Short lengths of synthetic DNA and RNA can trick cells into changing single bases in their genomes, possibly opening a new route to gene therapy

Repairing the Genome's Spelling Mistakes

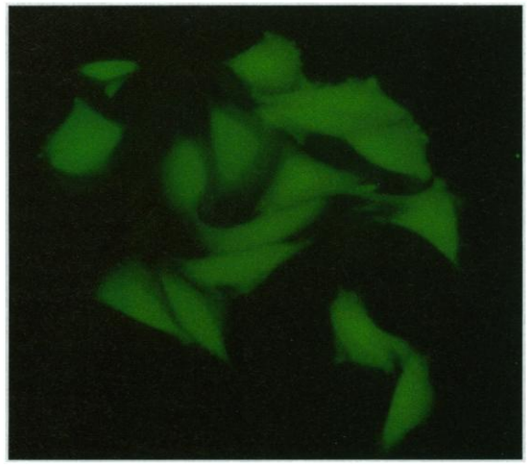
On the computer, correcting spelling errors takes nothing but a quick keystroke or two. Now, researchers are trying to harness the cell's own spell-check program—its DNA repair machinery—to tackle a much more difficult problem: fixing errors in the flawed genes that cause such hereditary diseases as sickle cell anemia and cystic fibrosis.

In recent work, some of it reported at this year's meeting of the American Society of Gene Therapy (9 to 13 June in Washington, D.C.), researchers have shown that they can remedy defects caused by single DNA spelling mistakes in both cultured cells and experimental animals. The technique has been dubbed chimeraplasty, because it relies on hybrid molecules of DNA and RNA called chimeras. In essence, these molecules contain DNA with the correct version of the misspelled letter flanked by RNA that perfectly mirrors the rest of the target gene segment. By pairing up with the defective gene, the chimeras can trick the cell's DNA repair machinery into replacing the wrong nucleotide in a gene with the right one.

If chimeraplasty works in human patients—and much more work will be required to show that it does—it could offer some significant advantages over current gene therapy strategies, which leave the defective gene in place and equip the cell with a spiffy new copy. Conventional gene therapy often relies on a virus to "infect" the diseased cells with the correct gene—a strategy that carries the risk of inflammation and other harmful reactions. What's more, because the replacement gene may land anywhere in the genome, it may not be subject to the same regulatory checks as the normal version, which could lead to its product being produced at the wrong time or in abnormal amounts.

Chimeraplasty, in contrast, can use nonviral carriers, such as the tiny membranous sacs known as liposomes, to shuttle the DNA/RNA molecule into the cell. And the hybrid itself doesn't hang around; it's degraded within 48 hours. But perhaps the biggest advantage of chimeraplasty is that it targets the endogenous gene. "All it does is change its spelling," says Michael Blaese, who left the National Institutes of Health last year to become chief scientific officer and president of the pharmaceutical division at the biotech firm Kimeragen in Newtown, Pennsylvania. "The gene's context, its regulatory regions, and its position on the chromosome all remain absolutely normal."

Even if clinical success turns out to be as elusive for chimeraplasty as it has been for conventional gene therapy, the technique could still prove useful in genetics research.



Corrected. These Chinese hamster ovary cells glow green after a DNA/RNA chimera corrected the mutated gene they carry for green fluorescent protein.

With appropriate changes in the DNA sequence of the DNA/RNA hybrid molecules, chimeraplasty can be used to create specific mutations as well as to cure them. Thus, it could aid in the development of animal models of human diseases and the generation of "knockout" mice, which help researchers probe the normal function of novel genes. The technology also works in plants, where researchers have begun applying it to crop development (see sidebar). "The beauty of chimeraplasty is that it appears to be a universal process," Blaese says.

The technique itself is the 6-year-old brainchild of molecular biologist Eric Kmiec at Thomas Jefferson University in Philadelphia, who studies homologous recombination, a natural process in which DNA strands with complementary sequences pair up and then swap closely matching segments. The process works very inefficiently in mammalian cells, except for germ cells undergoing meiosis. When doing routine assays, however, Kmiec found that the rate of recombination rises for active genes being copied into messenger RNAs—the recipes for proteins. He began toying with the idea that synthetic RNA molecules might be put to use in gene repair. They might trick the cell into recombining good DNA into mutant sites if added to organisms with genetic defects.

