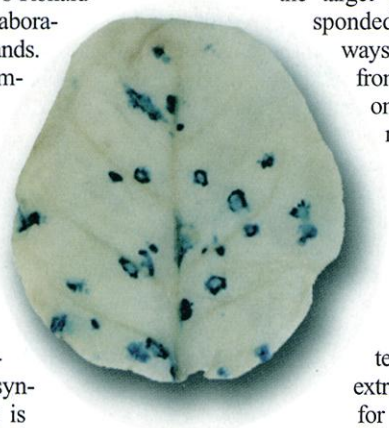
Interfering With Gene Expression

An explosion of recent evidence is revealing a new cellular pathway for silencing specific genes at the messenger RNA level that may protect organisms against viruses and genetic damage

In some ways, it was right under researchers' noses. For a decade, various groups had been seeing clues that cells have some novel way of shutting down or "silencing" genes. But they had been working in seemingly different fields and in different organisms, and they had given the murky mechanism a variety of names: cosuppression, quelling, and RNA interference (RNAi), among others. Only in the past year or so have researchers realized that they are all working on the same puzzle—and that they have stumbled upon what seems to be a critical pathway that cells use to protect themselves against viruses and certain kinds of genetic damage, and possibly to control normal gene expression as well. "Only recently, we've all come to realize that we are working on the same thing," says Ronald Plasterk of the Hubrecht Laboratory in Utrecht, the Netherlands. "We have a new field coming together."

Cell biologists have known for some time that genes can be silenced directly by chemical modifications that prevent the first step of gene expression: the transcription of the gene into the messenger RNA (mRNA) that ultimately directs protein synthesis. But the new work is revealing a novel method of silencing that kicks in later—preventing gene expression by degrading the mRNAs. This mechanism, which is very widespread—it occurs in organisms ranging from the mold *Neurospora* to plants, worms, simple vertebrates like the zebrafish, and perhaps even mammals—is apparently triggered when the cell senses some kind of danger. This might be, for example, an invading virus, or the mobilization of the bits of repetitive DNA called transposons, which can jump about the genome causing mutations if they happen to land in a gene, or possibly the production of an abnormal mRNA.

Whatever the exact cause, the cell directs an RNA-cutting enzyme (ribonuclease) to specifically degrade just the RNAs related to the trigger while other genes remain unaffected. What's more, by learning how the cell performs this feat, researchers have been able to devise a new method of inactivating specific genes—an ability that should be very useful for studying gene function and might also be used to create genetically modified plants and other organisms. "I believe RNA interference is going to be a very general and very exciting phenomenon," says Phillip Sharp of the Massachusetts Institute of Technology (MIT).



Silencing silenced. The plant at upper right has been silenced for a gene carried by a potyvirus and resists infection. But the leaf above carries a viral gene that blocks silencing and is susceptible, as shown by the blue spots where the virus is replicating.

Clues to the existence of the new gene-silencing mechanism began surfacing about 10 years ago, when researchers trying to perform various genetic manipulations found that the target organisms sometimes responded in totally unexpected ways. Early examples came from two independent groups, one led by Rich Jorgensen, now at the University of Arizona in Tucson, and the other by Joseph Mol of the Free University in Amsterdam, the Netherlands. These researchers were trying to create petunia plants with a more intense purple color by adding extra copies of genes needed for pigment synthesis. Their experiments failed in an intriguing way: Some of the resulting transgenic plants turned out to have blooms that were all or part white, indicating that pigment production had been shut down rather than ratcheted up. Indeed, not only had the introduced gene, or transgene, not been expressed, but somehow the plant's own pigment-synthesizing gene had been inactivated as well—a phenomenon that came to be known as cosuppression.

