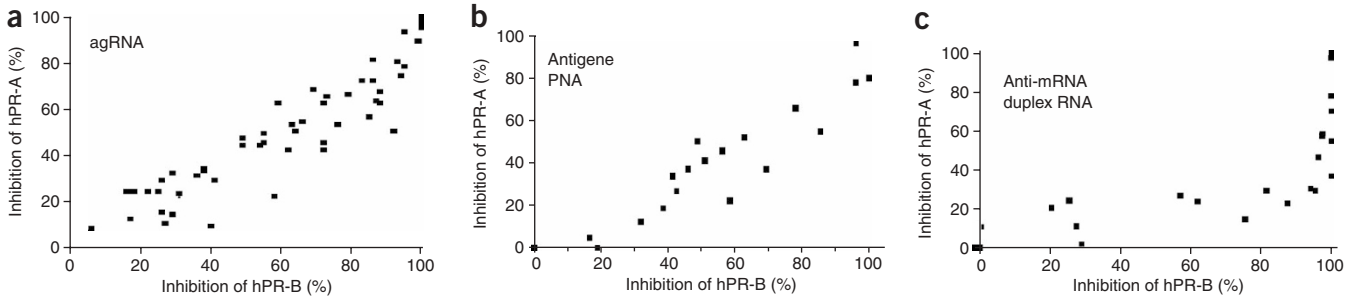

Figure 3 Linkage profiles between expression of hPR-B and hPR-A upon treatment with (**a**) agRNAs that target the –49 to +17 region of the hPR-B promoter, (**b**) agPNAs targeting the hPR-B start site[17] and (**c**) duplex RNAs that target hPR-B mRNA[17].

Table 1 Duplex RNAs and DNAs, T_m and inhibition of gene expression

agRNA	Target	Strand sequence	T_m	Inhibition of hPR-B/A (%) 100 nM	25 nM
Mismatch-containing duplex RNAs based on agRNA PR9					
MM1		UGUCUGGGCAGUCCACAGCTT	76	91/67	75/53
MM2		UGUCUCGCCAGUCGACAGCTT	71	ni/ni	–
MM3A		UCUCUGGGCAGUCCAGAGCTT	75	ni/ni	–
MM3B		UCUCUCGCGAGUCCACAGCTT	75	ni/ni	–
MM4		UCUCUCGCCAGUGCACACCTT	71	ni/ni	–
agRNAs that are fully complementary to hPR					
PR2	−2/+17	CCAGUCCACAGCUGUCACUTT	77	86/56	38/27
PR6	−6/+13	CUGGCCAGUCCACAGCUGUTT	84	71/26	ni/ni
PR7	−7/+12	UCUGGCCAGUCCACAGCUGTT	73	100/97	50/38
PR8	−8/+11	GUCUGGCCAGUCCACAGCUTT	75	88/67	73/67
PR9	−9/+10	UGUCUGGCCAGUCCACAGCTT	83	100/95	88/79
PR10	−10/+9	CUGUCUGGCCAGUCCACAGTT	73	100/99	55/50
PR11[a]	−11/+8	GCUGUCUGGCCAGUCCACATT	75	28/ni	ni/ni
PR12	−12/+7	AGCUGUCUGGCCAGUCCACTT	76	87/63	40/34
PR13	−13/+6	AAGCUGUCUGGCCAGUCCATT	73	100/100	80/72
PR14	−14/+5	AAAGCUGUCUGGCCAGUCCTT	81	ni/ni	ni/ni
PR19	−19/−1	GUUAGAAAGCUGUCUGGCCTT	75	ni/ni	ni/ni
PR22	−22/−3	GUUGUUAGAAAGCUGUCUGTT	62	ni/ni	ni/ni
PR23	−23/−4	CGUUGUUAGAAAGCUGUCUTT	62	26/29	ni/ni
PR24	−24/−5	GCGUUGUUAGAAAGCUGUCTT	70	96/88	68/62
PR25[a]	−25/−6	GGCGUUGUUAGAAAGCUGUTT	57	ni/ni	ni/ni
PR26	−26/−7	AGGCGUUGUUAGAAAGCUGTT	64	99/80	73/57
PR29	−29/−10	AGGAGGCGUUGUUAGAAAGTT	70	68/52	34/ni
PR34	−34/−15	AGAGGAGGAGGCGUUGUUATT	71	ni/ni	ni/ni
PR39	−39/−20	UCCCUAGAGGAGGAGGCGUTT	76	48/41	ni/ni
PR44	−44/−25	GGGCCUCCCUAGAGGAGGATT	78	ni/ni	ni/ni
PR49	−49/−30	GGGCGGGGCCUCCCUAGAGTT	84	52/45	36/ni
DNA duplexes that are fully complementary to hPR					
DNA1	−9/+10	TGTCTGGCCAGTCCACAGCTT	72	ni/ni	–
DNA2	−9/+10	TGTCTGGCCAGTCCACAGC	70	ni/ni	–
Duplex RNAs and agRNAs that target MVP					
MVP6	−6/+13	AGGCAGGGUGAGAGUUCCCTT	71	81	
MVP9	−9/+10	GGGUGAGAGUUCCCCAUCUTT	75	91	
MVP14	−14/+5	AGGCAGGGUGAGAGUUCCCTT	78	ni	
MVPRNA		UAGGAGUCACCAUGGCAACTT	70	99	
MVRSCR		GUUGCCAUGUCCUAGUGACTT	68	ni	
agRNAs that are fully complementary to AR					
AR8A	−8/+11	CACCUCCCAGCGCCCCCUCTT	89	100	
AR9A	−9/+10	CCACCUCCCAGCGCCCCCUTT	86	82	
AR10A	−10/+9	UCCACCUCCCAGCGCCCCCTT	89	34	
AR11A	−11/+8	GGGGCGCUGGGAGGUGGAGTT	87	39	
AR12A	−12/+7	UCUCCACCUCCCAGCGCCCTT	87	ni	
AR13A	−13/+6	CUCUCCACCUCCCAGCGCCTT	85	100	
AR14A	−14/+5	GCUCUCCACCUCCCAGCGCTT	86	92	
agRNA that is fully complementary to COX-2					
COX9	−9/+10	UUAGCGACCAAUUGUCAUATT	64	81	
COX13	−13/+6	UCGGUUAGCGACCAAUUGUTT	66	71	

