CENSORS of the

Biologists have been surprised to discover that most animal and plant cells contain a built-in system to silence individual genes by shredding the RNA they produce. Biotech companies are already working to exploit it

Genome

By Nelson C. Lau and David P. Bartel

Observed on a microscope slide, a living cell appears serene. But underneath its tranquil facade, it buzzes with biochemical chatter. The DNA genome inside every cell of a plant or animal contains many thousands of genes. Left to its own devices, the transcription machinery of the cell would express every gene in the genome at once: unwinding the DNA double helix, transcribing each gene into single-stranded messenger RNA and, finally, translating the RNA messages into their protein forms.

No cell could function amid the resulting cacophony. So cells muzzle most genes, allowing an appropriate subset to be heard. In most cases, a gene's DNA code is transcribed into messenger RNA only if a particular protein assemblage has docked onto a special regulatory region in the gene.

Some genes, however, are so subversive that they should never be given freedom of expression. If the genes from mobile genetic elements were to successfully broadcast their RNA messages, they could jump from spot to spot on the DNA, causing cancer or other diseases. Similarly, viruses, if allowed to express their messages unchecked, will hijack the cell's protein production facilities to crank out viral proteins.

Cells have ways of fighting back. For example, biologists long ago identified a system, the interferon response, that human cells deploy when viral genes enter a cell. This response can shut off almost all gene expression, analogous to stopping the presses. And just within the past several years, scientists have discovered a more precise and—for the purposes of research and medicine—more powerful security apparatus built into nearly all plant and animal cells. Called RNA interference, or RNAi, this system acts like a censor. When a threatening gene is expressed, the RNAi machinery silences it by intercepting and destroying only the offender's messenger RNA, without disturbing the messages of other genes.

As biologists probe the modus operandi of this cellular censor and the stimuli that spur it into action, their fascination and excitement are growing. In principle, scientists might be able to invent ways to direct RNA interference to stifle genes involved in cancer, viral infection or other diseases. If so, the technology could form the basis for a new class of medicines.

Meanwhile researchers working with plants, worms, flies and other experimental organisms have already learned how to co-opt RNAi to suppress nearly any gene they want to study, allowing them to begin to deduce the gene's purpose. As a research tool, RNAi has been an immediate success, allowing hundreds of laboratories to tackle questions that were far beyond their reach just a few years ago.

JANA BRENNING

PURPLE PETUNIAS offered the first clues to the existence of gene censors in plants. When extra pigment genes were inserted into normal plants (*left*), the flowers that emerged ended up with areas that strangely lacked color (*center* and *right*).

Whereas most research groups are using RNA interference as a means to an end, some are investigating exactly how the phenomenon works. Other labs (including our own) are uncovering roles for the RNAi machinery in the normal growth and development of plants, fungi and animals—humans among them.

A Strange Silence

THE FIRST HINTS of the RNAi phenomenon surfaced 13 years ago. Richard A. Jorgensen, now at the University of Arizona, and, independently, Joseph Mol of the Free University of Amsterdam inserted into purple-flowered petunias additional copies of their native pigment gene. They were expecting the engineered plants to grow flowers that were even more vibrantly violet. But instead they obtained blooms having patches of white.

Jorgensen and Mol concluded that the extra copies were somehow triggering censorship of the purple pigment genes—including those natural to the petunias—resulting in variegated or even albino-like flowers. This dual censorship of an inserted gene and its native counterpart, called co-suppression, was later seen in fungi, fruit flies and other organisms.

Clues to the mystery of how genes were being silenced came a few years later from William G. Dougherty's lab at Oregon State University. Dougherty and his colleagues started with tobacco plants that had been engineered to contain within their DNA several copies of the CP (coat protein) gene from tobacco etch virus. When these plants were exposed to the virus, some of the plants proved immune to infection. Dougherty proposed that this immunity arose through co-suppression. The plants apparently reacted to the initial expression of their foreign CP genes by shutting down this expression and subsequently also blocking expression of the CP gene of the invading virus (which needed the coat protein to produce an infection). Dougherty's lab went on to show that the immunity did not require synthesis of the coat protein by the plants; something about the RNA transcribed from the CP gene accounted for the plants' resistance to infection.

