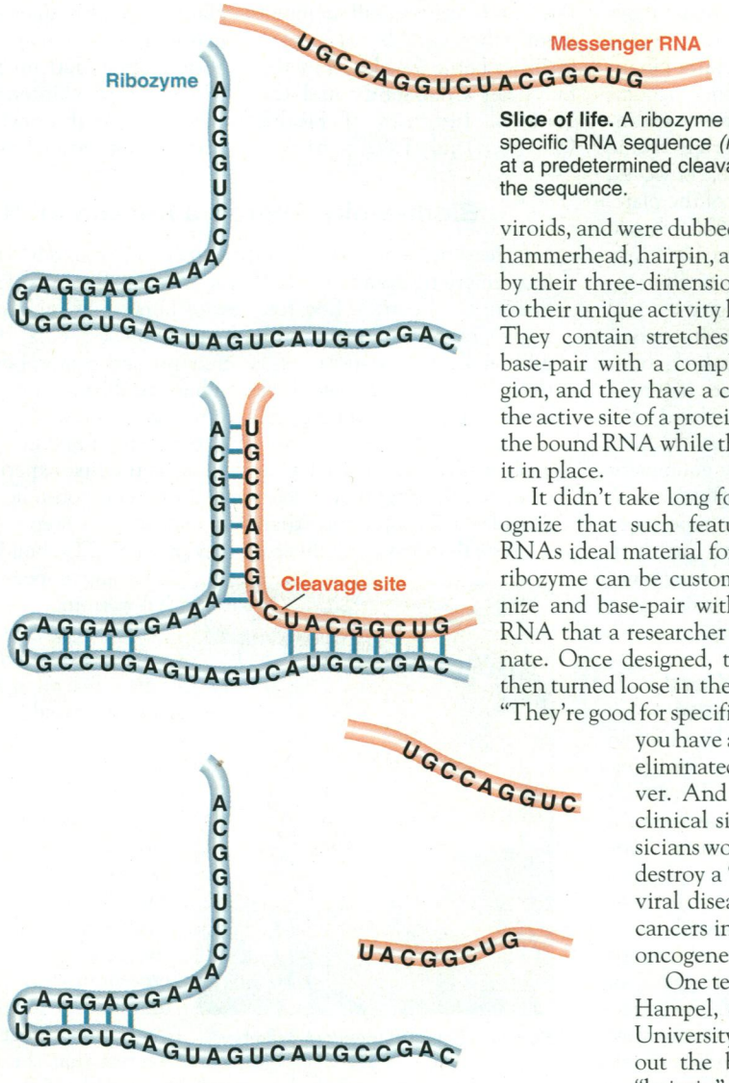
Ribozymes: Killing the Messenger

By destroying RNA that carries the message of disease, ribozymes—RNA molecules that act as enzymes—are on the verge of leaving the lab for the clinic

Sometime next summer, if the Food and Drug Administration (FDA) approves, AIDS researcher Flossie Wong-Staal from the University of California, San Diego, and biochemist Arnold Hampel of Northern Illinois University expect to insert the gene for a specific RNA sequence into the cells of HIV-infected patients. It won't be just any old RNA, but a specially designed ribozyme —an RNA molecule engineered to seek out and destroy the RNA genome of HIV by cutting it in two. The hope is that lymphocytes containing the ribozyme gene will have a better chance of surviving HIV infection.

This particular approach has been in development for several years, but the work on which the trial is based goes back a decade to the discovery of ribozymes by biochemists Tom Cech of the University of Colorado and Sidney Altman of Yale. Cech and Altman turned the molecular biology world upside-down with their finding that RNA molecules—once thought to be primarily passive carriers of genetic information—can, in fact, carry out functions that had been ascribed solely to proteins: acting as enzymes to catalyze their own cleavage or that of other RNA molecules. The work won Cech and Altman the 1989 Nobel Prize in chemistry, and boosted interest in RNA, both academic and applied. While basic biologists were speculating that catalytic RNA—with its combination of information-carrying and functional properties—may have been the primordial molecule from which life evolved, biotech types were looking to the future of RNA enzymes, or ribozymes, as potential therapies for diseases such as AIDS, cancer, and chronic hepatitis.

In the intervening 10 years, their vision has moved a long way toward reality. "There has been a tremendous degree of progress over the past few years," says Nava Sarver, chief of the targeted drug discovery section in the Division of AIDS at the National Institute of Allergy and Infectious Diseases (NIAID). "When we first proposed [ribozymes] as an anti-infective [in 1989], it was just a proposal. Now I am very optimistic that [they] will wind up in the clinic." That progress has been the result of a solid foundation laid by researchers in many labs who have worked to identify the features of ribozymes that allow them to bind, cut, and release specific targets. But as these advances bring ribozymes close to clinical use, some concerns remain that what's effective in a test tube won't work as well in a cell.