Sequences are listed 5′ to 3′ and correspond to GenBank sequences. T_m values are averages of four determinations. Activity values are normalized to a mismatch-containing duplex RNA and an actin-loading control. Mismatched bases in MM1–MM4 are underlined. All experiments have been repeated multiple times and values are averages. ni, no significant inhibition (measured values of inhibition are <20%).
[a]PR11 and PR25 were re-synthesized with similar results.

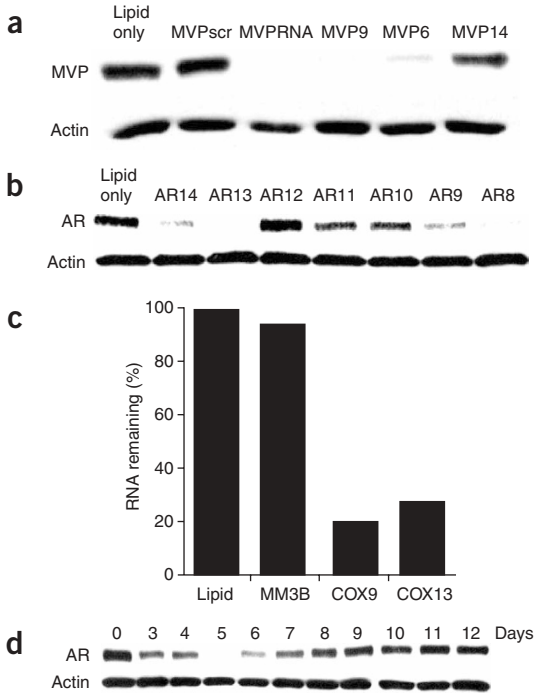

Figure 4 Inhibition of MVP, AR and COX-2 expression. (**a**) Inhibition of MVP expression by agRNAs MVP9, MVP6 and MVP14. MVPscr, a scrambled-sequence RNA duplex. MVPRNA, RNA duplex that targets MVP mRNA. (**b**) Inhibition of AR expression by agRNAs AR8–AR14. (**c**) Inhibition of COX-2 expression by agRNAs COX9 and COX13 analyzed by quantitative PCR. COX-2 expression in T47D cells was induced by addition of 10 ng ml^{-1} interleukin 1β to media lacking FBS. (**d**) Inhibition of AR expression from 0–12 days by AR9. The day 0 point is from cells treated with lipid only, no RNA. agRNAs targeted against MVP and AR were used at 100 nM. agRNAs targeted to COX-2 were used at 25 nM.