The group also showed that not only could plants shut off specific genes in viruses, viruses could trigger the silencing of selected genes. Some of Dougherty's plants did not suppress their CP genes on their own and became infected by the virus, which replicated happily in the plant cells. When the researchers later measured the RNA being produced from the CP genes of the affected plants, they saw that these messages had nearly vanished—infection had led to the CP genes' inactivation.

Meanwhile biologists experimenting with the nematode *Caenorhabditis elegans,* a tiny, transparent worm, were puzzling over their attempts to use "antisense" RNA to inactivate the genes they were studying. Antisense RNA is designed to pair up with a particular messenger RNA sequence in the same way that two complementary strands of DNA mesh to form a double helix. Each strand in DNA or RNA is a chain of nucleotides, genetic building blocks represented by the letters A, C, G and either U (in RNA) or T (in DNA). C nucleotides link up with Gs, and As pair with Us or Ts. A strand of antisense RNA binds to a complementary messenger RNA strand to form a double-stranded structure that cannot be translated into a useful protein.

Over the years, antisense experiments in various organisms have had only spotty success. In worms, injecting antisense RNAs seemed to work. To everyone's bewilderment, however, "sense" RNA also blocked gene expression. Sense RNA has the same sequence as the target messenger RNA and is therefore unable to lock up the messenger RNA within a double helix.

The stage was now set for the eureka experiment, performed five years ago in the labs of Andrew Z. Fire of the Carnegie Institution of Washington and Craig C. Mello of the University of Massachusetts Medical School. Fire and Mello guessed that the previous preparations of antisense and sense RNAs that were being injected into worms were not totally pure. Both mixtures probably contained trace amounts of double-stranded RNA. They

Overview/*RNA Interference*

- Scientists have long had the ability to introduce altered genes into experimental organisms. But only within the past few years have they discovered a convenient and effective way to turn off a specific gene inside a cell.
- It turns out that nearly all plant and animal cells have internal machinery that uses unusual forms of RNA, the genetic messenger molecule, to naturally silence particular genes.
- This machinery has evolved both to protect cells from hostile genes and to regulate the activity of normal genes during growth and development. Medicines might also be developed to exploit the RNA interference machinery to prevent or treat diseases.

RICHARD A. JORGENSEN *University of Arizona*

suspected that the double-stranded RNA was alerting the censors.

To test their idea, Fire, Mello and their colleagues inoculated nematodes with either single- or double-stranded RNAs that corresponded to the gene *unc-22*, which is important for muscle function. Relatively large amounts of single-stranded *unc-22* RNA, whether sense or antisense, had little effect on the nematodes. But surprisingly few molecules of double-stranded *unc-22* RNA caused the worms—and even the worms' offspring—to twitch uncontrollably, an unmistakable sign that something had started interfering with *unc-22* gene expression. Fire and Mello observed the same amazingly potent silencing effect on nearly every gene they targeted, from muscle genes to fertility and viability genes. They dubbed the phenomenon "RNA interference" to convey the key role of double-stranded RNA in initiating censorship of the corresponding gene.

Investigators studying plants and fungi were also closing in on double-stranded RNA as the trigger for silencing. They showed that RNA strands that could fold back on themselves to form long stretches of double-stranded RNA were potent inducers of silencing. And other analyses revealed that a gene that enables cells to convert single-stranded RNA into double-stranded RNA was needed for co-suppression. These findings suggested that Jorgensen and Mol's petunias recognized the extra pigment genes as unusual (through a mechanism that is still mysterious) and converted their messenger RNAs into double-stranded RNA, which then triggered the silencing of both the extra and native genes. The concept of a double-stranded RNA trigger also explains why viral infection muzzled the CP genes in Dougherty's plants. The tobacco etch virus had created double-stranded RNA of its entire viral genome as it reproduced, as happens with many viruses. The plant cells responded by cutting off the RNA messages of all genes associated with the virus, including the CP genes incorporated into the plant DNA.