Slice of life. A ribozyme *(blue)* bonds to a specific RNA sequence *(red)*, and then cuts it at a predetermined cleavage point, destroying the sequence.

That concern wasn't even on the horizon in 1982, when Cech discovered that RNA molecules from a protozoan called *Tetrahymena* could "splice" themselves, clipping out unnecessary parts without the aid of any protein enzymes. Cech's discovery of self-cleaving RNAs was followed the next year by Altman's finding that some RNA molecules can act in a truly catalytic way, cleaving molecules other than themselves.

The initial discovery was revolutionary, and over the next few years the list of catalytic RNAs exploded. Many came from obscure plant and human virus-like particles called viroids, and were dubbed with names such as hammerhead, hairpin, and axehead, inspired by their three-dimensional shapes. The key to their unique activity lies in their structure: They contain stretches of nucleotides that base-pair with a complementary RNA region, and they have a catalytic section, like the active site of a protein enzyme, that chops the bound RNA while the base-pairing holds it in place.

It didn't take long for researchers to recognize that such features make catalytic RNAs ideal material for bio-engineering: A ribozyme can be custom-designed to recognize and base-pair with a specific cellular RNA that a researcher would like to eliminate. Once designed, the ribozyme can be then turned loose in the cell to kill its target. "They're good for specific applications where you have a bad RNA you want eliminated," says NIAID's Sarver. And there are plenty of clinical situations where physicians would like to target and destroy a "bad RNA": chronic viral diseases, for instance, or cancers initiated by a mutated oncogene.

One tempting target is HIV. Hampel, of Northern Illinois University, spent years puzzling out the biochemistry of the "hairpin" ribozyme, which comes from a viroid that accompanies the tobacco ringspot virus. Then he teamed up with Wong-Staal to modify that ribozyme to recognize and cleave a sequence near the beginning of the HIV RNA that is conserved among many different HIV strains. When it was tested in cell culture, the ribozyme reduced the amount of virus produced by HIV-infected cells by 10,000-fold. That was "far beyond our wildest expectations," says Hampel, and the success contributed to the approval from the National Institute of Health's Recombinant DNA Advisory Committee to begin a trial in HIV-infected patients.

Wong-Staal says the team hopes to receive FDA approval within the next 6

Placing Ribozymes on Target

On paper, in 1990, Bruce Sullenger's ribozyme looked fine. Sullenger, a graduate student working in Eli Gilboa's gene therapy lab at Memorial Sloan Kettering Cancer Center in New York, had designed a "hammerhead ribozyme" to target HIV RNA. Despite its attractions in theory, when it was put into cells, the ribozyme just didn't work. It was a frustrating situation, but one familiar to any ribozyme researcher: molecules that theoretically should find and cut a specific target seem to miss it completely.

The usual solution is trial and error, but, having been frustrated once by researchers' lack of understanding of the principles of ribozyme operation, Sullenger didn't feel like just starting over with a different molecule. "What you need to understand is what may be limiting [the ribozyme's success] inside of a cell," he says. "If you try to improve something that's not the limiting factor, it's never going to help you." So he moved as a postdoc to the University of Colorado lab of someone who'd been pondering this very problem: Tom Cech.

Cech thought the answer might lie in the cell's ability to direct internal traffic. "People aren't taking the cell biology into account," he says. "RNA is going to have traffic-control patterns in the cell, and these can either work against you or work for you." So, he and Sullenger set out to see if they could make it work for them. "We wanted to create a system by which you could intentionally get a ribozyme near its target inside a cell and see if that helps," says Sullenger. On page 000 of this issue, they show that indeed it does.

With HIV therapy as their long-term goal, Sullenger and Cech chose another retrovirus, Moloney murine leukemia virus, for their pilot experiments. They engineered the virus to express the gene for the enzyme β-galactosidase as a test. Then they designed a ribozyme to target β-galactosidase RNA; included in that ribozyme was a part of the Moloney sequence that packages the viral RNA into viral particles. Their reasoning was that the packaging sequence, which apparently steers the Moloney RNA to sites in the cytoplasm where the virus is assembled, would direct the ribozyme along the same route in the cell, giving it the best chance to meet and cut the β-galactosidase-containing Moloney genomes.

The test had a built-in control, says Sullenger, because cells infected with the Moloney virus make β-galactosidase RNA in two forms: messenger RNA, which is translated into beta-galactosidase enzyme within the cell, and a Moloney genomic RNA, which is packaged into virus particles. The ribozyme eliminated the Moloney genomes, without putting a dent in β-galactosidase enzyme expression.

