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An RNA targeted to the HIV-1 LTR promoter modulates indiscriminate off-target gene activation

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ABSTRACT

Transcriptional gene silencing (TGS) can be achieved by small RNAs targeted to upstream promoter regions. Previously we characterized siRNAs targeted to the HIV-1 long terminal repeat (LTR) promoter at site 247, and found that a 21-base antisense strand of siRNA-247 (LTR-247as) suppressed LTR-mediated expression. To characterize the specificity of LTR-247as, vectors expressing antisense RNAs targeted to a region spanning 50 bases up- and downstream of the 247 target site were generated. LTR-247as+7, a ~22 base antisense RNA that is shifted by only seven bases upstream of LTR-247as, showed a significant increase in LTR-driven reporter gene expression that was independent of cell type and active chromatin methyl-marks. Promoter-targeting siRNAs have been recently shown to induce gene activation. However, here we demonstrate gene activation via a sequence-specific off-target effect. Microarray analysis of LTR-247as+7-treated cultures resulted in the deregulation of ~185 genes. A gene of unknown function, C10orf76, was responsive to inhibition by LTR-247as+7 and the loss of C10orf76 resulted in the upregulation of several genes that were activated by LTR-247as+7. These data suggest caution when using short antisense RNAs or siRNAs designed to target promoter sequences, since promoter-targeted RNAs may have unintended inhibitory effects against factors with suppressive gene activity.

INTRODUCTION

RNA interference (RNAi) is a ubiquitous and conserved eukaryotic cellular pathway whereby double-stranded (ds) RNA triggers specific and potent inhibition of gene expression. RNAi appears to behave via two different mechanistic pathways: transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) (1,2). Each pathway involves the action of small interfering RNAs (siRNAs). PTGS involves siRNA-mediated targeting and degradation of mRNA, which in human cells occurs predominantly in the cytoplasm (3,4). TGS, however, occurs exclusively at the promoter region of the siRNA-targeted gene in the nucleus resulting in transcriptional suppression via the recruitment of silent state epigenetic marks on DNA and chromatin (5–15). Recently, synthetic siRNAs or short dsRNAs targeted to the promoters for E-cadherin, $p21^{WAF1/CIP1}$ (p21), VEGF (16) and progesterone (PR) (17) demonstrated target-specific gene activation, or RNA activation (RNAa). Although RNAa appears to be a robust sequence-specific phenomenon, at present little is known about its underlying endogenous function and biological mechanism.

We have previously shown that siRNAs targeted to the HIV-1 subtype B LTR promoter mediate TGS via the action of the antisense strand of the siRNA (18). These data are supported by the observation that antisense RNAs (asRNA) are also involved in human genetic diseases (19) and point to a biological role for short RNAs in the epigenetic control of gene expression in human cells (20,21). To further investigate the effects of 21 base asRNAs in transcriptional silencing, and to define additional asRNAs that target the HIV-1 LTR promoter, we generated U6 snRNA RNA Pol III asRNA constructs

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that span approximately 50 bases up- and downstream of the previously defined suppressive asRNA target site, 247 (targeted by LTR-247as) (18). Site 247 specifically spans the LTR of HIV-1 from bp 247-268 (HIV reference sequence HXB2, accession K03455), and was previously shown to be an effective site for both siRNA- and asRNA-mediated TGS of HIV-1 (18). Although the asRNA screen did not produce any new suppressive asRNAs, a significant increase in LTR-mediated transcription of a luciferase reporter occurred by shifting the target site seven bases downstream of site 247 (LTR-247as + 7). This result at first glance appeared to be similar to that observed for RNAa (16,17). Microarray results revealed that several other genes were activated by the presence of LTR-247as + 7. Here we show that LTR-247as + 7, an antisense RNA directed to the LTR promoter of HIV-1, is capable of sequence-specific indiscriminate gene activation by suppressing C10orf76, a candidate gene of unknown function which may operate as a generalized transcriptional regulator. Although our data for RNA-dependent gene activation differs in a number of ways from that observed recently by Li, Janowski and co-workers (16,17), we suggest a measure of caution when interpreting RNA activation data, which may be the result of non-specific off-target effects.

MATERIALS AND METHODS

Cell culture

The 1G5 cell line (AIDS Research and Reagent Reference Program) was used to assess the efficacy of U6 expressed asRNAs (Figure 1a) to target the HIV-1 LTR/promoter (18). The 1G5 cell line is a Jurkat-based cell line with the HIV-1 subtype B LTR driving the expression of firefly luciferase followed by an SV40 Poly A strong stop signal (23,24). To determine the ability of the various U6 expressed HIV-1 subtype B LTR-specific asRNAs to induce off-target enhanced transcription, a CCR5 promoter expressing GFP construct was used to develop the reporter cell line 293-CCR5-GFP. The vector pR5-GFPsg143 contains ~3 kb of CCR5 promoter, intron and exons 1 and 2 (25–27) and drives the expression of red-shifted GFP (a gift from Dr G.N. Pavlakis) (9,28). A total of 4.0×10^6 HEK293 cells were transfected with vector pR5-GFPsg143 (5 μg, Lipofectamine 2000™) and Neomycin-selected (800 μg/ml) to generate the stable cell population (293-CCR5-GFP) (9).

