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Model systems: how chemical biologists study RNA

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Abstract

Ribonucleic acids are structurally and functionally sophisticated biomolecules and the use of models, frequently truncated or modified sequences representing functional domains of the natural systems, is essential to their exploration. Functional non-coding RNAs such as miRNAs, riboswitches, and, in particular, ribozymes, have changed the view of RNA's role in biology and its catalytic potential. The well-known truncated hammerhead model has recently been refined and new data provide a clearer molecular picture of the elements responsible for its catalytic power. A model for the spliceosome, a massive and highly intricate ribonucleoprotein, is also emerging, although its true utility is yet to be cemented. Such catalytic model systems could also serve as “chemo-paleontological” tools, further refining the RNA world hypothesis and its relevance to the origin and evolution of life.

Introduction

Ribonucleic acids (RNAs) are now known to perform diverse cellular functions, far beyond their “classical” roles as mediators of protein synthesis. Importantly, the role of non-coding regions, including microRNAs and riboswitches, continues to be the subject of intense current research [1–4]. RNA-based enzymes, or ribozymes, were the first functional non-coding RNAs to be discovered and have intrigued the scientific community ever since [5,6]. In particular, understanding the molecular intricacies of RNA catalysis remains of contemporary interest and relevance to molecular and chemical biology, as well as to prebiotic chemistry and evolution [7,8]. Given the large sizes of the first ribozymes, it initially appeared daunting to study them from physical organic chemistry and mechanistic perspectives. Subsequent discoveries of smaller ribozymes triggered, however, the development of model systems, which allowed chemists to advance the fundamental understanding of RNA as a genuine catalytic molecule.

The term ‘model systems’ embraces numerous and somewhat ambiguous meanings and might elicit different connotations among scientists in distinct disciplines. For an RNA biochemist, a model system could imply the truncation of a large RNA to a smaller and manageable core sequence largely capable of mimicking the structure and function of the biological macromolecule. Despite inherent challenges, such minimized constructs have found great utility in RNA biochemistry. Alternatively, one might view the fabrication of RNA sequence

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Dedication

Dedicated to the memories of **Leslie E. Orgel** (1927–2007) and **Stanley L. Miller** (1930–2007).

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that contain modified or reporter nucleobases as model systems that, in addition to mimicking the unmodified truncated RNA, come with the added value of built in probes. The fabrication of such systems could be more challenging, but has the potential to provide information that is not accessible by more classical means of traditional biochemistry. Yet, model systems could also further deviate from their natural counterparts. Such reductionism, popularized in the zenith of biomimetic chemistry, would view, for example, bis-dinitrophenyl phosphate as a model substrate for exploring RNA hydrolysis. Here we consider model systems that are truncated or isolated parts of the natural entities. They offer the advantage of retaining biological relevance and function, while at the same time serving the chemists' need for facile synthesis and chemical modification.

In this review we discuss two biologically relevant catalytic RNA systems of contrasting levels of understanding. The first is the well-known hammerhead ribozyme, where only recently biochemical data using model systems have been congruent with updated structural work. Recent studies are highlighted using the original and updated model systems that are finally producing detailed mechanistic views of the hammerhead's catalysis. The second system is the spliceosome. This ribonucleoprotein complex dwarfs the hammerhead in size and its RNA components are the least understood in terms of their role in catalysis. Given the spliceosome's large size and dependence on many protein components, one wonders whether or not simple RNA model systems can be considered valid. We look at a recent emerging model and its ability to shed light on the catalytic role of the RNA component.

The hammerhead ribozyme: the model that gained weight

The hammerhead ribozyme is a small motif within plant RNA viroids that is responsible for processing the genome via a self-cleaving phosphodiester transesterification reaction [9–11]. Understanding how the hammerhead increases the rate of this transformation by a million fold compared to the corresponding background uncatalyzed reaction continues to be of fundamental interest. This section focuses on the evolution of hammerhead model systems, largely favored due to their manageable size, and not on detailed analyses of self-cleaving ribozymes and their mechanisms, which have been reviewed [12,13].

Boundary experiments, defining the essential regions of the hammerhead motif [10], coupled to Uhlenbeck's hammerhead generated by hybridizing two oligonucleotides [14], ultimately led to a working model system known as the "minimal" (45 nt) hammerhead (Figure 1A) [15]. While the natural transformation takes place intramolecularly (or in *cis*), the bimolecular, *trans*-cleaving version shown in Figure 1 became the commonly used one [16]. In addition to the conserved 15 nt core, the ribozyme necessitates surrounding sequences shown as helices I, II, and III that presumably provide structural and conformational support. These sequences are adjustable both in length and nucleotide composition, but require Watson-Crick complementarity. The loop capping off helix II, while not conserved, was identified in all natural motifs and thus included in the minimal hammerhead construct. This synthetically simple model system was used to generate most of the early biochemical functional data. In particular, mutagenesis studies showed that replacing all core residues, except for nucleotide 7, impacts reaction rates, confirming their role in the assembly of the active site [16].