blockade mechanism through RNA-mediated recognition of DNA is consistent with our observation that inhibition is potent and transient and does not require methylation. The susceptibility of transcription start sites to a blockade mechanism is also suggested by the ability of antigene PNAs (agPNAs) to block expression[17].

For inhibition of transcription by agPNAs, it is difficult to envision a mechanism of action that does not involve direct hybridization of the PNA with chromosomal DNA. Direct binding of RNA to DNA may also be responsible for the inhibition of transcription by agRNAs that we observed, but it is only one of at least two possible mechanisms.

An alternative mechanism for our results is that proteins mediate binding of agRNAs to undetected transcripts initiated upstream of the major transcription start sites. This RNA-RNA-protein complex then interacts with promoter DNA, forming a blockade that prevents transcription from the major start sites.

This RNA-RNA-protein-DNA bridging mechanism has been proposed for RNA-mediated silencing in both *Schizosaccharomyces pombe*[24] and human cells[25]. The strength of this potential mechanism is that it does not require protein machinery capable of pairing duplex RNA with DNA—the RNA-induced silencing complex (RISC) that is known to promote RNA-RNA interactions would suffice. Resolving whether a DNA-RNA intermediate is formed and identifying the proteins involved in RNA-mediated recognition will be major goals for future research.

Off-target effects (that is, artifactual phenotypic effects caused by interactions unrelated to binding of the intended nucleic acid target) are an important concern for any strategy for silencing gene expression. To date, we have not observed any off-target effects upon introduction of agRNAs into cells. For example, regardless of whether we inhibit expression of hPR with antisense PNAs[17], antigene PNAs[17], agRNA (**Supplementary Fig. 6** online) or standard mRNA-directed siRNAs[17], we observe the same effects on cell morphology, cell growth and expression of downstream genes (morphological changes are described in detail in the accompanying article describing agPNAs[17]). agRNAs are not toxic to dividing cells, indicating that the blockade they induce is readily relieved during replication. We expect that off-target effects can be induced by agRNAs, as they can be for every other silencing strategy. It is likely that these effects can be minimized by chemical modifications that further enhance agRNA potency, and improving agRNAs will be an important object for future research.

Methylation-independent silencing by RNA provides a new mechanism for transcriptional silencing in human cells. MicroRNAs (miRNAs) are short, expressed RNA hairpins that have been shown to control gene expression by binding to mRNA[26]. Many miRNAs show strong evolutionary conservation, but have no known mRNA targets. These orphan miRNAs may use methylation-independent

