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Activating gene expression in mammalian cells with promoter-targeted duplex RNAs

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The ability to selectively activate or inhibit gene expression is fundamental to understanding complex cellular systems and developing therapeutics. Recent studies have demonstrated that duplex RNAs complementary to promoters within chromosomal DNA are potent gene silencing agents in mammalian cells. Here we report that chromosome-targeted RNAs also activate gene expression. We have identified multiple duplex RNAs complementary to the progesterone receptor (PR) promoter that increase expression of PR protein and RNA after transfection into cultured T47D or MCF7 human breast cancer cells. Upregulation of PR protein reduced expression of the downstream gene encoding cyclooygenase 2 but did not change concentrations of estrogen receptor, which demonstrates that activating RNAs can predictably manipulate physiologically relevant cellular pathways. Activation decreased over time and was sequence specific. Chromatin immunoprecipitation assays indicated that activation is accompanied by reduced acetylation at histones H3K9 and H3K14 and by increased di- and trimethylation at histone H3K4. These data show that, like proteins, hormones and small molecules, small duplex RNAs interact at promoters and can activate or repress gene expression.

Duplex RNAs are powerful tools for silencing gene expression and are being evaluated for therapeutic efficacy in clinical trials[1–3]. Synthetic agents that increase gene expression would be equally valuable and would create new opportunities for laboratory investigation and therapeutic development. However, in contrast to the rapid improvement of methods for silencing gene expression, progress toward sequence-specific activation of gene expression has been more limited[4–7].

We have shown that 19- to 21-base-pair duplex antigene RNAs (agRNAs) that are complementary to chromosomal DNA upstream from the +1 transcription start site can block gene expression inside cells by inhibiting transcription[8–10]. Other laboratories have observed similar results[11–17]. During the course of our experiments[8] with agRNAs targeting the PR promoter[18,19], we observed two unexpected phenomena. First, in T47D cells (a breast cancer cell line that expresses high levels of PR), a single base shift in the agRNA target sequence transformed a highly potent inhibitory agRNA into one that did not silence PR expression. Second, rather than inhibiting gene expression, some agRNAs reproducibly caused small (1.5- to 2-fold) increases in gene expression above the already high levels observed in T47D cells (**Supplementary Fig. 1** online).

One explanation for these unexpected observations is that some agRNAs were activating gene expression. However, the already high basal levels of PR expression in T47D cells made RNA-mediated gene activation difficult to evaluate. We reasoned that gene activation could be more readily observed against a low basal level of gene expression. Therefore, to address our hypothesis, we introduced duplex RNAs into MCF7 cells (a breast cancer cell line with a much lower basal level of PR protein expression than that observed in T47D cells[9,20]). We observed that RNAs targeting the PR promoter are able to activate expression of PR, which suggests a new dimension to the ability of RNA to regulate complex cellular processes.

RESULTS

agRNAs activate gene expression

We initiated testing with RNA PR11, a duplex complementary to the PR promoter sequence from –11 to +8 (RNA sequences are listed in **Table 1** (**Supplementary Methods** online)). We chose PR11 because it does not inhibit PR expression in T47D cells but is surrounded by agRNAs that are potent inhibitors. For comparison, we also tested RNAs PR9 and PR26, which we previously showed to be potent inhibitors of PR expression in T47D cells.

We introduced duplex RNA PR11 into MCF7 cells using cationic lipid[10] and observed an 18-fold increase in concentrations of PR protein relative to controls (**Fig. 1a**), which suggests that agRNAs can produce substantial upregulation of gene expression when tested in an appropriate cellular context. Addition of PR9 did not affect PR expression, whereas PR26 yielded a modest 2-fold increase in PR concentrations. Two small interfering RNAs (siRNAs) that are complementary to downstream coding sequences within PR mRNA inhibited expression of PR protein, which demonstrates that PR concentrations can be reduced by standard post-transcriptional silencing in MCF7 cells.

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Table 1 Duplex RNAs used in these studies

RNA		(+) strand sequence
Noncomplementary control RNAs		
PRMM4		UCUCUCGCCAGUGCACACCTT
PR11MM3		GCUGUCUGGCCACUCGACUTT
PR11MM4		GCAGACAGACCAGUCCACATT
PR11MM6		GCUUGGGUCCCAGUCCACATT
PR11SCR		ACAGCCAGCUGGUCCUCUGTT
MVPMM3		GCGUCAGACUUCCCCAUCUGA
MVRSCR		GUUGCCAUGUCCUAGUGACTT
PNAs (N terminus to C terminus)		
PNAPR11AS	–9/+10	GCTGTCTGGCCAGTCCACA
PNAPR11S	+10/–9	TGTGGACTGGCCAGACAGC
PNASCR		AGTCCTGTCACAGCTGGCC
RNAs complementary to progesterone receptor		
PR2	–2/+17	CCAGUCCACAGCUGUCACUTT
PR6	–6/+13	CUGGCCAGUCCACAGCUGUTT
PR7	–7/+12	UCUGGCCAGUCCACAGCUGTT
PR8	–8/+11	GUCUGGCCAGUCCACAGCUTT
PR9	–9/+10	UGUCUGGCCAGUCCACAGCTT
PR10	–10/+9	CUGUCUGGCCAGUCCACAGTT
PR11	–11/+8	GCUGUCUGGCCAGUCCACATT
PR12	–12/+7	AGCUGUCUGGCCAGUCCACTT
PR13	–13/+6	AAGCUGUCUGGCCAGUCCATT
PR14	–14/+5	AAAGCUGUCUGGCCAGUCCTT
PR19	–19/–1	GUUAGAAAGCUGUCUGGCCTT
PR22	–22/–3	GUUGUUAGAAAGCUGUCUGTT
PR23	–23/–4	CGUUGUUAGAAAGCUGUCUTT
PR24	–24/–5	GCGUUGUUAGAAAGCUGUCTT
PR25	–25/–6	GGCGUUGUUAGAAAGCUGUTT
PR26	–26/–7	AGGCGUUGUUAGAAAGCUGTT
PR29	–29/–10	AGGAGGCGUUGUUAGAAAGTT
PR34	–34/–15	AGAGGAGGAGGCGUUGUUATT
PR39	–39/–20	UCCCUAGAGGAGGAGGCGUTT
PR44	–44/–25	GGGCCUCCCUAGAGGAGGATT
PR49	–49/–30	GGGCGGGGCCUCCCUAGAGTT
PR56	–56/–38	GGGCUUUGGGCGGGGCCUCTT
PRrna1	3237–3255	AUGGAAGGGCAGCACAACUTT
PRrna2	1931–1949	GGUGUUGUCCCCGCUCAUGTT
RNAs complementary to MVP (variant 1)		
MVP9A	–9/+10	GGGUGAGAGUUCCCCAUCUTT
MVP9B	–9/+12	GGGUGAGAGUUCCCCAUCUGA
MVP13	–13/+6	AGGCAGGGUGAGAGUUCCCTT
MVP16	–16/+2	CAAGGCAGGGUGAGAGUUCTT
MVP19	–19/–2	CCCCAAGGCAGGGUGAGAGTT
MVP24	–24/–6	GUGAUCCCCAAGGCAGGGUTT
MVP25	–25/–7	AGUGAUCCCCAAGGCAGGGTT
MVP32	–32/–14	GCCGGGAAGUGAUCCCCAATT
MVP33	–33/–15	UGCCGGGAAGUGAUCCCCATT
MVP35	–35/–17	CCUGCCGGGAAGUGAUCCCTT
MVP54	–54/–36	UGGGCUUGGCCUGCCUUGCTT
MVP70	–70/–52	UCCCAAGCCCCACCCCUGGTT
MVP82	–82/–64	GGGCCCUUUAACUCCCAAGTT
MVPrna	110/128	UAGGAGUCACCAUGGCAACTT