A unique predicament, not encountered with other ribozymes, emerged with the publication of the first hammerhead crystal structures, [17,18] where the structural and biochemical data were in conflict [19]. In particular, the hammerhead conformation, as appeared in the solid-state structures, appears inconsistent with the proposed mechanism and involvement of key residues as established by systematic biochemical analysis. An especially troubling issue was the role of residue G12 in hammerhead catalysis. Modifications to G12 were known to drastically affect cleavage rates, suggesting its participation in assembling the transition state.

Yet, G12 was nowhere near the cleavage site in the crystal structure of the minimal hammerhead.

A potential resolution to this problem surfaced when a larger version of the minimal hammerhead, which contained an additional loop off helix I, was shown to display enhanced ribozyme activity (Figure 1B) [20]. This key discovery followed observations showing that the minimal hammerhead was not active *in vivo* while the natural motifs were. Expanding helix I with a domain first thought to be inconsequential resurrected ribozyme activity. The expanded hammerhead was indeed 100 to 1000 times faster than the minimal hammerhead due to tertiary interactions that populate the proper conformation necessary for cleavage [21]. An essential crystal structure of the hammerhead containing the additional loop [22], which is now termed an “extended” hammerhead (Figure 1B), showed a dramatically different hammerhead transition state-like conformation in the crystalline state and offered a solution to the structure–function dilemma [23].

The refined view of the hammerhead raised a crucial question regarding the validity of the biochemical data gathered with the minimized motif over decades of explorations. Nelson and Uhlenbeck have recently shown that essentially all previous cleavage kinetics data generated by modifying the active site nucleotides are consistent with the proposed transition state structure of the extended hammerhead [24]. Even the troubling quandary with residue G12 mentioned above was resolved, as this residue is perfectly positioned near the cleavage site in the extended hammerhead structure [22].

So what does the crystal structure of the minimal hammerhead show? It is now established that the minimal hammerhead has at least two primary conformations, the first being the active cleaving conformation that is representative of natural hammerheads and a second, more prevalent inactive conformation that had consistently been observed in crystal structures [25, 24]. Does this imply that the minimal hammerhead is no longer a good model system? This is likely to be the case if one is concerned with transition state structures. It remains, however, quite informative for biochemical investigations. It was, after all, results generated with the minimal hammerhead that suggested nucleotides G8 and G12 as the general acid and base residues, respectively (Figure 2A) [26]. Furthermore, a new tertiary base pair was identified between G8 and C3 and was suspected to facilitate the correct orientation of the 2'-OH of G8 for general acid catalysis using the minimal hammerhead construct (Figure 2) [27]. Indeed, mutations of G8 and C3 residues were shown to impact cleavage rates in both the minimal and expanded hammerhead constructs [27].

While the minimal hammerhead still remains a relevant model system for biochemical studies, there has been a definite shift towards the use of the expanded construct. This has particularly been the case for exploring the role of residue G12 as a general base (Figure 2) [25]. An extended hammerhead, modified at the C17 cleavage site, was recently used in affinity labeling studies. Specifically, replacing the 2'-OH with a 2'-bromoacetamide residue created a potent electrophilic trap for the suspected anionic G12 general base (Figure 2B). The pH dependence of the alkylation reaction rate mirrored that of the hammerhead cleavage reaction rate, supporting the proposal that G12 acts as a general base and suggesting that its pK_a is significantly modulated in the context of the ribozyme [28].

The specific roles of G8 and Mg^{2+} in acid catalysis have also been recently addressed. As shown in Figure 2, it is the 2'-OH of G8 and not the nucleobase itself that serves as a general acid. Even with pK_a perturbations within the folded ribozyme, this hydroxyl is unlikely to become acidic enough, unless influenced by a metal ion cofactor. Divalent metal ions such as Mg^{2+} have long been known to facilitate catalysis. Less clear are their specific effects as general or Lewis acids, or their impact on structural stability and global folding [29,30]. In a recent

FRET study with the extended hammerhead, different metal ions produced minimal variability in global folding but caused dramatically different cleavage rates [31], suggesting a metal ion participation in the transition state (Figure 2C) [32]. The metal ion, however, could also be serving as a Lewis acid coordinated to the 5' scissile oxygen (labeled X in Figure 2C). To discern this potential ambiguity Thomas and Perrin used a modified extended hammerhead containing a 5'-sulfur-based leaving group (called the S-link substrate) along with modifications to the 2'-OH of G8 (deoxyG8, and 2'-OMe versions) [33]. It was demonstrated that divalent metals lower the pK_a of the G8 2'-OH via direct coordination as shown in Figure 2C and not by acting on the 5'-scissile residue [25,32].

