

# Ribozymes:

## Catalytic RNAs that cut things, make things, and do odd and useful jobs

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Catalytic RNAs, or ribozymes, are a fossil record of the ancient molecular evolution of life on Earth and still provide the essential core of macromolecule synthesis in all life forms today. Are they also an avenue to the development of new catalysts to recreate evolution, or to use as therapeutics and molecule sensors?

Twenty years ago, it became clear that ribonucleic acids, or RNAs, are used as catalysts in living cells, in addition to their known roles in information storage and as molecular architectural frameworks. This idea was so profoundly contrary to the central dogma of molecular biology that it resulted in the award of a Nobel prize to two of the early proponents, Thomas Cech and Sidney Altman. It has inspired what is now itself the dogmatic view of pre-biotic macromolecules, the 'RNA World' hypothesis (Gesteland *et al.*, 1999). This hypothesis states that RNA, or something like it, was the original macromolecule, capable of both encoding its own reproductive information and catalysing the essential chemical reactions that effect reproduction. The RNA World is a compelling vision in that it offers a solution to the 'chicken-and-egg' problem of how to simultaneously bring into existence the complex processes of encoding genetic information in nucleic acids while decoding them into their 'functional' products, the enzymes. If a single macromolecule could be both the genetic carrier and the catalyst, decoding into the more modern catalysts, proteins, could have evolved later.

While proteins have largely taken over as the biological catalysts of the modern world, there are still remnants of the RNA World to be found. Cech, Altman, and others originally characterised them in isolated RNA processing reactions, but recent work suggests that RNA

catalysts are still at the heart of modern RNA and protein synthesis.

### Ribozymes that cut themselves or other RNAs

The original discovery of ribozymes by Cech and Altman was twofold – RNA segments that cut themselves out of larger RNAs (self-splicing introns) and a protein-assisted RNA enzyme (ribonuclease P) that cuts the leader sequences off all transfer RNAs throughout the three organismal domains. (The first two are the Nobel lectures: Altman, 1990; Cech, 1990; Gesteland *et al.*, 1999). This instigated a hunt for catalytic RNAs with a function in the biological processing of RNA. Quickly, a large number of self-splicing or self-cleaving RNAs were identified that make single cuts in precisely defined RNA sequences (the difference being that self-splicing RNAs go further and rejoin the ends of two single cuts) (Table 1). Although the reactions are often facilitated by protein cofactors *in vivo*, the active enzyme in each case is the RNA component. Two general mechanisms are recognised for splicing and cleavage reactions, characterised by the kinds of intermediates and products they generate. These mechanisms, shown in Figure 1A and B, differ primarily in that the 'A' mechanism uses an external hydroxyl group (from water or a nucleoside) as the backbone cutting nucleophile, whereas the 'B' reaction uses the 2'-hydroxyl from the sugar immediately preceding the scissile phosphodiester.

Table 1. Natural ribozymes and what they do

Ribozyme	Number identified	Biological source	Reaction catalysed (product)
Group I introns	>1000	Eukaryotes (nucleus and mitochondria), prokaryotes, bacteriophages	Self-splicing transesterification (3'-OH)
Group II introns	>700	Eukaryotes (organelles), prokaryotes	Self-splicing transesterification (3'-OH)
Group-I intron like	6	<i>Didymium</i> , <i>Naeglaria</i>	Hydrolysis (3'-OH)
RNase P RNA	>300	Eukaryotes (nucleus and organelles), prokaryotes	Hydrolysis (3'-OH)
Hammerhead ribozyme	11	Plant viroids and satellite RNAs, newt	Self-cleaving transesterification (2',3'-cyclic phosphate)
Hairpin ribozyme	4	Plant viroids and satellite RNAs	Self-cleaving transesterification (2',3'-cyclic phosphate)
Hepatitis delta virus ribozyme	2	Human hepatitis delta virus	Self-cleaving transesterification (2',3'-cyclic phosphate)
VS ribozyme	1	<i>Neurospora</i> mitochondria	Self-cleaving transesterification (2',3'-cyclic phosphate)
Ribosomal RNAs	>5000	Eukaryotes, prokaryotes	Peptidyl transfer (peptide bond)
Spliceosomal RNAs	>100	Eukaryotes	Trans-splicing transesterification (3'-OH)