Generation of target reporter- and U6 antisense RNA expression constructs

Plasmids expressing antisense sequences targeted to the HIV-1 U3 LTR were constructed. PCR fragments were cloned into pCR2.1-TOPO (Invitrogen) using the U6 + 1 snRNA promoter as PCR template. This method was described previously by Lee *et al*. (29) using a universal U6 forward primer: 5′ AAG GTC GGG CAG GAA GAG GGC CT 3′. The following reverse primers were used: LTR-247s + 7 oligo 5′ *AAA AA*A GAG TGG AGG TTT GAC AGC CCG GTG TTT CGT CCT TTC CAC AA 3′, LTR-247s + 13 oligo 5′*AAA AA*A GGT TTG ACA GCC GCC TAG CGG TGT TTC GTC CTT TCC ACA A 3′, LTR-247s + 19 oligo 5′*AAA AA*A GAC AGC CGC CTA GCA TTT CCG GTG TTT CGT CCT TTC CAC AA 3′, LTR-247s-7 oligo 5′ *AAA AA*A GAG AGA AGT GTT AGA GTG GCG GTG TTT CGT CCT TTC CAC AA 3′, LTR-247s-14 oligo 5′ *AAA AAA* ACC CTG AGA GAG AAG TGT TCG GTG TTT CGT CCT TTC CAC AA 3′, LTR-247s-21 oligo 5′ *AAA AAA* ATG GAT GAC CCT GAG AGA GCG GTG TTT CGT CCT TTC CAC AA 3′, LTR-247s-21/ + 19 oligo 5′ *AAA AAA* ATG GAT GAC CCT GAG AGA GAA GTG TTA GAG TGG AGG TTT GAC AGC CGC CTA GCA TTT CCG GTG TTT CGT CCT TTC CAC AA 3′ and LTR-247s-12/ + 10 oligo 5′ *AAA AAA* CCT GAG AGA GAA GTG TTA GAG TGG AGG TTT GAC AGC CGC CCG GTG TTT CGT CCT TTC CAC AA 3′. The construction of pCR2.1-LTR-247as −50/ + 50 required two separate sequential PCR reactions as described previously (30). The following reverse primers were used: LTR-247s-50/ + 50 R1 oligo 5′ AGT GGA GGT TTG ACA GCC GCC TAG CAT TTC ATC ACG TGG CCC GAG AGC TGC ATC CGG AGT ACG GTG TTT CGT CCT TTC CAC AA 3′ and LTR-247s-50/ + 50 R2 oligo 5′ *AAA AAA* CAG CTT GTT ACA CCC TGT GAG CCT GCA TGG AAT GGA TGA CCC TGA GAG AGA AGT GTT AGA GTG GAG GTT TGA CAG CCG 3′. The following oligos were used to generate pCR2.1-LTR-247as mutants (mA and mB) as well as the sense RNA, LTR-247as: LTR-247s + 7 oligo 5′ *AAA AAA* GGG CTG TCA AAC CTC CAC TCG GTG TTT CGT CCT TTC CAC AA 3′, LTR-247as + 7mA oligo 5′ *AAA AAA* GAG TGG AGG TTT GAC AGC GCG GTG TTT CGT CCT TTC CAC AA 3′ and LTR-247as + 7mB oligo 5á *AAA AAA* GAG TTG AGG TTT GAC AGC CCG GTG TTT CGT CCT TTC CAC AA 3′. For the above oligos, underlined regions correspond to 3′ end of the U6 + 1 promoter, and the polyT termination signal is italicized.

To generate luciferase reporter constructs containing putative LTR-247as + 7 target sequences within the C10orf76 mRNA (Supplemental Figure 4), complementary oligonucleotides were treated with polynucleotide kinase (Promega, WI, USA), annealed, and cloned directly into the XhoI-NotI sites of the dual-luciferase vector psiCheck2 (Promega, WI, USA). To facilitate screening, an EcoRV site was inserted within each annealed dsDNA insert. The oligonucleotides used include: LTR-247as + 7 F target 5′ TCG AGA TAT CGA GTG GAG GTT TGA CAG CCC GC 3′ and LTR-247as + 7 R target 5′ GGC CGC GGG CTG TCA AAC CTC CAC TCG ATA TC 3′; C10orf76 1049 F 5′ TCG AGA TAT CCA GAG TGG GCT CCC TAA ACA GCC CCG C 3′ and C10orf76 1049 R 5′ GGC CGC GGG GCT GTT TAG GGA GCC CAC TCT GGA TAT C 3′; C10orf76 2230 F 5′ TCG AGA TAT CGG GGA GTT GGC TTG TGG TTC CTC CCT TGG ATA GCC TCG C 3′ and C10orf76 2230 R 5′ GGC CGC GAG GCT ATC CAA GGG AGG AAC CAC AAG CCA ACT CCC CGA TAT C 3′; C10orf76 463 F 5′ TCG AGA TAT CCA GTG GAA GGA AGC AGC TAG C 3′ and C10orf76 463 R 5′ GGC CGC TAG CTG CTT CCT TCC ACT GGA TAT C 3′; C10orf76 1534 F 5′ TCG AGA TAT

