Hot Prospect for New Gene Amplifier

Ligase chain reaction, a combination DNA amplifier and genetic screen, could do for DNA diagnostics what PCR has done for basic molecular biology

HAD MOLECULAR BIOLOGIST FRANCIS Barany been superstitious, he might have taken a break after obtaining twelve water samples on his collecting trip in Yellowstone National Park this past August. But instead, he edged forward to secure his thirteenth specimen and disaster struck: The ground crumbled beneath his feet and sent him sliding into a scalding hot spring. His sudden spill into the 158°F pool left Barany with excruciating second- and third-degree burns on one leg; for weeks he used a cane to hobble about his lab on Cornell University's medical campus in Manhattan.

Barany's wounds are now healing, the nightmares about being boiled alive have stopped, and recent experiments suggest that bacteria in his Yellowstone samples may provide a healthy payoff for his painful research. Along with a handful of other university and corporate researchers, Barany is investigating one of the hottest areas (quite literally) in biotechnology: a gene-amplification technique called ligase chain reaction, or LCR. Already scientists have used LCR to detect the tiny mutation that causes sickle cell anemia and have adapted it to screen for a handful of other genetic diseases simultaneously—in a single test-tube. Some experts, in fact, are predicting that LCR will supplement that superstar of the 1980s, polymerase chain reaction (PCR), and in some cases even supplant it.

Indeed, if the technology moves successfully from the research laboratory to the clinical arena, LCR could revolutionize DNA diagnostics just as PCR transformed basic molecular biology following its introduction 6 years ago. With its ease of automation and ability to produce useful quantitative results—two areas in which PCR does not score well—LCR could become a major player in the rapidly growing market for DNA diagnostics, according to biotechnology analyst Jim McCamant, editor of the *Medical Technology Stock Letter* in Berkeley, California.

That prospect has made LCR a hot commercial property—so hot that a major battle over ownership of the technology is brewing (see box, p. 1293). A half-dozen companies and universities already have staked claims to various aspects of the technology, and three companies are currently negotiating with Cornell for the rights to Barany's version of LCR with an eye toward developing commercial diagnostic kits.

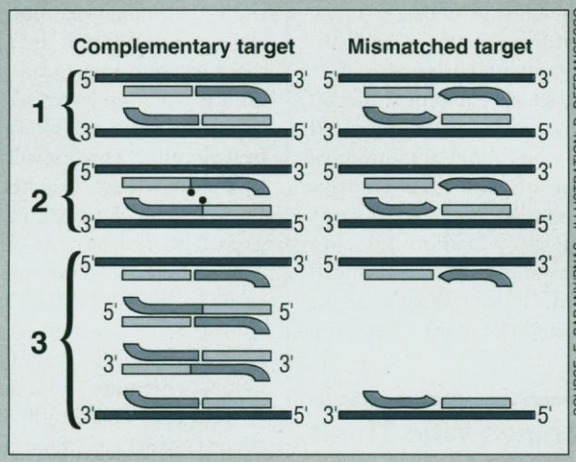
The competition centers on a process that, like PCR, uses a heat-stable enzyme isolated and cloned from the hot-springs bacterium *Thermus aquaticus* to amplify specific genetic sequences a million or more times in a matter of hours. But the two techniques differ in an important way. PCR amplifies a stretch of DNA between two primers but tells nothing about the precise sequence of the amplified fragment; to find out exactly what you have amplified, and whether a mutation resides in that stretch, requires restriction enzyme analysis or DNA

How LCR Works

LCR, like PCR, uses snippets of nucleic acid, or oligonucleotides, that anneal to a specific, complementary sequence on the target DNA to be amplified. But where PCR uses oligos that bracket the stretch to be amplified, LCR uses pairs of oligos that completely cover the target sequence. Here's how it works: One set of oligos is designed to be perfectly complementary to the left half of a sequence being sought and a second set matches the right half. Both sets are added to the test sample along with some ligase cloned from hot-springs bacteria. If the target sequence is present, the oligos blanket their respective halves of that stretch, with their ends barely abutting at the center. Interpreting the tiny break between those ends as a nick in need of repair, the ligase steps in and welds the oligo ends together with a permanent, covalent bond, creating a full-length stretch of DNA complementary to the target sequence. From here, amplification is achieved in much the same way as in PCR: The solution is heated to separate the new, full-length oligo strand from the original target strand, and both then serve as targets for additional oligos.

After running enough cycles to boost their numbers sufficiently, one simply screens for full-length, ligated oligos. This can be achieved in a variety of ways; one simple method is to construct the oligos such that all the left-half oligos have tiny "hooks" that prevent them from washing through a sieve-like column, and all the right-half oligos have fluorescent markers. Fluorescence within the column indicates the presence of full-length oligos, signifying that the target sequence being screened for was indeed present in the initial sample.

The key to LCR's usefulness is that ligase will ignore abutting oligos that are not lying perfectly flat upon their target sequences. So while oligos may manage to bind—albeit a little awkwardly—to an imperfectly matched target, the ligase won't weld those adjoining oligos together. Thus when the solution is heated, each oligo falls away from its target independently, no amplification occurs, and the test comes up negative. ■ R.W.



SOURCE: F. BARANY/PNAS ILLUSTRATION: D. DEFRANCESCO

Step 1: *Separate DNA strands, 94°C. Anneal oligonucleotides, 65°C.* **Step 2:** *Ligate with thermostable ligase, 65°C.* **Step 3:** *Repeat cycle 20 to 30 times.*

sequencing. The beauty of LCR, in contrast, is that it amplifies only stretches of DNA that have the exact sequence you are looking for, and so effectively performs both the amplification step and the detection step simultaneously (see box p. 1292).