In a catalytic RNA up to hundreds of nucleotides in length, only a single bond is cut. How can an RNA catalyst achieve this level of precision? Soon RNA biochemists started to employ tools extensively developed for protein enzymology to explore this conundrum. One of the first 'tricks' they taught their new pets was to cut an external substrate in 'trans', rather than themselves in 'cis'. With this advent, these ribozymes became true catalysts, facilitating repeated substrate reactions without being modified themselves (Figure 2). Besides providing a powerful avenue to ribozyme-mediated gene inactivation (see below), *trans*-acting ribozymes are amenable to the

complete arsenal of enzymology, including pre-steady-state and steady-state kinetic analysis.

Quickly, 'ribozymology' focused our attention on one of the most dominant properties of RNA as a biopolymer, its high negative charge (one charge per nucleotide). As a consequence, the presence of positively charged cations is essential for the catalytic activity of all ribozymes. In the cell, the cation of choice is  $Mg^{2+}$ , as it has a high affinity for the negatively charged phosphate backbone of RNA and is the most abundant divalent metal ion (one to two mM free concentration). Its double charge enables bridging of two phosphates from distant RNA regions, facilitating long-range structure formation. Indeed, RNA catalysts can form intricate three-dimensional structures that rival in complexity those of protein enzymes, allowing for the precise positioning of a substrate in a catalytic core (Figure 3). In addition,  $Mg^{2+}$  in aqueous solution can form the deprotonated  $Mg(OH)(aq)^+$  complex that, due to its residual positive charge, still binds to RNA. This relieved the chemists' concern that, at first sight, the four nucleobases of RNA (guanine, adenine, cytosine, uracil) seemed quite limited in their ability to do all the chemistry outlined in Figure 1. A properly positioned  $Mg(OH)(aq)^+$  ion, acting as a base catalyst, could easily be envisioned as an essential cofactor for most of these reactions. Much to the dismay of RNA biochemists, ribozymes were thus quickly dismissed as chemically inferior to proteins, which sport 20 versatile aminoacid side chains.

However, in recent years, several ribozymes have taught us new lessons about their catalytic

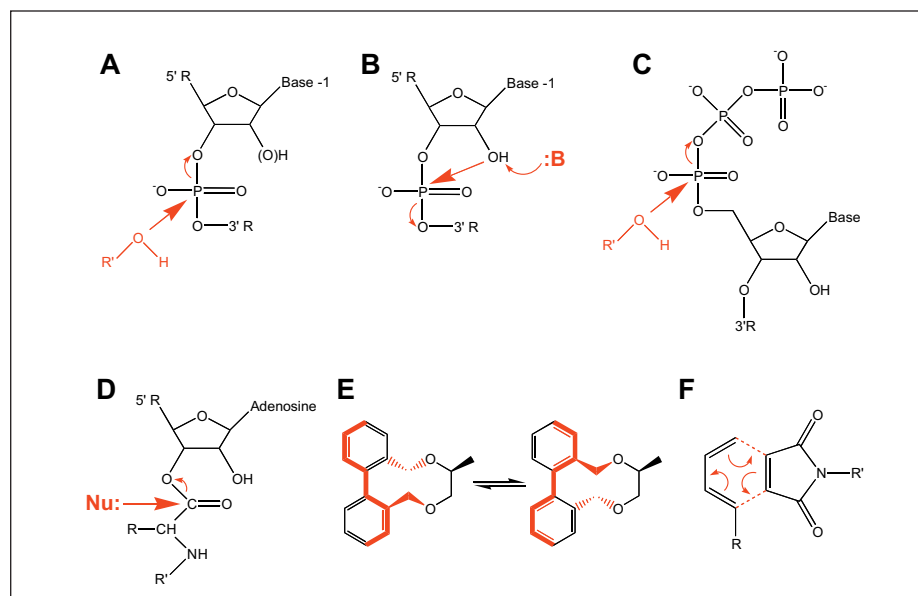


Figure 1. The catalytic portfolio of ribozymes. (A) Hydrolysis ( $R' = H$ ) or transesterification ( $R' =$  organic residue) of an RNA or DNA phosphoester linkage.  $R' = H$  for RNase P,  $R' =$  guanosine for group I introns,  $R' =$  internal adenosine for group II introns. (B) RNA cleavage as catalysed by the small hammerhead, hairpin, and HDV ribozymes ( $B =$  base). (C) RNA chain elongation. (D) Peptidyl transfer ( $Nu = NH_2$  group of another amino acid), amide bond formation ( $Nu = 5'-NH_2$  of a modified RNA), or ester hydrolysis ( $Nu =$  water). (E) Isomerisation. (F) Diels-Alder cycloaddition.