But, as hopeful as it sounded, that idea also presented a dilemma: In the cell, RNA degrades faster than DNA and thus might not stick around long enough to be useful for gene repair. It was only after months of pondering that Kmiec hit on the idea of making a hybrid. "Sometimes you get so close to the problem that you just don't see the answer," he notes.

Hybrid vigor

It later turned out that the chimeras did not work quite the way Kmiec thought, but initial tests showed that they do work, nonetheless. For his first target, Kmiec chose a gene called *ras*, which can be converted into a cancer-causing oncogene by changing a particular thymine base to a guanine. The molecule he designed to do this consisted of a five-base DNA segment flanked by two 10-base RNA segments that were modified to boost their stability. The sequence of this DNA/RNA hybrid mirrored that of the critical *ras* gene segment, except for the thymine-to-guanine change.

Kmiec then introduced these chimeric molecules, or "oligos," as small nucleic acids are sometimes called, into normal cells in culture. He subsequently found that some of the cells began showing the characteristic signs of cancerous transformation—apparently because the chimera had paired up with the normal *ras* gene in the cells and introduced the oncogenic mutation.

Soon after, Kyonggeun Yoon, a chemist and molecular biologist in Kmiec's lab, showed that a DNA/RNA chimera could correct mutations as well as introduce them. She corrected a single-base mutation in a

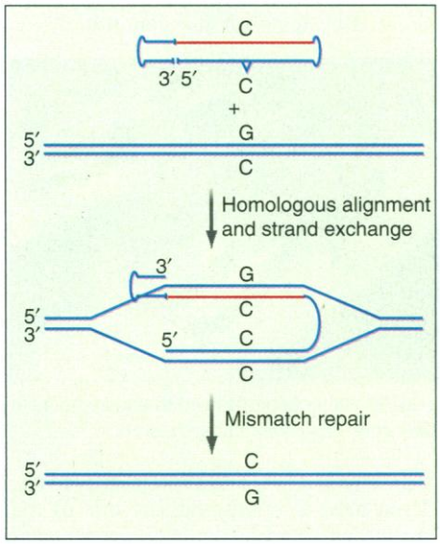
CREDIT: ERIC KMIEC

human gene for the liver enzyme alkaline phosphatase, which had been introduced into hamster cells. What's more, her results indicated that the change occurred in up to 30% of the cells. In contrast, traditional gene therapy works in less than 2% of cultured cells. "I wouldn't have believed it if I hadn't done it myself," says Yoon, who notes that she spent months verifying the work.

And less than a year after the initial hamster cell experiments, Allyson Cole-Strauss, Yoon, and others in Kmiec's lab extended the work, showing that they could correct a known disease-causing mutation, the single-base change in the β-globin gene that causes sickle cell anemia, in cells from patients with the disease (*Science*, 6 September 1996, p. 1386). The researchers estimated that 5% to 11% of the treated cells ended up with the normal gene.

To many gene-therapy experts, these early results sounded just too good to be true. As gene-therapy pioneer Mario Capecchi of the University of Utah, Salt Lake City, and his colleague Kirk Thomas pointed out in a letter in *Science*, the gene correction rates were three to six orders of magnitude higher than would be expected from the proposed mechanism of homologous recombination. A second letter from a team of European and U.S. researchers suggested that contamination with normal cells could have skewed the results, making the "cure" rate appear higher than it was. (The letters appeared in the issues of 7 March 1997 and 25 July 1997.)

Since then, Yoon has addressed the doubts with a series of experiments that were published in the December 1998 issue of *Nature Biotechnology*. After moving to the dermatology department of Jefferson Medical College in Philadelphia, she introduced a DNA/RNA chimera into albino mouse cells that can't make the pigment melanin because they have a single-letter mutation in a gene needed for melanin synthesis. Because those cells are colorless and normal melanin-producing cells are black, cells in which the chimera has corrected the gene defect are easy to spot.



Getting to the point. In the proposed chimeraplasty mechanism, the RNA/DNA chimera (top) binds to double-stranded DNA, and then the mismatch where two cytosines (Cs) pair is corrected by replacement of one cytosine with a guanine (G).

Yoon simply picked up the cells that had turned black and grew them individually in cultures to show that the chimeras did create a working gene and that it could be passed on to daughter cells. "It was reproof of principle," says Tom Wolfe, vice president of technology development at Sequitur, a biotech company located in Natick, Massachusetts.

