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17. I thank C. J. Mora, J. C. McElwain, W. G. Chaloner, C. Yapp, K. Moulton, and T. E. Cerling for discussions and S. G. Driese for making unpublished data available. Supported by NSF grant EAR 9417325, Department of Energy grant FG02-95ER14522, and American Chemical Society Petroleum Research Fund grant 29132-ACS.

IN VITRO EVOLUTION

Ribozymes in Wonderland

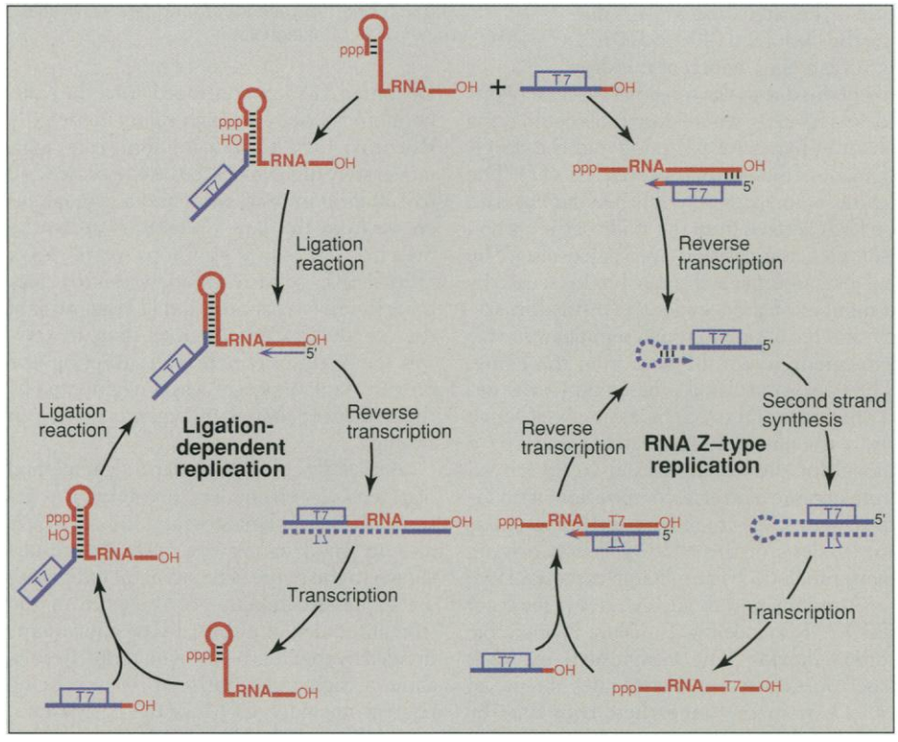
Andrew D. Ellington, Michael P. Robertson, Jim Bull

When Alice followed the White Rabbit down into Lewis Carroll's world of changing perspectives, she was confronted with edibles that made her bigger or smaller. At first, she could not generally predict whether a cake would make her shrink or a mushroom would make her as big as a house, but once she got the hang of things, she was able to change her size at will. A similar adventure comes to a happy conclusion on page 614 of this issue: Several years ago Breaker and Joyce tried to feed ribozymes and make them larger, but the diet did not take, and they grew smaller (*1*). Now Wright and Joyce have cleverly developed a different menu of substrates for different ribozyme diners, and this time the ribozymes grew larger (*2*).

The diverse possible courses of ribozyme evolution were not initially anticipated. Breaker and Joyce (*1*) attempted to develop a scheme for continuous evolution in which a group II ribozyme would ligate a primer sequence to itself and thus become fodder for a retroviral-like amplification reaction (see figure). Amplification required two steps: cDNA and second-strand synthesis from the RNA ribozyme template, then transcription from this double-stranded molecule to create new, descendant ribozymes (*3*). The primer sequence to be ligated was chosen to carry the T7 promoter sequence, and T7 RNA polymerase was provided in the reaction mix; hence, only ligated ribozymes could be amplified. The end product of the amplification cascade should have been the original ribozyme without the primer sequence, and any variants that arose during amplification would have competed for primer sequences in subsequent rounds of ligation and amplification. These reactions thus potentially enabled the continuous evolution of progressively better ligases.

Much to their surprise, this scheme did not evolve ligases. Within hours of starting the reaction, a short, self-replicating entity appeared that self-primed its own cDNA synthesis; a hairpin in the single-stranded DNA constituted the T7 promoter site, and the RNA polymerase generated an RNA complement to the cDNA, completing the cycle. This small molecule (dubbed RNA Z) had bypassed any need for ligation to a primer sequence, and from Breaker and Joyce's perspective, was a parasite of the reaction. Alice had gotten smaller. The rapid ascendance of RNA Z in the reaction mixture thwarted the more deliberate evolution of a better ligase.

In today's report, Wright and Joyce finally got Alice to grow larger—by ligation. Because the group II ribozyme appeared unsuited to continuous evolution, Wright and Joyce chose a faster ribozyme, a ligase selected from a completely random sequence pool by Bartel and Szostak (*4*) and optimized for function by



An evolutionary decision. The left-hand cycle depicts the ligation-dependent replication of a ribozyme, as described in this issue (*2*). The right-hand cycle depicts the ligation-independent replication of a parasite, RNA Z (*1*). The group II intron on the right "devolved" into RNA Z in part because its initial rate of ligation was slower than the formation of RNA Z–like intermediates; the Bartel ligase on the left evolved faster and faster variants in part because its initial rate of ligation was faster than the formation of RNA Z–like intermediates.