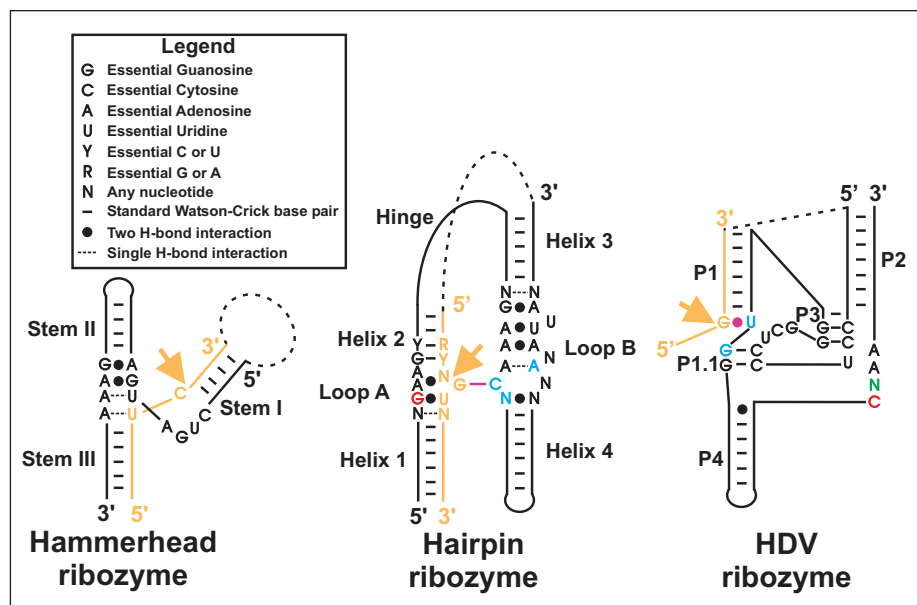


Figure 2. What they look like: Schematic representations of the three-dimensional structures of three small ribozymes, engineered to act *in trans* on external substrates of choice. Long dashed lines, sequences removed to generate the *trans*-acting ribozymes; gold, substrates; arrows, cut sites. Additional colours used in the hairpin and HDV ribozymes correlate with those used in Figure 3.

proceed. One such lesson is that larger ribozymes can not only precisely position an RNA substrate in a pre-organised active site, but also similarly position a second substrate such as a nucleoside to react it with as a nucleophile. Hence, RNA can drive catalysis by forced proximity of reaction partners (Gesteland *et al.*, 1999).

A second and completely unexpected lesson of recent years is the fact that ribozymes have found ways to utilise their own side chains directly to do chemistry (Butcher, 2001). Two examples from small catalytic RNAs, the hairpin and hepatitis delta virus (HDV) ribozymes, are shown in Figures 2 and 3. In both cases, the ribozyme precisely fixes the location of its substrate by hydrogen bonding in a specific binding pocket. It then employs one of its nucleobases, whose protonation equilibrium it fine-tunes for maximum reactivity around physiologic pH, to exchange a particular proton with the substrate, inducing a specific cut. The human analogy that comes to mind is that of an able blacksmith, holding his metal to an anvil to ply it with his hammer in a particular spot. No wonder that catalytic RNA is so precise and adept.

One particularly interesting aspect of these small ribozymes that are engineered to cut *in trans* is their straightforward targeting mechanism – Watson-Crick base-pairing between sequences flanking the catalytic domain and sequences surrounding the cleavage site. This means that the target RNA substrate can be varied by simply changing these targeting sequences (Figure 2). This provides the ability to easily create enzymes, ‘designer ribozymes’, to repeatedly cleave and inactivate specific RNA sequences. This idea has particularly caught the attention of researchers hoping to inhibit virus infection or oncogene function by providing these tailored ribozymes to human cells (Lewin & Hauswirth, 2001).

