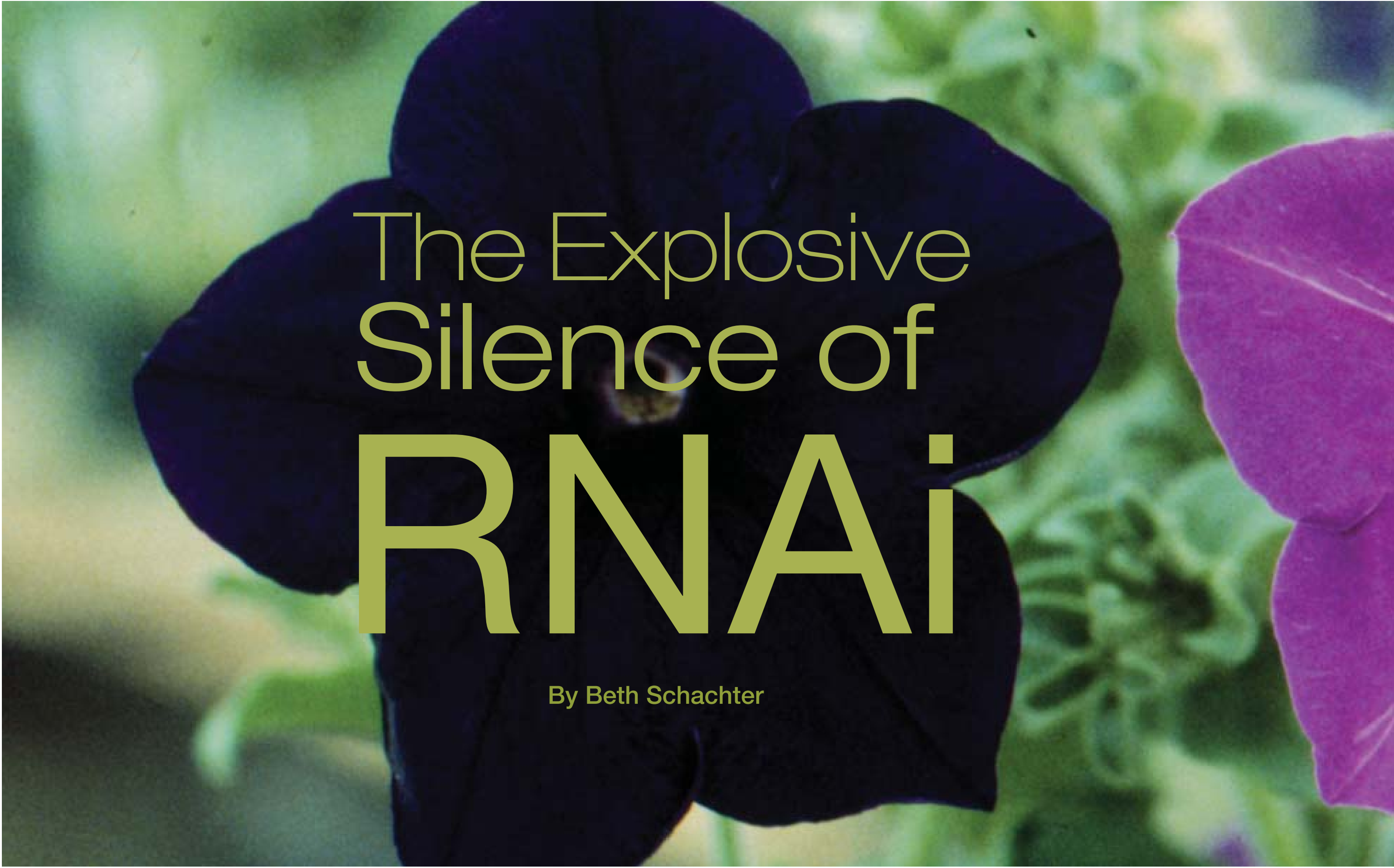

The Explosive Silence of RNAi

By Beth Schachter

When researchers come upon an unexpected but fundamental biological mechanism, it can define a scientific era. Consider the period between 1975 and 1985, when scientists were discovering oncogenes—genes in animal genomes that, when mutated or dysregulated, encode proteins that can cause tumor development. This startling revelation sparked new insights into the regulation of cell proliferation and pointed to new strategies for treating cancers.