The spliceosome: can this RNP machine be deciphered using model systems?

Eukaryotic RNA transcripts contain exons (coding regions) separated by introns (non-coding regions). These precursor-mRNAs (pre-mRNAs) are processed to the corresponding mature mRNAs by the spliceosome [34–38]. This massive ribonucleoprotein (RNP) complex, containing hundreds of proteins and five small nuclear RNAs (snRNA), catalyzes the two transesterification reactions that encompass the splicing and ligation processes [39–44]. It is certainly justifiable to ask whether or not RNA models can be considered biologically relevant where the native system is dependent on both proteins and RNA. Here we discuss attempts to model individual events of this inherently complex process using truncated RNA systems.

The spliceosome is an assembly of five RNP complexes (U1, U2, U4, U5, and U6) that interact with pre-mRNAs at different stages (Figure 3). It was shown that the catalytically essential complexes were the U2, U5 and U6 RNPs, which specifically bind to reactive regions. More importantly, the snRNAs of U2 and U6 were discovered to be necessary for catalysis in addition to their base-pairing recognition and binding roles [45–49]. Exactly how they participate in catalysis is the subject of current research, but these early observations led to the hypothesis that the spliceosome is a ribozyme [50–52]. The similarity of the spliceosome to group II intron ribozymes furthered this view. Both follow nearly identical splicing mechanisms. Also, the Intermolecular Stem-Loop (ISL), which contains a highly conserved AGC sequence in the U6 snRNA is strikingly similar to Domain V of the ribozyme [53–56]. Further evidence that supported the hypothesis of reactive snRNAs without the assistance of proteins came from *in vitro* evolution studies. Ribozyme activity was identified when a library of various RNAs based on the core sequences of U2 and U6 were subjected to rounds of selection and amplification [57,58]. But it was the publication of a protein free, catalytically active, minimal U2–U6 snRNA complex that proved most compelling [59].

The minimal splicing complex is able to assemble and recognize a small branch point oligonucleotide *in vitro* and catalyze a phosphoesterification reaction using a branch point adenosine similar to the natural system (Figure 4A) [59]. It consists of two RNA strands (70–80nt) containing the conserved ACAGAGA box, AGC triad and ISL that are essential for catalysis. While attempts were made to include a model 5' splice site intron, catalysis was only observed with the branch point RNA strand. Consequently, this system does not catalyze the native reaction; instead the branch point strand attaches to the conserved G of the AGC triad forming a non-natural phosphotriester bond and eliminating water. Despite this difference, the minimal complex appears to be structurally and functionally similar since mutagenesis and other biochemical data match the natural system. To further support the inherent reactivity and relevance of the U2–U6 complex, a follow up study used purified natural snRNAs to demonstrate the production of the same phosphotriester product [60].

A relevant model needs to demonstrate that a truncated system can utilize the 5' splice site and branch point pre-mRNA regions to catalyze the native reaction. Modifications to the minimal

U2–U6 splicing complex were made to include a 5' splice site intron in a covalent addition to the 5' domain of U6 via a stabilized linker to ensure correct folding and reactivity (Figure 4B) [61]. This proved necessary because, in the spliceosome, the U5 snRNA and proteins facilitate binding of the intron and the U6 domains. This model system catalyzes a reaction resembling the one *in vivo*, and is also shown to be sequence dependent at the 5'-splice site as in the natural system. The product is the addition of the branch point oligonucleotide via a covalent 2',5' bond of the adenosine to the splice site of the intron concomitant with the loss of the small exon sequence [61]. Mutagenesis of the conserved and non-conserved regions provided evidence that the complex adopts an active site conformation similar to the natural system. While it appears that a biologically relevant model has been identified, a recent study demonstrated that caution must be taken when such modifications are made [62].

Another attempt to use the minimal U2–U6 complex on the well-studied yeast spliceosome by constructing a modified version in which the U2 and U6 were embedded in one RNA strand, but separated with a polyU loop and stabilized with additional GC residues, was made (Figure 4C) [62]. The branch-site strand was extended for enhanced base pair stabilization to U2. New to this construct was the incorporation of a third RNA strand to represent the 5' splice site intron. While a reaction took place, it did not produce the expected product. It was discovered that the complex did not use the 5' splice site substrate, nor did it use any of the conserved sequences, including the ISL in U6, in its catalysis. Systematic truncation and labeling studies indicated that the system shown in Figure 4C could be reduced to a minimal active version, which catalyzes a unique phosphodiester-based reaction (Figure 4D) [62]. The complete branch site oligonucleotide inserts itself into the U6 region, but not with the usual branching A residue. Instead it utilizes the 2' OH of the residue on the 5' end, forming a unique 2'–3' phosphodiester bond while excising the remaining U6 construct. Do the consequences of the seemingly conservative modification made to the U2–U6 minimal system foreshadow future problems with this model? A recent discussion concerning the validity of protein free splicing systems has surfaced, and a more thorough process to fabricating U2–U6 models might be needed [63,64].