This means that LCR can more readily screen for rare point mutations responsible for inherited diseases, identify DNA polymorphisms useful for genetic mapping, reveal microbial sequences hidden in blood or tissues, and even differentiate between drug-resistant and drug-sensitive strains of viruses or bacteria without the need to culture the bugs first. "It's a wonderful way of screening," says Saul Silverstein, chairman of microbiology at Columbia University, who has been experimenting with Barany's version of LCR to detect the genetic deletion causing congenital adrenal hyperplasia. "We're just beginning to look at it," he cautions, "so we're not completely sold. But I have to say it is looking good."

Barany appears to have been the first to describe in a scientific journal the use of LCR as a genetic screen for a human disease, successfully detecting the single base substitution in hemoglobin that causes sickle cell trait (*PNAS*, 1 January 1991, p. 189). But others are going further. Taking advantage of the ease with which different LCR probes can be combined, or multiplexed, Deborah Nickerson and colleagues in Leroy Hood's lab at Caltech have developed a version of LCR that screens simultaneously for 10 different mutations in human DNA—including the three most common mutations causing cystic fibrosis, the error responsible for sickle cell anemia, and a handful of other single-base deletions. The bottleneck at this point, says Nickerson, is developing enough independent markers to keep track of all the LCR probes they'd like to multiplex together.

Preliminary tests in other labs, mostly using Barany's system, seem to confirm LCR's potential. Olen Kew, chief of the molecular virology section of the Centers for Disease Control, has been using LCR to screen for point mutations known to affect viral attenuation and virulence in poliovirus vaccine strains. He says the technique has the potential to detect those rare poliovirus revertants that cause polio in a few unfortunate vaccine recipients each year. And Vincent Wilson, director of molecular genetics at Children's Hospital in Denver, has begun using LCR to detect point mutations associated with certain cancers. He foresees using multiplexed LCR probes to detect the major mutations that accumulate in the course of tumor formation, providing an unprecedented natural history of carcinogenesis on a molecular scale.

Another likely convert is Joseph Prchal, professor of medicine at the University of Alabama in Birmingham. He says LCR "works quite well" as a means of establishing the clonality, or relatedness, of various tumors and has successfully sniffed out a rare point mutation that causes a congenital defect in red blood cell membranes.

Despite such testimonials, LCR's rise by no means portends the death of PCR. For speed and sensitivity, PCR still has no equal, amplifying much faster than LCR and requiring fewer copies of target DNA to get started. That has led some scientists to experiment with using the two techniques in combination—a recipe that Barany predicts may prove to be the best of all. Combining PCR's sensitivity and LCR's specificity in a single genetic screen "is akin to lining up the crosshairs," says Barany. "We're talking about a new era of DNA diagnostics in which people will come in with an infectious disease and before they leave the doctor's office we'll know exactly what they have and how to treat it." ■ RICK WEISS

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A Scramble for Patent Rights

In addition to being the only researcher known to have taken a plunge in a scalding hot spring to pursue research with the ligase chain reaction (LCR), Francis Barany claims to be the only one to have sufficiently boosted LCR's signal-to-noise ratio to a level that enables identification of single nucleotide mutations. Banking on a favorable ruling from the U.S. Patent Office, which has yet to determine ownership of the novel process, Cornell is currently negotiating to license the technology Barany has developed. But the university's grip on LCR technology is less than secure. In July Abbott Laboratories acquired certain rights to LCR from BioTechnica International of Overland Park, Kansas, which has been quietly developing LCR methods for several years. And Caltech, where Barany did some of his early ligase work and where Leroy Hood and others continue to develop the art, has licensed its LCR rights to Applied Biosystems Inc. (ABI) of Foster City, California, which already holds one patent on an aspect of the technology and which has been actively developing LCR-related instruments. Three other California companies—SIBIA/Salk in La Jolla, Beckman Research Institute of the City of Hope, and Amgen in Thousand Oaks—also have LCR-related patents pending. So intense is the competition for rights to LCR that some have suggested renaming the technology Litigase Chain Reaction.

The controversy stems largely from the gradual nature of LCR's development. Back in 1988, for example, Leroy Hood and colleagues at Caltech published a paper in *Science* describing a gene detection technique that used a heat-labile ligase. Later that year, Dan Y. Wu and R. Bruce Wallace at the Beckman Research Institute took the technology a bit further and made the important proposal that use of a heat-stable ligase would eliminate the need to add fresh ligase after each cycle of heating and open the door to an automated methodology. Barany was first to publish in a scientific journal the cloning of a thermostable ligase and its use in a gene-detection system. But by then both Amgen and Applied Biosystems had obtained sequence detection patents that the companies claim are broad enough to cover Barany's process. And others, including Abbott, have applied for their own.

Although Abbott officials in Illinois are mum about their plans for LCR, sources say the company may be ahead of everybody when it comes to adapting LCR for clinical use, with prototype LCR-based tests already made for chlamydia, human papilloma virus, and HIV. Moreover, Abbott has designed its LCR methodology to be compatible with the company's line of automated analyzers already working in clinical laboratories around the world. "Abbott is in [LCR] in a very big way," confirms Keith Backman, who helped develop Abbott's technology at BioTechnica. And although the company has yet to hear from the U.S. Patent Office, he says, "If there is patent protection to be had, we believe we have got it." Backman claims that Abbott's cloning of a heat-stabile ligase gene was done "years ago... in the Dark Ages" compared to when Barany published his work, though he acknowledges that the accomplishment seems never to have appeared in a scientific journal. As for Applied Biosystems, he adds, their patent "is not as pertinent to LCR as they'd like to think."

In the end, it will be up to the patent office—and probably the courts—to decide who gets custody of LCR. It won't be an easy case, says Beckman's Bruce Wallace. "The real story," he says, "is that everybody has something and nobody has everything." ■ R.W.