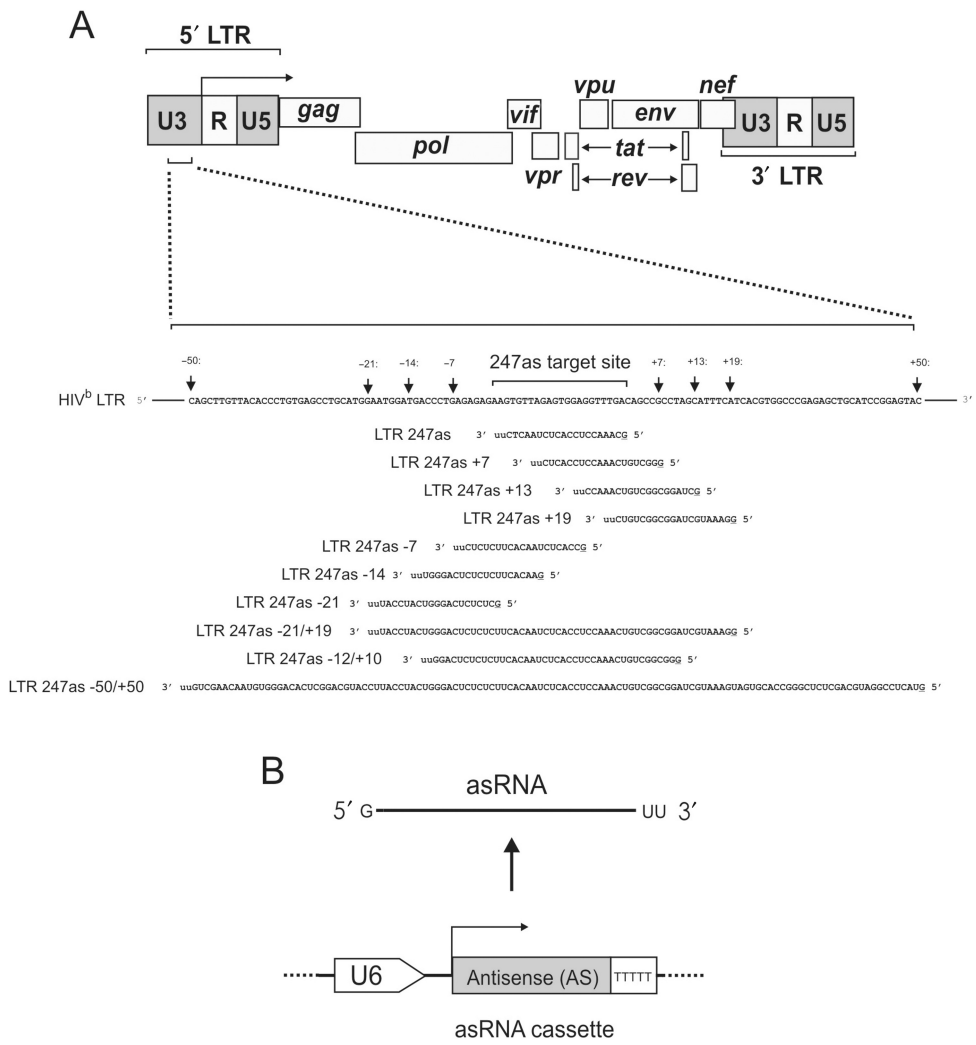

Figure 1. U6-antisense RNA cassettes generated to target regions flanking the LTR 247 site. (**A**) A set of nine asRNAs were selected that targeted either site 247, or target regions which span upstream (+) and/or downstream (−) of 247. The respective asRNAs are shown aligned with the HIV-1 subtype B LTR and site 247 (targeted by LTR-247as). (**B**) Selected U6-expressing asRNAs were cloned into the pTopoTA-based vector system as described (18).

CTA GAG ATG GAG TTT TTC ACC ATG TTA CCC AGG ATG GTC TCG C 3′ and C10orf76 1534 R 5′ GGC CGC GAG ACC ATC CTG GGT AAC ATG GTG AAA AAC TCC ATC TCT AGA TAT C 3′; C10orf76 210 F 5′ TCG AGA TAT CGG CAC AGU GGG GAG AGG CCU GC 3′ and C10orf76 210 R 5′ GGC CGC AGG CCT CTC CCC ACT GTG CCG ATA TC 3′.

Transfections and detection of transcriptional silencing

To determine the efficacy of the various U6 expressed HIV-1 subtype B LTR-specific asRNAs to suppress Tat-mediated expression of LTR-driven luciferase, 1G5 cells ($\sim$3–4 $\times$ 10^6) were transfected with 2.5 µg of the respective asRNA expressing vectors and 2.5 µg of pTat-dsRed (described previously) using the Bio-Rad Genepulser. Briefly, cells were washed in 1$\times$ PBS without Ca^{2+} or Mg^{2+} and then washed once with ice-cold Viaspan^{TM} (DuPont Pharma). Cultures were then re-suspended in 800 µl of ice-cold Viaspan^{TM}, transferred to a 0.4 cm gap 5 Cuvette (Bio-Rad), and the total 5 µg of plasmid DNA was added. The samples were then electroporated using 0.3 kV and 960 µF (time constant = 11 ms and field strength 0.95 kV/cm), before being transferred to 4 ml of warm RPMI media, and cultured. Twenty-four hours later, cultures were collected, total cellular RNA isolated (Qiagen RNeasy), DNase treated (Ambion Turbo DNA-free^{TM}) and then 1 µg of RNA was converted to cDNA (BioRad iScript^{TM}). Next, $\sim$50 ng of cDNA and non-amplified RNA (control) were subjected to PCR to detect the ratio of luciferase mRNAs normalized to *GAPDH*. The PCR primers used were: Luc_F 5′ CCT GGA ACA ATT GCT TTT AC 3′ and Luc_R 5′ GTT TCA TAG CTT CTG CCA AC 3′, GAPDH_F 5′ CCA CCC ATG GCA AAT TCC 3′ and GAPDH_R 5′ TGG GAT TTC CAT TGA TGA CAA G 3′.

Chromatin immunoprecipitation assay (ChIP)

Previous work has shown that siRNA-mediated transcriptional gene silencing in human cells involves histone methylation, specifically H3K9me2 or H3K27me3, at the siRNA-targeted promoter (9,18,20). To determine whether the observed increase in transcription was the result of the asRNAs directing histone modifications associated with increased gene expression, we performed a ChIP assay specifically for H3K4me2. Briefly, 1G5 cells were co-transfected with 2.5 μg of the U6 expressing 21 bp asRNA and 2.5 μg of pTat-dsRed (31). Twenty-four hours later the cultures were collected and utilized in ChIP assays as described previously (18) using an antibody against H3K4me2 (Upstate #07-030, Lot#26355). The final ChIP elutes were assayed by qPCR (BioRad Syber Green™) using HIV-1 LTR-specific primers as follows: LTR_F 5′CAC ACA AGG CTA CTT CCC TGA 3′ and LTR_R 5′ GGC CAT GTG ATG AAA TGC TA 3′.

Microarray analysis

Microarray analysis was used to specifically determine the genes involved in the LTR-247as + 7-mediated increase in gene expression. 1G5 cells were transfected in triplicate with either 2.5 μg LTR-247as and 2.5 μg of pTat-dsRed or 2.5 μg GFPas and 2.5 μg pTat-dsRed. Twenty-four hours later genomic RNA was isolated and 1 μg/sample was labeled and hybridized to the Affymetrix Human HG-U133 Plus 2.0 array. This array includes probes for ~52 000 human probe-IDs. The Affymetrix chips were then scanned using the Affymetrix GeneChip Scanner 3000. Chips have a background of less than 50 intensity units and a GAPDH 3′/5′ ratio of less than 3. Next, Robust Multichip Average (RMA) was used to convert the intensity values to expression values (32,33). RMA consists of a three-step approach which uses a background correction on the Perfect Match (PM) probes, a quantile normalization and summarization of the probe set information by using Tukey's median polish algorithm. Present and absent calls were calculated in the R software package as implemented in the Affymetrix Microarray Suite version 5. This algorithm uses a Wilcoxon signed rank-based calculation to assign presence or absence of probe sets. Probe set were filtered out if they were assigned absent for all the samples, which left ~30 000 probes for further analysis. ANOVA was performed using BRB Array Tools, developed by Dr Richard Simon and Amy Peng Lam. BRB utilizes multivariate permutation tests to ensure that the number or proportion of false discoveries is controlled and is effective when the number of samples is at least three per treatment. The ANOVA analysis identified 185 human genes on the chip that were significantly altered at a *P*-value level of 0.05, which can be considered as false positives. Heatmaps were generated with Cluster and TreeView programs written by Michael Eisen (34). The microarray analysis was validated on the same cellular RNA collected and utilized for the microarray specifically for 4 of the 20 most distinctly deregulated genes by RT-PCR (Table S1) using primers NSBP1 F 5′ AGG CAC CAG CTT CTG AAA AA 3′ and NSBP1 R 5′ GCT GCC ACT GCT TCT TTC TT 3′, C10orf 76 F 5′ ATG GCC TGG ACC AGT ATG AG 3′ and C10orf76 R 5′CCT TGA GCA GGA CTT CTT GG 3, NFYB F 5′ GGA ATT GGT GGA GCA GTC AC 3′ and NFYB R 5′ TGT TGT TGA CCG TCT GTG GT 3′ with the above described RT PCR methodology.

