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The RNA World on Ice: A New Scenario for the Emergence of RNA Information

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Abstract. The RNA world hypothesis refers to a hypothetical era prior to coded peptide synthesis, where RNA was the major structural, genetic, and catalytic agent. Though it is a widely accepted scenario, a number of vexing difficulties remain. In this review we focus on a missing link of the RNA world hypothesis—primitive miniribozymes, in particular ligases, and discuss the role of these molecules in the evolution of RNA size and complexity. We argue that prebiotic conditions associated with freezing, rather than "warm and wet" conditions, could have been of key importance in the early RNA world.

Key words: RNA world — Miniribozymes — RNA evolution — Freezing catalysis

Introduction

The question of how life began on Earth or elsewhere in the Universe remains one of the greatest scientific mysteries. The ubiquity of proteins as biological catalysts today, and the fact that peptides are relatively easy to synthesize under presumed prebiotic conditions (e.g., Leman et al. 2004), including −20°C (Liu and Orgel 1997), led to the long-held view that proteins evolved early in prebiotic evolution. Although amino acids were undoubtedly present on the early Earth (Miller 1953; Weber and Miller 1981) and diamino acids are barely detectable in meteorites (Meierhenrich et al. 2004), proteins cannot replicate themselves. Indeed, molecular mechanisms for self-replication of proteins do not exist, except either through an RNA-mediated mechanism or in highly specialized laboratory settings (Lee et al. 1996). Also, proteins-first models cannot explain why the evolution of nucleic acids (presumably for information storage) insinuated RNA into catalysis (Szathmary 1999).

The discovery of RNA catalysis offered a way to circumvent the difficulties of the proteins-first view. The RNA world hypothesis (Gilbert 1986; Joyce 2002), referring to an era prior to coded peptide synthesis where RNA was the major structural, genetic, and catalytic agent, is now a widely accepted scenario. There are four processes whose explanation in terms of RNA chemistry would constitute a satisfying account of evolution in an RNA world—replication, cellularization, translation, and metabolism (Yarus 1999). At present, RNA-catalyzed RNA synthesis has been experimentally demonstrated (Johnston et al. 2001; McGinness and Joyce 2003), and the combined results of many experimental studies of RNA, plus observations from modern metabolism, are consistent with its derivation from a complex RNA-based metabolism (Joyce 2002; Jadhav and Yarus 2002; Landweber 1999). All three translational reactions—amino acid activation, aminoacyl-RNA synthesis, and peptide bond formation—can be catalyzed by RNA (Lacey et al. 1990; Zhang and Cech 1997; Yarus 1999; Kumar and Yarus 2001; Tamura and Alexander 2004).

However, even if RNA is the best candidate for an immediate predecessor of DNA and encoded proteins, numerous unanswered questions remain. To

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name a few: How were the rather complex RNA building blocks synthesized under prebiotic conditions? Although progress continues to be made (for example, the recent proposal of a plausible pathway for ribose accumulation [Ricardo et al. 2004]), this question remains a challenge. How was membrane permeability controlled in the RNA world? Indeed, permeability of typical phospholipid bilayers to molecules that are polar or bulky is very low, and there were no channel proteins at this evolutionary stage, leaving RNA the improbable but only candidate for this job (Vlassov et al. 2001). How could long, complex RNA molecules, which were presumably required to perform multiple complex functions, be formed and survive under the "warm and wet" conditions commonly assumed to have been present early in evolution? In this article we focus on the last of these questions, reviewing recent findings that support the alternative, cold-origins view, particularly the ability of RNA to catalyze synthetic reactions under freezing conditions that favor RNA stability, and the role that very small, primitive ribozymes may have played in the generation of complex RNA molecules.

The Problem of RNA Instability

One of the biggest questions is how complex RNAs could evolve, survive, and replicate under typically assumed aqueous conditions, taking into account that the RNA phosphodiester backbone is chemically unstable under warm aqueous conditions, undergoing cleavage through transesterification (Pace 1991; Larralde et al. 1995; Levy and Miller 1998). The cleavage reaction is accelerated by the presence of di- or multivalent metal ions ($M^{\geq 2+}$), the same cofactors that are usually considered vitally important for RNA catalysis (Pyle 2002; Fedor 2002; Kazakov 1996; Lilley 2003). Unchecked, this cleavage reaction would clearly limit the length and complexity of RNA that could be made via spontaneous polymerization (Pace 1991).

