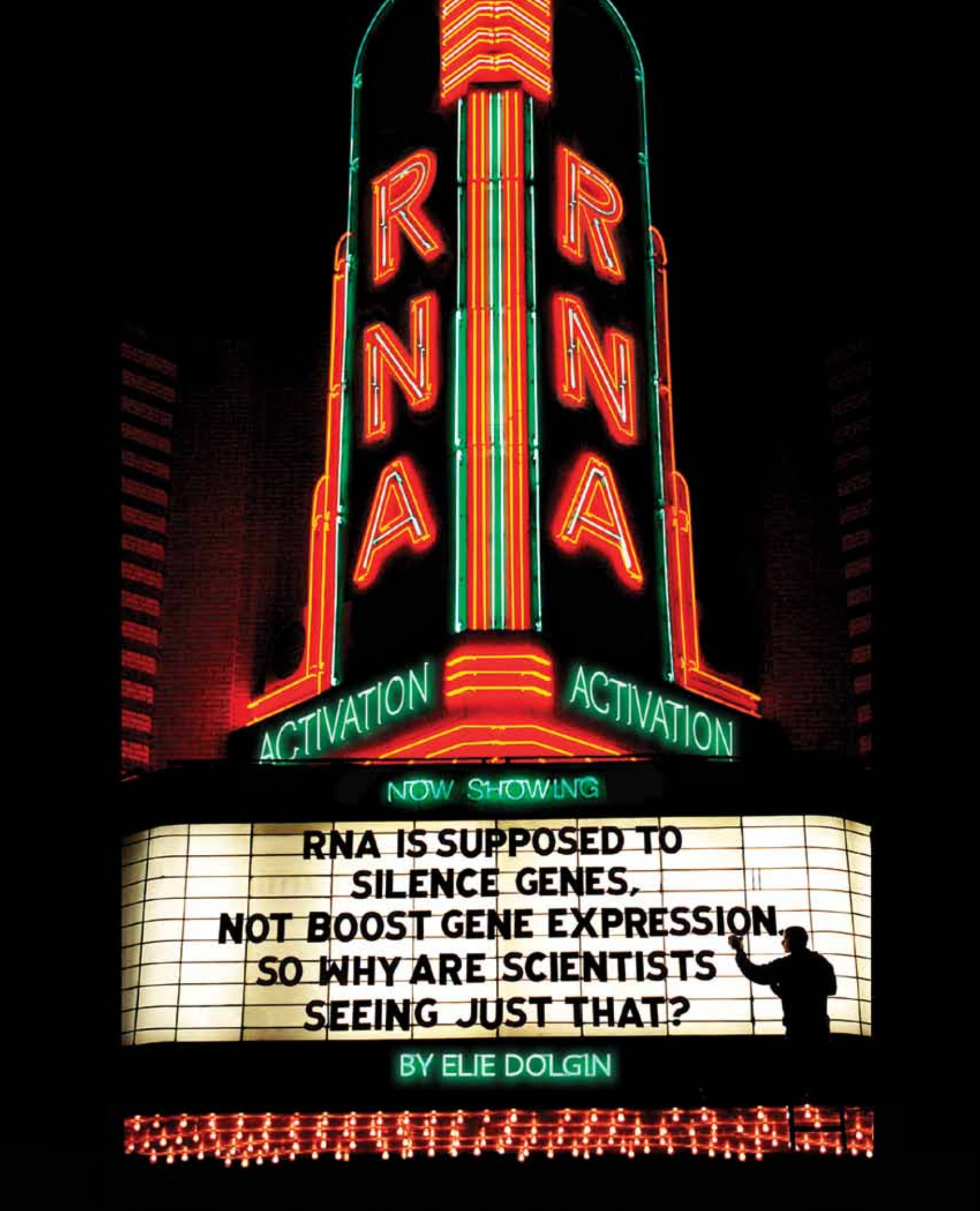
RNA
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NOW SHOWING
RNA IS SUPPOSED TO
SILENCE GENES,
NOT BOOST GENE EXPRESSION.
SO WHY ARE SCIENTISTS
SEEING JUST THAT?
BY ELIE DOLGIN

After getting the data back from the very first experiment at her new job, Rosalyn Ram, a lab technician at the University of Texas Southwestern Medical Center at Dallas, was convinced she had messed something up. The results were decidedly "weird," she recalls. Her lab heads, the husband-and-wife research duo David Corey and Bethany Janowski, had already shown that synthetic DNA molecules with protein-like backbones, known as peptide nucleic acids, could block gene transcription. And as a long shot, in October 2004 they had tasked the new lab tech with trying to do the same with small RNA molecules, fully expecting it not to work.