Only the sense strand is shown. Sequences are listed 5′ to 3′. The other strands of the RNA duplex are complementary and include two thymidine bases at the 3′ termini as indicated. Underlined bases are mismatched.

We then reexamined gene activation by duplex RNAs in T47D cells. To facilitate unambiguous observation of activation, we reduced the basal level of PR expression by growing the cells in culture medium containing charcoal-treated serum[21]. As expected, use of serum-stripped medium lacking hormones reduced PR expression (**Fig. 1b**). Addition of RNA PR11 induced PR expression to levels observed for T47D cells in normal medium (**Fig. 1b**). These results demonstrate that PR11 has the same physiologic effect in two different breast cancer cell types and that PR11 is able to counteract a well-established mechanism for manipulating hormone receptor expression.

Activation is potent and specific

After observing RNA-mediated activation of gene expression by PR11 we assayed the specificity and potency of the phenomenon. We tested a battery of mismatch and scrambled control duplexes (**Table 1**), including mismatches that preserve complementarity at either end of the duplex. These control duplexes did not increase expression of PR (**Fig. 1a**,**c**), thereby demonstrating that upregulation is sequence specific. We also tested the effect of adding peptide nucleic acid (PNA) strands, a nucleic acid mimic that contains amide linkages in place of the ribose phosphate backbone. PNAs can be introduced into cells, and they inhibit gene expression by targeting mRNA or chromosomal DNA[1,22]. PNAs analogous to the sense and antisense strands of PR11 were inactive, which suggests that complementarity to the target site is not sufficient for activation (**Table 1** and **Fig. 1d**). Addition of PR11 at varied concentrations demonstrated that activation is potent, with 17-fold activation achieved at a 12 nM concentration (**Fig. 1e**).

agRNAs upregulate PRB and PRA isoforms

PR protein is expressed as two isoforms, PRA and PRB, that have differing roles in physiologic processes[23]. The promoter for PRB is upstream from the promoter for PRA, and the RNAs used in this study target the PRB promoter. We previously observed that agRNAs, siRNAs, antisense PNAs and antigene PNAs that target either the PRB promoter (agRNA and antigene PNA) or PRB mRNA (siRNA and antisense PNA) also reduce concentrations of PRA[8–10,22], which suggests that expression of PRA is linked to expression of PRB. We now observe that RNAs targeting the PRB promoter can also enhance expression of both PRB and PRA protein (**Fig. 1** and **Fig. 2a**), thereby providing complementary evidence that expression of the isoforms is linked.

A cluster of agRNAs activate PR expression

To correlate activity with target sequence, we tested a series of duplex RNAs targeted to sequences throughout the region –56 to +17 within the PR promoter (**Table 1**). Several of these duplex RNAs induced expression of PR to levels that were five-fold (or more) greater than those of controls (**Fig. 2a,b** and **Supplementary Fig. 2** online). Small shifts in target sequence had large consequences for activation. For example, a single base shift upstream (PR12) or downstream (PR10) from PR11 substantially reduced activation. Experiments were repeated several times with similar results (**Supplementary Fig. 2**). These data suggest that sequences throughout the promoter are suitable targets and that the requirements for RNA-mediated gene activation are flexible.

