between RNAs or between RNA and a selected chelator in the presence of a moderately slowly exchanging metal like $Ni^{2+}$. The simultaneous presence of the usual mono- and divalent ions would not hamper such studies, since they will aid general RNA folding without effectively competing with slow exchanging ion at its rare binding sites. When molecules have been selected that stably bind the first (slow exchange) metal, the binding domain must be defined. This domain can then be used as a linker to join multiple RNAs in the presence of a second (very slowly exchanging) divalent, let's say $Pt^{2+}$, with the same coordination geometry as your first ion (both $Ni^{2+}$ and $Pt^{2+}$ favor square planar arrangements for their ligands). Selection, which requires reversal or disassembly of the metal-mediated complex, could be simplified using this strategy without sacrificing the ultimate production of stable multimolecular structures. If you are feeling a bit short of development time, funds, or motivation, you might well use the binding domain of SHR1 RNA, as shown above in Figure 1 (Figure 3 of Hati et al.) [7].

**Michael Yarus**
Department of Molecular, Cellular and Developmental Biology
University of Colorado
Boulder, Colorado 80302

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# RNA Sex

**Recombination of genetic information is a major driving force in evolution, today catalyzed by protein enzymes. In this issue of *Chemistry & Biology*, a paper by Riley and Lehman [1] demonstrates that RNA can perform general recombination of RNA strands, thus supporting the scenario of a prebiotic RNA world.**

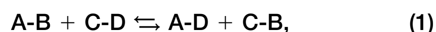
Advances in evolution have for some time been viewed by many as a process of continuing occurrence of minor mutations under the constant scrutiny of selection pressure, blocking the amplification of deleterious mutations and favoring a few punctual improvements. Besides the apparent painful slowness of such a process, mathematical models have clearly indicated that populations under a certain number of individuals would suffer a continuous loss of fitness from mildly deleterious mutations rather than producing a series of champions [2]. Sexual recombination offers a way out, since "good" mutations can also arise in, and propagate from, strains with a number of deleterious mutations. Moreover, rather than effecting single point mutations, recombination can generate, by error or design, sequence changes to a much larger extent. Such events enlarge the complexity of a given sequence pool by leaps and bounds in comparison.

All but a few of today's replicating entities try to improve their evolutionary flexibility by employing recombination, sometimes even by borrowing genetic information from outside their species, as in the case of horizontal gene transfer in bacteria. Failure or inability to perform recombination may lead to serious drawbacks. A case in point are animal mitochondria, which have resorted to a number of complicated mechanisms to compensate for deleterious effects of slowly accumulated point mutations in the mitochondrial genome [3].

Recombination at the level of RNA has been reported for viruses [4]. Splicing of mRNAs to generate a variety of different exons from a pre-mRNA is a mechanism bearing characteristics akin to recombination, although the newly generated sequence information is not handed down to the next generation. RNA from certain organisms is capable of performing the splicing reaction without the help of cofactors, protein or other, by sequential execution of a cleavage and a ligation reaction. Incidentally, these properties present the first reported catalytic activities of RNA, the discovery of which, by Cech and coworkers in 1982 [5], was later awarded the Nobel price.

In their paper "Generalized RNA-Directed Recombination of RNA," Riley and Lehman [1] make use of the catalytic properties of such introns to catalyze a reaction resembling a metathesis reaction:

$$\text{A-B} + \text{C-D} \underset{}{\overset{\text{RNA catalysis}}{\leftrightarrows}} \text{A-D} + \text{C-B}, \quad (1)$$



where A-B is a first RNA comprising a head and a tail part, and C-D is a second RNA composed similarly.

Based on previous observations by several other groups, the authors have turned, through proficient mo-

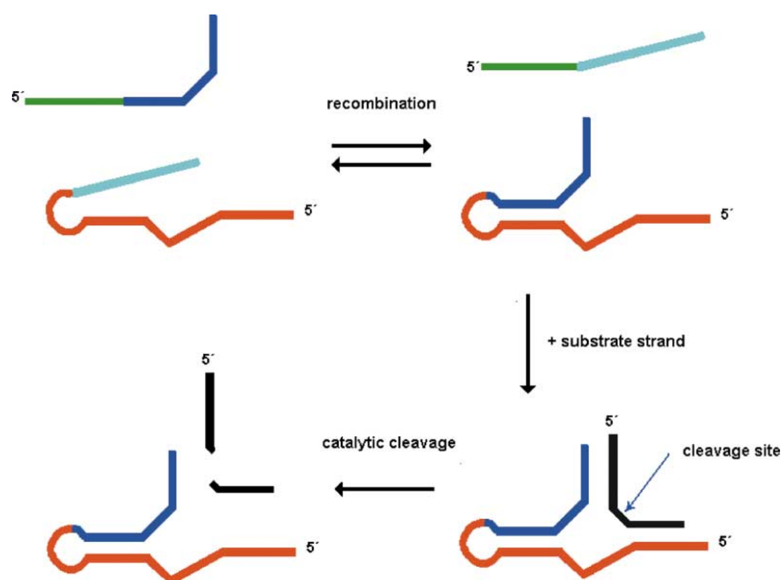



**Figure 1. Creation of New Functional RNA Domains by RNA Recombination, as Exemplified by Riley and Lehman with the Hammerhead Ribozyme**