But it did work: Like the peptide nucleic acids, the RNAs targeted to the same promoter also silenced gene expression at the level of transcription. "When [Ram] saw the silencing, she thought she had done something wrong," says Janowski. "She didn't want to show me the data because she thought it was supposed to be a negative result."

The data just didn't make sense: Single-stranded peptide nucleic acids bind directly to unwound DNA at the transcription start site, and double-stranded RNAs were thought only to target messenger RNA (mRNA) to prevent translation—a well characterized process known as RNA interference, or RNAi. So how could they both be causing the same effect? With this one finding, "all those things that you thought you could predict just flew out the window," Janowski says.

"It took months before we convinced ourselves that the results were real," says Corey. But eventually they did. They tested several different double-stranded RNAs—each 21 nucleotides long, just like standard, small interfering RNAs used in RNAi. All of these RNAs perfectly matched regions of the DNA promoter but had little to no overlap with the gene's mRNA, to ensure the RNAs were acting on transcription, not translation. Ultimately, in September 2005 Corey and Janowski showed that introduced RNAs could inhibit transcription by as much as 90%.[1] Interestingly, the findings confirmed work by Kevin Morris, now at the Scripps Research Institute in La Jolla, Calif., who had published the first evidence of this phenomenon in human cells a year earlier.

But Corey and Janowski also noticed something in their data that, if correct, would be even more unbelievable than what Ram saw on her first day at the bench. A few of Corey and Janowski's seemingly "inactive" RNAs that did not reduce gene expression reproducibly enhanced transcription by around 25% to 50%. Such relatively small changes weren't enough to say definitively that the researchers had observed gene activation, "but it planted the seeds in our minds that maybe activation could be occurring," says Corey. To investigate the trend further, the researchers switched to a cell line with much lower background activity levels of their gene of interest, the human progesterone receptor, and the data became glaringly obvious: The same RNAs that did not inhibit transcription could now trigger as much as 10- to 20-fold increases in gene expression. The activation effect was real.

"RNA activation went against the grain of everything we knew about how small RNAs regulate gene expression."
—John Rossi

Before they could publish the findings, however, another lab published results showing essentially the same effect.[2] A team led by Long-Cheng Li and Robert Place at the University of California, San Francisco, discovered that small RNAs targeting the DNA promoters of three other genes could also switch on gene transcription. Like Corey and Janowski, Li and Place were expecting inactivation, not the opposite. "We were surprised that we didn't observe gene silencing by the double-stranded RNA," says Li. "Instead, we observed strong gene activation."

RNA activation flew in the face of everyone's perceived wisdom regarding RNA-based regulation. RNA was thought to silence genes only by cutting up mRNA via the RNAi pathway, not at the point of gene transcription and definitely not through activation. As such, many of the big names in the RNA world dismissed the findings out of hand.

"It was so hard to get this work published," says Janowski. "It was like a pitchfork coming out. The RNAi community was so hostile."

Both groups had trouble publishing their papers. Corey and Janowski's work was rejected by *Science* before it was published in *Nature Chemical Biology* in January 2007,[3] while Li and Place battled for two years and faced four rejections before they finally published their paper in the *Proceedings of the National Academy of Sciences* in November 2006.[2] So, when Corey and Janowski's paper came out two months later showing the same basic observation, "it was kind of a relief," Li says. "It felt good that someone else also saw a phenomenon that was similar to ours," adds Place.

However, neither group offered a plausible mechanism for how RNA activation might occur. "I didn't think that the evidence that [Li and Place] had really supported the mechanism they were reporting," says John Rossi, a molecular geneticist at City of Hope Comprehensive Cancer Center in Duarte, Calif., who was a reviewer for the paper. "It went against the grain of everything we knew about how small RNAs regulate gene expression. The data were clear, but were they looking at something indirect? We wanted more experiments to validate the mechanism and they [Li and Place] never did them."

Nonreviewers alike were unconvinced. "I have a hard time to explain the findings, ▶

and what mechanism can explain it," says Thomas Tuschl, an RNA expert at New York's Rockefeller University, in an email. "It seems so idiosyncratic and special at the moment," adds Timothy Nilsen, an RNAi researcher at Case Western Reserve University in Cleveland, Ohio. "It's just not a generalizable phenomenon like RNAi. With RNAi, you know it's going to work."