Now Howard Gamper in Kmiec's lab is taking a closer look at the mechanism by which the chimeras correct genes and has concluded that it's not homologous recombination after all. Instead, he and Kmiec suggest that chimeraplasty works via mismatch repair, an error-correcting mechanism that detects when one strand of newly synthesized DNA doesn't pair properly with the complementary strand because it contains an incorrect base. The mismatch repair enzymes snip out the offending base and replace it with the correct one. The two investigators believe that chimeraplasty, by creating an intentional mismatch, may co-opt this mechanism to change a single letter in the original DNA strand. Supporting this picture, Kmiec's team found that cells in which mismatch repair genes are mutated perform chimeraplasty less efficiently than normal cells.

Toward the clinic

Researchers are now finding that the technique works in whole animals. In 1998, for example, liver expert Clifford Steer of the University of Minnesota, Minneapolis, and his colleagues showed that they could create rats with a hemophilia-like condition by us-

Surgically Altering Plant Genes

While gene therapists seek to correct the mutations that cause genetic disease, plant geneticists are more interested in creating new mutations—ones that might improve crop plants, making them more resistant to spoilage or herbicides, say, or boosting their nutritional value. Now plant researchers have a potential shortcut to that goal: a new technique called chimeraplasty, which is also being explored as a possible strategy for human gene therapy (see main text).

One standard method to induce mutations in plants is to expose plant cells in culture to a mutagen, such as radiation, and then screen them to see if any have acquired the desired trait, such as herbicide resistance. But that is a slow, imprecise process. A more direct approach is to genetically engineer the plant to carry a foreign gene for the trait, but that approach has come under fire, particularly in Europe, because the foreign DNA remains a permanent part of the plant. Chimeraplasty, in contrast, should allow researchers to specifically mutate whatever gene they want—without permanent introduction of foreign DNA.

The technique relies on hybrid DNA/RNA molecules—chimeras—that match the target gene region, except for the one base to be changed. By binding to the target gene, a chimera apparently triggers the plant cell's own DNA repair machinery to "correct" the mismatch between the target gene and the hybrid. As a result, the base change gets introduced into the gene.

Plant molecular biologists, including Gregory May of the Samuel Roberts Noble Foundation in Ardmore, Oklahoma, and Charles Arntzen of the Boyce Thompson Institute for Plant Research at Cornell University in Ithaca, New York, have tested the technique, delivering the chimeras to plant cells by shooting them in with a gene gun or applying an electric current to open pores in the cell walls, through which the molecules can slip. The results are largely unpublished. But in the book *Methods in Molecular Biology: Gene Targeting Protocols*,* May and his colleagues report that they used chimeraplasty to make tobacco cells resistant to sulfonylurea herbicides.

By altering a single base in the gene coding for an enzyme called acetolactate synthase, which is needed for amino acid synthesis, the chimera destroyed the enzyme responsible for herbicide sensitivity. The investigators also tried the technique in plant cells carrying a mutated version of the gene for the green fluorescent protein and found they could correct the defect, causing the cells to glow green.

Because the chimeras, which are small molecules, eventually break down after altering the gene, the technique doesn't leave any foreign DNA behind. As a result, chimeraplasty may raise fewer hackles among opponents of genetic engineering. "What we are seeing is a different tool for genetically changing crops," says Arntzen. "You can surgically inactivate some genes and either change the processing or nutritional value of others. It's really wonderful." **–T.G.**

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SOURCE: ERIC KMIEC

ing chimeras, transported in liposomes, to introduce a mutation into the animals' gene for a factor needed for normal blood clotting. In preliminary experiments, the group also did the reverse; they used DNA/RNA chimeras to reverse the hemophilia-like state in dog liver cells with a clotting-factor mutation.

And at the gene therapy meeting, Li-Wen Lai, a geneticist and molecular biologist at the University of Arizona Health Sciences Center in Tucson, reported that she and her husband, nephrologist Yeong-Hau H. Lien, have used DNA/RNA chimeras to correct a metabolic disease in mice. The mutation disables a kidney enzyme called carbonic anhydrase II and results in dangerously high acid levels in the bloodstream. Lai and her colleagues designed a chimera to reverse it, bound the molecules in liposomes, and then injected them into the ureters of the mice. The gene was corrected in 1% to 15% of the animals' kidney cells. Lai says she and Lien are now working with the animals to see if their blood acidity decreases and if the change lasts over time.