A few years later, plant researchers David Baulcombe of the Sainsbury Laboratory in Norwich, United Kingdom, and William Dougherty of Oregon State University in Corvallis noted something similar while trying to genetically engineer virus-resistant plants. For example, Baulcombe and his colleagues inserted into tobacco plants the gene encoding the replicase enzyme that potato virus X needs to reproduce. They hoped that excess replicase would check the growth of the virus by disrupting its life cycle. But while some of Baulcombe's plants became resistant to the virus, others didn't.

On further examination, the group found that the resistant plants were making very little of the replicase, while the susceptible ones were making a lot. "That was odd," Baulcombe recalls. "You'd expect it to be the other way around." Eventually, the researchers worked out that those plants that became resistant did so because they had silenced both the replicase gene in potato X virus and the transgene the researchers had put into the plants—a situation, Baulcombe says, that looked a lot like cosuppression.

Plant researchers weren't the only ones getting odd results from their genetic manipulations. Carlo Cogoni and Guiseppe Macino of the Università di Roma La Sapienza in Italy found that introduction of a gene needed for carotenoid synthesis in the mold *Neurospora crassa* led to inactivation of the mold's own gene in about 30% of the transformed cells. They called this gene inactivation "quelling."

Anomalous results also turned up in experiments in which researchers such as Su Guo and Kenneth Kemphues of Cornell University in Ithaca, New York, put so-called antisense RNA or DNA into cells from various organisms. These nucleic acids are constructed to have the reverse sequence of an active gene or mRNA—thus the name antisense—with the idea that they would bind to the gene or the mRNA, thus blocking its activity and preventing the synthesis of the corresponding protein. But researchers often found that the "sense" nucleic acids they used as controls, which shouldn't bind to the active genes or mRNAs, proved just as effective as the antisense constructs in blocking gene expression.

Solving the puzzle

By the mid-1990s, several teams had evidence that nucleic acids introduced into cells could specifically silence genes with similar sequences. The question was, how? By tracking down the answer, researchers found the new gene-silencing mechanism. The plant researchers provided one clue: Their measurements showed that the silenced gene was transcribed, even though the corresponding mRNA all but disappeared from the cell. In other words, the silencing was posttranscriptional, and it somehow led to the degradation of the mRNA so that the gene's protein prod-

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uct could no longer be made.

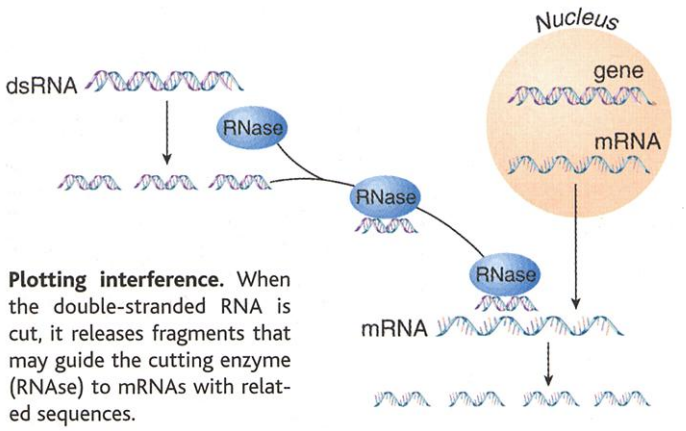
Another clue came from researchers working on both the nematode worm *Caenorhabditis elegans* and on the fruit fly. While trying to figure out what was going on with antisense RNA in the worm, Andrew Fire of the Carnegie Institution of Washington in Baltimore, Maryland, and Craig Mello of the University of Massachusetts Cancer Center in Worcester, with other colleagues, injected worms with either an antisense RNA, the sense version, or a double-stranded molecule in which the sense and antisense RNAs were bound together. Much to their surprise, Mello says, "the double-stranded RNA was much more effective than either the sense or antisense RNA" in silencing the corresponding *C. elegans* gene. Similarly, Jason Kennerdell and Richard Carthew of the University of Pittsburgh found that injecting fly embryos with double-stranded RNAs was a highly effective way to inhibit specific genes. Even now, though, researchers don't fully understand why double-stranded RNA has this effect, which came to be known as RNA interference.