The concerns are valid. Introducing nucleic acids into cells is a notoriously artifact-prone experiment, so the observed effects could easily be due to the introduced RNA interacting with nontarget molecules, such as unintended proteins or RNA that in turn cause the activation, the skeptics argued.

Another part of the problem was that unlike RNAi, in which the introduced RNA always perfectly matches the mRNA targets, RNA activation did not seem to follow any predictable set of rules. The researchers constructed the activating RNAs to match stretches of the promoter DNA, but couldn't devise a reliable design scheme: Single-base differences in the RNA's target sequence could turn an activator into a repressor and vice versa, they found. What's more, the most effective activator RNAs for some genes matched DNA sequences right at the transcriptional start site, but for others the target site was way upstream.

The RNA community adopted a wait-and-see attitude. "Whenever some controversial findings like these ones emerge, I will not touch the topic and I wait for follow-up by the labs that brought the topic up," says Tuschl. Despite the wide-ranging skepticism, the investigators kept pursuing the mechanism behind RNA activation while battling to publish their activation studies. In back-to-back papers in *Nature Structural & Molecular Biology* published in September 2006, Corey's group and a team led by Morris and Rossi implicated proteins from the Argonaute family in small RNA-mediated transcriptional silencing. Then two months later, Li and Place also showed that Argonaute was involved in activation. Although there was some disagreement about which Argonaute proteins were most important, the researchers were on to something.

Argonaute proteins—known members of the RNAi pathway—help facilitiate RNA-RNA interactions. Thus, the newfound connection between transcriptional regulation and Argonaute proteins indicated that RNA activation's target was naturally occurring RNA, not DNA, as was the case for peptide nucleic acids (and which the researchers had naively assumed would be true of activating RNAs, too). Morris then tested this idea directly and confirmed that RNAs were, indeed, interacting with each other.[4]

All together, "it established the link between transcriptional and post-transcriptional gene silencing," says Janowski. Maybe, they reasoned, this wasn't such a new and implausible phenomenon after all. But the question still remained: How could their introduced RNAs influence transcription if they were only interacting with other RNAs?

"One can be picky about the mechanism, but it's early days. What's clear is that RNA activation exists."
—John Mattick

While the scientists searched for a mechanism, it was becoming increasingly evident that the vast majority of the human genome is transcribed into RNA, even if only a sliver of these RNAs are translated into proteins. Some of these noncoding RNA transcripts—so-called because they don't produce proteins—were even located in regions of the genome that code for proteins. What's more, much of this noncoding transcription takes place on both strands of chromosomes, both in the same direction as mRNA transcription as well as in the opposite—or "antisense"—orientation. These ubiquitous noncoding RNAs probably served some purpose, perhaps even in RNA activation, the scientists reasoned.