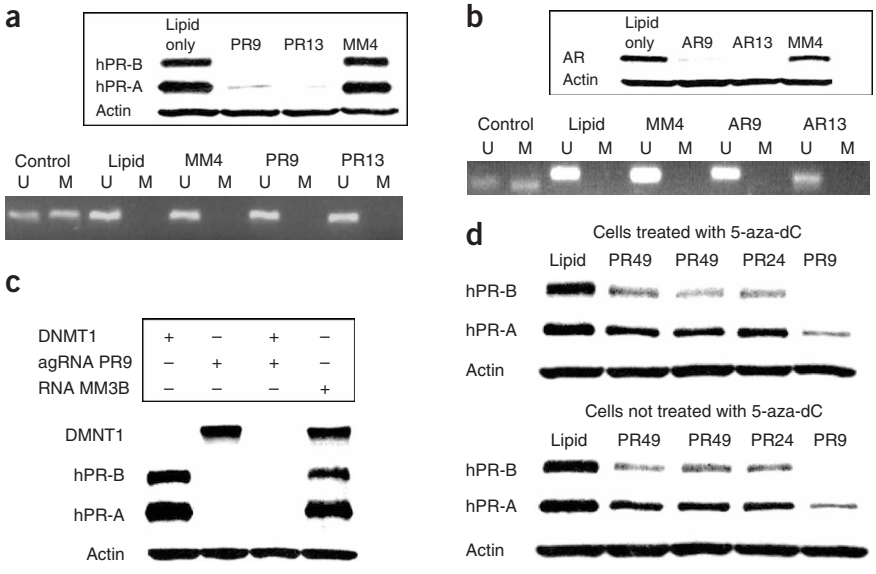

Figure 5 Gene silencing by agRNAs is independent of methylation. (**a**) Methylation-specific PCR (MSP) for hPR. (**b**) MSP for AR. U, unmethylated. M, methylated. Western analysis shows hPR levels of cells used for MSP. (**c**) Western analysis showing effect of inhibiting DNMT1 expression on agRNA PR9 activity. An RNA targeting DNMT1 mRNA or mismatch-containing agRNA MM3B were present at 100 nM. agRNA PR9 was present at 25 nM. (**d**) Effect of 5-aza-dC treatment (4 μM) on inhibition of hPR by agRNAs PR49, PR24 and PR9. agRNAs were used at 25 nM. Fresh 5-aza-dC was replenished at each medium change.

silencing to target chromosomal DNA. This targeting would offer a new layer of regulation for transcription that could exploit the generality of Watson-Crick base pairing. The existence of natural biological roles for agRNAs in mammalian cells remains to be tested, but if such roles were identified, their existence would have important implications for virology, cancer, development and evolution.

It is possible to envision many uses for agRNAs. Because an open complex is formed during the transcription of every gene, it should be straightforward to design agRNAs to any gene that has a characterized transcription start site. Because agRNAs recognize transcription start sites in the nucleus, they will be valuable agents for investigating transcription in living cells. Some genes are resistant to inhibition by standard mRNA-directed duplex RNAs. In these cases, more efficient silencing would be achieved by agRNAs. For genes with complex or uncharacterized splicing patterns, it may be difficult to use mRNA-directed siRNAs to successfully target every splice variant. agRNAs would abolish expression of each isoform equally. Finally, efficient silencing at transcription start sites suggests that agRNAs are a promising platform for therapeutic development.

METHODS

Preparation of siRNAs. 21-nucleotide RNAs or DNAs were synthesized at the Center for Biomedical Inventions at the University of Texas Southwestern Medical Center. Oligonucleotides contained two 2′-deoxythymidines on the 3′ end and were deprotected and desalted. Sequences are listed in **Table 1** and identified relative to the transcription start site as described for PR, AR and MVP. Melting temperature (T_m) values were determined with perfectly complementary RNA or DNA duplexes in 0.1 M NaH_2PO_4 buffer. T_m was measured as the inflection point for the curve showing the rise in optical density at 260 nm (OD_{260}) versus rise in temperature. Measurements were repeated at least four times and the results were averaged. Ranges in T_m for the same sample never varied more than ± 2 °C.

Cell culture. For experiments with hPR and AR, T47D cells (America Type Cell Culture Collection, ATCC) were maintained in RPMI-1640 medium (ATCC) supplemented with 10% fetal bovine serum (FBS), 0.5% nonessential amino acids (NEAA), 0.4 units (μ) bovine insulin, 100 μ ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin (P/S). For experiments with MVP, SW1573/2R120 cells (non-small-cell lung carcinoma from George Scheffer from the Free University Hospital Amsterdam, The Netherlands) were grown in RPMI-1640 supplemented with 10% FBS and P/S[27]. Cells were cultured at 37 °C and 5% CO_2.

Lipid-mediated transfection. Cells were plated at 80,000 cells per well in six-well plates (Costar) two days before transfection without antibiotics. Transfection with agRNA was performed with Oligofectamine (Invitrogen) according to the manufacturer's instructions. Per well, 25 nM duplex (0.9 μl lipid) or 100 nM duplex (3.6 μl lipid) in Optimem (Invitrogen) were added to a final volume of 250 μl. Medium was added to the duplex–lipid mixture for a final volume of 1.25 ml, then added to cells. Medium was changed 24 h later, and cells were harvested five days after transfection unless otherwise indicated.