RNA model systems: Tools for understanding the origin of life and chemical evolution?

This year marks the 150th anniversary of Darwin's publication *On the Origin of Species*. While he was largely concerned with the evolutionary mechanisms of new species, his interest in the origin of life is famously documented in the 1871 letter to his friend, botanist Joseph Hooker [66]:

"It is often said that all the conditions for the first production of a living organism are now present which could ever have been present. If (and oh! What a big if!) we could conceive in some warm little pond, with all sorts of ammonia and phosphoric salts, light, heat, electricity present, that a protein compound was chemically formed, ready to undergo still more complex changes. At the present day, such matter would be instantly devoured or absorbed, which would not have been the case before living creatures were formed".

While our understanding of the molecular basis of biology has since been significantly advanced, shedding light on the origin of life remains, as Darwin knew it, a fascinating and frequently frustrating endeavor.

Over the last three decades RNA has become the most relevant biomolecule for understanding Life's origin. Discoveries that point to RNA's diverse roles in biology [67], advances made in its prebiotic synthesis [68,69] and the ongoing study of catalytic RNAs continue to provide compelling evidence for the RNA World hypothesis [70–73]. The hammerhead and the U2–

U6 model systems discussed above are good examples for catalytic models that serve as “chemo-paleontological” tools in learning how RNA could have sustained an ancient biochemical world. Specifically, the discovery that nucleobases serve as catalytic moieties in small nucleolytic ribozymes such as the hammerhead beautifully demonstrates that chemically limited biomolecules (compared to the diverse building blocks found in proteins) can be quite resourceful in performing functional and genetic roles [74]. In terms of evolution, the spliceosome, while not likely to have originated in an RNA world, is still considered to have originally been an RNA machine which was either a relative to the group II intron or a product of evolutionary convergence [75,76]. It is likely the U2–U6 minimal complex, in some way, represents a vestige of the early RNA based spliceosome. Utilizing it as a starting point for directed evolution experiments or exploring the impact of systematic additions of RNA and/or protein components on catalysis, could provide a glimpse into the chemical evolution of this minimal system and the complexity of contemporary spliceosomes [77].

Interestingly, the ribosome, a more ancient RNP, which carries out the process of translation in contemporary cells, has been hypothesized to emerge from an RNA-only core structure termed a *proto-ribosome* [78–81]. At the heart of the peptidyl transferase center is a ribozyme which undoubtedly originated in the RNA world, albeit, with more primitive functionalities [82]. Identifying and experimentally corroborating the proto-ribosome hypothesis is likely to illuminate how RNA-catalyzed peptide bond formation took place over 3.8 billion years ago. Using a proto-ribosome as an RNA model system could also clarify how proteins have contributed and refined this machine over the course of evolution [83].

Conclusions

While RNA model systems have been widely exploited, their use with catalytically active RNA molecules is particularly informative. RNA domains, primarily involved in ligand binding, can be frequently truncated with little loss of function. In contrast, catalytically active RNAs could be exquisitely sensitive to modifications. Substrate specificity and product identity, as well as stereochemical and kinetic features, can all be monitored and used to assess the validity and quality of a proposed model system. Indeed, the minimal hammerhead has served as the workhorse model system for over two decades. Its size and simplicity facilitated the probing of virtually every aspect of its function. Even though early structural work is now considered to reveal inactive conformations, most, if not all, previous biochemical data has been reconciled with the updated extended hammerhead structure. The extended variant is presently taking over as the system of choice for mechanistic studies on ribozyme catalysis.

Employing RNA model system to the spliceosome, in comparison, is in its embryonic stages. Given the relatively recent identification of the U2–U6 complex as a model system, its true utility for exploring the first splicing reaction and its possible participation in the ligation step [38,65], remain to be established. It appears to be sufficiently relevant to the natural complex, provided that modifications are critically investigated. Since this RNA model lacks any protein counterparts, certain aspects, most notably its kinetic parameters, are unlikely to match the natural spliceosome.

The study of non-coding RNAs using model systems will undoubtedly continue to shed light on our biochemical origins. It is important to appreciate how young this field actually is and the likely existence of other biologically important non-coding RNAs we have yet to discover [84]. Future findings of RNA’s role in biology may prove more beneficial to our understanding of early and extant life. While 150 years have passed since the birth of a major biological revolution, RNA is clearly providing us with another.