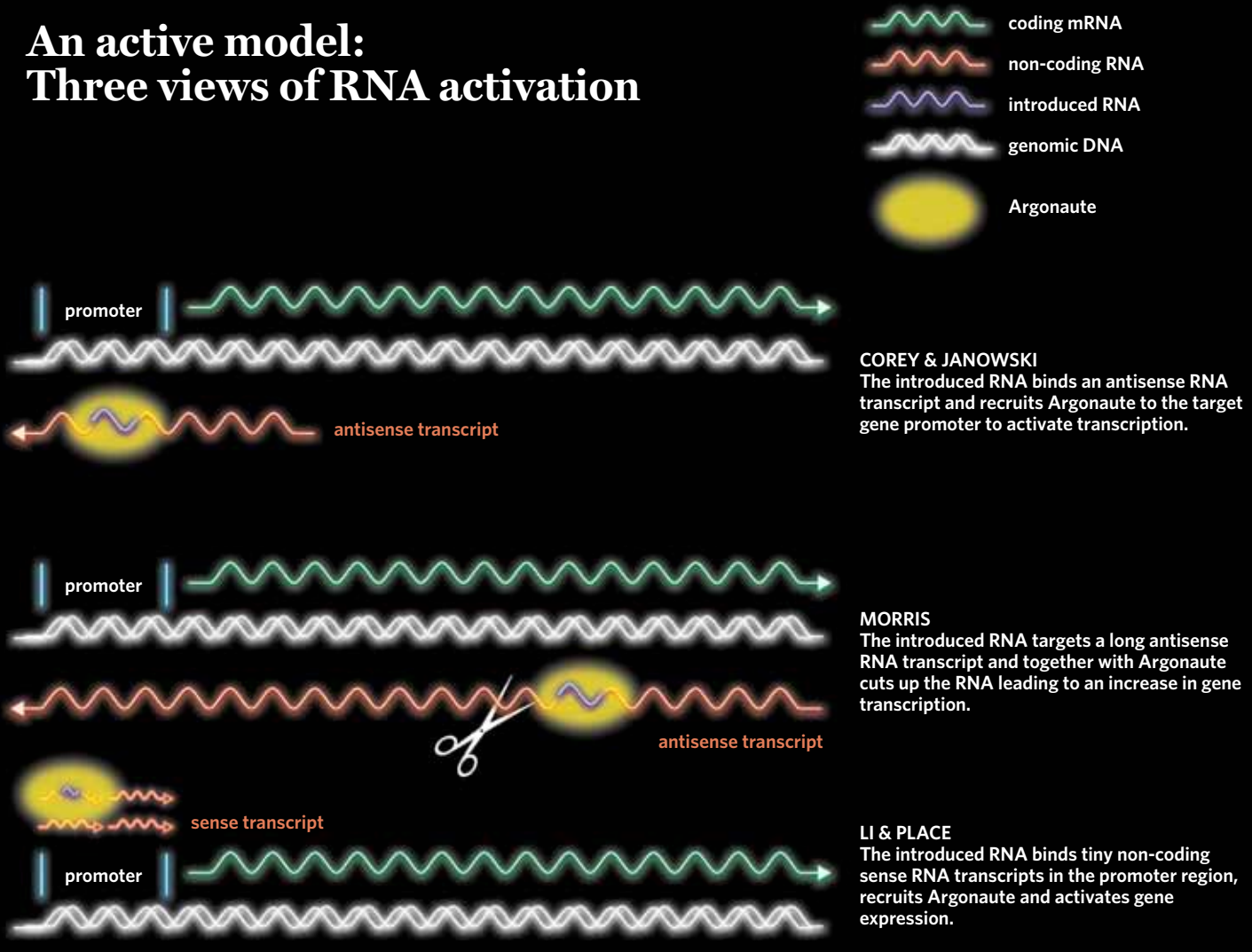
Using reverse transcription–PCR, Corey's grad student Jacob Schwartz scanned for noncoding RNAs around the progesterone receptor promoter. He couldn't find any RNA coded in the direction of transcription, but did discover three antisense transcripts spanning the promoter region. At first, however, Schwartz didn't believe his own results. Schwartz's "first year graduate course perspective of transcription" led him to believe that there wasn't going to be any RNA transcripts in the promoter region, he says, so he thought he was just seeing genomic DNA contamination. For two weeks, he tried "every which way" to get rid of the bands in his PCR before he convinced himself otherwise. In the end, Schwartz showed that activating RNAs, together with Argonaute, bound to these antisense transcripts in the vicinity of the promoter. This RNA-protein complex then acted as a scaffold to recruit and redirect other protein modifiers to either crank up or slow down transcription.[5]

"You really have to think of these promoters as very dynamic," explains Corey. "Genes are poised to either be turned on or off." In his view, antisense transcripts act as the regulators of this delicate balance. So by introducing RNAs that interacted with these RNA gatekeepers, his team tipped the balance toward activation or silencing. "I may be wrong, but it's the simplest explanation that's consistent with the data that we have," Corey says.

Notably, Corey proposes that the phenomenon is somewhat distinct from RNAi—even though Argonaute proteins are involved in both processes—because he never observes the slicing and dicing of any RNA transcripts. What's more, his data suggest that transcriptional activation and silencing both operate in fundamentally the same way, through interactions with antisense transcripts.

Morris, who showed that small RNAs could inhibit transcription a

An active model: Three views of RNA activation



year before Corey and Janowski, has an alternative explanation for the mechanism, however. He reanalyzed the activating RNAs originally used by Li and Place, and concluded that they didn't target the DNA promoter, as the UCSF researchers had supposed (but never shown). Rather, the activating RNAs matched sections of long antisense RNA transcripts that spanned the promoters, though he found the target sequences actually lay several hundred base pairs downstream of the transcriptional start sites, and further down from the sites Corey and Janowski located.