Several naturally occurring classes of catalytic RNA have been identified. These classes can be considered relics from the hypothetical RNA world, particularly as the phylogenetic distribution of some ribozymes, most notably ribosomal RNA, is wide (Walter and Engelke 2002). All the naturally occurring nonribosomal ribozymes catalyze cleavage and ligation of RNA molecules. The hairpin, hammerhead, hepatitis delta virus (HDV), and *Neurospora* Varkud satellite (VS) ribozymes are 50- to 150-nucleotide (nt) RNA molecules, found in viral, virusoid, or satellite RNA genomes (Harris and Elder 2000; Collins 2002). Their biological function is to generate unit-length genomes from rolling circle replication intermediates by site-specific self-cleavage of multimers and circularization of monomers, with the aid of proteins. Group I and group II introns and prokaryotic ribonuclease P (RNase P) are larger (several hundred nucleotides) and more structurally complex ribozymes. Group I and group II introns catalyze two-step self-splicing reactions via transesterification, while RNase P cleaves precursor RNA substrates via hydrolysis at specific sites to generate functional 5′-termini (Westhof 2002; Fedorova et al. 2002; Kirsebom 2002).

The invention of selection/amplification techniques (SELEX) allowed researchers to successfully isolate far more classes of ribozymes than are preserved in nature (Landweber et al. 1998; Joyce 2004). As these experiments were designed to probe the range of natural catalytic RNA, most selected molecules derive from libraries of 50–100 random nt, and thus the active molecules are the same length, although their functional domains may be much smaller. It is a challenge to imagine how such long RNAs could emerge, withstand degradation, and replicate in prebiotic conditions.

There are two obvious solutions to the problem of RNA instability. First, if smaller RNAs that could be generated by random polymerization can have synthetic activity, particularly the ability to ligate other small RNAs into larger RNAs, then this would provide a plausible path to an RNA world. The second solution would be to provide a unique environment (combination of solvent, surface, temperature) that favors RNA stability over hydrolysis. We will start from the description of natural and in vitro selected miniribozymes, focusing on a few examples. However, we note that conditions supporting RNA stability would have been obligatory anyway, because at a certain point there had to be a transition to sophisticated catalysts, even if vulnerable to degradation. Indeed, small ribozymes may only support simple reactions (like cleavage and ligation), while more complex reactions (like polymerization and peptide bond formation) require larger ribozymes. In addition, small ribozymes typically have modest rate enhancements. Thus, the second part of the review discusses environments that could promote RNA stability, along with preliminary experiments supporting our ideas.

Miniribozymes: Small Is Beautiful

A Trinucleotide Promoting Metal Ion-Dependent Specific Cleavage of RNA

Dange et al., in 1990, found that a 31-nt RNA, which is normally excised from the 5′ end of the *Tetrahymena* group I rRNA intron during autocyc-

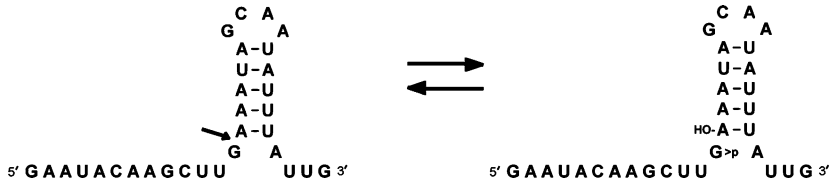

Fig. 1. Mn-dependent ribozyme, with cleaved form shown at right and ligated form shown at left. Small arrow indicates the "cleavage/ligation" site. G > p is 2′,3′-cyclic phosphate.

lization, can further undergo self-cleavage at a specific site in the presence of Mn^{2+} cations, generating 5′-hydroxyl and 2′, 3′-cyclic phosphate termini (Fig. 1) (Dange et al. 1990). More detailed investigation of the Mn-induced specific cleavage of this RNA explored various mutant versions, as well as fragments and shorter derivatives of the 31-mer (Kazakov and Altman 1992; Kazakov 1996). This work revealed a minimal active complex consisting of only seven nucleotides (UUU/GAAA) that promote specific cleavage between the G and the A at 37°C and pH 7.5. Additional nucleotides flanking the oligonucleotides in the minimal complex were unnecessary for the cleavage reaction to take place but can affect the rate of the reaction. The 2′-OH groups of uridine residues do not participate in catalysis, because both poly(U) and poly(dU) can promote the specific cleavage reaction in trans. Cd^{2+} ions can also promote the specific cleavage reaction (Kazakov and Altman 1992; Van Atta and Hecht 1994), and under certain conditions Mg^{2+} ions, while inactive alone, can enhance the Mn^{2+}-induced cleavage of RNA (Kuo, Landweber, Johnston, and Kazakov, unpublished). It is worth noting that the Mn^{2+} concentration might have been two to five times higher in the Archaean oceans than today, and the anoxic environment would have prevented the manganese from oxidizing until ~2 Byr (billion years) ago (Holland 1984).