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References and recommended reading

1. Bartel DP. MicroRNAs: Target Recognition and Regulatory Functions. *Cell* 2009;136:215–233. [PubMed: 19167326]
2. Sotiropoulou G, Pampalakis G, Lianidou E. Emerging roles of microRNAs as molecular switches in the integrated circuit of the cancer cell. *RNA* 2009;15:1443–1461. [PubMed: 19561119]
3. Montange RK, Batey RT. Riboswitches: Emerging Themes in RNA Structure and Function. *Annu Rev Biophys* 2008;37:117–133. [PubMed: 18573075]
4. Shabalina SA, Koonin EV. Origins and evolution of eukaryotic RNA interference. *Trends in Ecology and Evolution* 2008;23:578–587. [PubMed: 18715673]
5. Kruger K, Grabowski PJ, Zaug AJ, Sands J, Gottschling DE, Cech TR. Self-Splicing RNA: Autoexcision and autocyclization of the ribosomal RNA intervening sequence of the Tetrahymena. *Cell* 1982;31:147–157. [PubMed: 6297745]
6. Guerrier-Takada C, Gardiner K, Marsh T, Pace N, Altman S. The RNA moiety of the ribonuclease P is catalytic subunit of the enzyme. *Cell* 1983;35:848–857.
7. Harris ME, Cassano AG. Experimental analyses of the chemical dynamics of ribozyme. *Curr Opin Chem Biol* 2008;12:626–639. [PubMed: 18952193]
8. Muller UF. Re-creating an RNA world. *Cell Mol Life Sci* 2006;63:1278–1293. [PubMed: 16649141]
9. Prody GA, Bakos JT, Buzayan JM, Schneider IR, Bruening G. Autolytic Processing of Dimeric Plant Virus Satellite RNA. *Science* 1986;231:1577–1580. [PubMed: 17833317]
10. Forster AC, Symons RH. Self-cleavage of plus and minus RNAs of a virusoid and a structural model for the active sites. *Cell* 1987;49:211–220. [PubMed: 2436805]
11. Symons RH. Small catalytic RNAs. *Annu Rev Biochem* 1992;61:641–671. [PubMed: 1497321]
12. Cochrane JC, Strobel SA. Catalytic strategies of self-cleaving ribozymes. *Acc Chem Res* 2008;41:1027–1035. [PubMed: 18652494]
13. Bevilacqua PC, Yajima R. Nucleobase catalysis in ribozyme mechanism. *Curr Opin Chem Biol* 2006;10:455–464. [PubMed: 16935552]
14. Uhlenbeck OC. A small catalytic oligoribonucleotide. *Nature* 1987;328:596–600. [PubMed: 2441261]
15. Haseloff J, Gerlach WL. Simple RNA ribozymes with new and highly specific endoribonuclease activities. *Nature* 1988;334:585–591. [PubMed: 2457170]
16. Birikh KR, Heaton PA, Eckstein F. The structure, function and application of the hammerhead ribozyme. *Eur J Biochem* 1997;245:1–16. [PubMed: 9128718]
17. Pley HW, Flaherty KM, McKay DB. Three-dimensional structure of a hammerhead ribozyme. *Nature* 1994;372:68–74. [PubMed: 7969422]
18. Scott WG, Finch JT, Klug A. The crystal structure of an all-RNA hammerhead ribozyme a proposed mechanism for RNA catalytic cleavage. *Cell* 1995;81:991–1002. [PubMed: 7541315]
19. Blount KB, Uhlenbeck OC. The structure-function dilemma of the hammerhead ribozyme. *Annu Rev Biophys Struct* 2005;34:415–440.
20. Khvorova A, Lescoute A, Westhof E, Jayasena SD. Sequence elements outside the hammerhead ribozyme catalytic core enable intracellular activity. *Nat Struct Biol* 2003;10:708–712. [PubMed: 12881719]
21. De la Pena M, Gago S, Flores R. Peripheral regions of natural hammerhead ribozymes greatly increase their self-cleavage activity. *EMBO J* 2003;22:5561–5570. [PubMed: 14532128]
22. Martick M, Scott WG. Tertiary contacts distant from the active site prime a ribozyme for catalysis. *Cell* 2006;126:309–319. [PubMed: 16859740]
23. Nelson JA, Uhlenbeck OC. When to believe what you see. *Molecular Cell* 2006;23:447–450. [PubMed: 16916633]