Rules have been developed for predicting the efficiency of small RNAs that target mRNA for RNA interference[24]. Favorable criteria for siRNA design, in order of influence, include the following: 30–52% C+G content, at least three adenosines or uridines at positions 15–19 of the sense strand, an adenosine at position 19 of the sense strand, a uridine at position 10 of the sense strand, a base other than cytidine or guanosine at position 19 of the sense strand, and a base other than guanosine at position 13. Before applying this analysis it is necessary

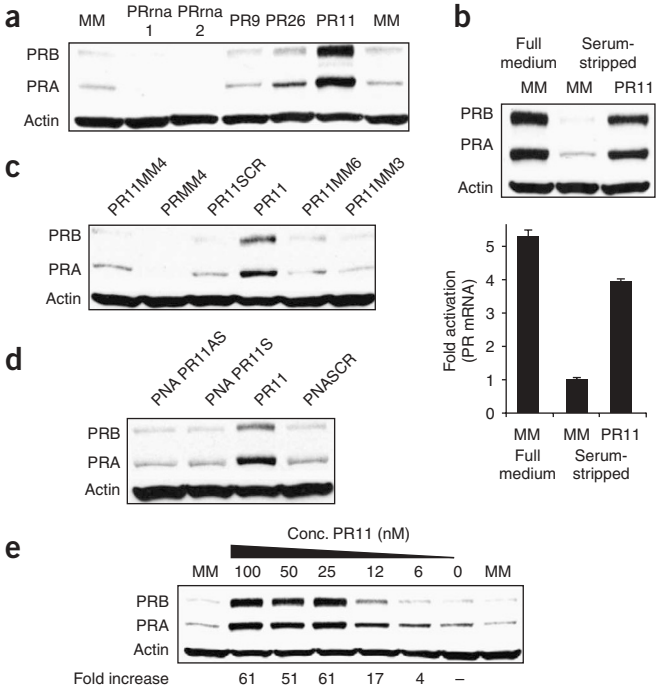

Figure 1 Increased expression of PR protein or mRNA upon transfection of duplex RNAs into MCF7 or T47D breast cancer cells. (**a**) Western analysis showing the effect of treating MCF7 cells with mismatch-containing RNA (PRMM4; shown as MM), RNAs targeting PR mRNA (PRrna1 and PRrna2), and RNAs targeting the PR promoter (PR9, PR26 and PR11, targeting nucleotides –9/+10, –26/–7 and –11/+8, respectively). (**b**) Western analysis and QPCR showing effect of adding mismatch-containing RNA (PRMM4) or PR11 to T47D cells grown in full medium or serum-stripped medium. Levels of mRNA are expressed as fold activation relative to PR expression in cells treated with mismatch RNA and grown in serum-stripped medium. (**c**) Western analysis comparing treatment of MCF7 cells with PR11, mismatch-containing RNAs (PR11MM4, PRMM4, PR11MM6, PR11MM3) and a scrambled control (PR11SCR) RNA. (**d**) Western analysis comparing treatment of MCF7 cells with PR11 or PNA oligomers analogous to the two strands of RNA duplex PR11. (**e**) Western analysis showing effect of treating MCF7 cells with varying concentrations of PR11. Unless otherwise noted, all RNAs were present at 100 nM. Unless otherwise noted, all mismatch controls (MM) are PRMM4. Error shown is s.e.m.

to realize that (i) the design of activating RNAs is constrained by the requirement for complementarity at the promoter, (ii) it is not known which strand of the agRNA duplex guides recognition, so both strands must be considered, and (iii) even for standard siRNAs that target mRNA, these rules are based on statistical analysis of the activity of many hundreds of duplexes and may not be an accurate predictor of the potency of an individual RNA.

We observed that activating RNAs can score poorly on one or more of these criteria (**Table 2**). For example, PR11 has a C+G content of 63% (too high), has fewer than three adenosines or uridines at positions 15–19 for both strands, and lacks a uridine at position 10. In general, inactive or less active RNAs had higher C+G content and higher T_m values than the most efficient activating RNAs, but this may simply reflect the fact that the PR promoter becomes more (C+G)-rich toward the upstream end of the targeted region. More data is needed to learn whether the rules developed to predict siRNA efficacy apply to agRNAs, but our data suggest that less-than-optimal scores should not discourage targeting of promoter sequences.

Active and inactive agRNAs compete for a target sequence

The differences between activating and inactive RNAs are substantial. The inactive RNAs may be ineffective because they are not mediating recognition of promoter DNA. Alternatively, they may mediate association with DNA, but this association may not be sufficient for activation. To address these possibilities, we performed order-of-addition experiments in which inactive RNAs PR8 and PR12