Ribozyme researchers say the results clearly point to a potential route for improving ribozyme efficiency. "RNA tends to track; it has routes through the nucleus, and the endoplasmic reticulum," says John Rossi of the City of Hope National Medical Center, who is working on a similar strategy in his lab. Without some sort of tracking signal built into the ribozyme, he adds, ribozymes may be failing because they simply "aren't in the right place at the right time." The signal could change all that.

Cech and Sullenger are already developing a ribozymé against HIV that contains the HIV packaging sequence. But whether the approach can be generalized beyond retroviruses remains to be seen, since trafficking signals have not been identified for other types of RNA. "Sorting of RNAs is still a new area," says Cech. "People don't know whether this is the right paradigm for all RNAs."

But if the tracking improvement works for HIV, it will catch on fast. Flossie Wong-Staal, of the University of California, San Diego, who together with Arnold Hampel of Northern Illinois University has an anti-HIV ribozyme poised to enter a human trial, welcomes new refinements like this one. "If they have data that shows that is the way to go, we're going to jump into it too," she says. "We'll change our strategy. We're not proud."

–M.B.

months to begin what she calls "an experiment in vivo," on six HIV patients, by next summer. The plan is to remove HIV-infected T lymphocytes from the subjects' bloodstream and infect them with a disabled retrovirus that contains a gene coding for the ribozyme. The gene will be inserted into the cells' genomes by the virus, causing them to produce the ribozyme. The scientists will return the modified cells to the subjects where, they hope, the ribozymes will inactivate HIV RNAs produced in the treated cells. As a Phase I trial, one main goal of the experiment is to test the safety of ribozyme treatment in humans, but the researchers will also investigate whether ribozyme-containing lymphocytes resist or survive HIV infection.

While Wong-Staal and Hampel's anti-HIV ribozyme may be the first to be tried in humans, it certainly won't be the last, as many other groups are working on related strategies. Some of these approaches take into account HIV's notorious talent for mutation—changing bases—which could help it evade the ribozyme assault. To get around this potential snag, some labs are linking together several ribozymes, each directed to a different part of the HIV genome. Such ribozymes will mount a multi-site attack that should be immune to blockage by a single mutation, says John Rossi, of the City of Hope National Medical Center, in Duarte, California, who heads one of the labs taking this approach.

Another concern is that the ribozyme has to find its target in order to bind to it, and for an RNA molecule a cell can be a large and confusing place. So Cech and postdoc Bruce Sullenger are developing a way to target a ribozyme to the site in the cell where the HIV RNA accumulates, thereby improving the ribozyme's chances of hitting home (see story, this page).

Delivering the goods, not only to the right site in the cell, but to the right cell in the first place, is another important challenge for ribozyme therapy, as it is for other types of gene therapy. For example, ribozymes are a potential therapy for chronic hepatitis B, a life-threatening form of hepatitis with few effective treatments. But unlike white blood cells, which can be removed from the body and reintroduced for treating HIV, the liver obviously cannot be temporarily removed, and therefore ribozymes need to be delivered to the organ while it's still in the body.

Innovir Laboratories in New York is approaching this problem by working with a ribozyme taken from a liver-infecting viroid called delta that often tags along with the hepatitis virus. Since delta has a natural affinity for liver cells, it might be able to be exploited not only as a source of ribozymes, but also as a delivery vehicle, according to Hugh Robertson of Cornell Medical College, an Innovir consultant who studies delta. "If you could just engineer delta to be a nonsymptomatic form that would deliver and express certain sequences to liver cells... you would have a self-limiting vector delivery system that could combat [hepatitis B]," he says.

If the delivery challenges can be overcome, the sequence-specific affinities of ribozymes makes them potentially useful for cancer, another disease that can be caused by the presence of unwanted messenger RNAs resulting from aberrant gene activity. With ribozymes, says molecular biologist Kevin Scanlon of the City of Hope, "you may have the ability to discriminate between a normal gene and a cancer gene," even if the cancer

gene differs from the normal gene by only one nucleotide. In theory that means ribozymes could combat the effects of an oncogene, without affecting the gene's normal counterpart in other cells.

Several years ago, Scanlon's laboratory showed the promise of this approach when they made a ribozyme designed to cleave the mutated form of the *ras* oncogene in a human bladder carcinoma cell line. The *ras* gene sequence differed from its normal counterpart by just one nucleotide, and Scanlon designed a hammerhead ribozyme to recognize that changed sequence. When they put a gene for the engineered ribozyme into the bladder carcinoma cells, they found that not only was the mutant Ras protein not produced, but "the ribozyme was able to reverse the metastatic, invasive, and tumorigenic properties of the bladder carcinoma," says Scanlon. When the scientists put the ribozyme-containing carcinoma cells into mice, the cells didn't cause tumors.