But even though evidence is building that chimeraplasty can work, the success rate can vary from cell type to cell type, and even from experiment to experiment. For example, Yoon repeated her melanocyte experiment 30 times and found cells turned black anywhere from 0.01% to 15% of the time. Such variations convince Capecchi that "it's a little early to talk about human trials," although he now says that chimeraplasty "certainly has potential."

Researchers also worry about safety, although that's a concern with standard gene therapy, too. Perhaps the chimeras will start "fixing" other parts of the genome that aren't broken, in essence creating mutations like those that lead to cancer. "Just how much less frequent is a nonspecific change than a specific change?" Blaese questions. "Those are the issues we are trying to address."

Even so, the first human trial of chimeraplasty may be on the horizon. At the gene-therapy meeting, Steer's group reported results from their recent work on Gunn rats, which carry a single-base deletion in the gene for a liver enzyme that detoxifies the yellow pigment bilirubin. The rats accurately model a rare human hereditary condition called Crigler-Najjar disease, in which patients can't metabolize bilirubin, which builds up to toxic levels. The patients end up severely jaundiced and have to spend 12 to 16 hours a day under blue light, which promotes bilirubin breakdown. If untreated, the disease is lethal, and the only cure is a liver transplant.

Steer and his colleagues have now found that an appropriate chimera corrects the gene defect in a substantial proportion of the Gunn rats' liver cells. Up to 40% of the cells revert to normal, Steer says, as indicated by tests for the genomic DNA, messenger RNA, and protein sequences. Even more encouraging, the bile of the treated rats contains telltale liver metabolites that signify normal enzyme activity.

Light therapy. Crigler-Najjar patients need to endure hours in blue light to break down their toxic bilirubin levels.

Steer attributes the success of the therapy to the system he used to shuttle the chimeras into the rats' liver cells. The molecules are encapsulated in liposomes carrying surface molecules that specifically target them to receptors on the liver cells. "I've had people get up at meetings and say, 'I don't believe your data,' " Steer recalls. "But as more labs are becoming successful, people are beginning to accept this."

Indeed, Blaese is gearing up with Steer to try the technique in three patients with Crigler-Najjar syndrome. The two groups are now doing safety studies in order to obtain Food and Drug Administration approval to go on into humans. If those studies show that the chimeras aren't targeting other DNA sequences and are safe in humans, then researchers could move on to target a very long list of human genetic diseases of the liver. "I wouldn't have to go back to drug discovery," Blaese notes. "I could just go to the human genome project, read off what the gene is, and change the spelling of our molecule."

–TRISHA GURA

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ASTRONOMY

Holes in the Sky Provide Cosmic Measuring Rod

The shadows cast by distant galaxy clusters against the microwave glow of the sky are offering a new gauge of the universe's expansion rate and makeup

CHICAGO—Most astronomers measuring the universe on the largest scales chase bright lights. They look for beacons called standard candles—flickering stars, supernovae, or certain galaxies—and turn their observed brightnesses into cosmic distances, clues to the universe's expansion rate, its age, and whether it is permeated by a strange energy called the cosmological constant. But one group of observers is chasing shadows instead: the dark silhouettes cast by distant clusters of galaxies against what John Carlstrom, a radio astronomer at the University of Chicago, calls "an amazing backlight": the glow known as the cosmic microwave background radiation (CMBR). The size of the shadows in the CMBR provides a cosmic measuring stick independent of any now in use.

Cosmic measuring sticks based on standard candles generally rely on a "distance ladder" in which short-range beacons are used to calibrate others that can reach deeper into the cosmos. But the shadows created by the so-called Sunyaev-Zeldovich (SZ) effect can be seen out almost to the edge of the universe, and they can be converted into distances without any intermediate steps. "This method goes straight out to very large distances in one go," says Mike Jones, an astronomer at Cambridge University in the United Kingdom.

Conceived decades ago by two Russian scientists, the technique is only now showing its potential, thanks to more sensitive instruments for measuring the microwave background and satellite x-ray images of clusters. At a recent American Astronomical Society (AAS) meeting here, Erik Reese of the University of Chicago and Brian Mason of the University of Pennsylvania each presented new results on distances to a half-dozen clusters, based on work by multi-institutional teams. Combined with separate observations of how fast those clusters are rushing away, the distances give the expansion rate, called the Hubble constant, which can be combined with other cosmic measurements to give an age. Uncertainties in the technique are still large, but results so far provide a comfortable fit with a recently announced value for the Hubble constant. And, as more and more SZ observations roll in, they could help determine not only the expansion rate but also how it has changed over billions of years of cosmic history.

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