GLOWING NEMATODES proved that RNA interference operates in animals as well as plants. When worms whose cells express a gene for a fluorescent protein (*left*) were treated with double-stranded RNA corresponding to the gene, the glow was extinguished (*right*).

Surprisingly FEW MOLECULES of double-stranded RNA made the worms—and even their offspring—TWITCH UNCONTROLLABLY.

Biologists were stunned that such a powerful and ubiquitous system for regulating gene expression had escaped their notice for so long. Now that the shroud had been lifted on the phenomenon, scientists were anxious to analyze its mechanism of action and put it to gainful employment.

Slicing and Dicing Genetic Messages

RNA INTERFERENCE was soon observed in algae, flatworms and fruit flies—diverse branches of the evolutionary tree. Demonstrating RNAi within typical cells of humans and other mammals was considerably trickier, however.

When a human cell is infected by viruses that make long double-stranded RNAs, it can slam into lockdown mode: an enzyme known as PKR blocks translation of all messenger RNAs—both normal and viral—and the enzyme RNAse L indiscriminately destroys the messenger RNAs. These responses to double-stranded RNA are considered components of the so-called interferon response because they are triggered more readily after the cells have been exposed to interferons, molecules that infected cells secrete to signal danger to neighboring cells.

Unfortunately, when researchers put artificial double-stranded RNAs (like those used to induce RNA interference in worms and flies) into the cells of mature mammals, the interferon response indiscriminately shuts down every gene in the cell. A deeper understanding of how RNA interference works was needed before it could be used routinely without setting off the interferon alarms. In addition to the pioneering researchers already mentioned, Thomas Tuschl of the Rockefeller University, Phillip D. Zamore of the University of Massachusetts Medical School, Gregory Hannon of Cold Spring Harbor Laboratory in New York State and many others have added to our current understanding of the RNA interference mechanism.

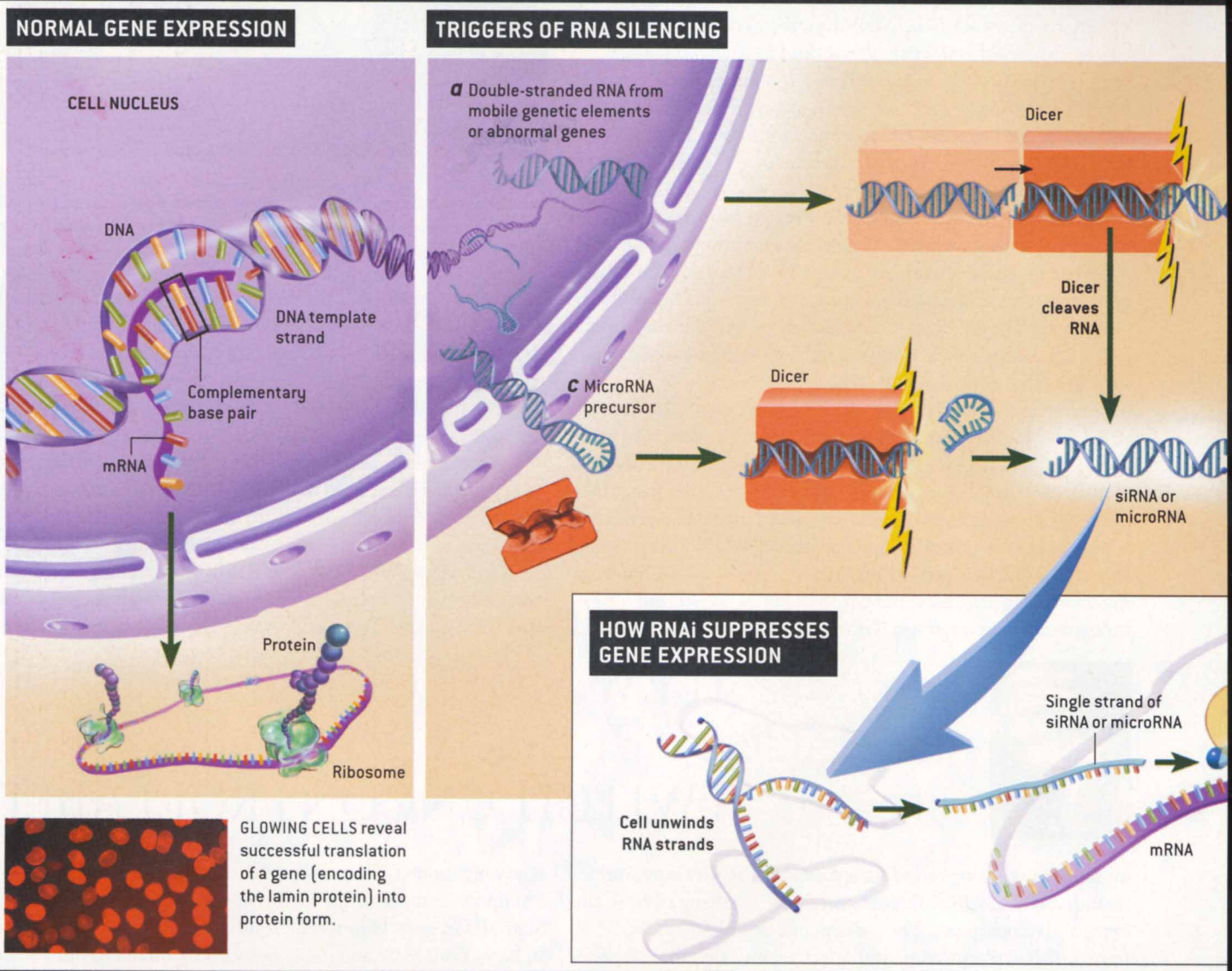
RNAi appears to work like this: Inside a cell, double-stranded RNA encounters an enzyme dubbed Dicer. Using the chemical process of hydrolysis, Dicer cleaves the long RNA into pieces, known as short (or small) interfering RNAs, or siRNAs. Each siRNA is about 22 nucleotides long.