Western blotting. Cell pellets were lysed and protein concentrations were quantified with BCA assay (Pierce). Western blots were performed on protein lysates (30 μg per well). Primary antibodies (Ab) included: PR-Ab (Cell Signaling Technology), AR-Ab (from Michael McPhaul, University of Texas Southwestern Medical Center, Dallas) and MVP-Ab (BD Transduction Laboratories). β-actin-Ab (Sigma) was used as an internal control and for quantitation. Protein was visualized with anti-mouse or anti-rabbit secondary antibody (Jackson Immunolabs) and Supersignal developing solution (Pierce). Cell culture and western analysis for MVP was performed as described[27].

RNA analysis. Total RNA from treated T47D cells was extracted withTRIzol (Invitrogen)[28]. RNA was treated with deoxyribonuclease to remove contaminating DNA, and 4 μg were reverse-transcribed by random primers with Superscript II RNase H-reverse transcriptase (Invitrogen).

Two primer sets directed against different regions of the PR (NM 000926) along with the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (G3PDH, BC 020308) were generated with Primer Express software (PE Applied Biosystems) based on published sequences. Primers were hPR-B FWD 5′-ACA CCT TGC CTG AAG TTT CG-3′, hPR-B REV 5′-CTG TCC TTT TCTGGG GGA CT-3′ hPR-A/B FWD 5′-GAG GAT AGC TCT GAG TCC GAG GA-3′, hPR-A/B REV 5′-TTT GCC CTT CAG AAG CGG-3′, COX-2 (FWD) 5′-TTCCAGATCCAGAGCTCATTAAA-3′, (REV) 5′-CCGGAGCGG-GAAGAACT-3′.

The first primer set, PR-B[29] was directed at the sequence specific for PR-B (upstream of the second ATG transcription initiation site), whereas the second primer set, PR-AB, was directed at the sequence downstream of the second ATG start site. Using known concentrations of cDNA, we showed that the PR primers had equal efficiency in priming their target sequences (data not shown).

The relative amount of mRNA encoding PR-A was calculated by subtraction of the relative abundance of PR-B from that of PR-AB. All primer sets produced amplicons of the expected size and sequence. For the quantitative analysis of mRNA expression, the ABI Prism 7700 Detection System (Applied Biosystems) was employed with the use of the DNA binding dye SYBER Green (Applied Biosystems) for the detection of PCR products. The cycling conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, and 60 °C for 1 min. The cycle threshold was set at a level at which the exponential increase in PCR was linear.

Methylation analysis. Genomic DNA was extracted with the DNeasy Tissue Kit (QIAGEN) as per the manufacturer's instructions. Sodium bisulfite modification of genomic DNA was performed as described previously[30]. PCR amplification of sodium bisulfite genomic DNA was performed with 1 unit of HotStarTaq DNA polymerase (QIAGEN) and 100 ng of treated DNA. Primers and PCR conditions may be found in **Supplementary Methods**[22,30]. MSP products were resolved on 2% agarose gel (Sigma) with ethidium bromide. PCR products for sequencing were gel purified with Qiaex II Gel extraction kit (QIAGEN) and cloned into the TOPO TA cloning vector (Invitrogen, CA). Transformed cells were selected on ampicillin (Sigma) LB plates supplemented with UltraPure Blue-Gal (Invitrogen). Between 5 and 15 colonies were selected from each plate. Plasmid DNA was purified with Qiaprep Spin Miniprep (QIAGEN). Purified plasmid DNA was sequenced in the McDermott DNA Sequencing Core facility at the University of Texas Southwestern Medical Center, Dallas.

Control bisulfite-treated DNA for the hPR MSP was a mixture of SssI-treated DNA and normal lymphocyte DNA. Control DNA for the AR MSP was normal lymphocyte DNA from a female donor. Heterozygosity and X-inactivation are indicated by different-sized bands for the methylated and unmethylated alleles in the control lanes. The absence of a second, different-sized, methylated band in the experimental samples confirms that T47D has a single unmethylated allele at the AR locus.

Accession codes. GenBank identifiers: PR, X51730; AR, M58158; MVP, AJ238509–AJ238519.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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