This GAAA/UUU complex is the smallest catalytic RNA system known to date (Pyle 1993; Feig 2000). However, the cleavage rate enhancement by this miniribozyme is very low—about three orders of magnitude less than for the hammerhead ribozyme, for example, an optimized product of evolution. This suggests clear trade-offs between size and efficiency; however, the smallest molecules are likely to arise first, and any rate enhancement would have been beneficial in a prebiotic setting.

Selection of a Miniligase

Bartel and Szostak's (1993) seminal experiment, demonstrating that one can recover new (artificial) ribozymes by in vitro evolution from random sequences, revealed an abundance of RNA ligase ribozymes in a pool of 10^{15} unique sequences. Because of the difficulty of sampling such a large sequence space (one can sample approximately $10^{15–16}$ molecules out of an astronomical 4^{100}–10^{60}), the initially selected ribozymes are generally suboptimal representatives of a class of catalytically active molecules (Bartel and Szostak 1993). Therefore, the addition of a second layer to this type of experiment, via more rounds of in vitro evolution and mutagenic PCR, frequently led to improvements in the reaction efficiency, such as a reduction in the required substrate or metal ion concentration or reaction time. Alignment of the molecules produced in such an experiment generally reveals a sequence consensus for one or more classes of evolved ribozymes, and the alignments sometimes suggest the presence of a small conserved region that is the catalytic core. Finding such regions aids the subsequent design of very small ribozymes based on the selected motif. Similar in vitro selection methods have also been used to optimize the activity of naturally occurring ribozymes and to infer the essential nucleotides in the RNA (e.g., Berzal-Herranz et al. 1992, 1993; Joseph and Burke 1993; Siwkowski et al. 1998).

One can also use in vitro evolution to ask how small an RNA catalyst can be. Landweber and Pokrovskaya (1999) selected a novel class of ligase ribozymes that can append a small RNA substrate molecule to their 5' ends. The surviving molecules in this experiment were minimal RNA ligases that require only two selected nucleotides for function, in the context of a series of apparent conventional base pairing interactions (the 29-nt ribozyme in Fig. 2 provides a reaction rate of approximately 3×10^{-4} min^{-1}) A consensus sequence within the random region provided a template to position the 2′-hydroxyl of the substrate RNA near the 5′-triphosphate of the pool RNA (Fig. 2). This extreme simplicity—requiring only a single bulged nucleotide in a mostly paired RNA duplex—suggests that such a motif would have likely been accessible in a pool of RNA sequences more than 3 Byr ago during prebiotic evolution.

Surprisingly, the catalytic core assembled from random sequences in this experiment actually contained precisely the 7-nt manganese-dependent self-cleavage motif present in the *Tetrahymena* group I intron (Kazakov and Altman 1992), making this miniligase the first dual-catalytic RNA isolated from purely random sequences. The RNA promotes both ligation at the selected site and cleavage at another, with possibly two folded conformations surrounding a divalent metal-ion binding pocket (Fig. 2). Because

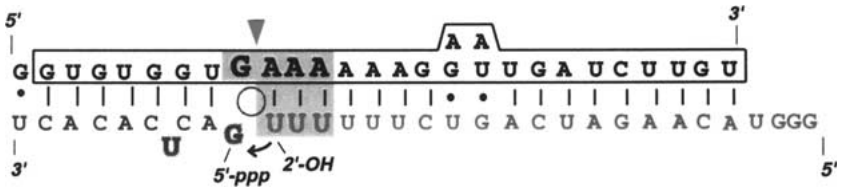

Fig. 2. A very simple RNA ligase ribozyme. The boxed region is the template strand which catalyzes 2′, 5′ ligation of the two lower strands at the curved arrow. Notice that most of the bases are involved in simple paired interactions, which can tolerate substitutions or even mismatches, except for a required bulged U in the lower left substrate and the G–G juxtaposition at the active center. Dots indicate potential G•U base pairs. This is one example of many simplified ligase reactions from Landweber and Pokrovskaya (1999). The shaded region in the center is the (UUU/GAAA) miniribozyme that undergoes self-cleavage at the arrowhead in the presence of manganese, and the circle represents a putative metal ion-binding site (or sites). (Adapted from Landweber 1999.)

the second activity (cleavage) arose during the experiment as a by-product of selection for ligation, and because manganese was never provided during the original experiment, these results provide an experimental RNA demonstration of Darwinian preadaptation. The final product of artificial evolution in this case was ultimately suited for a second catalytic function in addition to the one for which it was selected, but this second function only revealed itself when presented with an altered chemical environment providing manganese.

