BACKWARD GENETICS

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Knocking some 'antisense' into wayward genes

By FAYE FLAM

Imagine a weapon designed using perfect knowledge of the enemy — one custom-made to destroy exclusively that enemy. Scientists are working on such a weapon to combat cancers and viruses. They call it "reverse" or "antisense" genetics.

The antisense strategy involves disabling a piece of genetic code by using an opposite, or complementary, piece of code. Antisense researchers base their approach on the principle that opposites attract — and, in this case, cancel each other out. Ideally, an antisense molecule should perform only its set mission: to bind and disarm a specific target sequence of RNA while leaving the rest of the cell's genetic machinery untouched.

As scientists learn more about the genes triggering cancer and other diseases, they are becoming increasingly optimistic that antisense genetics could eventually provide a highly specific therapy. Already, researchers are using this approach to neutralize the activity of various plant and animal genes. In doing so, they are discovering the ways in which both "good" and "bad" genes dictate the characteristics of living things.

Every genetic sequence, whether DNA or RNA, has an opposite sequence, because the four components of its chemical code pair up in complementary partnerships bound with a specific lock-and-key fit. Known as bases, the four components are represented by the letters A, T, G and C (for adenine, thymine, guanine and cytosine). A and T make up one complementary pair, C and G the other.

This coupling of opposites is what holds together the two strands of the DNA double helix in a cell nucleus. Only one DNA strand — the "sense" strand — actually sends out its genetic message. The antisense strand remains a silent partner.

RNA, on the other hand, has only a sense strand — and so lies vulnerable to attack from an antisense sequence. Both DNA and RNA carry some configuration of the four-character code, but while DNA is the actual material of genes, RNA plays an essential role in expressing the information coded in those genes. The code-transfer process begins when the double-stranded DNA unwinds a bit and its sense strand acts as a template to mold single-stranded messenger RNA. The messenger RNA detaches and carries the code from the DNA out of the nucleus to other parts of the cell, where the genetic information is used to assemble proteins.

Messenger RNA works only as a single strand, but scientists can add a complementary sequence of DNA or RNA to serve as an antisense strand. The added sequence will bind up the messenger RNA with the same powerful bonding that holds together a DNA double helix, thus creating a freak double-stranded RNA or a DNA-RNA hybrid. In such a state, the messenger RNA can no longer perform its job of passing on the genetic code.

Researchers studying antisense genetics follow two different paths. Molecular biologists are manipulating cells' own DNA to induce them to produce their own antisense molecules. Scientists do this by taking duplicates of a cell's DNA strands, altering them and then returning the whole strands of altered DNA to the cell nucleus, where they become incorporated with the cell's regular DNA. The added DNA codes for production of antisense RNA, and it is this RNA that acts against a target sequence, binding up that sequence on the normal RNA. This genetic engineering approach gives the cell a tiny antisense factory, continually churning out antisense RNA.

On the other path, biochemists are synthesizing small pieces of antisense DNA in the laboratory that match known sequences of DNA. Unlike the whole strands of DNA added to cells by the genetic engineers, these short segments consist of only single strands of synthetic DNA. They never get into the nucleus or masquerade as normal DNA.

These single DNA strands accomplish the same goal as the biologists' antisense RNA. Researchers have found that cells take in small pieces of the synthetic DNA and that these pieces can recognize, bind to and disable target sequences on messenger RNA. Because they can use the small pieces to "knock" antisense into many cells, biochemists say these molecules hold promise as future drugs.

Though sometimes skeptical of each other's work, the biologists and biochemists are starting to share ideas. "The two streams are beginning to come together," says Paul C. Zamecnik of the Worcester Foundation for Experimental Biology in Shrewsbury, Mass., who did some of the first antisense experiments in the late 1970s.

Zamecnik began with the biochemical strategy of synthesizing small DNA pieces, although he lacked the synthesizing equipment benefiting scientists today. He delivered the first successful antisense attack against a virus by adding small pieces of synthesized antisense DNA to a cell culture to block the RNA of the virus causing

Rous sarcoma, a cancer of chickens. "It's not that we liked chickens so much," he says, "but this was a virus for which we knew the genetic sequence."