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Ekland *et al.* (5). The ligation junction of the Bartel ligase was altered to provide the appropriate start site for T7 RNA polymerase. Unfortunately, this modification reduced the activity of the ligase by 10,000-fold, once again rendering it too slow for continuous evolution. Thus, a partially randomized population was generated, and Wright and Joyce carried the population through 15 cycles of conventional selection (ligation products purified before amplification) and 100 cycles of a simplified selection protocol in which ribozymes were allowed to react and then were directly transferred to the amplification mixture. At the end, the ribozyme was finally fast enough to successfully grow (ligate) rather than shrink (internally prime).

Roughly 10^{11} copies of the pre-evolved ligase were used to seed a continuous-evolution reaction (see figure). The ribozyme mass increased at the expense of primers and nucleotides, and each individual produced roughly 1000 copies of itself. After 60 minutes, the carrying capacity of the tube was nearly reached and the now-starving population was serially transferred to a new source of food. The ribozyme mass regrew, was transferred, regrew, was transferred, and so forth over 100 serial transfers. Overall, the evolutionary potential of the continuous-evolution system was enormous: In a little more than 2 days, the net amplification was nearly 1000 doublings. The catalytic efficiency of an evolved ribozyme variant was 14,000 times that of the parent.

Wright and Joyce's success required intelligent use of evolutionary principles. The initial reaction conditions enforced discrete amplification, whereas the final reaction conditions allowed continuous amplification. Discrete protocols proceed step by step, with the reaction being halted at specific points, and thereby allow tight control over what is selected at each step. [The polymerase chain reaction (PCR) uses discrete amplification, for example, because each replication step must await the proper temperature.] The downside of discrete amplification is that it provides for sluggish evolution; molecules have to await the researcher's permission to reproduce each time, and those with a fitness advantage can only realize that advantage once per cycle (6). In contrast, continuous amplification intimately couples variation in amplification to reproductive success: Each molecule reproduces at its own pace, so faster ones continually outpace all slower ones, and their numerical advantage grows disproportionately over time. Paradoxically, although continuous evolution is faster, it is a double-edged sword because it denies much investigator control over the outcome: amplification parasites (for example, RNA Z) can accumulate at the expense of slow catalysts. In contrast, during discrete evolution any variants that are catalytically competent can be efficiently amplified. Moreover, because the relatively faithful PCR is frequently used for amplification, rather than the more evolutionarily prone isothermal protocol, amplification parasites cannot as easily arise and overrun the selected population. Thus, discrete evolution may for the time being be better suited to produce novel nucleic acid catalysts.

Perhaps ironically, in vitro biochemical systems such as these may provide a new dimension to studies of classic problems in organismal ecology and evolution. For example, the contrast of today's study by Wright and Joyce with the earlier one by Breaker and Joyce mirrors a fundamental issue in classical evolutionary biology. Like Alice early in her adventure, evolutionary biologists have yet to achieve much power to predict the course of evolution (as opposed to explaining it once it happens). One group argues that an understanding of natural selection is sufficient to predict the course of evolution; a much smaller group (championed by Richard Lewontin) argues that the course of evolution is buried in the "details" and often depends on nuances of the biology rather than predominantly on natural selection. The contrast between these two ribozyme studies strengthens both views and suggests that they are not necessarily exclusive: The evolutionary trajectory was sensitive to the starting conditions, profoundly so, yet both outcomes were consequences of intense natural selection.

Ribozyme studies like these should enable further comparative studies of evolution—an approach previously reserved for the most ancient of evolutionary processes. Comparative studies of in vitro evolution even offer the possibility of experimental tests. The benefit to organismal biology of in vitro biochemical reactions may not even be limited to molecular evolution. A recently proposed coupled amplification mimics predator-prey dynamics (7). Nor should it be assumed that the relation between organismal biology and in vitro evolution is purely one-sided. Just as the intelligent mix of discrete and continuous amplification protocols contributed to Wright and Joyce's success, other principles of evolutionary biology may increase the versatility of these systems. Recombination can be used to funnel evolutionary pathways toward a desired outcome, or functions can be indirectly evolved through intermediates rather than by selecting outright for the final goal.

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NOTA BENE: NEUROSCIENCE

Seeing the Synapse

A neuron is studded with thousands of synapses, membranous gateways through which the cell sends messages to other neurons in the form of tiny pulses of neurotransmitter molecules. To monitor the action at these synapses, investigators have had to be content with electrical recording methods, which usually yield an aggregate measure of synapse behavior. An article in a recent issue of the *Proceedings of the National Academy of Sciences* reports a method by which this invisible cell-to-cell communication can be dramatically visualized at all of the synapses on a single cell.

The authors have constructed fusion proteins, called synaptolucins, from the enzyme luciferase—which makes a chemiluminescent product—and the synaptic proteins synaptotagmin or synaptobrevin. When cultured neurons from rat hippocampus were infected with recombinant viruses containing these artificial proteins, the synaptolucins traveled to the synapse and took up residence. Upon electrical stimulation, the cells communicated with their neighbors, releasing packets of neurotransmitter from the synapse by exocytosis and exposing the synaptolucins to the extracellular fluid. The luciferase part of the artificial molecule reacted with its substrate luciferin in the fluid at each active synapse, emitting light that was easily recorded.

The present sensitivity of the method is about five quanta (or packets) of neurotransmitter. But with expected improvements, it should be possible to see single exocytotic events. The resulting ability to know precisely the behavior of a cells' synapses could greatly increase our understanding of the cell's computation rules.

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