More surprising still, silencing is not limited to the cells where it's initiated. For example, Mello's group found that when they injected double-stranded RNA into the intestines of worms, it not only triggered RNAi in all parts of the animals, but the silencing effects could even be transmitted through the germ line to one or more additional generations before petering out. Interference spreads in plants as well, as shown independently by Baulcombe's team and by that of Hervé Vaucheret of the Agricultural Research Institute in Versailles, France. The fact that the specificity of gene silencing is retained even as the interference spreads suggests that the signal is carried by a nucleic acid, Baulcombe notes.

Within the past 6 months or so, researchers have begun to identify the biochemical machinery that brings about RNAi. They are tackling the problem from two directions, one genetic, screening for genes that, when mutated, lead to loss of posttranscriptional silencing in the various organisms, and the other biochemical, trying to isolate the various molecules involved.

For starters, the genetic work is providing concrete evidence that quelling, cosuppression, and RNAi are likely to be one and the same. In the 16 March issue of *Nature*, for example, Plasterk's team described work showing that genetic mutations that block RNAi in *C. elegans* also block cosuppression in the worm—indicating that they both use the same biochemical machinery. And in that same issue, Cogoni and Macino reported that a gene they called *quelling defective 2* (*qde2*), because mutations in the gene block quelling in *Neurospora*, is in fact the mold's equivalent of a gene called *rde1* (*RNA1-defective 1*) originally identified by Mello's team in *C. elegans*.

So far, researchers have identified roughly a half-dozen genes that are needed for RNAi. Their exact functions aren't yet known, because they were found by submitting the various organisms to mutagenic treatments and then screening to see whether RNAi still works. But the sequences of the genes that have been cloned provide some intriguing clues. For example, Plasterk's team in Utrecht cloned a gene called *mut-7* and found that its sequence suggests that it's a ribonuclease, an enzyme that cleaves RNA. This makes sense, because mRNA degradation is apparently the final step of the RNAi process.



Plotting interference. When the double-stranded RNA is cut, it releases fragments that may guide the cutting enzyme (RNAse) to mRNAs with related sequences.

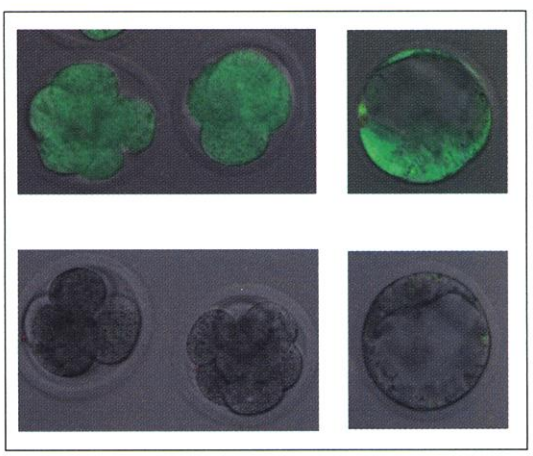
The biochemical work also suggests that ribonucleases are involved—and that the enzyme has a novel way of recognizing its specific mRNA targets. Some of this work comes from Baulcombe and his Sainsbury colleague Andrew Hamilton. As reported last fall, they found that short pieces of antisense RNAs, corresponding to the particular gene being silenced and only about 25 nucleotides long, were present in tomato plants where posttranscriptional gene silencing was occurring (*Science*, 29 October 1999, p. 950). More recent results, described in the 31 March issue of *Cell* by a team including Phillip Zamore of the University of Massachusetts Medical School in Worcester, Thomas Tuschl of the Max Planck Institute for Biophysical Chemistry in Göttingen, Germany, and MIT's Sharp and David Bartel, point to short RNAs playing a role in RNAi in *Drosophila* as well.