Dicer cuts through both strands of the long double-stranded RNA at slightly staggered positions so that each resulting siRNA has two overhanging nucleotides on one strand at either

THE AUTHORS

NELSON C. LAU and *DAVID P. BARTEL* have been studying microRNAs and other small RNAs that regulate the expression of genes. Lau is completing a doctoral degree at the Whitehead Institute and the Massachusetts Institute of Technology. Bartel started his research group at the Whitehead Institute in 1994, after earning a Ph.D. at Harvard University. Bartel is also an associate professor at M.I.T. and a co-founder of Alnylam Pharmaceuticals, which is developing RNAi-based therapeutics. Lau and Bartel are among the recipients of the 2002 AAAS Newcomb Cleveland Prize.

GENETIC CENSORSHIP: HOW IT WORKS



GLOWING CELLS reveal successful translation of a gene (encoding the lamin protein) into protein form.

end [*see box above*]. The siRNA duplex is then unwound, and one strand of the duplex is loaded into an assembly of proteins to form the RNA-induced silencing complex (RISC).

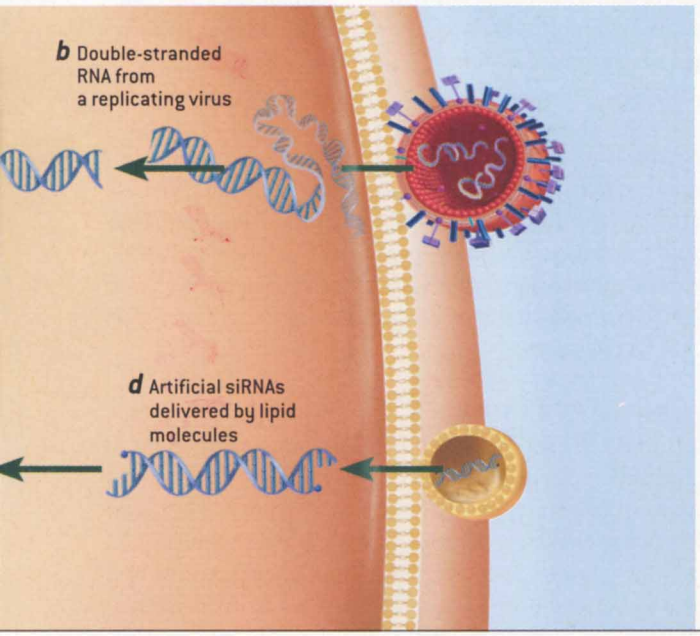
Within the silencing complex, the siRNA molecule is positioned so that messenger RNAs can bump into it. The RISC will encounter thousands of different messenger RNAs that are in a typical cell at any given moment. But the siRNA of the RISC will adhere well only to a messenger RNA that closely complements its own nucleotide sequence. So, unlike the interferon response, the silencing complex is highly selective in choosing its target messenger RNAs.