We are now experiencing another such period of dramatic breakthroughs, this time with the focus on a suite of biological processes collectively called RNA interference (RNAi). Only recently have scientists started to understand this ancient and vital regulatory mechanism for selectively turning down gene expression.

The discovery of RNAi garnered two of its pioneers, **Craig Mello** (University of Massachusetts) and **Andrew Fire** (Stanford), the 2006 Nobel Prize in Physiology or Medicine. That award came just eight years after the pair published their landmark paper in the February 19, 1998 issue of *Nature*, identifying double-stranded RNA (dsRNA) as a key component of the gene-silencing process in worms. (By comparison, the Nobel Prize awarded to **James Watson** and **Francis Crick**, for their discovery of the double helical structure of DNA, came nine years after they published their findings in the April 25, 1953 issue of *Nature*.)

Very quickly, RNAi has become not just a new textbook topic but also a powerful strategy for studying gene function; or, as plant geneticist **David Baulcombe** (Sainsbury Laboratory) put it, "God's gift to 21st century molecular biology." RNAi is also a promising platform for a whole new type of therapeutic drug.

A SUITE OF REGULATORY MECHANISMS

RNAi refers to a set of molecular mechanisms in which tiny RNA snippets prevent the expression of specific genes. Most, if not all, eukaryotes use one or more RNAi pathways, implying that RNAi arose in an ancient common ancestor. Most frequent-

Figure 1. The lavender petunia was derived by silencing the gene that converts the reddish-blue pigment to deep purple. USED BY PERMISSION © CSIRO PUBLISHING

A Brief History of the Basic and Applied Science of Antisense Oligonucleotides

The notion that antisense RNA or DNA could silence genes post-transcriptionally (i.e., block mRNA translation into protein) came from 1978 reports by **Paul Zamecnik** and **Mary Stephenson** (Harvard). Their work showed that small antisense DNA, when added to a cell extract, bound specifically to its complement on mRNA in the extract and selectively inhibited translation of that mRNA. Extending that finding, researchers showed that antisense oligodeoxynucleotides (ASOs) could sometimes block mRNA function in living cells and even in intact organisms.

During the 1980s, ASOs captured the imagination of many scientists: Some aimed to use the synthetic ASOs as tools for knocking down expression of their genes of choice, to study their functions. Other investigators tried to design and develop ASO drugs. Indeed, the recent RNAi "applications revolution" had its conceptual origins in ASO research. A major hurdle has been that ASOs rarely worked well. As RNAi researchers like to mention, RNAi strategies are robust and specific precisely because applied RNAi exploits an endogenous cellular mechanism, something ASOs do not do.

ly, the RNAi mechanism involves either blocking the translation of specific messenger RNAs (post-transcriptional gene silencing; PTGS) or preventing transcription of specific regions of DNA into RNA (transcriptional gene silencing; TGS).

What biological function does RNAi play? Answers are starting to emerge. One RNAi pathway governs distinct aspects of development, tissue-specific differentiation, and maintenance of differentiated functions in both animals and plants. This regulatory process uses microRNAs, single-stranded RNAs that can form double-stranded hairpinlike structures. (See Fig. 2, right side.) The hairpinned microRNAs are converted enzymatically into short dsRNAs that are part of the ribonucleoprotein machinery for silencing messenger RNAs (mRNAs). MicroRNAs may also be part of evolutionary mechanisms that promote or maintain differences between species.

A different RNAi pathway seems to be part of an antiviral defense mechanism and a means of stabilizing transposable elements within genomes. This pathway, in which long dsRNAs become converted to small interfering RNAs (siRNAs), occurs in plants and in some invertebrate animals. (See Fig. 2, left side.)

DISCOVERING A NEW MECHANISM

Mello and Fire were not alone in seeing that dsRNA triggers PTGS. In 1998, **Peter Waterhouse** (CSIRO, Canberra), studying plants and using a different experimental strategy, reported the same conclusion. Indeed, as **Tom Tuschl** (Rockefeller University), another RNAi leader, noted, the similar findings about dsRNA silencing mRNAs in worms and plants "propelled the field forward," reinforcing the notion that these results might be pointing to a fundamental regulatory mechanism.

Soon after its discovery, RNAi became an invaluable laboratory tool. Researchers now use commercial or homemade libraries of double-stranded siRNAs to silence the expression of any gene they choose. This greatly speeds up their ability to determine the function of individual genes and to dissect complex biochemical pathways.