Working with a test tube system they previously developed for studying RNAi, the researchers put in a double-stranded RNA that triggers the breakdown of the mRNA encoding the luciferase protein. They found that the double-stranded RNA is broken down into short segments of 21 to 23 nucleotides. Furthermore, Zamore says, "when we looked at the fate of the mRNA, we found that it is also cleaved roughly every 22 nucleotides." This suggests, he says, that the fragments from the double-stranded RNA are directing the cleavage.

An indication of how the fragments are doing that comes from Gregory Hannon's team at Cold Spring Harbor Laboratory on Long Island. These workers partially purified a ribonuclease from *Drosophila* cells in which they had induced RNAi. Intriguingly, their findings suggest that the enzyme associates with an RNA roughly 25 nucleotides long—the same length as the RNAs detected by Baulcombe and the Zamore-Tuschl team.

Putting all this together, it appears that a ribonuclease first cleaves the double-stranded RNA, producing the short fragments. Then the enzyme picks up the fragments, which direct it to any mRNA whose sequence matches, and the enzyme breaks down the messenger as well. "Here we have an enzyme that essentially has a guide sequence—a piece of RNA bound to it—that determines its specificity," Zamore says.

Another gene whose function is at least tentatively known may aid posttranscriptional gene silencing by making extra copies of the RNA triggers. This is the *qde1* gene of *Neurospora*, cloned last year by the Cogoni-Macino team. The sequence of the protein encoded by this gene indicates that it is an RNA-directed RNA polymerase. As such, its activi-



Turned off. The mouse embryos above (four- to six-cell stage at left and a blastula at right) make green fluorescent protein (GFP). But those below were injected at the single-cell stage with a double-stranded RNA that silences the GFP gene.

CREDIT: F. WIANNY AND M. ZERNICKA-GOETZ

ty may lead to the synthesis of more of the small RNAs that guide the mRNA-degrading ribonuclease to its targets.

Such RNA amplification could help explain how RNAi spreads through plants and other organisms. As Hannon notes, "You either have to have amplification, or the enzyme [degrading the mRNA] has to be ferocious." More work will be needed to establish exactly what the RNA polymerase does in RNA, but other investigators now have evidence pointing to some involvement. In today's issue of *Cell*, both Baulcombe's team and Vaucheret's describe an RNA polymerase from *Arabidopsis* plants that is needed for RNAi.

How RNAi helps the organism

Although much of the evidence for RNAi comes from experiments in which researchers have artificially perturbed cells by putting in foreign nucleic acids, they are finding that it provides essential services for the organism. Several groups, including Baulcombe's and that of Vicki Vance of the University of South Carolina in Columbia, have evidence that plants use RNAi as a defense against infection with viruses.

It turns out that when viruses invade plant cells, the cells silence the viral genes needed for reproducing and spreading. Such silencing may be triggered by the double-stranded RNAs that plant viruses produce as part of their life cycle. Indeed, Baulcombe, Vance, and others have shown that, in the continuing evolutionary war to survive and reproduce, plant viruses have evolved genes that enable them to suppress silencing.

RNAi may also help keep the transposable elements that litter genomes from jumping around and causing harmful mutations. Both Plasterk's team and Mello, Fire, and their colleagues found that mutations that knocked out RNAi in *C. elegans* led to abnormal transposon movements. "Transposons were jumping out all over the place," Plasterk says. "These experiments tell us that RNAi's function is to protect your genome from transposons."

There are also hints that RNAi may be important in embryonic development. For example, Eleanor Maine of Syracuse University in New York and her colleagues found that the Ego1 protein, which is needed for germ line development in *C. elegans*, is structurally related to the RNA polymerase made by the *Neurospora qde1* gene. Her evidence also suggests that Ego1 participates in RNAi in worms. When she knocks out the gene, she finds the resulting animals are defective in RNAi directed at some genes expressed in the worm germ line.

Moreover, Florence Wianny and Magdalena Zernicka-Goetz of the University of Cambridge, U.K., have shown that they can elicit RNAi against certain genes in early mouse embryos by injecting them with the corresponding double-stranded RNAs. This indicates that RNAi could be used to inactivate specific genes in mammals, just as in the worm and fly, and could thus be a valuable tool for studying gene function in mammalian development.