Dual luciferase reporter assay

To evaluate the effects LTR-247as + 7 encoding plasmids on a reporter target, HEK293 cells were seeded 24 h prior to transfection at 1.2×10^5 cells per well in 24 well culture dishes. Cells were transfected with 150 ng of psiCheck2 target plasmid, 750 ng of pCR2.1-LTR-247as or pTZ-U6 + 1 (Mock) and 100 ng of pCI-eGFP. Forty-eight hours post-transfection, cells were lysed and luciferase determined according to the manufacturer's instructions (Promega, WI, USA) using a Veritas dual-injection luminometer (Turner Biosystems, C A, USA).

The siRNAs for C10orf76 targeting and for the RT primer assay

The C10orf76 siRNAs (C10-si1 5′ 5′ GCU GUG AAU CAC AUA UCC CAA 3′, C10-si2 5′ CCA GAG CTT TGA CAA CCT CAA 3′ and C10-si3 5′ CCT ACT TTG CTG GTT CCC TAA 3′) and the control AKT-19 5′ AAC ACC ATG GAC AGG GAG AGC 3′ were transfected (50 nM, Lipofectamine 2000) into 293-CCR5-GFP cells as described previously and 24 h later culture mRNA assessed for C10or76, *GFP*, *NFYB* and *NSBP1* expression, each relative to *GAPDH* expression by qRT PCR. To determine whether or not LTR247as + 7 can directly interact with C10orf76 the siRNA 247as + 7 or an oligo specific for C10orf76 mRNA or a negative control siRNA targeted to AKT19 were incubated with total DNase treated (Ambion TurboDNAse, TX) cellular RNA (293T cells) at 95°C for 5 min, room temperature cooled and converted to cDNA in the absence of a degenerate or poly A primer (Superscript III, Invitrogen, Carlsbad, CA). The converted cDNA was PCR amplified using C10orf76 gene-specific primers C10orf76 F and C10orf76 R. The miRNA RT Primer assay is based on work done by Vatolin *et al*. (35).

RESULTS

Selection of HIV-1 subtype B LTR targeted asRNAs

Previously we have demonstrated that only the antisense strand of the promoter-directed siRNA is required to initiate transcriptional silencing in human cells (18). Specifically, a U6 expressed 21 base asRNA targeted to site 247 (denoted as LTR-247as), which lies 205 bp upstream of the HIV-1 transcription start site, was capable of suppressing Tat-mediated transcription of HIV-1 LTR-luciferase expression in 1G5 cells (18). 1G5 cells possess an integrated LTR-driven luciferase reporter gene construct, which is responsive to the HIV transactivating protein Tat (22). In order to comprehensively characterize the suppressive efficacy of LTR-247as to the 247 target site, a series of U6 expressed asRNAs were generated to target the region surrounding site

247 (Figure 1a). Antisense RNAs of 22 nucleotides (nt) and 100 nt were generated to span site 247, and 21 nt asRNAs were constructed such that they are shifted 50 bases upstream and downstream in relation to LTR-247as. We hypothesized that site 247 (Figure 1a) is intrinsically unique with regards to siRNA and/or asRNA targeted TGS.

Screening of HIV-1 LTR targeted U6 expressed antisense RNAs

The asRNA expression plasmids (Figure 1a and b) were transiently co-transfected into 1G5 cells with a Tat expression plasmid, pTat-dsRed (23,24). To determine asRNA-mediated promoter suppression, 24 h later the cultures were collected and firefly luciferase (h*Luc*) mRNA relative to *GAPDH* was assessed. Interestingly, one of the U6 expressed asRNA constructs, LTR-247as + 7, which is shifted seven bases downstream relative to LTR-247as (Figure 1a), produced a significant increase in luciferase mRNA expression when compared to an asRNA targeted to the green fluorescent protein (GFP) open reading frame. LTR-247as and LTR^{c}-247as [targeted to the same sequence on HIV subtype C, isolate Du151 (accession DQ411851)], suppressed Tat-mediated activation of the LTR similarly to what had been shown previously (18) (Figure 2a and Supplemental Figure 1a). Moreover, the observed increase in luciferase expression did not appear to be the result of varied cell numbers or an increase in cell death and was also observed at 48 h post-transfection (Supplemental Figure 1b). Thus, to determine if the increased gene activation was independent of the activity of HIV-1 Tat, 1G5 cells were co-transfected with either LTR-247as + 7 and the HIV-1 Tat expression plasmid, pTat-dsRed, or LTR-247as + 7 and an irrelevant plasmid, pBluescript. There was a ~1.5-fold enhancement of LTR-mediated luciferase expression in the absence of HIV-1 Tat (Figure 2b), suggesting that the previously observed increase in LTR activity (Figure 2a and Supplemental Figure 1a and b) was specifically mediated by LTR-247as + 7. These observations were not exclusively related to integrated transgenes, since LTR-247as + 7 was additionally shown to modulate episomal activation (Supplemental Figure 2a and b). Activation of an episomal HIV-1 subtype B and subtype C LTR-expressed luciferase reporter was determined by transient co-transfections in HEK293 cells (Supplemental Figure 2a). Treatment with U6-expressed LTR-247as + 7 plasmids resulted in a significant increase in episomal gene expression in a Tat-independent manner (Supplemental Figure 2b), albeit to a lesser degree than when targeting LTR-247as + 7 to an integrated target.