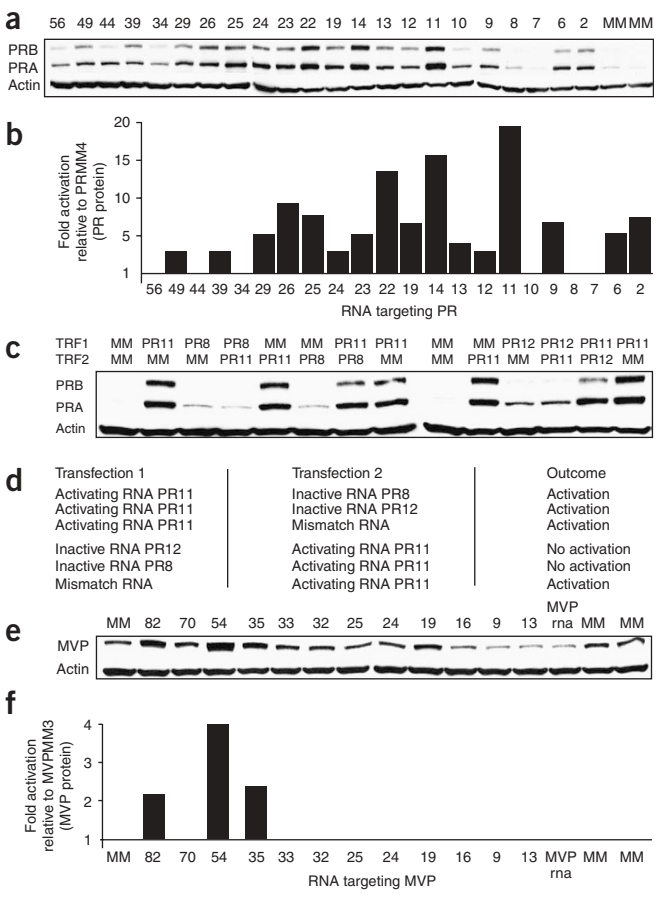

Figure 2 Probing the PR and MVP promoters with duplex RNAs. (**a**) Western analysis showing the effect of adding duplex RNAs complementary to sequences throughout the –56 to +17 region of the PR promoter. (**b**) Relative PR protein levels shown in **a**. Values from mismatch-containing RNA PRMM4 controls were averaged to calculate fold activation. (**c**) Western analysis showing the effects of competition between activating (PR11), inactive (PR8 or PR12) and mismatch-containing duplex RNAs (PRMM4). RNAs were added to cells in an initial transfection (transfection 1, TRF1) or a subsequent transfection (transfection 2, TRF2) 3 d later. All RNAs were added at 100 nM. (**d**) Summary of double transfection results with activating RNA PR11 and inactive RNAs PR8 or PR12. (**e**) Western analysis showing the effect of adding duplex RNAs complementary to sequences throughout the –82 to +6 region of the MVP promoter. (**f**) Relative MVP protein levels shown in **e**. Values for the triplicate treatments with mismatch-containing RNA PRMM3 were averaged to calculate fold activation. Results from replicate experiments (three for PR, one for MVP) are shown in **Supplementary Figure 2**. Duplexes were present at 100 nM. Numbering denotes the most upstream base of the RNA relative to the (+1) transcription start site. RNA sequences are in **Table 1**. MCF7 cells were used for these experiments.

Table 2 Characteristics of RNAs targeting PR

RNA	C+G content (%)	T_m (°C)	At least 3 A/U bases at positions 15–19 (sense/antisense strand)	Base at position 19 (sense/antisense strand)	Base at position 3 (sense/antisense strand)	Base at position 10 (sense/antisense strand)	Base other than G at position 13 (sense/antisense strand)
Activation > five-fold in MCF7 cells							
PR2	57	77	no/no	U/G	A/U	A/U	yes/yes
PR6	63	84	no/no	U/G	G/A	C/G	yes/yes
PR9	63	83	no/no	C/A	U/U	A/U	yes/no
PR11	63	75	no/no	A/C	U/U	C/G	no/yes
PR14	57	81	yes/no	C/U	A/A	U/A	yes/no
PR19	53	75	no/no	C/C	U/C	C/G	yes/yes
PR22	42	62	no/no	G/C	U/G	A/U	yes/yes
PR23	42	62	no/no	U/G	U/A	A/U	no/no
PR25	47	57	no/no	U/C	C/A	A/U	yes/no
PR26	47	64	no/no	G/U	G/G	U/A	yes/yes
PR29	47	70	no/yes	C/U	G/U	U/A	yes/no
Activation < five-fold in MCF7 cells							
PR7	63	73	no/no	G/A	U/G	U/A	yes/yes
PR8	63	75	no/no	U/C	C/C	G/C	yes/yes
PR10	63	73	no/no	G/G	G/G	C/G	yes/yes
PR12	63	76	no/no	C/U	C/G	G/C	yes/no
PR13	57	73	no/no	A/U	G/G	G/C	yes/yes
PR24	47	70	no/no	C/C	G/C	G/C	yes/yes
PR34	53	71	no/yes	A/U	A/A	G/C	no/no
PR39	63	76	no/no	U/A	C/G	G/C	no/no
PR44	68	78	no/no	A/C	G/C	U/A	yes/yes
PR49	79	84	no/no	G/C	G/C	C/G	yes/no
PR56	79	≥90	no/no	C/C	G/G	G/C	no/yes

were transfected either before or after transfection with activating RNA PR11 (**Fig. 2c**,**d**). When PR8 or PR12 was added to cells first, subsequent addition of PR11 did not result in activation. When PR11 was added to cells first, neither PR8 nor PR12 blocked gene activation. Addition of mismatch-containing RNAs in either the first or the second transfection did not affect activation by PR11.

These competition assays suggest that inactive RNAs PR8 and PR12 bind at the same target sequence as PR11. Recognition is sufficient to block binding of PR11 and prevent activation of PR expression. Competition of PR11 with PR8 and PR12 provides further evidence for the target and sequence specificity of RNA-mediated activation of PR. The finding that PR8, PR11 and PR12 compete for closely related target sequences but produce much different levels of gene activation suggests that the geometry of recognition is critical for activating gene expression.

agRNAs activate expression of major vault protein

To determine whether duplex RNAs can activate expression of other genes, we examined a series of RNAs targeted to major vault protein (MVP)[25] (**Fig. 2e**,**f**, **Table 1** and **Supplementary Fig. 2**). We chose MVP because we had previously silenced its expression using agRNAs[8]. MVP9 inhibited gene expression, a result that we had reported previously[8]. By contrast, MVP35, MVP54 and MVP82 increased expression by two-fold to four-fold above normal levels. These data suggest that duplex RNAs can enhance expression of genes with relatively high basal expression, which agrees with our initial observation of RNA-mediated upregulation of PR in T47D cells (**Supplementary Fig. 1**). Activation of a second gene suggests that RNA-mediated gene activation may be a general phenomenon. We note that even a two-fold activation of some genes might be valuable for treatment of some diseases.

Activating RNAs produce a relevant physiologic response

Quantitative PCR (QPCR) revealed that treatment of MCF7 cells with PR11 enhances expression of PR mRNA under a variety of cell culture conditions (**Fig. 3a**). We had previously shown that inhibition of PR expression in T47D cells by siRNAs[26] or agRNAs (B.A.J. and D.B.H., unpublished data) substantially increases expression of cyclooxygenase 2 (COX-2) after induction with interleukin 1β (IL-1β). We now observe that activation of PR gene expression in MCF7 cells after treatment with RNA PR11 reduces COX-2 expression in the presence or absence of IL-1β (**Fig. 3a**). These data demonstrate that activating RNAs can be used to manipulate expression of physiologically relevant downstream target genes in a predictable manner and that the induced PR is fully functional. Treatment of cells with PR11 did not alter concentrations of estrogen receptor-α (ERα), a key regulator of PR expression (**Fig. 3a**).