Although the explosion of recent results has provided a good start toward understanding RNAi, researchers know that many questions still remain to be answered. They have to pin down the functions of the genes they have identified so far, and they say there are additional genes waiting in the wings to be identified.

Then there is the big question of whether RNAi, which is posttranscriptional and occurs in the cytoplasm, ties together in any way with the transcriptional silencing known to happen in the nucleus. Again, there are hints that it might. Plant researchers in particular have found that genetic manipulations that trigger RNAi often correlate with addition of methyl groups to the corresponding genes. Such methylation can lead to transcriptional shutdown of genes, but it's unclear which comes first in this situation.

Baulcombe and his colleagues have suggested that methylation of transgenes that have become inserted in the genome might lead to formation of abnormal transcripts, rather than complete transcriptional inhibition. These aberrant RNAs might then be selected for copying by an RNA polymerase to make double-stranded RNAs, thus triggering posttranscriptional silencing. But other researchers, such as Michael Wassenegger of the Max Planck Institute for Biochemistry in Martinsreid, Germany, and Marjorie Matzke of the Austrian Academy of Sciences in Salzburg, have found that certain RNA constructs can lead to methylation of the corresponding gene—an indication that the RNA is somehow talking back to the DNA. If confirmed, Mello says, "such retrograde flow of information would be really remarkable."

But much of what researchers have already learned about RNAi has been remarkable. As Vance puts it, "It's been so incredibly cool."

—JEAN MARX

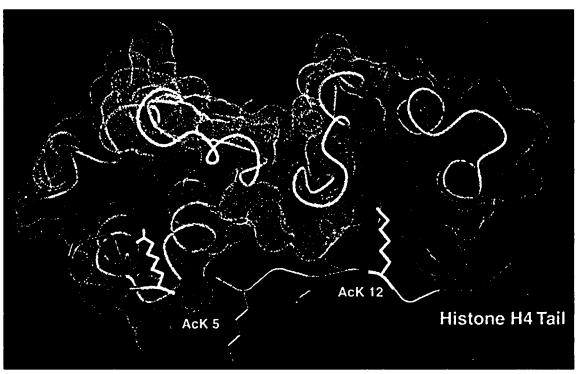
NEWS

Matching the Transcription Machinery to the Right DNA

The structure of a tandem set of folds called bromodomains reveals how they help set the stage for transcription

Gene transcription in the nucleus is a bit like an elaborate wedding at St. Patrick's Cathedral in New York City. Imagine that the "aisle" is the DNA of a gene that's going to be transcribed—that is, copied into a messenger RNA, as the first step in protein synthesis. The ushers and bridesmaids are the proteins that line up along the DNA to prepare it for the enzymes that will do the copying. In either case, before the walk down the aisle can occur, all sorts of players must get in their proper places—despite what often seems like total chaos.

Cell biologists have long wondered how this molecular event is choreographed. Now they have a new clue about how one key member of the transcription wedding party, a protein called $TAF_{II}250$, knows where to stand. Researchers knew that this protein is an essential part of the transcribing machinery—a complex of many proteins, some in common for all genes and some unique to particular gene targets—but its role was unclear. Work described on page 1422 by structural biologist Robert Tjian and his colleagues at the University of California, Berkeley, may now provide an answer: The protein helps direct the transcription machinery to the right DNA targets and gets the DNA into the correct configuration for transcription to occur.



Close connection. The configuration of paired bromodomains lets them bind to the acetylated histone tail. AcK 5 and 12 are acetylated histone lysines.

CREDIT: JACOBSON ET AL.

Combining both structural analysis of a portion of $TAF_{II}250$ and biochemical studies of its behavior, the Berkeley workers find that the protein apparently homes in on one of the histone proteins that wraps the DNA and forms