When a matched messenger RNA finally docks onto the siRNA, an enzyme known as Slicer cuts the captured messenger RNA strand in two. The RISC then releases the two messenger RNA pieces (now rendered incapable of directing protein synthesis) and moves on. The RISC itself stays intact, free to find and cleave another messenger RNA. In this way, the RNAi censor uses bits of the double-stranded RNA as a blacklist to identify and mute corresponding messenger RNAs.

David C. Baulcombe and his co-workers at the Sainsbury Laboratory in Norwich, England, were the first to spot siRNAs, in plants. Tuschl's group later isolated them from fruit fly embryos and demonstrated their role in gene silencing by synthesizing artificial siRNAs and using them to direct the destruction of messenger RNA targets. When that succeeded, Tuschl wondered whether these short snippets of RNA might slip under the radar of mammalian cells without setting off the interferon response, which generally ignores double-stranded RNAs that are shorter than 30 nucleotide pairs. He and his co-workers put synthetic siRNAs into cultured mammalian cells, and the experiment went just as they expected. The target genes were silenced; the interferon response never occurred.

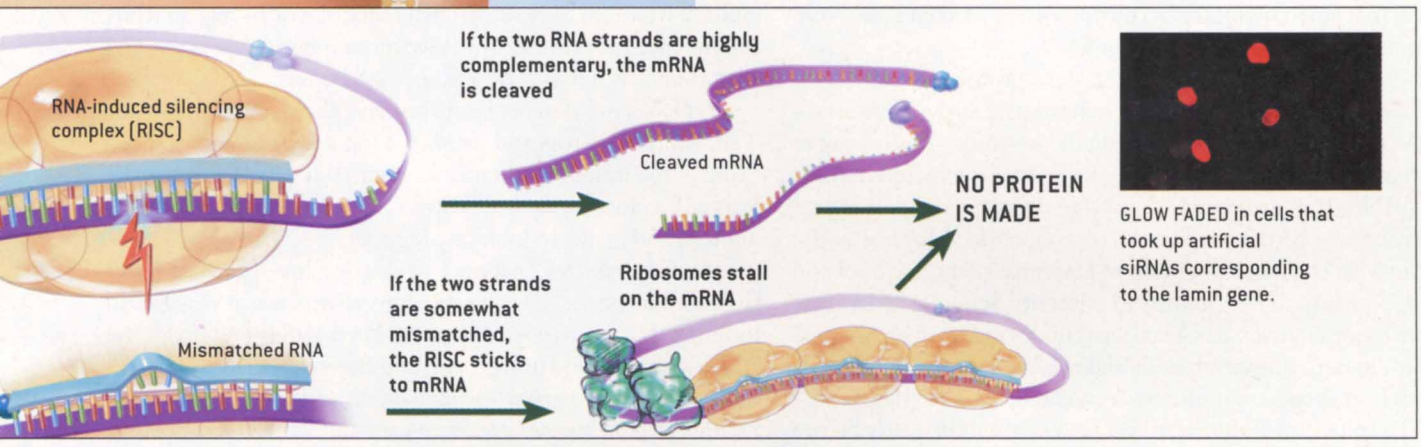
Tuschl's findings rocked the biomedical community. Geneticists had long been able to introduce a new gene into mammalian cells by, for example, using viruses to ferry the gene into cells. But it would take labs months of labor to knock out a gene