These antisense RNAs normally recruit proteins that limit transcription. But the introduced RNAs, with the help of Argonaute, cut up the antisense transcripts, thereby releasing the suppressive factors, and producing an overall increase in mRNA transcription.[6] In his model, the phenomenon of RNA activation would not be all that different from traditional RNAi, only the target is antisense RNA instead of mRNA transcripts. "It's a ying-yang sort of thing," says Morris.

The flipside—transcription silencing—does not work the same way, however, Morris believes. Turning a gene off involves introducing RNA—either double-stranded or just the single antisense strand—to target rare extensions of the mRNA transcript that span the promoter region and are transcribed but are not ultimately translated.[4] Altering these mRNA extensions remodels the DNA's configuration, blocking the RNA polymerase enzyme from doing its normal job of gene transcription.

Morris's ideas for RNA activation "seem perfectly reasonable," says Place, "but his model doesn't explain all of our data." Li and Place created a visually trackable system by combining their promoters ▶

A natural turn-on

Tiny snippets of genetic code called microRNAs (miRNAs) regularly whir around the cell, fine-tuning gene expression. More than 400 miRNAs have been identified in the human genome, and each one can regulate hundreds of different genes, says David Bartel, an RNA biologist at the Whitehead Institute in Cambridge, Mass. Like most noncoding RNAs, these small, cellular supervisors were long thought only to repress genes by interfering with messenger RNA and reducing protein production. But recent research shows that miRNAs are much more versatile.

Last year, UCSF's Robert Place and Long-Cheng Li followed up on their initial observation of RNA activation by scanning the gene promoter region for natural miRNA target sites. They found one matching the miRNA-373 and showed that this endogenous RNA could significantly upregulate transcription in the same way as synthetic RNA (*Proc Natl Acad Sci*, 105:1608–13, 2008). "MicroRNAs had only been considered to suppress gene activity," says Place. This study was "proof of principle that microRNAs can have the opposite effect and can lead to gene activation."

Other researchers are also seeing the same effects. For example, Raghu Vemuganti, a neuroscientist at the University of Wisconsin–Madison, profiled miRNAs in rat brains and discovered that several appeared to activate transcription (*J Cereb Blood Flow Metab*, 29:675–87, 2009). "Bioinformatics-wise, I can already say confidently that this is happening in the brain," says Vemuganti. "Experimentally, we still need to prove that."

These weren't the first examples of naturally occurring RNAs that could get transcription revved up, however. In 2004, Fred Gage, a neuroscientist at the Salk Institute in La Jolla, Calif., found a small, double-stranded RNA in rat neural stem cells that interacted with proteins to activate genes important for neuron function (*Cell*, 116:779–93, 2004). "There's lots and lots of RNA that exists in the nucleus," says Gage. "Now we think of it as junk or background noise, but it may not be. There's enormous room here for mischief given the fact that there's this plethora of RNA that's there."

What's more, endogenous miRNAs can also stimulate gene expression at the post-transcriptional level, after the mRNA has been produced. Yale University molecular biologists Joan Steitz and Shobha Vasudevan discovered that miRNAs can switch from turning protein production on and off depending on the activity of the cell as a whole: miRNAs repressed translation in proliferating or cycling cells, but activated translation in quiescent cells, they found (*Science*, 318:1931–34, 2007).

The same basic silencing components—including Argonaute and RNA binding proteins—were involved in both translational activation and repression, suggesting, yet again, that gene activation and gene silencing might simply be two sides of the same coin. "Maybe the associations are different, but the basic Argonautes and the basic microRNAs are the same in activation and repression," says Vasudevan. "It looks like microRNA can do a lot of other things and the mechanism is still mysterious."

of interest with the green-fluorescence protein gene. This allowed them to quantify levels of transcription and pinpoint the target site for RNA activation. After mutating the promoter DNA and looking at changes in the intensity of the green glow, they showed that their activating RNAs were homing in on the promoter region itself, similar to what Corey and Janowski propose, and not a downstream target, as Morris argues. However, Place believes that his activating RNAs are actually targeting tiny, noncoding RNA transcripts in the promoter region that code in the same direction as mRNA, not antisense RNA. "Our activating RNAs are not targeting antisense transcripts and we're getting activation," Place says. "That offsets their [Corey and Morris's] models right away."

Thus, Li and Place are working with a third, as yet unpublished explanation for RNA activation. And they're making the most of it. "We've designed a pretty standard set of rules for acquiring activating, double-stranded RNA that we can now apply to any promoter that we want," Place says. To date, his team has activated around 15 to 20 genes, often with several different RNAs that all work for the same gene, he adds.

