

NOVEMBER 01, 2001

## Evidence that a Ribozyme Evolved Multiple Times

Laboratory experiments designed to evolve new catalytic RNA molecules, called ribozymes, have demonstrated that a type of self-cleaving ribozyme found in highly divergent organisms might have evolved independently multiple times.

Thomas R. Cech, who is currently president of the Howard Hughes Medical Institute (HHMI), and colleagues at the University of Colorado, Boulder, discovered RNA catalysis in the early 1980s. Prior to Cech's research on RNA, many scientists believed that proteins were the only catalysts in living cells. A series of experiments done independently by Cech and Sidney Altman at Yale University ultimately revealed that RNA could also act as a biologic catalyst, a ribozyme.

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The evidence for multiple origins of the hammerhead ribozyme, which is found in organisms as diverse as plant viruses, newts, schistosomes and cave crickets, was published in the November 1, 2001, issue of the journal *Nature* by HHMI investigator Jack W. Szostak and Kourosh Salehi-Ashtiani at Massachusetts General Hospital.

According to Szostak, the evidence that the hammerhead ribozyme might have had multiple origins arose from experiments in which the scientists were attempting to create a faster self-cleaving ribozyme for use in research. "There were two alternatives that had been proposed for the evolution of this ribozyme," said Szostak. "Since the hammerhead is a very simple motif, if any structure was going to arise independently multiple times, it would be something like this," he said. "But there was always the possibility that all these enzymes descended from a single ancestral progenitor."

According to Szostak, one way to explore the origin of the ribozyme is to use *in vitro* directed evolution, a technique pioneered by Szostak to develop and isolate molecules with specific functions. *In vitro* directed evolution involves generating large numbers of DNA molecules with different sequences and imposing a selective pressure on the mixture. Alternately, the scientists can impose selective pressure on RNA or protein enzymes, to winnow out those with desired properties. Then, they can work backward to determine the original DNA sequences from which the selected molecules were derived.

In "evolving" self-cleaving ribozymes, Szostak and Salehi-Ashtiani first generated a large random pool of DNA molecules that would code for random-sequence RNAs. To prevent self-cleavage in this large pool of ribozymes, they added a short piece of DNA to the cleavage site on the RNA, thus protecting this site from the action of the ribozyme. The scientists then started the reaction by adding magnesium ions and "pushed" the selection for faster and faster cleavage by allowing less and less time for cleavage in subsequent rounds of selection. They purified those RNAs that had undergone self-cleavage. Next, they reverse-transcribed the original full-length, self-cleaving RNAs back into DNAs, replicated the DNAs, made large numbers of copies, and repeated the process of inducing the new batch of RNAs to self-cleave. To reveal the structures that had evolved, the researchers sequenced RNAs from sixteen successive rounds of selection.

"We were quite surprised when we found that the selection was completely dominated by hammerhead motif when we got up to the activity levels found in biological molecules," said Szostak. The evolution of the hammerhead motif likely resulted from the design of their experiments, he said. "While a number of other laboratories have done such selection experiments, they hadn't pushed as hard on the activities, so most of the published results had shown lower-activity ribozymes," he said.

"Clearly, once we required a certain level of activity, almost all the sequences we saw emerge from our selection process were consistent with formation of a hammerhead ribozyme," said Szostak.

Commenting on the studies, HHMI investigator Jennifer A. Doudna, who studies RNA structure and catalysis at Yale University, said, "It's a very interesting finding, and not necessarily one that would have been expected. It's particularly interesting that they set out initially to do a very different kind of experiment to try to identify fast self-cleaving sequences. And while one might have thought they would find something perhaps completely different than any of the ribozymes found to occur biologically, the surprising result is that the hammerhead is the best there is."

Szostak's and Salehi-Ashtiani's selection experiments also yielded some unusual RNA ribozyme sequences, in which structural elements expected to be conserved as necessary for self-cleaving functioning were altered. Closer study of those aberrant RNA structures might reveal insight into the self-cleaving mechanism of the hammerhead ribozyme.

According to Doudna, basic understanding of the hammerhead ribozyme's catalytic mechanism has been lacking. "The hammerhead was the first ribozyme structure determined by x-ray crystallography," said Doudna. "Since then, there has been some very elegant work – by people such as Bill Scott of the University of California, Santa Cruz, Olke Uhlenbeck at the University of Colorado, Boulder, and Dan Herschlag at Stanford – to address the mechanism of catalysis. But the frustrating thing is that, despite all that work, it's been very hard to nail that mechanism down exactly. As 'simple' as this ribozyme would appear, it's actually quite complex," she said.

Szostak and his colleagues plan to continue their efforts to achieve faster self-cleaving ribozymes, which might be useful for in vivo studies of cell function as well as therapeutics. Some ribozyme-based therapies seek to use hammerhead ribozymes to block the production of an unwanted protein in a metabolic disease by cleaving its messenger RNA before the protein can be produced. However, said Szostak, ribozymes have not proven active enough to be clinically useful.