TERESE WINSLOW; THOMAS TUSCHL *The Rockefeller University [micrographs]*



A CELL CAN CENSOR the expression of an individual gene inside it by interfering with the messenger RNA (mRNA) transcribed from the offending gene, thus preventing the RNA from being decoded by ribosomes into active protein, as normally happens (*left panel*). The censorship machinery is triggered by small, double-stranded RNA molecules with ragged ends. An enzyme called Dicer chemically snips such short interfering RNAs (siRNAs) from longer double-stranded RNAs produced by self-copying genetic sequences (*a*) or viruses (*b*). Regulatory RNA sequences known as microRNA precursors (*c*) are also cleaved by Dicer into this short form. And scientists can use lipid molecules to insert artificial siRNAs into cells (*d*).

The RNA fragments separate into individual strands (*bottom panel*), which combine with proteins to form an RNA-induced silencing complex (RISC). The RISC then captures mRNA that complements the short RNA sequence. If the match is essentially perfect, the captive message is sliced into useless fragments (*top row*); less perfect matches elicit a different response. For instance, they may cause the RISC to block ribosome movements and thus halt translation of the message into protein form (*bottom row*).



GLOW FADED in cells that took up artificial siRNAs corresponding to the lamin gene.

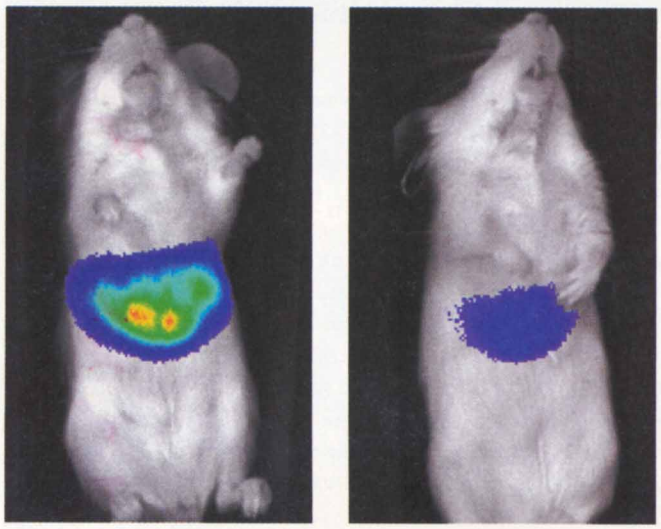
of interest to ascertain the gene's function. Now the dream of easily silencing a single, selected gene in mammalian cells was suddenly attainable. With siRNAs, almost any gene of interest can be turned off in mammalian cell cultures—including human cell lines—within a matter of hours. And the effect persists for days, long enough to complete an experiment.

A Dream Tool

AS HELPFUL AS RNA interference has become to mammal biologists, it is even more useful at the moment to those who study lower organisms. A particular bonus for those studying worms and plants is that in these organisms the censorship effect is amplified and spread far from the site where the double-stranded RNA was introduced. This systemic phenomenon has allowed biologists to exploit RNAi in worms simply by feeding them bacteria engineered to make double-stranded RNA corresponding to the gene that should be shut down.

Because RNA interference is so easy to induce and yet so powerful, scientists are thinking big. Now that complete genomes—all the genes in the DNA—have been sequenced for a variety of organisms, scientists can use RNA interference to explore systematically what each gene does by turning it off. Recently four groups did just that in thousands of parallel experiments, each disabling a different gene of *C. elegans*. A similar genome-wide study is under way in plants, and several consortia are planning large RNAi studies of mammalian cells.

RNA interference is being used by pharmaceutical companies as well. Some drug designers are exploiting the effect as a shortcut to screen all genes of a certain kind in search of promising targets for new medicines. For instance, the systematic silencing of genes using RNAi could allow scientists to find a gene that is critical for the growth of certain cancer cells but not so important for the growth of normal cells. They could then develop a drug candidate that interferes with the protein product of this gene and then test the compound against cancer. Biotech firms have also been founded on the bet that gene silencing by



MICE LIGHT UP when injected with DNA containing the luciferase gene (*left*). But scientists took the shine off the mice by also injecting siRNAs that match the gene (*right*), thus demonstrating one way to exploit RNAi in mammals.