### Pervasive use of RNA in synthesis of macromolecules

The use of RNA in protein synthesis has long been part of the central dogma. Not only is information carried in messenger RNA (mRNA) triplet codons, but transfer

RNA (tRNA) serves as the adapter to interpret codons into amino acids; and the enormously complex ribosome, containing both ribosomal RNA (rRNA) and protein components, serves as the factory for decoding the message into protein chains. In addition, the last 20 years have seen a steady rise in the number of distinct small RNAs that are known to be used in macromolecular synthesis and regulation. Small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) are essential subunits of a large and diverse group of RNA-protein complexes (RNPs) that process pre-mRNAs and pre-rRNAs, respectively. Small cytoplasmic RNAs (scRNAs) include the 7SL subunit of the signal recognition particle, an RNP involved in nascent protein translocation from ribosomes into the endoplasmic reticulum. Finally, complexes between proteins and small double-stranded RNAs have recently been discovered

as the basis of powerful and universal pathways of cellular gene regulation and antiviral defense, called RNA interference (RNAi).

Although a high percentage of RNA processing reactions appear to involve RNPs, it is unclear exactly what catalytic elements may reside in the essential RNA

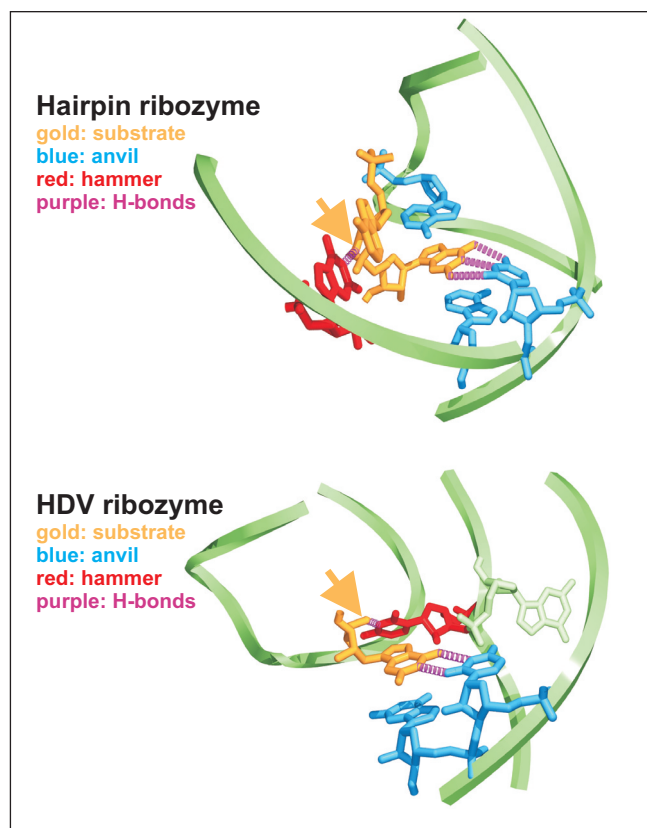


Figure 3. Caught in the act: Details of the catalytic core of the hairpin and HDV ribozymes. The colour scheme is the same as in Figure 2. The red nucleotides are poised to act on the substrate by abstracting or donating a proton. Dashed purple tubes, hydrogen bonds to fix the substrate in place.