Much of the current excitement surrounding RNAi stems from the anticipation that a new class of medicines will emerge from it. New siRNA therapeutic companies have sprung to life and some existing firms have morphed into RNAi-based entities. Already, a few have moved products into clinical trials. The speed of these developments has prompted pharmaceutical giants to invest heavily in RNAi; last year Novartis put $700M into the RNAi-based company called Alnylam. Recently Merck spent $1.1B to buy the company aptly called Sirna.

The New York Academy of Sciences has been a forum for sharing work in the RNAi field. Leaders in the field or members of their groups have presented new findings at the quarterly RNAi discussion group meetings, organized by Tuschl and colleagues, and at the Academy-sponsored Oligonucleotide Therapeutics Society meetings.

A COUNTERINTUITIVE WORLD

After Fire and Mello described dsRNA's role in worms as a necessary silencing

agent, Baulcombe and **Andrew Hamilton** showed that uniformly short RNAs, 25 nucleotides long, facilitated PTGS in plants. Baulcombe recently recounted how Hamilton, his postdoc, hunted for antisense RNAs in the RNA-mediated silencing process they were studying in a flowering plant. (mRNA, because it encodes proteins, is the "sense strand" molecule. Nucleotide strings—either RNA or DNA—that carry the sequence complementary to the sense strands are "antisense" molecules.)

According to Baulcombe, Hamilton screened the moderately sized RNAs for antisense molecules that might be silencing agents. Failing to find any, he inspected the tiny RNAs, which were thought to be nonspecific waste products. Here he found discrete and specific antisense RNAs, complementary to the mRNAs that were silenced in their experimental systems. Controls, in which the same genes were not silenced, lacked the tiny antisense RNAs. Curiously, the preparations that had the antisense RNA snippets also had complementary 25-nucleotide sense strand RNAs. Seeing the Fire/Mello paper, Baulcombe reports that "the scales fell from my eyes" upon realizing that PTGS in plants, as in worms, might be triggered by a small sense-antisense RNA silencing.

THE PREVIOUSLY PURPLE PETUNIA SAGA

Baulcombe's studies had their roots in **Richard Jorgensen**'s now famous "previously purple petunia" findings. In the early 1990s, Jorgensen (University of Arizona) wanted to produce petunias with a rich, dark purple color, using a transgenic strategy that over-expressed a key enzyme involved in coloration. However, instead of making darker flowers, his transgenic lines silenced the enzyme's expression, bleaching rather than deepening the petal color. As a follow-up, Jorgensen found that this silencing blocked neither transcription of the transgene nor of its endogenous counterpart. Instead, it acted post-transcriptionally; that is, on the mRNA. But the mechanism of this PTGS remained elusive until the studies by Fire and Mello, and then Baulcombe and Hamilton, gave clues to the puzzle.

MAKING SENSE OF THE ANTISENSE/SENSE STRAND PUZZLE

What is the RNAi mechanism, which involves a double-stranded silencing trigger, that interacts with, and consequently silences, the target mRNA? Tuschl and **Philip Zamore** (University of Massachusetts) took on the challenge of addressing that question. At that time, Tuschl was a postdoctoral fellow with **Phillip Sharp** (MIT) and **David Bartel** (Whitehead Institute) and Zamore was a postdoc in Bartel's group. Tuschl recalls that he was initially puzzled by the Fire/Mello finding. "It sounded so mysterious and unbelievable." How might this double-stranded RNA trigger be disassembled, and the sense strand discarded so that the antisense could find its complement in mRNA and take the latter out of commission?

Years before, Andy Fire had worked in the Sharp lab and was known to be careful about confirming his work before publishing it. Therefore, as Tuschl tells it, Sharp could make the necessary intellectual leap, seeing that his former student was accumulating evidence pointing to a naturally occurring genetic regulatory mechanism.

Sharp kept mentioning these findings, trying to encourage someone in the lab to undertake biochemical experiments that might uncover the silencing mechanisms. "That's how Phillip Sharp directed people in his laboratory," Tuschl recalls, "never coming into the lab to tell someone what to do, but bringing up an observation and waiting until someone took it on for themselves." After half a year of hearing Sharp bring up RNAi, Tuschl opted to investigate the topic.

Zamore had developed an in vitro biochemical assay, using a *Drosophila* embryo extract, which seemed suitable for a test tube experiment because by then it was known that flies had an RNAi mechanism. (As experienced scientists will confirm, the life or death of one's research may depend on choosing a good experimental system to investigate the question at hand.)