Addition of the hormones 17β-estradiol (estrogen, **1**) and progesterone (**2**) can affect normal expression of PR[27–29]. Estrogen raises PR concentrations by activating an estrogen receptor–mediated response pathway in MCF7 cells, and, as expected, cells treated with mismatch-containing RNA showed slight increases in PR expression upon addition of estrogen. Addition of estrogen did not further enhance PR mRNA levels in cells treated with RNA PR11, which suggests that the two mechanisms for activation are not additive (**Fig. 3b**). Addition of progesterone is known to induce an inhibitory feedback mechanism on PR gene transcription in cells with high PR expression[28], and we observed that addition of progesterone reduces the level of activation by PR11 (**Fig. 3b**). These data further indicate that the upregulated PR

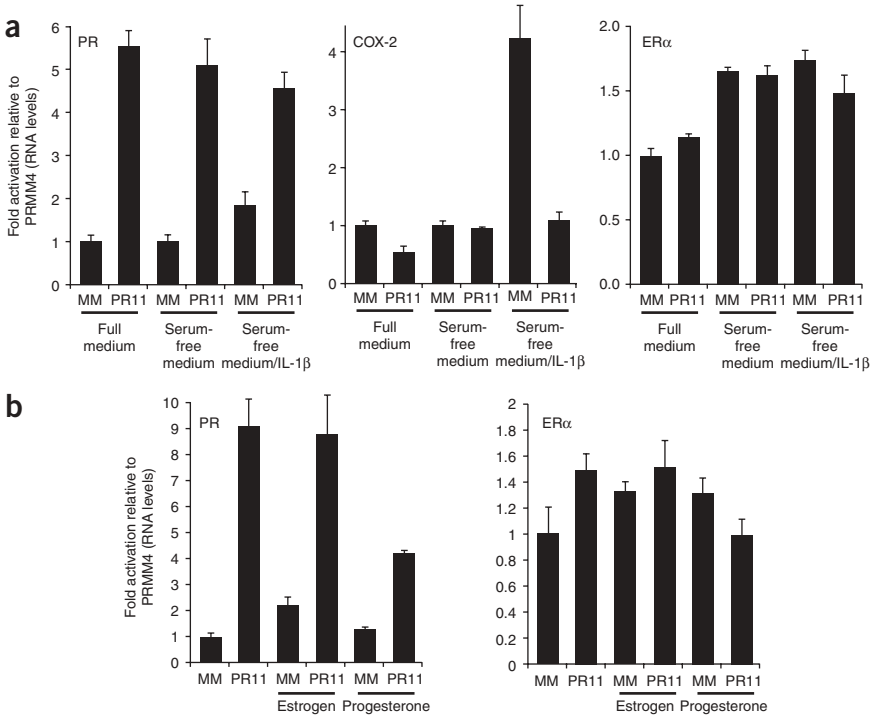

Figure 3 QPCR analysis showing effects of adding activating RNA PR11 on mRNA levels of selected genes in varied media. (**a**) Measurement of PR, COX-2 and ERα levels in full medium, in serum-free medium and in the absence or presence of IL-1β. Fold activation is relative to mismatch RNA in full medium. (**b**) Measurement of PR or ERα levels in full medium in the presence or absence of estrogen or progesterone. Fold activation is relative to mismatch RNA in full medium lacking added hormones. All mismatch duplexes were PRMM4. MCF7 cells were used for these experiments. Error shown is s.e.m.

is fully functional and susceptible to ligand-dependent downregulation characteristic of many nuclear hormone receptors.

Activation time course

Gene activation can be modulated through modifications of histones and other nuclear proteins[30]. These modifications can produce transient or long-lasting changes in chromatin structure and either activate or inhibit gene expression. To determine the effect of time on RNA-mediated activation we monitored the effect of adding RNAs PR11 and PR22 to MCF7 cells over a 15- or 16-d period (**Fig. 4**).

We observed an increase in PR protein expression after addition of either PR11 or PR22 at the initial day 3 time point, maximal activation after day 4, and a return to basal levels by day 9 or 10 (**Fig. 4a**,**b**). The similarity between the results observed with PR11 and PR22, which contain dissimilar sequences but share complementarity to the PR gene, suggests that initial activation and subsequent reduced expression are due to recognition of the target sequence within the PR promoter. During the 16-d period of the experiment, the cells were passaged several times and doubled every 3 d. It is likely that this dilution of RNA contributed to reduced activity at later time points.

To further investigate the effect of cell passaging on gene activation, we grew cells to confluence without passaging for 10 d. These unpassaged cells retained a high level of activation at day 10 (**Fig. 4c**). Subsequent passaging at days 11 and 14 resulted in reduced expression of PR, which supports the hypothesis that passaging and dilution of RNA contributes to reduced gene activation. However, we note that some activation above background continued to be observed after 15 d.

Activation and histone modifications

To determine the potential role of histone modification in the activation of PR expression we examined the effect of adding trichostatin A (TSA, **3**), a histone deacetylase inhibitor, to cells treated with either activating RNA PR11 or a mismatch-containing RNA. As previously reported[31], addition of TSA increased COX-2 expression, thereby demonstrating that we were using an effective concentration of TSA (**Supplementary Fig. 3** online). Also as noted previously[32], TSA had no effect on basal PR expression in MCF7 cells. By contrast, addition of TSA reversed activation in cells treated with PR11 (**Fig. 5a**), which suggests that deacetylation of histone proteins may be required for activation of the PR promoter by PR11. Consistent with this suggestion, efficient