The role of histone modifications in LTR-247as + 7 mediated increase in LTR-luciferase expression

Methylation of histone 3 at lysines 9 and 27 (H3K9me2 or H3K27me3) is observed in genes silenced by siRNAs targeted to their respective promoter regions in human cells (5,7–10,12,13,18,20), *Saccharomyces Pombe* and plants [reviewed in (36,37)]. While H3K9me2 and H3K27me3 correlates with transcriptionally silent genes

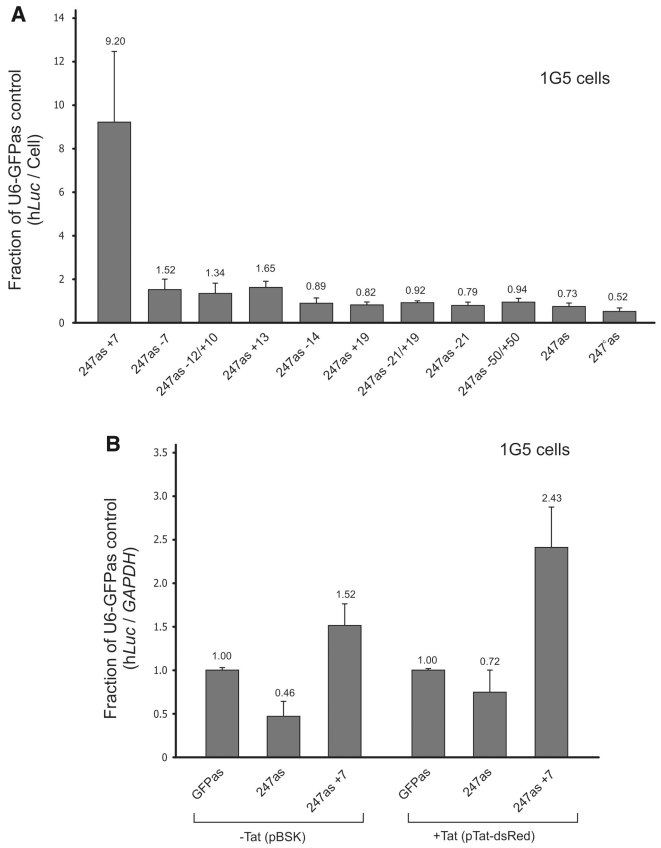

Figure 2. Effects of various U6 expressed antisense RNAs on luciferase expression. 1G5 cells containing an integrated HIV-1 subtype B LTR-Luciferase-SV40 poly A cassette were co-electroporated with pTat-dsRed and the respective LTR-247as vectors (2.5 μg of each plasmid respectively) and assessed for luciferase expression. (**A**) Relative luciferase expression standardized to *GAPDH* per cell presented relative to the control treated cultures (pU6 GFPas) as determined by real-time RT-PCR 24 h post-transfection. Data represent three independent experiments with the standard deviations shown. LTR-247as + 7-mediated increased transcription ($P = 0.06093$) relative to the control GFPas based on a single-sided *f*-test. (**B**) Non-specific LTR-247as + 7-mediated enhancement of luciferase expression. 1G5 cells (3×10^6) were co-transfected with either pBSK + or pTat-dsRed and either pU6 LTR-247as + 7, pU6 LTR-247as or the control pU6 GFPas. Twenty-four hours later cultures were collected, qRT-PCR was performed and luciferase expression determined and normalized to *GAPDH*. Results represent the mean ± standard deviations of three independent experiments.

(38), H3K4me2 associates with transcriptionally active genes (39). Recently, siRNAs have been shown to participate in the activation of genes via epigenetic modifications that include the acetylation and/or methylation of histone N-terminal residues (16,17). In light of this observation, we hypothesized that the increase in LTR-luciferase reporter gene expression observed with asRNA LTR-247as + 7 may be mediated by asRNA-directed H3K4me2. We assessed levels of enrichment of H3K4me2 at the targeted LTR 247 promoter site with asRNAs LTR-247as, LTR-247as + 7, LTR-247as-7 and LTR-247as + 13 by chromatin immunoprecipitation (ChIP). Relative to the suppressive effects of asRNA LTR-247as, which did not enrich for H3K4me2,

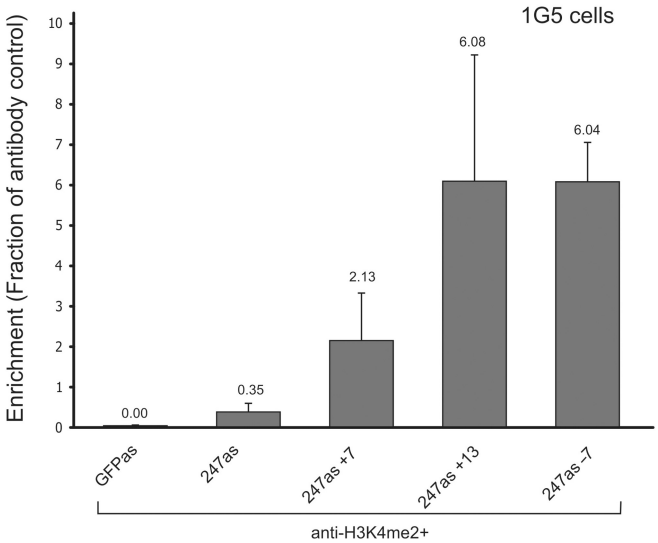

Figure 3. H3K4me2 in various asRNA-treated 1G5 cells. A total of 4×10^6 1G5 cells were co-transfected with pTatDSRed or one of the various U6 expressed asRNA constructs [pU6 GFPas (control), pU6 LTR-247as, pU6 LTR-247as + 7, pU6 LTR-247as + 13 and pU6 LTR-247as-7]. Twenty-four hours later the cultures were collected and ChIP analysis performed specifically for H3K4me2 as described (18). The relative enrichment following ChIP was determined by qPCR (SYBR green, BioRad^{TM}) with LTR-specific oligonucleotides (18). Data represent four independent experiments standardized to no antibody controls with the standard deviation shown.

LTR-247as + 7, LTR-247as-7 and LTR-247as + 13 were enriched for H3K4me2 in 1G5 cells (Figure 3). Although LTR-247as-7 and LTR-247as + 13 showed a 6-fold increased enrichment of H3K4me2 (relative to 2-fold enrichment by LTR-247as + 7), these asRNAs were only capable of marginal gene activation when compared to LTR-247as + 7 (Figure 2a, Supplemental Figure 1a and b). The presence of activating H3K4me2 marks may be a contributing factor in LTR-247as + 7-mediated gene activation. However, this alone is not sufficient to explain the marked activation observed relative to LTR-247as-7 and LTR-247as + 13.

Single changes in LTR-247as + 7 sequence can abrogate the LTR-targeted activation

To determine whether LTR-247as + 7 activation is sequence-specific, we generated two U6 expressed LTR-247as + 7 RNAs containing single mutations (LTR-247as + 7mA and mB) aimed at disrupting sequence-specific interactions between the asRNA and the target. Construct LTR-247as + 7mA includes nucleotide changes within the first seven bases, since this region acts as a 'seed' region defining typical microRNA target specificity (40) and also because this sequence discriminates LTR-247as + 7 from the suppressive asRNA LTR-247as. We additionally generated a sense sequence construct (LTR-247s + 7) as a control (Figure 4a). When these expression vectors were co-transfected into 1G5 cells with HIV-1 Tat, a single mutation (G to C) at position 2 in LTR-247as + 7 (mutant LTR-247as + 7mA) was enough to partially reverse the activation potential of LTR-247as + 7 in 1G5 cells (Figure 4a and b).