Tuschl and Zamore decided to collaborate, and in a series of publications, they showed that they could recapitulate RNAi in a test tube. From these studies, they deduced that long dsRNA is enzymatically cleaved to short dsRNA products that become part of the mRNA silencing machinery. (See Fig. 2.) A key paper from the Tuschl lab showed that the short dsRNAs, but not the antisense or the sense strand alone, were needed to cleave the target mRNA in the fly embryo extract. Thus, the dsRNA (at that point dubbed siRNA) is a necessary intermediate in the RNAi pathway.

ENTER THE MICRORNAS

In parallel, **Victor Ambros** (Dartmouth) was studying PTGS in worms. In 1993, he described a regulatory gene involved in the developmental timing of certain differentiation events. Curiously, this "gene" didn't encode a protein. Instead, it produced a 22-nucleotide, non-coding RNA. Ambros and **Gary Ruvkun** (Harvard) together found that this RNA—now called a microRNA–used an antisense silencing mechanism that involved base-pairing with a specific mRNA. Then, Ruvkun found another 22-nucleotide worm microRNA, which governed a different developmental event through PTGS. In a key finding, Ruvkun showed that his microRNA was perfectly conserved among a wide range of different animal species; this finding supported the idea that it was ancient.

Each of these microRNAs that Ambros and Ruvkun described came from a longer RNA transcript with a nucleotide sequence that could form a "hairpin" structure, a double-stranded RNA connected by a loop at one end. However, the self-complementarity was imperfect. Similarly, the pairing between these microRNAs and complements in the target RNAs was imperfect. Therefore, the researchers at first did not make the connection between the microRNAs and double-stranded RNA, muses Ambros.

Considering Ruvkun's findings alongside those of Hamilton and Baulcombe, Ambros finally had what he calls his "holy

on the web

Much more information is available on the Academy's RNAi channel, www.nyas.org/rnai and in the eBriefings for the annual meetings of the Oligonucleotide Therapeutics Society. The eBriefing for the first meeting, featuring presentations by John Rossi and Tom Tuschl, can be found at www.nyas.org/ots, the second at www.nyas.org/ots2006.

You may also find these eBriefings of particular interest:

- Mandatory Morphogens at www.nyas.org/morphogens
- Running Interference: Tactics for a Gene Silencing Strategy at www.nyas.org/running
- Many Facets of RNAi Machinery: Structural & Mechanistic Insights at www.nyas.org/machinery

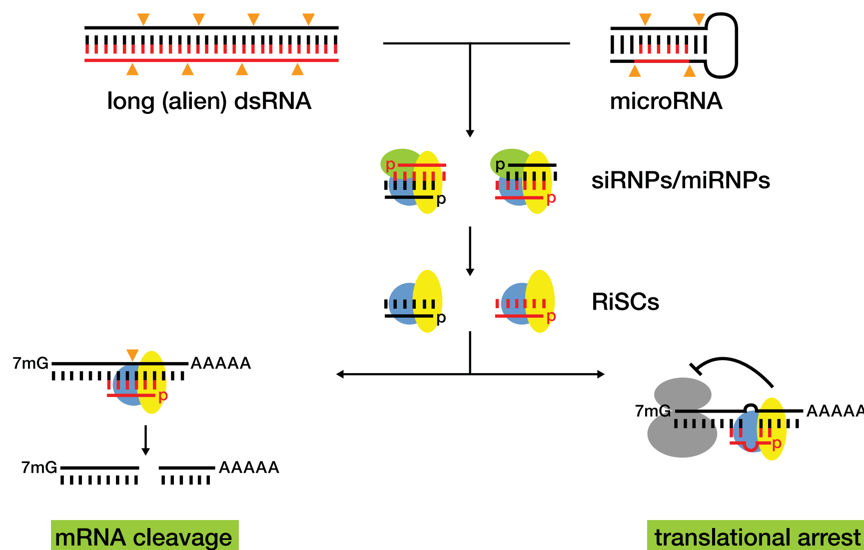

Figure 2. Mechanisms of dsRNA-mediated post-transcriptional gene silencing.
ADAPTED AND USED BY PERMISSION FROM TOM TUSCHL

cow!" experience: "This is the same deeply conserved phenomenon!" he realized. "Something has constrained the siRNAs and the microRNAs to almost the same length." This realization led Ambros's group to identify (simultaneously with the Bartel and Tuschl labs) many small, non-coding RNAs with regulatory functions in worms, flies and human cells. We now know that eukaryotic genomes each carry hundreds of microRNA genes.

