of the small-molecule switch. Although the current work is at the level of a proof of concept, the chemical regulation of gene expression, combined with the ability to conduct high-throughput screening of embryos, may open a unique systematic functional genomic platform for investigating general vertebrate physiology and disease.

COMPETING INTERESTS STATEMENT
The author declares that he has no competing financial interests.

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Transcriptional activation by small RNA duplexes

John J Rossi

Short double-stranded RNA duplexes are the triggers for post-transcriptional gene silencing and can also induce epigenetic silencing of genes at the level of transcription. A surprising new finding is that short RNA duplexes targeted to promoter regions can also mediate potent enhancement of transcription.

RNA interference (RNAi) was first described as a post-transcriptional gene silencing process triggered by small double-stranded RNAs 21 to 28 nucleotides in length[1]. Initial reports found a role for these small RNAs in DNA methylation in plants[2,3], with subsequent experiments in *Schizosaccharomyces pombe* and *Drosophila melanogaster* providing further proof of the generality of this phenomenon and additional mechanistic insight into the process with the identification of RNAi components and methylated histones[4–6]. Recent results indicate that synthetic small interfering RNAs (siRNAs) are also able to mediate gene silencing in human cells[7–11]. Two new papers now demonstrate that synthetic antigene RNAs (agRNAs) can also potently activate gene expression[12,13] in human cancer cell lines. The two papers, by Li *et al.*[12] and Janowski *et al.*[13], explore the process of RNAa, or activating RNA, and demonstrate increases in gene expression of 10- to 20-fold for the studied genes. Taken together, these two studies reveal important new observations about the chromatin-remodeling potential of small, promoter-directed RNAs.

RNAi is mediated by key RNA binding and processing proteins[1]. One of these proteins, called Dicer, is an RNase that processes double-stranded RNA precursors into shorter 19- to 21-base duplexes with 2-base overhangs at the 3′ termini. The processed products of Dicer are referred to as siRNAs or microRNAs (miRNAs) depending on their functional role of either directing sequence-specific cleavage of a target mRNA (siRNAs) or inhibiting translation (miRNAs). For both classes of RNA, one of the two duplex strands is selected as the guide strand and is bound by one of the phylogenetically conserved Argonaute (Ago) family protein members. In humans, only Argonaute 2 has RNase activity, and it uses the short antisense RNA to guide cleavage of complementary sequences in targeted mRNAs, thereby resulting in destruction of the mRNA. The functional roles of other members of the mammalian Ago family are poorly understood. Dicer and various Ago family proteins are essential for heterochromatin remodeling in *S. pombe*, *D. melanogaster* and *Arabidopsis thaliana*[4–6], and recent reports of mammalian gene silencing demonstrate additional roles for Ago1 and Ago2 (refs. 7,11).

The discovery that small double-stranded RNAs structured like siRNAs can also activate gene expression raises some exciting and challenging new questions about the mechanistic differences between silencing and activation[12,13]. Li *et al.*[12] and Janowski *et al.*[13] (the latter in this issue) were initially studying small RNA–directed gene silencing when they serendipitously discovered that small RNA duplexes can trigger as much as 10- to 20-fold activation of transcription of a diverse set of genes. These findings add to the gene regulatory mechanisms that short RNA duplexes can trigger in mammalian cells. A primary question is whether both mechanisms rely on the RNAi machinery. Previous reports of mammalian gene silencing with agRNAs have demonstrated the involvement of the RNAi components Ago1 and Ago2 in this process[7,11]. In contrast, the two studies of RNAa have somewhat contradictory findings with regards to the role of RNAi machinery. Li *et al.* used siRNAs to deplete Ago1, Ago2, Ago3 and Ago4 from their cell line and found that depletion of Ago2 abrogates RNAa. In contrast, Janowski *et al.* performed anti-Ago1 and anti-Ago2 chromatin immunoprecipitation (ChIP) assays in extracts prepared from cells treated with one of the activating double-stranded RNAs, but they found no enrichment of either Ago protein at the double-stranded RNA target site. Thus, it remains to be seen whether other Ago family proteins have a role in this process (**Fig. 1**).

What about the methylation status of histones in the agRNA-directed silencing versus activation pathways? It had previously been shown that agRNA-directed transcriptional gene silencing is accompanied by dimethylation of Lys9 in histone H3 (H3K9)[7,9], which is consistent with the known state of H3 methylation in heterochromatin[14]. Li *et al.* observed that RNAa is accompanied by demethylation of H3K9, and Jankowski *et al.* demonstrated increased di- and trimethylation of H3K4. In general, increased transcriptional activity is accompanied by di- and trimethylation of H3K4 (ref. 2). Thus, both of these results are consistent with the status of H3 lysine methylation for active versus inactive chromatin[14]. In agreement with these observations, Li *et al.* further showed that agRNA targeting of epigenetically silenced promoters does not result in transcriptional activation.

Is there a position dependence within the promoter region that results in silencing versus activation? The differences between RNAa and silencing relative to the positioning of the agRNAs within the respective promoter-region targets are very intriguing. Janowski *et al.* found that single-base differences in the positioning of the agRNA within the progesterone receptor promoter can lead to either activation or repression of transcription, and they were not able to

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find a general consensus of a silencing versus activation site. They carried out extensive target-site analyses for RNAa in two different promoters (the progesterone receptor and major vault protein) and observed little correlation of the relative positions of the most effective agRNA targets. The most effective agRNA targeting the progesterone receptor promoter spanned the transcriptional initiation site from position –11 to position +8, whereas the most effective agRNA for the major vault promoter spanned positions –54 to –36 relative to the transcriptional start site. Moreover, shifting the effective progesterone receptor agRNA by a single nucleotide completely abrogated RNAa, a result similar to what is often observed for siRNAs in post-transcriptional gene silencing. In the studies by Li *et al.*, the most effective RNAa targets were far upstream of the transcriptional initiation sites of the E-cadherin, p21 and vascular endothelial growth factor promoters they studied, and the major difference they observed between activation and silencing targets was the lack of CpG islands in the former, and their inclusion in the latter. Thus, it remains to be seen whether the optimal binding site varies with each new gene or whether rules can be developed to describe what sites are prone to RNAa upregulation.