24. Nelson JA, Uhlenbeck OC. Hammerhead redux: Does the new structure fit the old biochemical data? *RNA* 2008;14:605–615. [PubMed: 18287565]An analysis/review of a variety of minimal hammerhead ribozymes that demonstrate an agreement from biochemical studies to the updated structure of the extended hammerhead.
25. Martick M, Lee TS, York DM, Scott WG. Solvent structure and hammerhead ribozyme. *Chemistry and Biology* 2008;15:332–342. [PubMed: 18420140]This article presents mechanistic hypotheses for the roles of the nucleobases in the active site
26. Han J, Burke JM. Model for General Acid-Base Catalysis by the Hammerhead Ribozyme: pH-Activity Relationships of G8 and G12 Variants at the Putative Active Site. *Biochemistry* 2005;44:7864–7870. [PubMed: 15910000]
27. Nelson JA, Uhlenbeck OC. Minimal and extended hammerheads utilize a similar dynamic reaction mechanism for catalysis. *RNA* 2008;14:43–54. [PubMed: 17998291]Nucleotide residues not previously known to have an interaction in the active site of the minimal hammerhead, are the main subject of this study along with the demonstration that the minimal hammerhead is still a relevant model.
28. Thomas JM, Perrin DM. Probing general base catalysis in the hammerhead ribozyme. *JACS* 2008;130:15467–15475. A paper that presents evidence for G12 serving as a general base.
29. Kisseleva N, Khvorova A, Westhof E, Schiemann O, Wolfson AD. The different role of high-affinity and low-affinity metal ions in cleavage by a tertiary stabilized *cis* hammerhead ribozyme from tobacco ringspot virus. *Oligonucleotides* 2008;18:101–110.
30. O'Rear JL, Wang S, Feig AL. Comparison of the hammerhead cleavage reactions stimulated by monovalent and divalent cations. *RNA* 2001;7:537–545. [PubMed: 11345432]
31. Boots JL, Canny MD, Azimi E. Metal ion specificities for folding and cleavage activity in the *Schistoma* hammerhead ribozyme. *RNA* 2008;14:2212–2222. [PubMed: 18755844]
32. Lee TS, Lopez CS, Giambasu GM, Martick M, Scott WG, York DM. Role of Mg^{2+} in hammerhead ribozyme catalysis from molecular simulation. *J Am Chem Soc* 2008;130:3053–3064. [PubMed: 18271579]
33. Thomas JM, Perrin DM. Probing general acid catalysis in the hammerhead ribozyme. *J Am Chem Soc* 2009;131:1135–1143. [PubMed: 19154176]This paper provides evidence for the specific role of divalent metal ions as general acids.
34. Sashital, DG.; Butcher, S. Is the Spliceosome a Ribozyme?. In: Lilley, DM.; Eckstein, F., editors. *Ribozymes and RNA catalysis*. RSC Publishing; 2008. p. 253-269.
35. Ritchie DB, Schellenberg MJ, Gesner EM, Raithatha SA, Stuart DT, MacMillan AM. Structural elucidation of a PRP8 core domain from the heart of the spliceosome. *Nature Structural and Molecular Biology* 2008;15:1199–1205.
36. Pena V, Rozov A, Fabrizio P, Luhrmann R, Wahl MC. Structure and function of an Rnase H domain at the heart of the spliceosome. *EMBO J* 2008;27:2929–2940. [PubMed: 18843295]
37. Wahl MC, Will CL, Luhrmann R. The Spliceosome: Design principles of a dynamic RNP machine. *Cell* 2009;136:701–718. [PubMed: 19239890]The most up to date review covering both the protein and RNA components of the spliceosome in structural and functional roles
38. Wachtel C, Manely JL. Splicing of mRNA precursors: the role of RNAs and proteins in catalysis. *Mol Biosyst* 2009;5:311–316. [PubMed: 19396366]A recent review focusing on the catalysis aspect of the spliceosome.
39. Nilsen, TW. RNA-RNA interaction in nuclear pre-mRNA splicing. In: Simon, RW.; Grunberg-Manago, M., editors. *RNA Structure and Function*. Cold Spring Harbor Laboratory Press; 1998. p. 279-307.
40. Graveley BR. Alternative splicing: increasing diversity in the proteomic world. *Trends in genetics* 2001;17:100–107. [PubMed: 11173120]
41. Patel AA, Steitz JA. Splicing double: insight from the second spliceosome. *Nature Reviews* 2003;4:960–970.
42. Will, CL.; Luhrmann, R. Spliceosome structure and function. In: Gesteland, R.; Cech, TR.; Atkins, JF., editors. *The RNA World*. Cold Spring Harbor Laboratory Press; 2006. p. 369-400.
43. Valadkhan S. The spliceosome: a ribozyme at heart? *Biol Chem* 2007;388:693–697. [PubMed: 17570821]

