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## Rapid Evolution of a New Fluorescent Protein

Fascinated by the efficient way the human immune system generates a rapid response to create a near-infinite variety of antibodies, researchers have “hijacked” that machinery and used it to evolve a new type of fluorescent protein.

The mutation process, called somatic hypermutation (SHM), normally acts on immunoglobulin genes, producing a large array of antibodies necessary to attack microbes and other foreign substances that the immune system may never have encountered before.

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— **Roger Y. Tsien**

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The researchers said their demonstration that SHM can be widely adaptable for research use opens the way for enormously faster mutation of genes to produce proteins with useful new properties, including research tools and disease therapeutics.

For example, the researchers used SHM to evolve a red fluorescent protein - which is used to track molecules inside cells - with improved stability and color emission properties beyond that which the researchers could create on their own. The properties of the new fluorescent protein, made by a mutant gene called *mPlum*, will make it a useful indicator of gene activity or protein trafficking when *mPlum* is attached to a specific gene in a living cell, they said.

The research team led by Roger Y. Tsien, a Howard Hughes Medical Institute investigator at the University of California, San Diego, reported its achievement in the November 30, 2004, issue of the *Proceedings of the National Academy of Sciences*. In related but separate studies, Tsien's group has used a different strategy to create an array of new colors of fluorescent protein. (To read about those studies, please go to

<http://www.hhmi.org/news/tsien2.html> ).

According to Tsien, the process of SHM, which takes place in antibody-producing B cells of the immune system, offers considerable advantages for researchers seeking to generate a large variety of mutant genes to produce proteins with new properties.

“The traditional approach to mutagenesis is to use a biochemical method to make changes in a gene in the test tube and then insert the gene into an organism to determine the properties of its protein,” he said. “You then have to grow it up and pull the gene out in order to do any further mutation cycles. So, you're constantly putting the gene in to find out its properties and pulling it out to make more changes. It's extremely tedious and time-consuming, and the size of the library of mutant genes is limited by the efficiency with which we can introduce at most one mutant copy of the gene per cell. If two different mutant genes end up in the same cell, the effect of the good mutant would be diluted and masked by the not-so-good version.”

Another approach to gene alteration, which is just as problematic, said Tsien, is to treat cells with x-rays or chemicals that generate mutations. “The problem there is that if you induce too much mutation, you kill the organisms because you're frying all the genes they need,” he said.

“What we really wanted was an organism with cells that could rapidly produce mutations and target them to one specific gene,” said Tsien. “To our surprise, that organism is us.” SHM produces mutant genes at roughly a million times the rate of mutation elsewhere in the genome, said Tsien. And in earlier studies, other researchers had demonstrated that SHM could be induced in B cells to repair a single mutation in a non-antibody gene.

“This finding hinted that the process might be useful for generating multiple mutations,” he said. “But it's like taking a perfectly typed manuscript, introducing one mistake, and giving it to a million bad typists to see whether any of them could accidentally fix it. They could eventually fix it, but what we were trying to do was take, say, one Shakespearean play and see if we could mutate it into a whole different story. It was unknown whether we could do it until we tried,” said Tsien.

To explore whether SHM could create a broad array of mutations, the researchers introduced a gene for a red fluorescent protein into a human B cell cancer line, called Ramos, that mutates immunoglobulin genes through SHM. The gene for the fluorescent protein was fitted with an “on-switch” that could be activated by the antibiotic doxycycline, so the researchers could control its activity in the cells. Since SHM affects only active genes, this technique allowed the researchers to also control the rate of mutation of the introduced gene.

The scientists' objective was to induce SHM to evolve new versions of the red fluorescent protein that would be more useful in the laboratory. Specifically, they hoped to create a molecule that fluoresced at longer wavelengths than the original protein when stimulated by laser light. Such a

fluorescent protein would help researchers use fluorescent proteins in intact mammals, because it would absorb and emit light at red wavelengths beyond those absorbed by blood.

Once they had allowed the B cells to carry out SHM on the gene for red fluorescent protein, the researchers used a cell-sorting technique based on fluorescence to separate out cells whose randomly mutated genes produced proteins that fluoresced at longer wavelengths. The researchers carried out 23 cycles in which they allowed SHM to mutate the gene. In each cycle they isolated cells that fluoresced at longer wavelengths. They then allowed those cells to proliferate, and then mutated the gene again with SHM.

At the end of these cycles, the process had yielded a mutant that the researchers named *mPlum* because of its purplish appearance under reflected light. In addition to its improved fluorescence, the new protein was also more resistant to bleaching by light than the original red fluorescent protein.

Particularly striking, said Tsien, was that SHM “outperformed” human researchers' efforts to design a protein that fluoresced at longer wavelengths based on their knowledge of protein structure.

“When people in the lab had used their best chemical intuition, they didn't get as far as SHM did. And SHM chose a much different route that arrived at a different structure than we would have,” said Tsien. “We were unable to think up *mPlum* ; it's something only SHM could have found. We are not patient enough to have gone through 23 rounds. And it would have taken us much more time and effort.”

And, the researchers found that SHM made a broad array of mutations in its production of new gene sequences - meaning that it can be a highly “creative” approach to producing improved genes.

Analysis of where the genes for the fluorescent proteins ended up when inserted into the B-cell genome revealed that initially many were integrated outside the region, or locus, that contained SHM's primary targets, immunoglobulin genes. However, the researchers found that the *mPlum* mutation was made on a gene that happened to land at an immunoglobulin locus.

“That makes us think that there is something special about the immunoglobulin locus that makes it a little better than other sites, and further efforts will aim at targeting such loci better in introducing genes,” said Tsien.

Overall, he said, SHM should prove to be extremely useful in directed protein evolution. “It will not be limited to the particular class of proteins that we studied,” he said. “It does require that you have a robust way to pick out the best cells of every generation without killing them. But if you have such a means, you should be able to select for practically any protein property you want. That means that the genes of the immune system—which has already proven to be smarter than us—can now be applied to produce a vast array of proteins other than antibodies.”