But bladder carcinoma is treatable with conventional chemotherapy, and only 5% to 10% of bladder cancers have the *ras* mutation. Hence, to be useful, the ribozyme-based therapy must be effective in other forms of cancer. Scanlon is now studying eight other types of cancer, including such deadly forms as melanoma and pancreatic cancer, in which a high percentage of tumors carry the *ras* mutation, to see whether blocking *ras* can reduce tumorigenicity singlehandedly or in combination with other treatments.

Before such treatments ever make it to the clinic, there are concerns about safety to be answered. Ribozymes' specificity should keep them from affecting anything but their target, but NIH's Sarver points out that specificity has been studied for the most part in test-tube experiments. In a cell, things may be different. "Will it lose some of its specificity in the cell and cleave other cellular RNAs?" she worries. "It doesn't have to lose much specificity for this to be a problem." So far in experiments in cells, this doesn't seem to be happening, she adds, although safety needs to be borne out, and in most cases this will be done in animal trials.

Given these uncertainties, it may seem premature for a ribozyme therapy to be headed for human trials, and Hampel admits that his ribozyme's spectacular success in the test tube can be attributed to some lucky guesses. But while other groups work on removing the guesswork from ribozyme design, Wong-Staal says she and Hampel chose to move ahead with what they have. "We feel like somebody has to push the technology forward to a stage where we can evaluate it in the clinic." That is appropriate, says Sarver, because of the desperate nature of AIDS. "The first clinical uses [of ribozyme technology] will be with HIV," she says, "because people are willing to take more risks with HIV. It will be important for other applications to see how it fares." And given all the hope and unanswered questions about ribozymes as therapy, plenty of attention will be on those early HIV trials.

–Marcia Barinaga

Additional Readings

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CONSERVATION BIOLOGY

An Avian Arch-Villain Gets Off Easy

The populations of many migratory songbirds in North America are dwindling alarmingly, with one in three Eastern species showing significant declines in the 1980s, and some biologists think cowbirds are a major reason. After all, the brown-headed cowbird (*Molothrus ater*), a close cousin of the blackbird, has a reputation as an arch-villain of the avian world. Female cowbirds slip their eggs into the nests of other songbird species; by competing for food and attention from their foster parents, the cowbird nestlings often starve out the parents' own young. With cowbirds common in North America, such nest parasitism became a prime suspect in the songbird decline. But now it seems cowbirds may not deserve so much blame.

K. MASLOWSKI/VISUALS UNLIMITED

Unhealthy appetite? Hooded warblers with cowbird nestlings.

When cowbird biologists shared their data last month in Austin, Texas, at a research workshop on cowbird ecology and management, they found little direct evidence that the cowbird problem has grown worse in recent decades. Songbirds that seem to be suffering because of cowbirds are usually on the ropes for other reasons as well, such as loss of woodland nesting habitats and tropical wintering grounds. Contrary to earlier hopes, therefore, cowbird control may not offer a quick fix to the disappearance of songbirds. "The cowbird problem has perhaps been exaggerated in the press," says workshop organizer Terry Cook of the Nature Conservancy of Texas. "The real problem is the destruction of habitat."

Like many villains, cowbirds gained their bad name from a lot of circumstantial evidence and a little eyewitness testimony. Ten years ago, for example, half of all the nests of the least Bell's vireo (*Vireo bellii pusillus*) on Southern California's Camp Pendleton military base fell victim to cowbirds, and the vireo population was near extinction. When a cowbird-trapping program reduced parasitism to near zero, the vireo population shot up tenfold. "The correlation is very clear," says wildlife biologist John Griffith, a consultant on the Camp Pendleton program. "We didn't expect to discuss at the cowbird conference whether or not parasitism is a threat."

Few examples place cowbirds so clearly at the scene of the crime, however. Most of the evidence that cowbirds are decimating songbird populations is indirect. For example, cowbirds feed in open, grassy areas but dump many of their eggs in songbird nests in woodlands. As a result, the cowbirds thrive where open spaces dot the forest. New subdivisions and industrial parks create just that kind of environment, and conservationists reasoned that the cowbird threat should be increasing as a result.

A few small-scale studies and anecdotal accounts suggest that parasitism rates may indeed have increased, at least in some regions. No one, however, has done the extensive random sampling required to estimate parasitism rates across the entire continent, says longtime cowbird researcher Stephen Rothstein of the University of California, Santa Barbara. As a substitute, some researchers looked to the U.S. Fish and Wildlife Service's Breeding Bird Survey, a series of annual nationwide population censuses dating back to 1966. At last month's meeting, they reported their conclusion: little evidence of a cowbird population boom. "One reads a lot about [cowbird] populations exploding everywhere," says Rothstein, "but the data clearly show that there have been at best small increases in cowbird numbers since 1966....That was a real surprise."

Results like these have led most researchers to downgrade cowbirds from a continent-wide scourge to a regional concern. "Cowbirds are a spotty problem," says James Smith