The sequence elements composing a hammerhead ribozyme are initially contained in two separate RNA molecules. The 5′ end of the hammerhead ribozyme (red) corresponds to the head A of RNA A-B in Equation 1, while the 3′ end of the hammerhead (blue) corresponds to the tail D of RNA C-D. RNA recombination generates the product A-D, a functional hammerhead which can bind and cleave a substrate RNA (black) strand *in trans*.

lecular engineering, the ribozymes into tool enzymes that cleave the first RNA at a defined site marked by a short recognition sequence. The (5′) head A of that first RNA is released from the active site, while the (3′) tail B gets covalently bound to the ribozyme (termed PUTT, *p*ick *u*p *t*he *t*ail reaction). Upon addition of the second RNA C-D, its cleavage is accompanied by the release of its tail D, while its head C is kept in the active center to be joined with the tail B of the first RNA. Owing to the actual event of recombination, this reaction was dubbed REC. The overall result is a combination of the head of the first RNA with the tail of the second RNA. The authors have first carried out PUTT and REC steps in separate reaction tubes, thus controlling the nature of the substrate in each step. This favored formation of C-B from A-B and C-D, since C-D was not permitted as a substrate in the PUTT reaction. They subsequently showed that PUTT and REC can be combined in a one-pot reaction. In that case, C-D is also available for the PUTT reaction, and the complementary recombination product must also be formed, so that the overall reaction equation corresponds to that cited above (Equation 1).

A recombination product can of course reenter the recombination cycle, and given the right proportions, substrates A-B and C-D can be reformed from products A-D and C-B, the whole scheme being a dynamic equilibrium. The reaction as such is energetically neutral and could theoretically go on forever, if there were not a competing hydrolytic side reaction producing single head or tail fragments A, B, C, and D.

The authors investigated various oligonucleotide combinations and thereby demonstrated the general nature of this mechanism. Finally, they assembled a hammerhead ribozyme by recombination from catalytically inactive precursors and found it to be enzymatically active (Figure 1). This demonstrates that RNA-directed RNA recombination can lead to the creation of completely new molecular function, which is of particular relevance to evolution in the hypothetical RNA world.

While the intrinsic capability of RNA introns for recombination has been pointed out before, the authors' major contribution is strictly separating the three players, namely ribozyme function and the two crossover substrate sequences, into three different molecules and adjusting both of the ribozyme's catalytic activities (PUTT and REC) to a *trans* reaction. The catalytic RNA should, in principle, perform multiple turnover on any pair of RNA molecules carrying a short recognition sequence.

In addition to its evolutionary implications, the method developed in the article by Riley and Lehman has great potential to enter the field of in vitro evolution as a ubiquitous tool. While an artificial variant of DNA recombination, DNA shuffling, has found successful application in the evolution of enzymes and other proteins [6], a comparable recombination method at the RNA level is still lacking. Such techniques would be particularly useful for scientists working on the in vitro evolution of new ribozymes or nucleic acid ligands (aptamers) [7]. In these fields, the goal is to identify a few sequences with whatever properties are desired, usually starting from a pool of $10^{14}$–$10^{15}$ sequences. To that end, a selection event is engineered to retain nucleic acids with the desired properties, and retained nucleic acids are enzymatically amplified. Repetition of this scheme increases the fitness of the sequence population for selective retention and concurrently reduces pool complexity [8].

To enrich the sequence pool and increase its complexity, mutations can be introduced by error-prone PCR [9]. During such "morphing," a large fraction of the selected sequences is destroyed in hope of generating a few better ones. Drawbacks are obvious: not only is a large portion of previously established fitness destroyed, but also mutagenic PCR rarely produces new properties, it mostly improves on a few chosen ones.

In contrast, recombination can combine two or more favorable preexisting sequence elements that have been retained in separate RNA molecules from the original pool into a single RNA sequence. More exciting yet, one might combine pools from different selections to merge

the separately evolved qualities. Assuming one wants to generate RNA aptamers binding two different targets simultaneously, one can envisage performing two selections on separate pools, then scrambling sequence pieces through recombination. This strategy will be even more efficient when three or more functional domains are combined. Functional domains are not restricted to aptamer-type ligand binding, as illustrated by recent advances leading to allosterically regulated ribozymes [10].

Many principal concepts of in vitro evolution have been modeled after natural evolution. If, likewise, the concept of in vitro RNA recombination is successfully implemented in practical protocols of in vitro evolution, many tasks currently out of reach can be tackled by this technique.

**Andres Jäschke and Mark Helm**
Ruprecht-Karls-Universität Heidelberg
Institut für Pharmazie und Molekulare Biotechnologie
Im Neuenheimer Feld 364
Abteilung Chemie
D-69120 Heidelberg
Germany

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