RNAi could itself become a viable therapy to treat cancer, viral infections, certain dominant genetic disorders and other diseases that could be controlled by preventing selected genes from giving rise to illness-causing proteins.

Numerous reports have hinted at the promise of siRNAs for therapy. At least six labs have temporarily stopped viruses—HIV, polio and hepatitis C among them—from proliferating in human cell cultures. In each case, the scientists exposed the cells to siRNAs that prompted cells to shut down production of proteins crucial to the pathogens' reproduction. More recently, groups led by Judy Lieberman of Harvard Medical School and Mark A. Kay of the Stanford University School of Medicine have reported that siRNAs injected under extremely high pressure into mice slowed hepatitis and rescued many of the animals from liver disease that otherwise would have killed them.

Despite these laboratory successes, it will be years before RNAi-based therapies can be used in hospitals. The most difficult challenge will probably be delivery. Although the RNAi effect can spread throughout a plant or worm, such spreading does not seem to occur in humans and other mammals. Also, siRNAs are very large compared with typical drugs and cannot be taken as pills, because the digestive tract will destroy them rather then absorb them. Researchers are testing various ways to disseminate siRNAs to many organs and to guide them through cells' outer membranes. But it is not yet clear whether any of the current strategies will work.

Another approach for solving the delivery problem is gene therapy. A novel gene that produces a particular siRNA might be loaded into a benign virus that will then bring the gene into the cells it infects. Beverly Davidson's group at the University of Iowa, for example, has used a modified adenovirus to deliver genes that produce siRNAs to the brain and liver of mice. Gene therapy in humans faces technical and regulatory difficulties, however.

Regardless of concerns about delivery, RNAi approaches have generated an excitement not currently seen for antisense and catalytic RNA techniques—other methods that, in principle, could treat disease by impeding harmful messenger RNAs. This excitement stems in part from the realization that RNA interference harnesses natural gene-censoring machinery that evolution has perfected over time.

Why Cells Have Censors

INDEED, THE GENE-CENSORING mechanism is thought to have emerged about a billion years ago to protect some common ancestor to plants, animals and fungi against viruses and mobile genetic elements. Supporting this idea, the groups of Ronald H. A. Plasterk at the Netherlands Cancer Institute and of Hervé Vaucheret at the French National Institute of Agricultural Research have shown that modern worms rely on RNA interference for protection against mobile genetic elements and that plants need it as a defense against viruses.

Yet RNA interference seems to play other biological roles as well. Mutant worms and weeds having an impaired Dicer enzyme or too little of it suffer from numerous developmental defects and cannot reproduce. Why should a Dicer deficiency cause animals and plants to look misshapen?

One hypothesis is that once nature developed such an effective mechanism for silencing the subversive genes in viruses and mobile DNA sequences, it started borrowing tools from the RNAi tool chest and using them for different purposes. Each cell has the same set of genes—what makes them different from one another is which genes are expressed and which ones are not. Most plants and animals start as a single embryonic cell that divides and eventually gives rise to a multitude of cells of various types. For this to occur, many of the genes expressed in the embryonic cells need to be turned off as the organ matures. Other genes that are off need to be turned on. When the RNAi machinery is not defending against attack, it apparently pitches in to help silence normal cellular genes during developmental transitions needed to form disparate cell types, such as neurons and muscle cells, or different organs, such as the brain and heart.

What then motivates the RNAi machinery to hush particular normal genes within the cell? In some cases, a cell may nat-

RNAi has temporarily STOPPED VIRUSES—HIV, polio and hepatitis C among them—from proliferating IN HUMAN CELLS.