Importantly, however, all three models of transcriptional activation share one key feature in common. They all zero in on the same central target of transcriptional regulation: noncoding RNA. Last December, a quartet of papers appeared in *Science* showing that more than 90% of the genome is transcribed—forward, backward, and all around—and that the promoters of active genes are not, in effect, "quiet." That means there's a lot of RNA to go around, possibly with many different hidden regulatory effects.

"It would be shortsighted to think that there's only one pathway to anything we're seeing; redundancy is built into the system," says Morris. On this point, all the researchers can agree. "It looks like there are many forms by which you can induce RNA activation," says Place. In the meantime, even without an agreed-upon explanation, the phenomenon is real, says Corey. "People can criticize our

work for opening up more questions than it answers, but another way of looking at that is that it's a good thing."

All of the researchers are now actively pursuing therapeutic and cellular applications of transcriptional activation. Li and Place designed RNAs that upregulate tumor suppressor genes in a mouse model containing human xenographed prostate cancer cells; Morris ramped up HIV's expression in hopes of drawing out hidden reservoirs of the virus so it can be attacked therapeutically; and Corey and Janowski activated a "significant metabolic gene" in cell culture using RNA (Corey declines to elaborate, as the results are unpublished and have commercial potential). In addition, Place and Morris say they have each activated several pluripotency-associated genes, such as Oct4 and Nanog, with an eye to reprogramming adult cells to an embryonic-like state.

Morris is now working together with the Scripps tech-transfer office to commercialize his approaches, while Corey and Place's teams both licensed their technologies to Alnylam Pharmaceuticals, a Cambridge, Mass.–based company specializing in RNAi-based therapeutics. "It's a very logical extension for us, but obviously it could represent a whole new platform," says Alnylam's chief executive John Maraganore. "It's still evolving in terms of how reliable and predictive the observations are, but there certainly are a number of gene targets we've been able to activate."

With a few working models to toss around, some researchers in the RNA community have now warmed up to the idea of RNA activation. "At face value, there's a very interesting phenomenon going on here," says John Mattick, an RNA genomicist at the University of Queensland, Australia. "One can be picky about the mechanism, but it's early days. What's clear is that [RNA activation] exists."

"I don't think there's as much of a controversy as people make it out to be," says Rossi. He argues that the phenomenon is simply an extension of classic, vanilla-flavored RNAi, which, as Morris points out, was also controversial when it was first discovered. RNA activation "is just like post-transcriptional gene silencing," says Rossi, only the introduced RNA targets noncoding RNA (in the case of activation) instead of mRNA (in the case of interference, or RNAi). Erik Sontheimer, an RNA researcher at Northwestern University in Evanston, Ill., agrees. "In terms of what small RNAs are actually doing, it's not all that fundamentally new—it's still RNAi," he says.

Corey, too, now concedes the point. "When we first started doing this people didn't know about these noncoding RNA transcripts, and now they do," he says. "So it's just another branch of the RNAi pathway. The RNAi pathway has many fingers reaching into many pies."

Still, not everyone is convinced. "The whole effect of RNA on transcription in mammals is viewed with a bit of skepticism by the whole field," says Nilsen. Tuschl is another one of the holdouts: "It needs more thorough biochemistry, and more genes to be targeted before I get sold on it." Thomas Gingeras, head of functional genomics at Cold Spring Harbor Laboratory, says that he's heard "around the bar" of a few other labs that have tried to repeat the activation experiments, but with only mixed success. "If what Morris and Corey have reported is a genuine phenomenon, then people would have seen this and people would have jumped on this very hard," he says. "Clearly there's something that's either missing or wrong in that model as they've done it, and until a mechanism or some missing element is identified, people will just wait." (Several other leading RNAi researchers declined to comment for this article.)

Corey says that he's pretty fed up with the naysayers who won't believe his findings until he's worked out every last detail of the mechanism. "I'm running out of patience with people who think that way; I think they're being hypocritical," he says. "If they can't provide an alternative explanation then they need to get in and do the experiments." Morris, whose lab counts only three people, including himself, says he would gladly welcome the competition, given how much untapped biology is left to explore. RNAi and post-transcriptional regulation "was just the beginning," he says. "There are so many more levels of complexity involved with [RNA-mediated transcriptional regulation]. The whole RNA world is far more complex than we even know." ■

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"People can criticize our work for opening up more questions than it answers, but another way of looking at that is that it's a good thing."
—David Corey

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