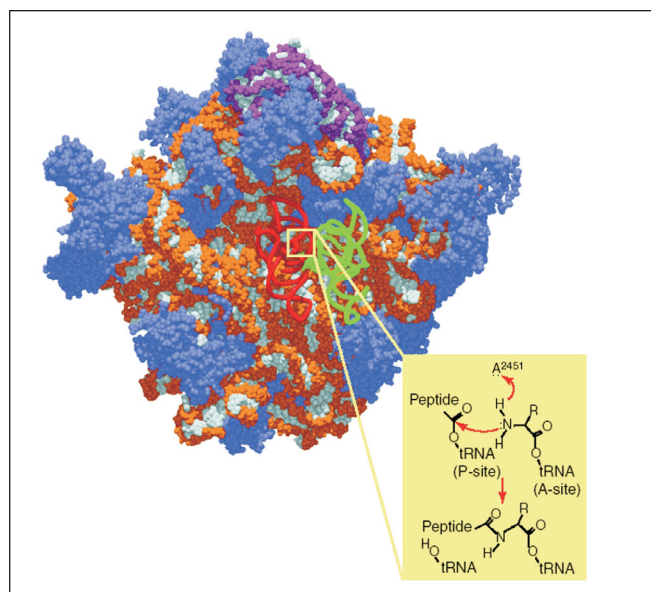


Figure 4. A ribosome's true colours. (Top) The large subunit of the ribosome, with proteins in blue and RNA components in orange, grey, and burgundy. Green and red ribbons, tRNAs. (Bottom) The peptidyl transfer mechanism catalysed by the RNA components of the ribosome. A particular adenosine (A2451 in *Escherichia coli*) is rendered unusually basic by its environment within the folded structure; it is presumed to act as a base and abstract a proton as shown. Reprinted with permission from T R Cech (2000). Copyright 2000, American Association for the Advancement of Science.

subunits of these RNPs. In the case of mRNA splicing (intron removal), the RNP involved, called the spliceosome, clearly reconstitutes the same reaction found in the simpler, self-splicing ribozymes of the group II introns (Table 1). In fact, recent evidence suggests that the snRNA components are ribozymes themselves and carry the catalytic activity of the spliceosome. Thus, an external RNA-containing complex has evolved to splice *in trans*, rather than each intron needing to carry its own ribozyme. Many of the modern functions of RNA might simply involve such cases, in which a function that originally was performed by an RNA on itself has become externalised.

For a while, interest in ribozymes had been limited somewhat due to the feeling that they were essentially a 'one-trick' class of enzymes – they simply cut other RNAs. While this left the aficionados with a lot of interesting RNA processing reactions, the RNA World hypothesis was waning in the general scientific community. For one thing, RNA would have had to accomplish a great many things, especially the synthesis of its eventual successors in molecular evolution, proteins. In this context, there was much rejoicing when a series of papers over the last few years established that the ribosome, that gigantic particle that was always thought to contain a large RNA framework for structural reasons only, is really a large ribozyme that catalyses peptide bond formation. While the modern ribosome has become dependent on proteins for efficient function, it is clear that the catalytic centre is still composed of RNA. Early work suggested that the deproteinised rRNA component could carry out the peptidyl synthetase reaction, and recently published crystal structures show that the region of the active center is solely composed of RNA (Figure 4), with protein components residing primarily on the periphery of the complex (Cech, 2000; Nissen *et al.*, 2000). As with the small hairpin and HDV ribozymes (Figure 3), a particular RNA nucleotide has been

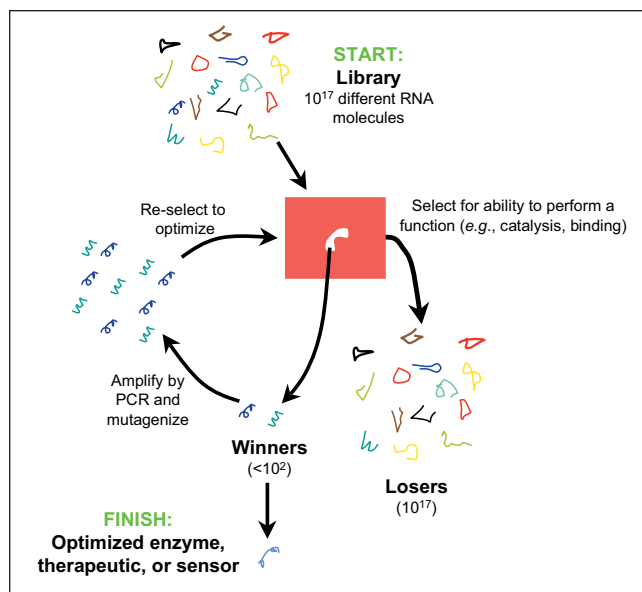


Figure 5. Teaching an ancient dog new tricks by harnessing nature's techniques: In vitro evolution of RNA. Starting from a library of many diverse molecules, the fittest ones for a certain function are selected, amplified, mutagenised, and re-selected until the winners fulfil a given task to perfection.

proposed to exchange a proton with the substrate (Figure 4, bottom), although this is still hotly debated within the field (Moore & Steitz, 2002). Be that as it may, at its core, protein synthesis is composed of two tRNA cofactors bringing together the growing peptide chain and the next amino acid in an RNA active site.