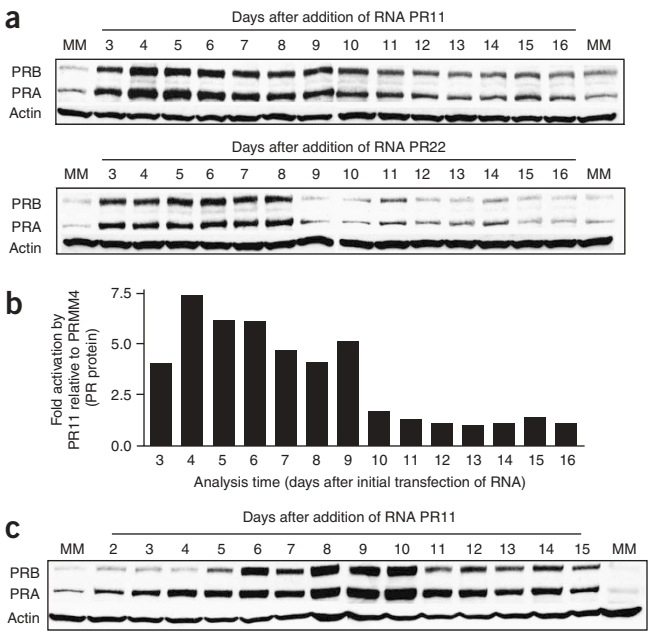

Figure 4 Time course of activation by PR11 or PR22 in MCF7 cells. (**a**) Western analysis of PR protein at various time points (3–16 d) after treatment with PR11 or PR22. Cells were split every 3 or 4 d. (**b**) Quantitation of protein levels for data in **a** from treatment with PR11. Quantitation of data from treatment of PR22 was similar. The two treatments with mismatch-containing RNA PRMM4 were averaged to calculate fold activation. (**c**) Western analysis of PR protein at various time points (3–16 d) after treatment with PR11. Cells were grown to confluence without passaging until day 10. Cells were passaged (split) on days 11 and 14.

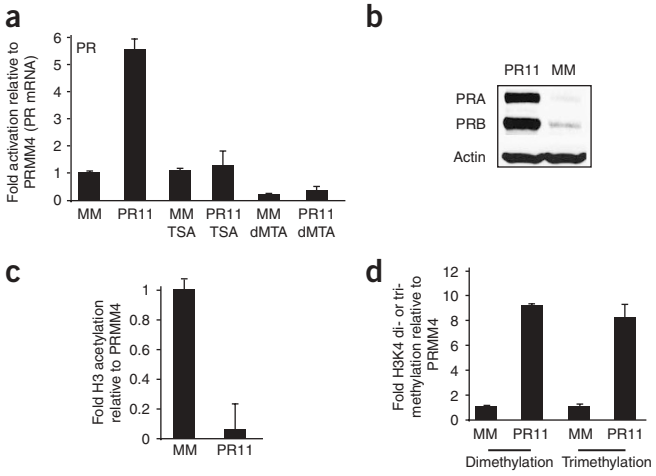

Figure 5 Effect of adding PR11 on histone modifications at the PR promoter in MCF7 cells. (**a**) Effect of adding TSA or dMTA on the activation of PR expression by PR11. (**b**) Typical western analysis of cells treated with PR11 and harvested for evaluation by ChIP. (**c**) Effect of PR11 on acetylation of histone H3. (**d**) Effect of adding PR11 on dimethylation and trimethylation of H3K4. All experiments were done in full medium and used 100 nM PR11 or mismatch RNA. All mismatch duplexes were PRMM4. Fold activation is relative to mismatch RNA in full medium. MCF7 cells were used for these experiments. Error shown is s.e.m.

activation of PR expression in MCF7 cells (**Fig. 5b**) followed by chromatin immunoprecipitation (ChIP) assays revealed that treatment of MCF7 cells with RNA PR11 reduces acetylation of histone residues H3K9 and H3K14 (**Fig. 5c**).

We also treated MCF7 cells with 5′-deoxy-5′-(methylthio)adenosine (dMTA, **4**), a protein methyltransferase inhibitor known to alter the methylation status of histones and other nuclear proteins[33–35]. Treatment with dMTA substantially reduced both basal and activated levels of PR expression (**Fig. 5a**), which suggests that histone methylation may be required for activation of PR expression by PR11. Consistent with this suggestion, ChIP assays showed an increase in both dimethylation and trimethylation of H3K4 after treatment with PR11 (**Fig. 5d**). Dimethylation and trimethylation of H3K4 are hallmarks of genes that have been activated for expression[36–39], which is consistent with their linkage to upregulated PR.

For gene silencing by promoter-targeted RNAs, we previously showed that addition of complementary RNAs leads to localization of two proteins, Argonaute 1 (AGO1) and Argonaute 2 (AGO2), to chromosomal DNA[9]. Another laboratory reported similar localization for AGO1 (ref. 15). For activating RNA PR11, our preliminary results using ChIP assays with antibodies complementary to AGO1 or AGO2 do not reveal increased localization of AGO1 or AGO2 to the PR promoter upon addition of PR11. These data suggest that the role of Argonaute proteins in RNA-mediated activation of gene expression may differ from that observed during RNA-mediated transcriptional silencing, and that the issue merits further examination.

DISCUSSION

Introduction of duplex RNA into cells can increase or decrease the expression of genes through "off-target" effects in which the observed phenotype is produced by interactions at sites other than the intended target sequence[40]. Several lines of evidence support the conclusion that duplex RNAs activate PR through sequence-specific interactions at the PR promoter: (i) potent activating RNAs PR11 and PR22 had minimal complementarity to RNA sequences in the human genome (**Supplementary Fig. 4** online), (ii) mismatch-containing RNAs related to PR11 were inactive (**Fig. 1**), (iii) multiple RNAs complementary to different sequences within the promoter for PR activated PR expression (**Fig. 2** and **Supplementary Fig. 2**), (iv) expression of ERα, a key regulator of PR expression and a prominent candidate for mediating off-target effects, was unchanged (**Fig. 3a**,**b**) and (v) PR11 raised PR concentrations in two breast cancer cell lines (T47D and MCF7, **Fig. 1**) that regulate PR expression differently. Taken together, these data suggest that activating RNAs mediate recognition at the targeted PR promoter.