The origin of an unexpected pair of ribozyme reactions in a single RNA molecule suggests that the emergence of new catalytic function can sometimes be a simple response to a changing environment, an event that would have likely been commonplace on the evolving early earth. The emergence of new catalytic function can also be the combinatorial product of small existing modules, such as metal ion binding sites, that evolution, the tinkerer, can mix, match, and vary. This experiment therefore provides a window into some of the most basic steps in early molecular evolution and, by revealing exquisitely small functional units, provides a glimpse into some of the components that may have contributed directly to forming the early RNA world.

The process of breaking and joining RNA sequences that the small RNA in Fig. 2 catalyzes would also help solve another dilemma in the RNA world, which is the problem of variation. How did complex mixtures of RNA polymers arise to provide the necessary ingredients for further evolution? Cascades of cleavage and ligation, catalyzed by simple molecules such as the one in Fig. 2, could have led to the production of longer, varied sequences in an RNA world. The prevalence of extremely simple ligase ribozyme motifs would have therefore been instrumental in the production of longer and more complex RNA polymers in prebiotic evolution, if the conditions were available.

The preference of this ligase for forming 2′-5′ linkages is a potential problem, however, in terms of modern day biological relevance. Naturally occurring ribozymes, such as the hairpin ribozyme, catalyze formation of 3′-5′ linkages from substrates with 2′, 3′-cyclic phosphate and 5′-OH groups. On the other hand, even a small, 6-nt RNA, 5′, 3′ (UUUUUU), is a substrate for the miniligase ribozyme. Because this hexamer is small enough to be readily synthesized by montmorillonite-catalyzed RNA condensation (Ding et al. 1996), this observation increases the prebiotic relevance of the discovery of miniligase ribozymes.

Freezing and Dehydration Provide RNA Stability

The evolution of RNA complexity can occur only in settings where either RNA synthetic reactions are efficient enough to overcome random degradation or random degradation is much less likely than in warm aqueous solutions. Since the primordial ribozymes are likely to have been inefficient (as in the examples described above), the most straightforward way for RNA synthesis to outpace random degradation would be if conditions were available to reduce rates of degradation. Such conditions might include minimal levels of accessible di- or multivalent metal cations ($M^{\geq 2+}$), reduced temperature, solution conditions that disfavor transesterification, adsorption to surfaces that stabilize phosphodiester bonds relative to cleavage products, or any combination of these (Pace 1991; Levy and Miller 1998; Ferris et al. 1996; Orgel and Lohrmann 1974; Lazcano and Miller 1996; Bada et al. 1994). Although many of these circumstances may have been available at the time of prebiotic evolution, freezing seems to be especially attractive for many reasons (see below). Indeed, a number of investigators have argued that much of the water in the oceans was frozen but underwent periodic melting due to large meteor impacts or volcanic activity (Lazcano and Miller 1996; Grant and Alburn 1965; Sanchez et al. 1966; Levy et al. 1999; Bada et al. 1994; Bada and Lazcano 2002).

It is worth noting that Jupiter's moon Europa and even Mars are also thought to contain large amounts of liquid water and ice now or at some time in the past (e.g., Psenner and Sattler 1998; Clifford and Parker 2001; Squyres et al. 2004). The possibility of

synthetic RNA reactions in freezing aqueous solutions lends some credibility to claims that the rather extreme environments of these extraterrestrial locations could have provided suitable conditions for the emergence of life.