Microarray analysis of LTR-247as + 7-treated cells

We noticed that the observed increase in gene expression by LTR-247as + 7 was not unique to the LTR-luciferase target in 1G5 cells. Co-transfections with LTR-247as + 7 were performed in a cell line (HEK293-CCR5-GFP) that contains an integrated CCR5 promoter that constitutively expresses GFP (9). Similar to observations in 1G5 cells, the addition of LTR-247as + 7 resulted in an increase in CCR5-expressed GFP mRNA (Supplemental Figure 3). Yet, no corresponding homologous target site for LTR-247as + 7 exists within the CCR5 promoter, indicating a possible off-target effect induced by LTR-247as + 7. Moreover, an off-target increase in GFP expression was also observed in cells treated with a synthetic siRNA of LTR-247as + 7 (data not shown). To determine which genes may be involved in LTR-247as + 7-mediated activation, microarray analysis was performed on 1G5 cells treated with LTR-247as + 7 or the control GFP asRNA (GFPas). A total of 185 genes showed significant alterations in their expression profile when compared to control-treated cultures (Table S1), with RNA-associated proteins such as La, DEAD box proteins (polypeptide 50 and 26B), and a cold inducible RNA binding protein, standing out as being of interest (Table S1). Of the 185 deregulated genes, we focused on 23 genes, which could be involved (based on their annotation) in producing the observed off-target effect (Figure 5a). The microarray analysis was further validated by RT PCR for 3 of these 23 genes which confirmed the corresponding trend (Figure 5b). The 23 deregulated genes were screened for microRNA-like characteristics with homology to the LTR-247as + 7 sequence. Based on multiple positive matches using the online miRNA target-site search algorithms: RNA22 (41) and RNAhybrid (42) (Supplemental Figure 4), we decided to investigate the role of C10orf76, a suspected non-coding gene expressing a ~2730 nt transcript from chromosome 10 of unknown function (accession no. NM_024541).

The effect of C10orf76 on 247as + 7-mediated gene activation

To assess if C10orf76 functions to modulate the suppression of genes that were deregulated in the microarray study, we targeted C10orf76 with three different siRNAs (Figure 6a). Interestingly, when C10orf76 was suppressed, increased expression of NSBP1, NFYB and CCR5-expressed GFP was observed that was inversely proportional to the suppression of C10orf76 by RNAi (Figure 6b). These results matched the increase seen in the microarray data for NSBP1, NFYB and CCR5-expressed GFP following treatment with LTR-247as + 7 (compare Figures 5b and 6b).

To determine if any of the putative predicted LTR-247as + 7 targeted sites on C10orf76 were susceptible to LTR-247as + 7-mediated inhibition, HEK293 cells were co-transfected with LTR-247as + 7 and various psiCheck dual-luciferase reporter constructs, each containing individual C10orf76 target sequences for LTR-247as + 7 in the

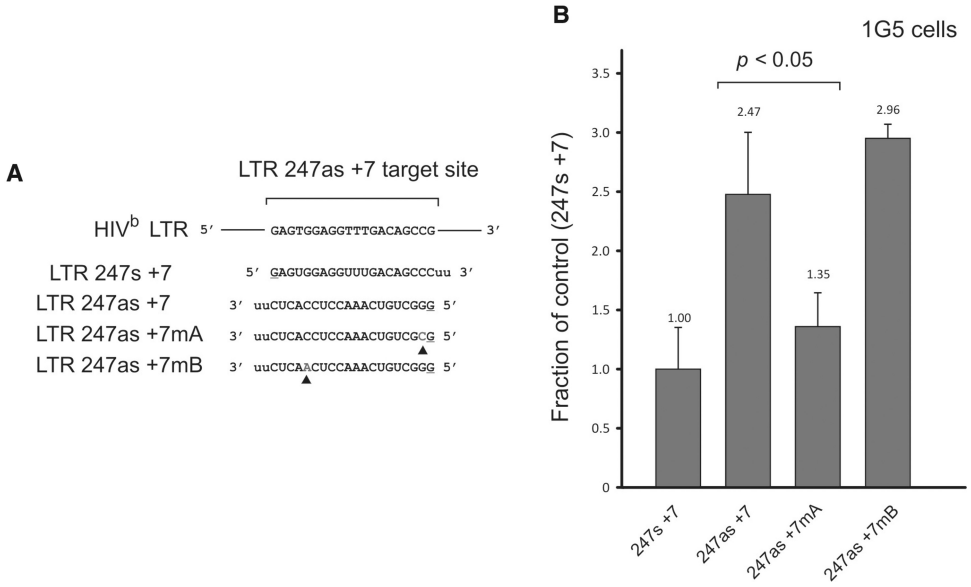

Figure 4. Characterization of LTR-247as+7 asRNA mutants. A single mutation in LTR-247as+7 at nucleotide 7 abrogates off-target gene activation in 1G5 cells. (**A**) Cultures were co-transfected with the various U6 expressed LTR-247as+7 RNAs containing different point mutations, along with HIV-1 Tat. *luciferase*/*GAPDH* expression was measured by qRT PCR 24 h later. (**B**) Triplicate transfected 1G5 cultures are shown with the respective ranges. A Student's *t*-test was performed using the web-based tool http://home.clara.net/sisa/t-test.htm and statistical significance was determined for $P < 0.05$.

3′ UTR of *Renilla* luciferase (Figure 6c and Supplemental Figure 4). Relative to a complete LTR-247as+7 target as a control, 2 of the 5 putative C10orf76 target sites tested were susceptible to LTR-247as+7, suggesting that C10orf76 is being directly suppressed via LTR-247as+7-mediated post-transcriptional inhibition (Figure 6d). Moreover, the C10orf76 transcript could be selectively reverse-transcribed using the priming of LTR-247as+7 siRNA (Figure 6e). No alteration in suppression was observed when Argonaut 2 (Ago2) was inhibited by an anti-Ago2 siRNA (Supplemental Figure 5), indicating that LTR-247as+7 is likely inhibiting C10orf76 through an antisense mechanism. However, we cannot completely rule-out a role for the RNAi-related pathway in suppressing C10orf76. Taken together these data suggest that off-target inhibition of C10orf76 expression is predominantly responsible for the observed gene activation.