Our data suggest several important features for the mechanism of gene activation by promoter-targeted RNAs. (i) Consistent with previous studies on RNAs that target promoter DNA and block expression[8–16], activity requires complementarity to sequences within promoter DNA. It is possible that the activating RNAs bind directly to DNA, but it is also possible that they bind to rare RNA transcripts that initiate upstream from the +1 transcription start site or to antisense transcripts; our data are consistent with either mechanism. (ii) Activation can occur in multiple cell lines and at different target genes, can be used to manipulate physiologically relevant cellular pathways, and can occur regardless of the basal level of gene expression. (iii) There is no evidence that activation is an off-target–mediated phenomenon. (iv) The PR protein produced is fully functional. (v) Activation is achieved by multiple RNAs targeting different sequences within promoters for PR and MVP. (vi) Activation is sensitive to small changes in target sequence. (vii) Inactive and active sequences compete for the same target. Recognition of the target sequence alone is not sufficient for activation; therefore interactions that occur after hybridization must be critical for gene activation. (viii) Activation decreases over time. (ix) The sequence of active RNAs can diverge substantially from the optimized criteria developed for siRNAs. (x) Activation correlates with histone modifications, and agents that influence histone modification can block activation.

Gene expression can also be controlled by methylation of promoter DNA, and there have been reports of an association between RNA-directed DNA methylation and gene silencing in plants[41] and human cells[13]. Reversal of methylation can reactivate gene expression, but this is not an explanation for our findings because the PR promoter is not methylated in MCF7 cells[42].

Key issues for research include (i) resolving whether the target for agRNAs is rare RNA transcripts or chromosomal DNA and (ii) understanding the molecular details of how this recognition is translated into activated expression. These molecular details may differ from one gene to the next and depend on the context of proteins and the preexisting regulation at the promoter. We note that a small RNA has been reported to increase transcription in neural stem cells[43], which suggests that RNA-mediated enhancement of gene expression may be a natural mechanism for controlling gene expression.

After completion of our studies, investigators reported that small double-stranded RNAs can also activate expression of the cell-adhesion molecule E-cadherin, the cyclin-dependent kinase inhibitor p21, and vascular endothelial growth factor[44]. Taken together with our data, these results suggest that small RNAs can be used to mediate activation of a wide array of genes and can target sequences throughout the promoter. Understanding the detailed mechanism of activation, and why RNAs activate expression in one context and inhibit expression in another, will require more research. However, one explanation is that RNAs associate with promoter DNA and alter

the composition or orientation of nearby proteins. These changes might then tip the balance from inhibition to activation or vice versa.

Activating RNAs targeting the PR promoter provide useful tools for elucidating the roles of PR protein in normal physiology and disease. More broadly, activating RNAs that are complementary to other genes should provide a class of agents for laboratory manipulation of gene expression and may extend the range of genes that can be productively targeted by RNA drugs. The significance of activating RNAs for natural mechanisms of gene regulation is unresolved. However, it seems reasonable that such a potent and evolutionarily flexible mechanism for gene regulation would be used by cells.

METHODS

Cell culture. MCF7 and T47D breast cancer cells (America Type Culture Collection, ATCC) were maintained in RPMI-1640 medium (ATCC) supplemented with 10% FBS (v/v), 0.5% nonessential amino acids (v/v), 0.4 units ml^{-1} bovine insulin, 100 units ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin (Pen/Strep solution). Cells were cultured at 37 °C and 5% CO_2.

Double-stranded RNAs. 21-nucleotide RNAs were synthesized by Integrated DNA Technologies or Dharmacon. 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. Duplex RNAs or PNA–DNA duplexes were made and melting temperatures of the duplexes measured as previously described[10].

Peptide nucleic acids. PNAs and DNAs were obtained as described[10]. The identities of the DNA oligonucleotides used to transport PNAs are as follows: carrier PR11as, 5′-CTG GCC AGA CAG CAC TGA CT-3′; carrier PR11s, 5′-TGG CCA GTC CAC AAC TGA CT-3′; carrier scr, 5-ATC AGC TGG CCA GCT GTG A-3′.

Lipid-mediated transfection. PNAs and RNA duplexes were introduced into cells as described[10]. Cells were plated at 200,000 cells per well in 6-well plates (Costar) 2 d before transfection without antibiotics. Transfection with agRNA or PNAs was performed using oligofectamine (Invitrogen), following manufacturer's instructions. For example, to test a 100 nM concentration of activating RNA, duplex RNA was mixed with 3.6 µl oligofectamine in optimem (Invitrogen) to achieve a final volume of 250 µl. Optimem was added to the mixture of duplex RNA and lipid for a final volume of 1.25 ml, then added to cells. Cells were plated for ChIP experiments at 6 million cells per plate. Per plate, 100 nM duplex and 57 µl oligofectamine in optimem were added to a final volume of 20 ml. Medium was changed 24 h later, and cells were harvested 5 d after transfection for western blot analysis or QPCR unless otherwise indicated.

Treatment with trichostatin A or 5′-deoxy-5′-(methylthio)adenosine. MCF7 cells were transfected with PR11 or mismatch-containing RNA duplex as described above. Cells were treated with 100 nM TSA or 300 µM dMTA (Sigma) in complete medium 24 h before harvest. After transfection, cells were harvested on day 3 for QPCR analysis or on day 5 for western blot analysis.

Treatment with hormones in T47D cells. T47D cells were transfected with PR11 or mismatch-containing RNA duplex. 24 h after transfection, cells received complete RPMI medium lacking phenol red and supplemented with 2.5% charcoal-stripped FBS (Hyclone) for the duration of the experiment. Cells were treated with 100 nM estradiol or 100 nM progesterone (Steraloids) 24 h before harvest. After transfection, cells were harvested on day 5 for QPCR or western blot analysis.