The combined transcriptional silencing and activation studies of Janowski *et al.* show that a single-base difference in the positioning of the agRNAs can activate or inhibit transcription. In the RNAa study they also showed that inactive agRNAs that overlap an active agRNA binding site can inhibit activation in a competitive cotransfection assay, but once an active agRNA is bound it cannot be competed with by overlapping inactive agRNAs. These results suggest that the transcriptional inhibitors and activators bind to the same target. What, then, differentiates the activation from the inhibition properties of these agRNAs? There are obviously some significant experimental questions that need to be addressed to achieve a better understanding of the mechanisms underlying agRNA-directed RNAa and gene silencing.

It is apparent that small double-stranded RNAs can trigger both silencing and activation of transcription. But what are the molecular targets for these events—DNA, promoter transcripts or transcription factors—and do they differ in silencing versus activation? An earlier study found that a naturally occurring 20-base-pair RNA duplex with complementarity to the neuron restrictive silencer element (NRSE; also known as RE1) is required to activate neuron-specific transcription through a potential interaction with the transcription factor NRSF (neural restrictive silencing factor; also referred to as REST) (ref. 15). Could such a mechanism underlie RNAa as well? For silencing, one report suggests that only one of the two agRNA strands is required (the one complementary to the sense orientation of the target-gene mRNA), and active transcription of the gene (perhaps a promoter-specific transcript) is also a requirement[10]. Future studies of RNAa should address the actual target for the agRNAs, as this will be important for the future design of agRNAs.

Finally, the mechanisms of transcriptional gene silencing and RNAa have both been proposed as possible therapeutic modalities for the treatment of disease[7,8,11–13]. The agRNA-mediated activation and silencing events seem to be transient in cell culture, perhaps owing to dilution of the agRNAs during cell proliferation. It is of great importance to test these events *in vivo* by targeting nondividing tissue to determine the duration of silencing or activation. Once we have a better understanding of the mechanisms involved in these two opposing pathways, it may be possible to simultaneously silence one gene and activate another for therapeutic purposes. Of course, many of the concerns that apply to systemic delivery of siRNAs will also apply to agRNA therapies, including efficiency of delivery, off-targeting and induction of interferon response pathways. Perhaps the most important take-home message from the silencing and activation studies is that we are on the tip of the iceberg with respect to our understanding of the multiple roles small RNAs can have in regulating gene expression. It seems that some of the most exciting times still lie ahead.

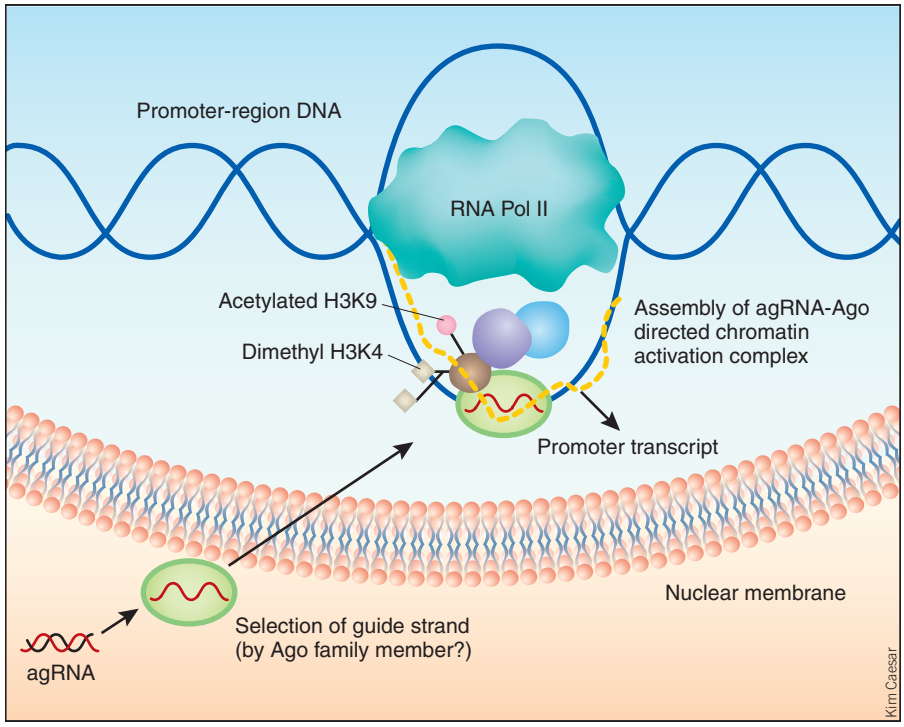

Figure 1 Hypothetical model for agRNA-triggered RNAa. The agRNAs are transfected into the cytoplasm, and one of the strands is selected as a guide strand by an Ago family member (oval). The complex moves across the nuclear membrane to the nucleus, and the guide strand (red) pairs with a nascent promoter transcript (yellow dashes), which results in recruitment of histone remodeling enzymes (blue and purple circles). Histone H3 (brown circle) Lys9 becomes acetylated (pink circle) and H3K4 is dimethylated (gray diamonds). The remodeling of the histones activates transcription from the agRNA-targeted promoter.

COMPETING INTERESTS STATEMENT
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