DISCUSSION

We previously identified a TGS-susceptible region, site 247, which when targeted by an asRNA (LTR-247as), was capable of suppressing a HIV-1 subtype B LTR promoter in the presence of the transactivator Tat (18). It was unexpected that a 22 nt asRNA sequence targeted only seven bases downstream of site 247 (LTR-247as+7), and still largely overlapping with the suppressor LTR-247as, showed the opposite effect of increased gene activation. Short dsRNAs of ~22 bp targeted to non-coding regulatory regions in gene promoters have been recently shown to induce potent gene activation, thus adding to the diversity of functions for short RNAs in gene regulation (16,17). However, since a mechanistic explanation

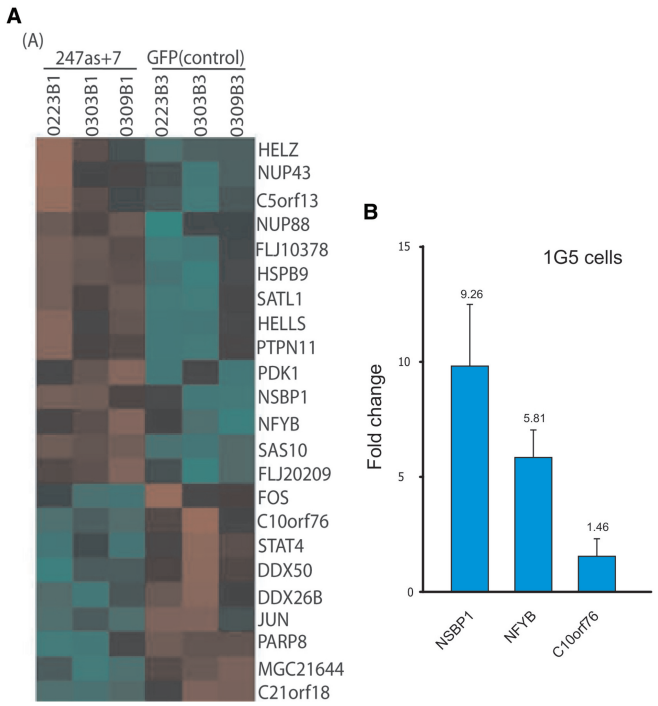

Figure 5. Microarray analysis of 23 deregulated genes. (**A**) A heatmap was produced depicting the variation in gene expression in triplicate treated LTR-247as+7 relative to the corresponding expression profile for the control GFPas treated 1G5 cells. (**B**) The microarray analysis was utilized to validate the increase in expression from 3 of the 23 candidate genes by real-time RT PCR analysis. Results represent the mean ± standard deviations of three independent transfection experiments.