ANTON P. McCAFFREY ET AL. IN *NATURE*, VOL. 418; JULY 4, 2002; IMAGING SYSTEM COURTESY OF CHRISTOPHER CONTAG

Efforts to Apply RNA Interference to Medicine

THE MACHINERY for RNA interference was discovered to operate in mammals just two years ago. Yet about 10 companies, including the sampling below, have already begun testing ways to exploit gene censoring to treat or prevent human disease. —*The Editors*

COMPANY	PROJECTS	STATUS
Alnylam Pharmaceuticals Cambridge, Mass.	Researching therapeutic applications of RNAi, but specific disease targets not yet announced	Founded in 2002 by Bartel, Tuschl, Sharp and Zamore, the firm has secured initial funding and several patents
Cenix Biosciences Dresden, Germany	Investigating the use of RNAi-based therapies for cancer and viral diseases	With Texas-based Ambion, Cenix is creating a library of siRNAs to cover the entire human genome
Ribopharma Kulmbach, Germany	Attempting to chemically modify siRNAs to make drugs for glioblastoma, pancreatic cancer and hepatitis C	Clinical trials in brain cancer patients are expected to begin this year
Sirna Therapeutics Boulder, Colo.	Testing a catalytic RNA medicine for advanced colon cancer in clinical trials; development of RNAi-based therapeutics is still in early stages	Changed name from Ribozyme Pharmaceuticals in April; recently secured $48 million in venture capital

urally produce long double-stranded RNA specifically for this purpose. But frequently the triggers are "microRNAs"—small RNA fragments that resemble siRNAs but differ in origin. Whereas siRNAs come from the same types of genes or genomic regions that ultimately become silenced, microRNAs come from genes whose sole mission is to produce these tiny regulatory RNAs.

The RNA molecule initially transcribed from a microRNA gene—the microRNA precursor—folds back on itself, forming a structure that resembles an old-fashioned hairpin. With the help of Dicer, the middle section is chopped out of the hairpin, and the resulting piece typically behaves very much like an siRNA—with the important exception that it does not censor a gene with any resemblance to the one that produced it but instead censors some other gene altogether.

As with the RNAi phenomenon in general, it has taken biologists time to appreciate the potential of microRNAs for regulating gene expression. Until recently, scientists knew of only two microRNAs, called *lin-4* RNA and *let-7* RNA, discovered by the groups of Victor Ambros of Dartmouth Medical School and Gary Ruvkun of Harvard Medical School. In the past two years we, Tuschl, Ambros and others have discovered hundreds of additional microRNA genes in worms, flies, plants and humans.

With Christopher Burge at M.I.T., we have estimated that humans have between 200 and 255 microRNA genes—nearly 1 percent of the total number of human genes. The microRNA genes had escaped detection because the computer programs designed to sift through the reams of genomic sequence data had not been trained to find this unusual type of gene, whose final product is an RNA rather than a protein.

Some microRNAs, particularly those in plants, guide the slicing of their mRNA targets, as was shown by James C. Carrington of Oregon State University and Zamore. We and Bonnie Bartel of Rice University have noted that plant microRNAs take aim primarily at genes important for development. By clearing their messages from certain cells during development, RNAi could help the cells mature into the correct type and form the proper structures.

Interestingly, the *lin-4* and *let-7* RNAs, first discovered in worms because of their crucial role in pacing development, can employ a second tactic as well. The messenger RNAs targeted by these microRNAs are only approximately complementary to the microRNAs, and these messages are not cleaved. Some other mechanism blocks translation of the messenger RNAs into productive proteins.

Faced with these different silencing mechanisms, biologists are keeping open minds about the roles of small RNAs and the RNAi machinery. Mounting evidence indicates that siRNAs not only capture messenger RNAs for destruction but can also direct the silencing of DNA—in the most extreme case, by literally editing genes right out of the genome. In most cases, however, the silenced DNA is not destroyed; instead it is more tightly packed so that it cannot be transcribed.

From its humble beginnings in white flowers and deformed worms, our understanding of RNA interference has come a long way. Almost all facets of biology, biomedicine and bioengineering are being touched by RNAi, as the gene-silencing technique spreads to more labs and experimental organisms.

Still, RNAi poses many fascinating questions. What is the span of biological processes that RNA interference, siRNAs and microRNAs influence? How does the RNAi molecular machinery operate at the level of atoms and chemical bonds? Do any diseases result from defects in the RNAi process and in microRNAs? As these questions yield to science, our understanding of the phenomenon will gradually solidify—perhaps into a foundation for an entirely new pillar of genetic medicine. SA

MORE TO EXPLORE

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