This "synthetic pieces" approach advanced slowly for the next few years. In the meantime, biologist Harold Weintraub of the Fred Hutchinson Cancer Research Center in Seattle took some of the first steps along the genetic engineering path. By the mid-1980s, he began using antisense RNA to stop mouse cells from producing thymidine kinase, an enzyme involved in DNA synthesis. He engineered DNA that, when added to a cell, made antisense RNA complementing the messenger RNA that normally codes for thymidine kinase, thereby blocking the enzyme's production.

To trick a gene into making antisense RNA that complements the RNA it produces normally, biologists take advantage of the natural antisense strand that forms the quiet half of the DNA double helix. They start with a copy of the double strand of DNA making up the original gene, and perform a role reversal so that instead of the sense strand molding sense RNA, the antisense strand molds antisense RNA.

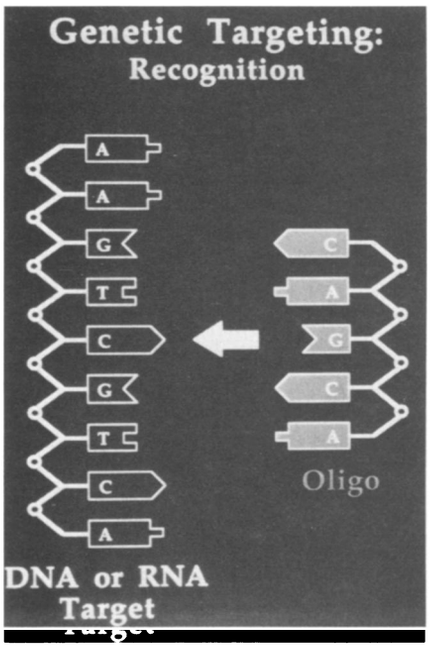
To accomplish this, they rely on the fact that genes have a forward and a backward direction, determined by the orientation of the "backbone" holding together the coding bases. The two strands of the DNA ladder fit together so that the forward direction for one strand is the backward direction for the other, as in opposing lanes of highway traffic. To assemble a piece of RNA, the double strand will unwind at a special sequence called a promoter. The strand extending forward from the promoter serves as the sense strand, assembling RNA. The other acts as the antisense strand.

To switch the roles of the two helix partners, researchers splice a promoter into the beginning of the sequence, then flop that piece of DNA so that the sense strand extends backward and the antisense strand extends forward. Then, if all goes well, the antisense strand takes on the active role.

A number of biologists have squelched the expression of various genes in plants and animals using this genetic engineering approach. In 1985, for instance, Douglas A. Melton of Harvard University used antisense RNA to alter the development of frogs. And in 1988, a group from Tokai University in Isehara, Japan, reproduced the effect of a mutation that makes mice shiver constantly. The shivering disorder usually results from damage to a gene coding for a nerve-protecting protein. The Japanese researchers mimicked the mutation's effect in mice with normal genes by adding a gene that makes antisense RNA, blocking the production of the nerve-protecting protein.

In 1987, David A. Knecht and William F. Loomis of the University of California, San Diego, used antisense genes in a slime mold to investigate cell specialization and movement. The slime mold has the unusual ability to exist both as a collection of single-celled organisms and, when starved, as a multicellular organism with some cells specializing in particular tasks. The San Diego researchers used antisense RNA to block the formation of myosin, a protein important in muscle contraction. The myosin-lacking cells could still move and congregate, but together couldn't form the same sort of multicellular animal, indicating myosin plays a crucial role in the way cells differentiate. "Antisense allows you to make a temporary mutation in an animal without changing existing genes," says Knecht.

Last summer, a practical application of antisense RNA came out of some work by researchers at the University of Nottingham in Loughborough, England, and Calgene, Inc., a Davis, Calif.-based biotechnology company, investigating how to produce better tomatoes. They successfully used antisense RNA to block the gene that makes tomatoes continue to soften after ripening and eventually causes them to spoil. While most commercially grown tomatoes are picked green to avoid spoilage in shipping, antisense tomatoes would ripen naturally and still hold up in their journey from the field to the supermarket.