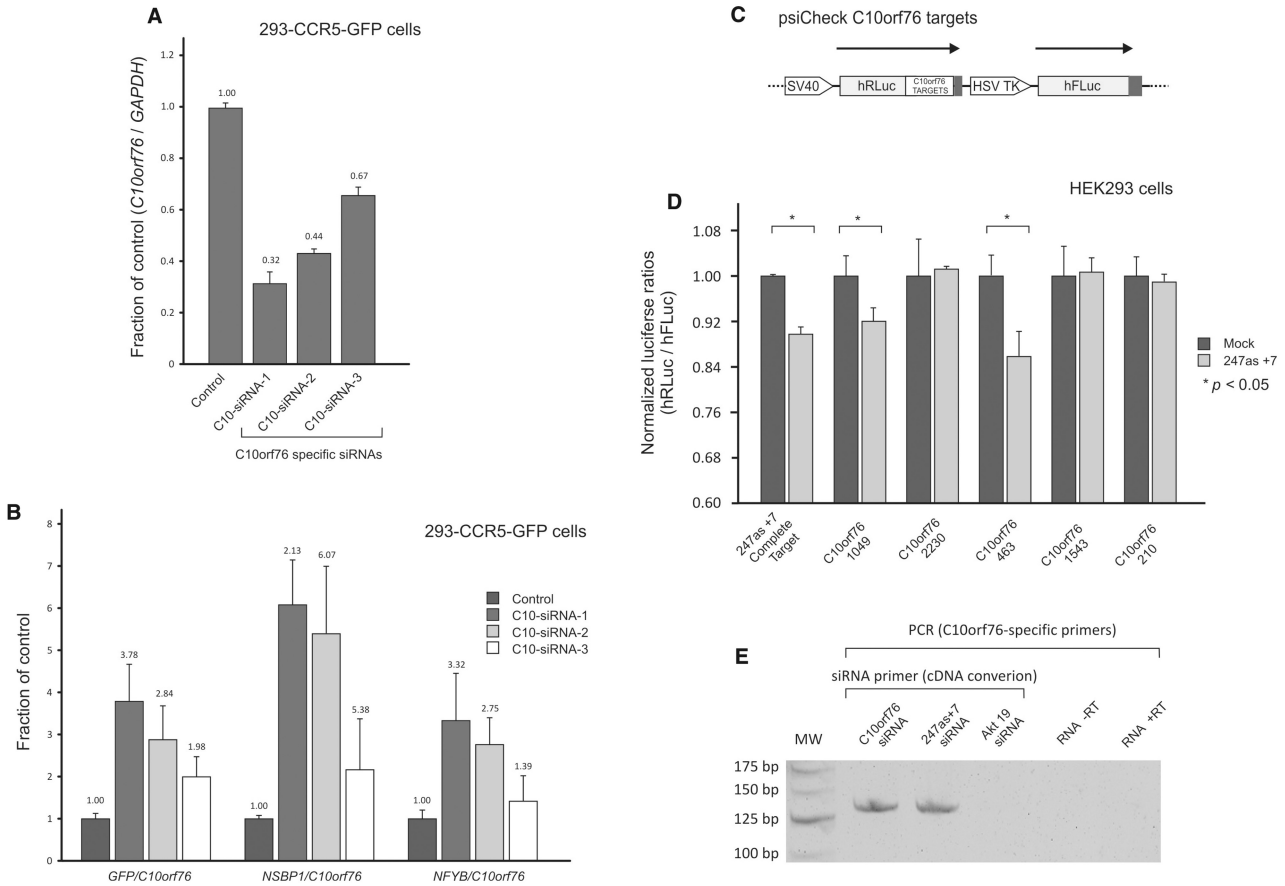

Figure 6. The effect of C10orf76 on 247as + 7-mediated gene activation. (**A**) Suppression of C10orf76 RNA by siRNAs. 293-CCR5-GFP cells were transfected with C10orf76-specific siRNAs (50 nM) in triplicate and the standard deviations are shown. (**B**) Suppression of C10orf76 is inversely proportional to gene activation of GFP, NSBP1 and NFYB. 293-R5-GFP cells. Results represent the mean ± standard deviations of three independent transfection experiments. (**C**) The complete LTR-247as + 7 target site and putative LTR-247as + 7 target sites within C10orf76 were inserted downstream of a *Renilla* luciferase 3′ UTR within the psiCheck vector. (**D**) Knockdown efficiency of LTR-247as + 7 was determined against each of the C10orf76 targets. Values are normalized to the ratio of *Renilla* to firefly luciferase activity in the Mock transfected cells and are presented as the mean ± SEM of three independent experiments. A single-tailed Student's *t*-test was used to determine statistical significance (where shown, for $P < 0.05$). (**E**) The LTR-247as + 7 siRNA is capable of reverse-priming C10orf76 mRNA. Cellular RNA from transfected HEK293T cells was converted to cDNA using siRNAs as the template primer for cDNA conversion (44). The siRNAs targeted to C10orf76 (C10-siRNA-1), LTR-247as + 7 (247as + 7) and targeted to Akt-19 (AKT-19) as well as control RNA in the absence of RT (RNA-RT) or RNA in the presence of RT (RNA + RT) were converted to cDNA and PCR was performed using C10orf76-specific oligos for an expected band ~136 bp.

for RNA activation remains to be elucidated, we were unsure whether this phenomenon represented a unique pathway, or formed part of an existing framework of post-transcriptional inhibitory effects caused by antisense RNAs or RNAi effector sequences. A number of mechanisms exist whereby RNAs can modulate gene activity. For example, gene activation has been observed for 20 bp dsRNAs containing neuron restrictive silencer element (*NRSE*/*RE1*) sequences. *NRSE* dsRNA activates expression of *NRSE*/*RE1*-containing genes by recognizing the NRSF/REST transcriptional regulator to stimulate neural stem cell differentiation (43).

The gene activation observed for LTR-247as + 7 is likely due to a sequence-specific off-target effect that is ubiquitously observed when using RNAi and antisense effector sequences for post-transcriptional inhibition (44), and explains why ~185 genes were deregulated by LTR-247as + 7 (Table S1). The data presented here suggests that a complex cascade exists which possibly includes several of the genes which have been shown to be deregulated by LTR-247as + 7. However, we have focused our attention on a gene of unknown function, C10orf76, which was downregulated by LTR-247as + 7. The link between C10orf76 and LTR247as + 7 was determined via an *in silico* analysis of putative microRNA-like binding sites, two of which were established to be susceptible to post-transcriptional inhibition by LTR-247as + 7.

Even though we ascribe the observed RNA-mediated gene activation to an off-target effect, some of the experimental criteria used by Li (16) and Janowski (17) in establishing the validity of RNA-mediated gene activation may also be true for off-target effects. These include the sequence specificity and length characteristics of the targeting asRNA or dsRNA. First, no notable increase in gene expression was observed with LTR-247as-50/ + 50, which includes the LTR-247as + 7 sequence, indicating that the LTR-247as + 7 sequence motif alone is not a determinant for off-target gene activation.

Moreover, a mutant version of LTR-247as + 7, with 'seed' region mismatches, can abrogate activation, similarly to what was observed by Li *et al.* (16). Since sequence specificity may not be a determinant for ruling out off-target or RNA–protein interactions, more stringent criteria may be necessary for determining sequence-dependent effects induced by RNA when targeted to promoter sequences. As was shown for antisense RNAs that induce TGS (18), direct association of effector RNAs at the targeted promoter would be of value, although this was attempted without success by Janowski *et al.*, for Ago1 and Ago2 (17). Secondly, RNA activation has been associated with the removal of silencing histone methylation marks and/or addition of activating acetylation and methylation marks (16,17). While we show some increase H3K4me2 elicited by LTR-247as + 7, even greater enrichment was elicited by upstream and downstream overlapping asRNAs, which do not show potent gene activation. It is possible that the off-target inhibition of a repressor may function to induce active chromatin. Our data suggest that showing active or suppressive chromatin marks are largely correlative, and do not provide mechanistic basis for the underlying result. Lastly, both Li and Janowski use siRNAs and not antisense RNAs to induce gene activation. Li *et al.* (16) show that the inhibition of Ago2 negatively affects the ability of siRNAs to inhibit RNAa. Taken together with the inability to localize Ago2 to the activating promoter (17), the data are consistent with the possibility that siRNAs are inadvertently inhibiting a transcriptional regulator. In addition, most studies that target siRNAs for TGS make use of very high concentrations of siRNAs (>30 nM) in order to direct nuclear localization of the siRNAs. However to date, much lower concentrations were used to elicit RNA-mediated gene activation [1 nM for Li *et al.* (16) and 12 nM for Janowski *et al.* (17)].

Even though the data presented here point to an off-target mechanism for RNA activation, previous reported observations of this phenomenon (17) do show some intriguing differences which warrant further explanation. Importantly, both studies by Li and Janwoski show that RNA activation activity lasts longer than traditional knockdown times established for siRNAs (16,17). This effect is difficult to explain using an antisense- or siRNA-based post-transcriptional inhibition hypothesis. Moreover, Janowski *et al.* (17) do show that peptide nucleic acid (PNA) mimics of their activating dsRNAs do not induce gene activation and that inactive siRNAs with overlapping sequences abrogate activating siRNAs when added sequentially. These latter experiments either suggest a direct link between the activating siRNA and the targeted promoter sequence or that Argonaut 2 is simply swamped out by the addition of competitive small RNAs for RISC.

Overall, our findings indicate that targeting promoter regions with short RNAs can have unintended effects, especially if there exists partial homology to transcripts in the treated cell. The targeting of promoter DNA sequences with RNAs has recently been described as a novel sequence-specific mechanism for activating downstream elements. Here we show that indiscriminate cell-wide gene activation can be elicited by off-target downregulation of a suppressor gene. Just as off-target effects mask the efficacy of siRNAs for post-transcriptional gene knockdown, caution should be taken when RNAs (either siRNAs and asRNAs) are generated to target promoter regions with the intention of inducing specific RNA-mediated transcriptional modulation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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Conflict of interest statement. None declared.

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