44. Smith DJ, Query CC, Konarska MM. "Nought may endure but mutability": Spliceosome dynamics and the regulation of splicing. *Molecular Cell* 2008;30:657–666. [PubMed: 18570869]
45. Sontheimer EJ, Steiz JA. The U5 and U6 small nuclear RNAs as active site components of the spliceosome. *Science* 1993;262:1989–1996. [PubMed: 8266094]
46. Lesser CF, Guthrie C. Mutations in U6 snRNA that alter splice site specificity: implications for the active site. *Science* 1993;262:1982–1988. [PubMed: 8266093]
47. Kandels-Lewis S, Seraphin B. Role of U6 snRNA in 5' splice site selection. *Science* 1993;262:2035–2039. [PubMed: 8266100]
48. Sun JS, Manley JL. A novel U2-U6 snRNA structure is necessary for mammalian mRNA splicing. *Genes Dev* 1995;9:843–854. [PubMed: 7705661]
49. Yean SL, Wuenschell G, Termini J, Lin RJ. Metal-ion coordination by U6 small nuclear RNA contributes to catalysis in the spliceosome. *Nature* 2000;408:881–884. [PubMed: 11130730]
50. Collins CA, Guthrie C. The question remains: Is the spliceosome a ribozyme? *Nature Structural Biology* 2000;7:850–854.
51. Brow DA. Allosteric cascade of spliceosome activation. *Annu Rev Genet* 2002;36:333–360. [PubMed: 12429696]
52. Butcher SE, Brow D. Towards understanding the catalytic core structure of the spliceosome. *Biochemical Society Transactions* 2005;33:447–449. [PubMed: 15916538]
53. Sharp PA. On the origin of RNA splicing and Introns. *Cell* 1985;42:397–400. [PubMed: 2411416]
54. Cech TR. The generality of self-splicing RNA: relationship to nuclear mRNA splicing. *Cell* 1986;44:207–210. [PubMed: 2417724]
55. Gordon PM, Sontheimer EJ, Piccirilli JA. Metal ion catalysis during the exonligation step of nuclear pre-mRNA splicing: extending the parallels between the spliceosome and group II introns. *RNA* 2000;6:199–205. [PubMed: 10688359]
56. Dayie KT, Padgett RA. A glimpse into the active site of a group II intron and maybe the spliceosome, too. *RNA* 2008;14:1697–1703. [PubMed: 18658120] Interesting paper that reviews the recent publication of the group II intron crystal structure and highlights the similarities with the U6 snRNA of the spliceosome.
57. Tuschel T, Sharp PA, Bartel DA. Selection in vitro of novel ribozymes from a partially randomized U2 and U6 snRNA library. *EMBO J* 1998;17:2637–2650. [PubMed: 9564046]
58. Tuschel T, Sharp PA, Bartel DA. A ribozyme selected from variants of U6 snRNA promotes 2',5'-branch formation. *RNA* 2001;7:29–43. [PubMed: 11214178]
59. Valadkhan S, Manley JL. Splicing-related catalysis by protein-free snRNAs. *Nature* 2001;413:701–707. [PubMed: 11607023]
60. Valadkhan S, Manley JL. Characterization of the catalytic activity of U2 and U6 snRNAs. *RNA* 2003;9:892–904. [PubMed: 12810922]
61. Valadkhan S, Mohammadi A, Wachtel C. Protein-free spliceosomal snRNAs catalyzes a reaction that resembles the first step of splicing. *RNA* 2007;13:2300–2311. [PubMed: 17940139]
62. Smith DJ, Konarska MM. Identification and characterization of short 2'-3' bond forming ribozyme. *RNA* 2009;15:8–13. [PubMed: 19029304] In attempting to use a modified version of the U2-U6 minimal version, a different ribozyme within the model itself is discovered.
63. Smith DJ, Konarska MM. A critical assessment of the utility of protein-free splicing systems. *RNA* 2009;15:1–3. [PubMed: 19029306]
64. Valadkhan S, Manley JL. The use of simple model systems to study spliceosomal catalysis. *RNA* 2009;15:4–7. [PubMed: 19029305]
65. Mefford MA, Staley JP. Evidence that U2/U6 helix I promotes both catalytic steps of pre-mRNA splicing and rearranges in between these steps. *RNA* 2009;15:1386–1397. [PubMed: 19458033]
66. Darwin, CR. The life and letters of Charles Darwin, including an autobiographical chapter. Vol. Vol 3. New York Johnson Reprint Corp; 1969.
67. Sharp PA. The centrality of RNA. *Cell* 2009;136:577–580. [PubMed: 19239877]
68. Szostak JW. Systems chemistry on early Earth. *Nature* 2009;459:171–172. [PubMed: 19444196]
69. Powner MW, Gerland B, Sutherland JD. Synthesis of activated pyrimidine ribonucleotides in prebiotically plausible conditions. *Nature* 2009;459:230–242. [PubMed: 19444211] Challenges in the

prebiotic syntheses of RNA nucleotides are shown to be overcome by some clever synthetic chemistry.