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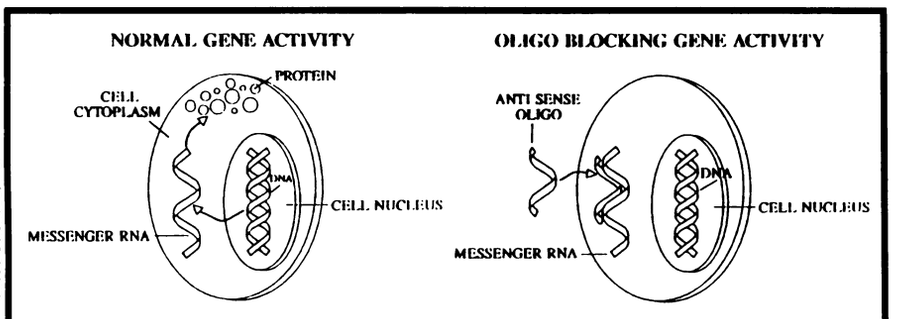
Each chemical component of the genetic code on an antisense molecule recognizes and locks into its complement on the target. G and C act as one complementary pair, A and T the other.

More recently, researchers used the genetic engineering approach to halt the effects of some cancer-inducing genes, or oncogenes. In the March 10 SCIENCE, Usha Kasid of Georgetown University in Washington, D.C., and George E. Mark of Merck, Sharpe, and Dohme Research Laboratories in Rahway, N.J., describe using antisense molecules directed against an oncogene called *raf*. The team engineered the DNA of cultured cells to produce antisense RNA, which neutralized the oncogene RNA in cultured human laryngeal cancer cells. Normally, laryngeal cancer strongly resists radiation treatment. When exposed to various levels of radation, engineered laryngeal cancer cells proved much more vulnerable than unaltered ones.

Kasid notes that if the experiments had worked according to theory, she and Mark would have seen some sense RNA joined to antisense RNA. Instead, her group observed a striking absence of such hybrids. She suggests the antisense RNA is working at a different stage, interrupting RNA production in the nucleus. Other researchers, observing a similar lack of hybrid messenger RNA in their experiments, have suggested the hybrids degrade quickly. Brenda Bass of Seattle's Hutchinson Center offers another explanation. Her experiments suggest antisense RNA may sometimes disable normal RNA not by permanently binding to it but by scrambling its message.



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In a normal cell (left), messenger RNA carries the genetic code of DNA from the nucleus out to the rest of the cell, where the code is used to assemble proteins. An antisense oligonucleotide (right) binds to a piece of messenger RNA, preventing it from translating its genetic information into proteins.

Experiments like Kasid's, using antisense to suppress oncogenes, provide new information about the role of these cancer-causing genes. Kasid notes that oncogenes are closely linked to other essential functions; some, in fact, are only slightly altered versions of useful genes. By blocking oncogene expression, researchers are learning about both the useful and damaging roles of these genes.

Applying scientific findings to cancer therapy, however, poses frustrating complications. For instance, a treatment based on antisense RNA would require the antisense to get into many malignant cells in a patient's body. Scientists envision purposely infecting a cancer patient with a benign virus that deposits antisense-producing DNA into cell nuclei. Ideally, the resulting antisense RNA would disrupt only the cancer-causing effects of the oncogene, leaving the gene's other functions intact. However, such a "curing" virus remains only an idea, scientists caution.

A more workable therapy might come from the other side of antisense research – the biochemists making synthetic antisense. Scientists in this area are experimenting with small pieces of single-stranded DNA called oligonucleotides, or "oligos." They can synthesize these in the laboratory, assembling sequences of single-stranded DNA complementary to a gene sequence they want to suppress. They use pieces with approximately 15 base pairs – long enough to code fairly unique sequences but small enough to get into the cell.

By observing in experiments that oligos somehow sneak into cells and suppress their complementary RNA sequences, researchers have opened up a whole range of interesting questions. "The way these molecules get into the cell is not understood at all," says Paul S. Miller, who works with oligos at Johns Hopkins University in Baltimore.

Many conventional drugs act like vandals, randomly destroying DNA and other parts of healthy cells in fighting disease. But antisense agents promise to work more like highly skilled assassins, fulfilling their assignments to kill specific RNA segments. Harvard's Melton, who did some of the first experiments with oligonucleotides, says these DNA sequences apparently bind to messenger DNA just as the antisense RNA made in the nucleus does – except that DNA oligos essentially embark on a suicide mission, because an enzyme called RNase-H quickly destroys the hybrid RNA-DNA.