### New RNA catalysts through forced molecular evolution

Can RNAs be used to catalyse additional reactions? The answer is emphatically yes, and new ribozymes are currently being identified through a process that is variously called *in vitro* evolution or SELEX (Systematic Evolution of Ligands by EXponential enrichment (Wilson & Szostak, 1999)) (Figure 5). The idea is a simple one, given current technology, and mimics natural evolution. A large pool ( $\sim 10^{15}$ – $10^{17}$ ) of RNA or DNA molecules is synthesised, in which the sequences at the ends are known, but the centre is randomised. In this huge sequence pool, there is something (usually a lot of things) that will bind to practically any molecular surface or fulfil any number of enzymatic tasks to at least some extent. In its simplest form, RNA or DNA that binds to a molecular target is separated by one of many methods from the great majority of molecules that do not bind. Because the end sequences of all molecules are the same and known, it is possible to amplify the binders by a variation of PCR (polymerase chain reaction) called RT-PCR (reverse transcription followed by PCR) to get sufficient physical quantities of the binding molecules to characterise. Typically, the winners of the first round are sent through at least several rounds of re-selection, often with deliberate mutagenesis, to obtain winners with optimised binding characteristics, so-called aptamers.

The main limitation when applying this technique to identify new ribozymes is the ability to sort the small number of molecules that perform a particular function from the large number that do not. This is relatively straightforward when the desired function is tight binding to a molecule that can be immobilised on a bead or surface. However, when searching for an enzyme, it is necessary to

be cleverer than that, and only a limited number of enzymatic activities have been identified to date (Wilson & Szostak, 1999). Again, the predominant classes are those that synthesise, chemically modify, or cut nucleic acids, important contributions for supporting the RNA World hypothesis. In addition, classes of ribozymes with unrelated enzymatic activities have been selected. One important demonstration has been the discovery of a peptidyl transferase enzyme (catalysing the reaction in Figure 1D). This is particularly interesting because the new enzyme does not necessarily mimic the ribosome's active site. The ability to catalyse other reactions, *e.g.*, alkylations, isomerisations, Diels-Alder cycloadditions, and metal transfers, has also been found (for some examples see Figure 1). This diversity of reaction types further encourages belief in a period of early molecular evolution on Earth where nucleic acids catalysed the full range of reactions necessary for their own reproduction and metabolism.

Although many of these nucleic acid enzymes are not as efficient as known protein counterparts, it is not clear that *in vitro* evolution of the individual enzymes has been pushed to its fullest efficiency yet, especially considering possible inclusion of cofactors. The beauty of this form of molecular evolution is that it can be pushed quite ruthlessly to attain the goals of the experimentalist. Conditions and accessory molecules can be varied while mutagenising heavily and applying stringent selection for the desired function. It is a form of evolution where it is possible to control all the rules (short of changing physical laws) and focus on a single outcome. The future of nucleic acid catalysts (and aptamers) therefore seems not limited to enzymes that might once have existed in an RNA World, but can include solutions to problems not encountered in nature. Scientists have already started to fathom their options by selecting high-affinity and -specificity aptamers, which can be used as therapeutics to block function of their targets *in vivo* or as diagnostic sensors to detect them *in vitro*. These applications promise a rich future for today's explorers of the RNA World.

### Further reading

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### Website

[www.imb-jena.de/RNA.html](http://www.imb-jena.de/RNA.html)

This 'RNA World Website' is maintained at the Institute for Molecular Biotechnology in Jena and has links to numerous databases, web tools, tutorials and scientific meetings revolving around RNA.

After training for his PhD from 1992 to 1995 with the 1967 Chemistry Nobel Laureate Manfred Eigen at the Max-Planck-Institute for Biophysical Chemistry (Göttingen, Germany) and as a postdoctoral fellow from 1995 to 1999 with John M Burke at the University of Vermont (Burlington, VT, USA), **Nils G Walter** is now Assistant Professor of Chemistry at the University of Michigan (Ann Arbor, MI, USA). His research interests are in the area of structure and dynamics of small catalytic RNA, using biophysical tools such as fluorescence spectroscopy. **David Engelke** is a Professor of Biological Chemistry at the University of Michigan and an Associate Editor of the journal of the international RNA Society, RNA. His laboratory investigates the biosynthesis and functions of small RNAs in eukaryotic nuclei.

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