70. Woese, C. The Evolution of the Genetic Code: Molecular basis for genetic expression. Harper & Row; 1967.
71. Crick FC. The origin of the genetic code. *J Mol Biol* 1968;38:367–379. [PubMed: 4887876]
72. Orgel LE. Evolution of the genetic apparatus. *J Mol Bio* 1968;38:381–393. [PubMed: 5718557]
73. Gilbert W. The RNA World. *Nature* 1986;319:618.
74. Wilson TJ, Lilley DM. The evolution of ribozyme chemistry. *Science* 2009;323:1436–1438. [PubMed: 19286542] The elucidation of ribozyme mechanisms is essential to understanding the RNA world hypothesis.
75. Pyle, AM. Group II introns: Catalysts for splicing, genomic change and evolution. In: Lilley, DM.; Eckstein, F., editors. *Ribozymes and RNA catalysis*. RSC Publishing; 2008. p. 201–228.
76. Cech TR. Crawling out of the RNA world. *Cell* 2009;136:599–602. [PubMed: 19239881] In considering the evolution of RNPs, they may have used proteins all along, even in the RNA world.
77. Ellington AD, Chen X, Robertson M, Syrett A. Evolutionary origins and directed evolution of RNA. *International Journal of Biochemistry and Cell Biology* 2009;41:254–265. [PubMed: 18775793]
78. Anderson RM, Kwoon M, Strobel SA. Toward Ribosomal RNA catalytic activity in the absence of protein. *J Mol Evol* 2007;64:472–483. [PubMed: 17417708]
79. Bokov K, Steinberg S. A hierarchical model for evolution of 23S ribosomal RNA. *Nature* 2009;457:977–980. [PubMed: 19225518]
80. Agmon I. The Dimeric Proto-Ribosome: Structural Details and Possible Implications on the Origin of Life. *Int J Mol Sci* 2009;10:2921–2934. [PubMed: 19742176]
81. Agmon I, Davidovich C, Bashan A, Yonath A. Identification of the prebiotic translation apparatus within the contemporary ribosome. *Nature Precedings*. 2009hdl:10101/npre.2009.2921.1:Posted 4 Mar 2009.
82. Noller, HF. Evolution of Ribosomes and translation from an RNA World. In: Gesteland, R.; Cech, TR.; Atkins, JF., editors. *The RNA World*. Cold Spring Harbor Laboratory Press; 2006. p. 287–307.
83. Woese C. Translation: in retrospect and prospect. *RNA* 2001;7:1055–1067. [PubMed: 11497425]
84. Petherick A. The Production Line. *Nature* 2008;454:1042–1045. [PubMed: 18756228]

Figure 1.

(A) A working model for the minimal hammerhead ribozyme based on early crystal structures [18,27]. The numbered nucleotides shown in helix I and II represent the highly conserved 15 nt catalytic core. The four colored nucleotides (A13, G8, C17, C3) appear in the catalytic core as observed in crystal structures. The red arrow marks the site of cleavage. (B) The extended hammerhead with the interacting loops 1 and 2. This populates the active conformation without high metal ion concentrations [22,27].

Figure 2.

(A) The proposed mechanism of the cleavage reaction facilitated by the general base G12 and general acid G8 [25,26]. (B) Affinity labeling approach used to probe the catalytic role of G12. The bromoacetamide moiety, replacing the 2'-OH group, serves as an electrophilic trap for the putative general base [28]. (C) The role of divalent metal ions was investigated using chemically modified extended hammerhead construct at the 5' scissile oxygen and with modifying the 2'-OH on G8 [33].

Figure 3.

The processing of pre-mRNA by the spliceosome, introns are shown in red, exons in blue [37,42]. The U1 and U2 snRNPs first attach via RNA–RNA interactions to the 5' splice site and the adenosine branch site intron regions respectively. A tri-snRNP complex, which includes an initial U4–U6 duplex along with U5 then approaches the pre-mRNA. U4 dissociates from U6, allowing U6 to replace U1 at the 5' splice site while also pairing with U2, which facilitates the splicing at the 5' exon site using the branch point adenosine. U5 binds to both exons and holds them together while the splicing occurs. After the first splicing, the 3'-OH of exon 1 is ligated to exon 2, releasing the lariat intron and the remaining four RNPs dissociate.

Figure 4.

(A) The original minimal splicing U2-U6 complex based on the truncated human spliceosome sequences [59]. Pre-mRNA introns are shown in red, and conserved sequences are pink. (B) The modified U2-U6 minimal complex contains the essential RNA components for the first splicing process. The 5' end of the U6 domain has been extended to become an intron with an added exon region, in blue [61]. (C) A modified version of the U2-U6 minimal complex based on the yeast spliceosome sequences [62]. (D) Truncation experiments identified a minimal ribozyme complex within the structure of (c). The branch site oligonucleotide uses the 5' end nucleotide to form a 2'-3' phosphodiester bond on a G, excising the remaining original U6 fragment [62].