Treatment with hormones or interleukin 1β in MCF7 cells. MCF7 cells were transfected with PR11 or mismatch-containing RNA duplex. Cells were treated with 100 nM estradiol, 100 nM progesterone or 10 ng ml^{-1} IL-1β (Steraloids) in either complete medium or phenol red–free medium supplemented with charcoal-stripped FBS 24 h before harvest. Cells were harvested 3 d after transfection for mRNA analysis by QPCR.

Western blot. Cell pellets were lysed and protein concentrations were quantified using BCA assay (Pierce). Westerns were performed on protein lysates (30 µg per well). Primary antibodies (Ab) included PR-Ab (Cell Signaling) and MVP-Ab (BD Transduction Laboratories). β-actin–Ab (Sigma) was used as an internal control and for quantitation. Protein was visualized using secondary antibody to mouse or rabbit (Jackson Immunolabs) and supersignal developing solution (Pierce).

Quantitative PCR. Total RNA from MCF7 cells was extracted by the one-step method of Chomczynski and Sacchi (Trizol, Invitrogen). RNA was treated with DNase to remove any contaminating DNA, and 4 µg were reverse transcribed using random primers and superscript II RNase H reverse transcriptase (Invitrogen). Primer sets directed against human COX-2, ERα and PR, along with h368B, a control primer set against ribosomal RNA, were generated using Primer Express (PE Applied Biosystems) based on published sequences (**Table 1**).

The relative abundance of each transcript was determined by QPCR. For the quantitative analysis of mRNA expression, the ABI Prism 7700 detection system (Applied Biosystems) was employed using the DNA-binding dye SYBR Green (PE Applied Biosystems) for the detection of PCR products. The cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The cycle threshold was set at a level at which the exponential increase in PCR amplification was approximately parallel between all samples. All primer sets produced amplicons of the expected size and sequence. Negative control experiments were performed lacking cDNA, and under these conditions no amplified products were observed (that is, cycle time values of >38). Error was calculated as s.e.m. based on two to four independent determinations. We calculated the relative fold changes using the comparative cycle times (Ct) method with cyclophilin as the reference guide. Over a wide range of known cDNA concentrations, all primer sets were demonstrated to have good linear correlation (slope $= -3.4$) and equal priming efficiency for the different dilutions compared to their Ct values (data not shown). Given that all primer sets had equal priming efficiency, the ΔCt values (primer–internal control) for each primer set were calibrated to the experimental samples with the lowest transcript abundance (highest Ct value), and the relative abundance of each primer set compared to calibrator was determined using the formula $2^{\Delta\Delta Ct}$, where ΔΔCt is the calibrated Ct value.

Chromatin immunoprecipitation. MCF7 cells were washed once with phosphate-buffered saline and incubated with 1% formaldehyde (in control medium) for 10 min at room temperature (20–23 °C) to cross-link proteins and DNA. Cross-linking was terminated by the addition of glycine (0.125 M, final concentration). The cells were washed twice with cold (32 °C) phosphate-buffered saline and placed in 500 µl of lysis buffer (50 mM Tris-HCl, pH 8.1, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, protease inhibitor cocktail (Roche) and 5 mM EDTA). The lysates were sonicated on ice to produce sheared, soluble chromatin. The soluble chromatin was precleared with Protein A/G Plus agarose beads (Upstate) (60 µl) at 4 °C for 1 h. The samples were microfuged at 14,000 r.p.m. to pellet the beads, and the supernatant containing the sheared chromatin was placed in new tubes. The precleared chromatin was aliquoted into 300 µl amounts and incubated with antibodies for dimethyl-histone H3 (Upstate) or histone H3 (Upstate) at 4 °C overnight. Two aliquots were reserved as controls—one incubated without antibody and the other with nonimmune IgG. Protein A/G Plus agarose beads (60 µl) were added to each tube, the mixtures were incubated for 2 h at 4 °C and the immune complexes were collected by centrifugation. The beads containing the immunoprecipitated complexes were washed sequentially for 5–10 min in wash buffer I (20 mM Tris-HCl, pH 8.1, 2 mM EDTA, 0.1% SDS, 1% Triton X-100 and 150 mM NaCl), wash buffer II (same as I, except containing 500 mM NaCl) and wash buffer III (10 mM Tris-HCl, pH 8.1, 1 mM EDTA, 1% NP-40, 1% deoxycholate and 0.25 M LiCl), and in 2 × TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The beads were eluted with 250 µl elution buffer (1% SDS, 0.1 mM $NaHCO_3$ and 20 µg salmon sperm DNA (Sigma)) at room temperature. This was repeated once and eluates were combined.

Cross-linking of the immunoprecipitated chromatin complexes and input controls (10% of the total soluble chromatin) was reversed by heating the samples at 65 °C for 4 h. Proteinase K (15 µg, Invitrogen) was added to each sample in buffer (50 mM Tris-HCl, pH 8.5, 1% SDS, 10 mM EDTA) and incubated for 1 h at 45 °C. The DNA was purified by phenol-chloroform extraction and precipitated in ethanol overnight at –20 °C. Samples and input

controls were diluted in 10–100 μl of TE buffer just before PCR. Real-time PCR was employed using forward (5′-CCTAGAGGAGGAGGCGTTGTT-3′) and reverse (5′-CATTGAGAATGCCACCCACAC-3′) primers that amplify a ~100-base-pair region surrounding the area in the human PR promoter that PR11 agRNAs target. Using serial dilutions of human chromosomal DNA, these primers were demonstrated to have equal efficiency in priming their target sequences.

Accession codes. GenBank: PR is listed as entry NM_000926; MVP is listed as entries AJ238509–AJ238519. All entries are derived from previous studies.

Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website.

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AUTHOR CONTRIBUTIONS

B.A.J., S.T.Y., D.B.H., R.R. and K.E.H. designed and performed experiments. B.A.J. and D.R.C. supervised experiments.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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