Different experiments, however, hint that oligos sometimes alter cells in more complicated ways. Jack S. Cohen of the National Cancer Institute in Bethesda, Md., has targeted antisense oligos against DNA sequences from the AIDS virus, HIV. He found he could slow the virus' spread *in vitro* by adding oligos not with antisense sequences but made with only one base, such as a row of all Gs or all Cs. Cohen suggests these oligos disrupt a process by which retroviruses, such as HIV, make new DNA starting with the single-stranded RNA, the reverse of the usual production of RNA from DNA. The oligos, he says, appear to interfere with the action of reverse transcriptase, an enzyme needed for this backward production of DNA from RNA.

However, Cohen observed that only oligos with a true antisense sequence worked in cells he calls "chronically infected" – those that start out with the virus and host it throughout its life cycle without themselves dying. In such cells, only antisense DNA – with sequences complementary to the HIV genes – stopped the spread of the virus and blocked production of telltale viral proteins. "No other compound has been found to kill the virus in chronically infected cells," Cohen says.

Other experiments have shown that, in addition to working against HIV, antisense oligos act against herpes simplex, influenza A and several oncogenes. French researcher Claude Helene, of the National Institute for Health and Medical Research in Paris, has used antisense DNA to interrupt the life cycle of the protozoan *Trypanosoma brucei,* which causes African sleeping sickness.

Drugs based on antisense oligonucleotides remain in an early stage of development, but scientists say they have good reasons to believe the antisense concept could someday yield improved treatments for such killers as cancer and AIDS. So promising is the field that a handful of private companies have sprung up, concentrating much of their resources on antisense drug research.

But drug researchers must clear some major hurdles to make synthetic antisense molecules practical. The oligos are expensive to synthesize and often require large doses to achieve any effect. Many oligos fail to work because they can't get into the cell, don't disperse or don't bind well to the target sequence. They must be somewhat fat-soluble to penetrate the fatty cell membranes, and water-soluble to dissolve in the watery cell interior. Once they get into a cell, they must stand up to degrading enzymes. While scientists want enzymes to destroy the oligos after they bind to the target, these weapons become useless if enzymes reach them first.

At Johns Hopkins, Miller and his colleagues have developed enzyme-proof oligos by altering the backbone of the DNA, replacing an oxygen atom with a methyl group. However, with improved stability comes another hurdle: The altered oligos dissolve less readily in water and may fail to bind as well with RNA. Another modification to the DNA backbone – substituting a sulfur atom for an oxygen – appears to improve both solubility and enzyme resistance, according to other researchers, including Cohen.

Some biologists involved in the genetic engineering approach still question the biochemists' work with synthetic oligos. Weintraub of the Hutchinson Center cautions that researchers need to be thorough in making sure they have hit their genetic target. Gene sequences hold the code for assembling specific "product" proteins, he notes – so if an experiment has really disabled some genetic sequence, the cell will stop making the product proteins. Researchers who don't check for these proteins are doing sloppy work, he says.

But many biochemists experimenting with oligos contend they see just that. "We have seen reductions of the specific product proteins as well as observed the expected DNA-RNA hybrids to show how the antisense is working," says Eric Wickstrom of the University of South Florida in Tampa. Last year, Wickstrom found that antisense oligos stop cells from making the protein product of the *mic* oncogene, and he is currently working on an antisense approach to the AIDS virus. He says HIV makes a protein called tat, which researchers can check to make sure they are suppressing HIV genes.

While some scientists explore medical uses for antisense genetics or seek to puzzle out just how antisense works, others are already steering the field in new directions. Some envision using antisense sequences not as destructive agents in themselves but as tools to bring various molecular weapons to just the right spot on an RNA strand. For instance, antisense sequences might deliver a molecule to cut up, chemically alter or scramble the message of a desired RNA strand – irreversibly changing the RNA. One such molecule under investigation is called a ribozyme, which researchers have nicknamed "molecular scissors" because it snips apart RNA. Part of the ribozyme contains antisense sequences, providing the "glue" it needs to stick to a particular piece of RNA. Another part contains an enzyme, acting as the scissors.

Heading in another direction, Peter B. Dervan of the California Institute of Technology in Pasadena is studying how oligos bind to the double helix of DNA itself, forming a triple helix.

As scientists in different disciplines begin to see the sense in each other's antisense work, they are moving forward in the field of backward genetics. With luck, the exchange of results and ideas will advance the hope of reversing the damage of unwanted genes. □