MULTIPLE RNAI-MEDIATED PTSG PATHWAYS

Subsequent investigation has shown that siRNAs and microRNAs are generated by similar cellular machinery. Two parallel schemes, using similar enzymes (called Dicer and RNAse III Drosha) have evolved—Dicer for processing long dsRNAs into siRNAs, and both Dicer and Drosha for converting hairpinned microRNA precursors into siRNA-like duplexes. (See Fig. 2.) In both cases, the short RNA duplex intermediate is part of a ribonucleoprotein (RNP) complex called RNA Interference Silencing Complex (RISC), which includes a factor that separates the two RNA strands, discarding one. The RNA remaining in RISC becomes the guide for this RISC "silencing machine", by base-pairing with an mRNA carrying a complementary sequence. So, indeed, RNAi does act via a sense-antisense mechanism after all!

The siRNAs and the microRNAs differ in how they silence the target mRNAs. (See Fig.2, bottom half.) The siRNA-derived antisense RNAs act on perfectly complementary sequences. An enzyme in the RISC complex (called Argonaute) cleaves the target mRNA, irreversibly silencing it. By contrast, the antisense component of microRNAs interacts with a partially complementary sequence within a target mRNA. Proteins in this complex prevent translation of the target mRNA by mechanisms that are still under investigation. What is clear is that in eukaryotes, microRNAs mediate silencing of many mRNAs (perhaps 1/3 of them).

MAGIC BULLETS FOR STUDYING GENE FUNCTION

The discovery of RNAi also revealed that living cells can take up short dsRNAs. Once inside, dsRNAs enter the RNAi pathway, and consequently can silence any mRNA an experimenter aims at. That finding prompted scientists to use synthetic siRNAs as research tools, to explore the function of genes by intentionally silencing them.

SiRNAs turned out to work better, and more consistently at selectively silencing the intended target mRNA, than did the synthetic, single-stranded antisense oligodeoxynucleotides (ASOs), which people had struggled with through the 1980s and 1990s. (See sidebar.) The reason? The synthetic siRNAs, which mimicked the naturally occurring ones, became incorporated into cellular RISC. The proteins in RISC presumably stabilize the interaction between the antisense siRNA and the mRNA target. By contrast, ASOs, which do not become part of RISC, may form relatively weak interactions with their target mRNAs and therefore may not be very potent silencers of those mRNAs.

By the turn of this century, scientists knew enough about natural RNAi mechanisms to develop industrial-scale siRNA tools. The speed with which siRNA suppliers appeared, and put their products to use shows that researchers were primed for them. They had experiments designed and ready to go from the failed ASO studies. Moreover, the confusion that surrounded ASO had helped some scientists develop the skepticism needed for doing well-controlled studies. Currently, large annual meetings focus entirely on strategies for using siRNAs for functional analysis and target validation.

A NEW PLATFORM FOR THERAPEUTIC DRUG DEVELOPMENT

The therapeutic application of RNAi-based compounds is also progressing. Here too, the industry builds on the foundation laid while attempting ASO drug development. As with the ASO compounds, delivery presents a major hurdle for broad-scale siRNA drug development. One sees evidence of that in the choice of siRNA drugs at the front of the development pipeline—drugs targeting diseases such as macular degeneration and respiratory infections, which have anatomically accessible targets. But, as **John Rossi** (City of Hope, and the first president of the Oligonucleotide Therapeutics Society) explained, because siRNA drugs are so robustly effective once inside cells, drug delivery experts are motivated to develop effective and safe methods for distributing them to all different parts of the body.

Thus, in a very short period of time, scientists have uncovered a new regulatory mechanism, used their new knowledge to make powerful investigatory tools and are well on their way to developing an entirely new class of drugs based on this discovery. When the Nobel Prize to Fire and Mello was announced, some people seemed surprised at how quickly this award came. But, a more common question was, "When will the next Nobel Prize for work in this exploding field of little RNA silencers be given?" ■

Beth Schachter is a New York-based science writer and a partner in Still Point Coaching & Consulting, a professional development firm for life scientists. In her previous career as a molecular and cell biologist, she was on the Mount Sinai School of Medicine faculty.