Although reduced temperatures inhibit most reactions, freezing does accelerate some chemical and enzymatic reactions that occur in aqueous solutions, perhaps owing in part to the organization of frozen water as well as to the concentrating of reactants. Examples include hydrolysis of penicillin (Grant et al. 1961), dehydration of 5-hydro-6-hydroxydeoxyuridine (Prusoff 1963; Butler and Bruice 1964), oxidation of ascorbic acid by hydrogen peroxide (Grant and Alburn 1965), tetramerization of hydrogen cyanide to form diaminomalonodinitrile (Sanchez et al. 1966), glucose mutarotation (Kiovsky and Pincock 1966), amino acid modification (Grant and Alburn 1966), and peptide synthesis (Schuster et al. 1991; Tõugu et al. 1995). Examples more relevant to RNA include chemical ligation of phosphorothioate and bromoacyl oligonucleotide derivatives (Gryaznov and Letsinger 1993), pyrimidine and purine synthesis from ammonium cyanide (Miyakawa et al. 2002), formation of dinucleotides from adenosine 2′, 3′-cyclic phosphate (Renz et al. 1971), and synthesis of polynucleotides from phosphor imidazole-activated mononucleotides (Kanavarioti et al. 2001; Monnard et al. 2003).

Little is known about the physicochemical properties of nucleic acids in frozen solution. Renz et al. (1971) attributed the effects of freezing in bimolecular reactions primarily to the concentrating of reactants. For freezing catalysis in nonnucleic acid systems, additional mechanisms besides concentration have been proposed to play a role: altered juxtaposition or orientation of reacting groups, catalysis at surfaces of the ice lattice, enhanced proton transfer resulting from high proton mobility in ice, and reduction of water activity (dehydration) (Grant et al. 1966; Tõugu et al. 1995).

Intramolecular and intermolecular interactions of polynucleotides require that interacting surfaces be partially dehydrated so that specific contacts can form (Rau et al. 1984; Lundbäck and Härd 1996). In other words, the formation of RNA–RNA contacts depends on competition between RNA–RNA and RNA–water interactions. For example, the folding of tRNA results in substantial decreases in hydration (Kuntz et al. 1969). By removing water from solution, freezing has the effect of both raising the concentration of RNA molecules and lowering the activity of water in the remaining liquid microinclusions, thereby favoring RNA–RNA interactions relative to water–RNA interactions. This effect should promote intra- as well as intermolecular interactions, as both require water molecule displacement from the interacting RNA surfaces. The concentrating of salts that occurs with freezing can also promote RNA–RNA interaction by shielding interphosphate repulsion through increased ion-pair formation (Murray 1998; Nesbitt et al. 1999). Altogether, freezing provides a unique environment, greatly reducing RNA degradation while also concentrating solvents and reducing water activity, which can promote many reactions.

The Hairpin Ribozyme as a Model Catalytic RNA Functioning Under Freezing Conditions

Ligation was likely a key primordial RNA reaction, providing a means for increasing RNA length and complexity by combining short fragments made by other processes. The hairpin ribozyme (HPR) has been the subject of studies of RNA catalysis under freezing conditions (Kazakov et al. 1998; Vlassov et al. 2004; S.A. Kazakov, S. Balatskaya, and B.H. Johnston, unpublished). Under standard solution conditions, the HPR can either cleave substrates to generate RNA fragments with 5′-hydroxyl and 2′, 3′-cyclic phosphate termini or ligate them in the reverse reaction; both reactions require either Mg^{2+} or high concentrations of monovalent ions to proceed (Walter and Burke 1998). Interestingly, the termini that are ligated by HPR are chemically the same as those formed through transesterification during ordinary (i.e., metal-, base-, or acid-catalyzed) ''random'' cleavage (Usher 1977)—and thus could have been available on the prebiotic Earth. It was first discovered that in frozen solutions, the HPR promotes efficient Mg^{2+}-independent ligation in cis (intramolecular ligation), while the cleavage reaction is very slow (Kazakov et al. 1998; Kazakov et al., unpublished). Because intermolecular reactions are more useful from an evolutionary point of view, further studies were performed to confirm that freezing-induced ligation by the HPR can also proceed efficiently in trans (Vlassov et al. 2004) (Figs. 3A and B). The reaction is relatively insensitive to environmental conditions as long as freezing occurs, underscoring the robustness of the system and increasing the likelihood that conditions on the prebiotic Earth could have supported freezing-induced ligation. Importantly, the natural 3′–5′ linkage is the major or only product formed under freezing conditions. The maximum initial rate observed at −8 to −10°C was 0.006 min^{-1} for the ligation in trans, which is several orders of magnitude lower than rates for the conventional ribozymes acting under standard solution conditions, at +37°C. This rather slow rate is the trade-off for the stabilization that freezing conditions provide.

If the solution was supercooled to −10°C without freezing, no ligation occurred; also, ligation in trans is very inefficient in solutions containing 3 *M* NaCl,

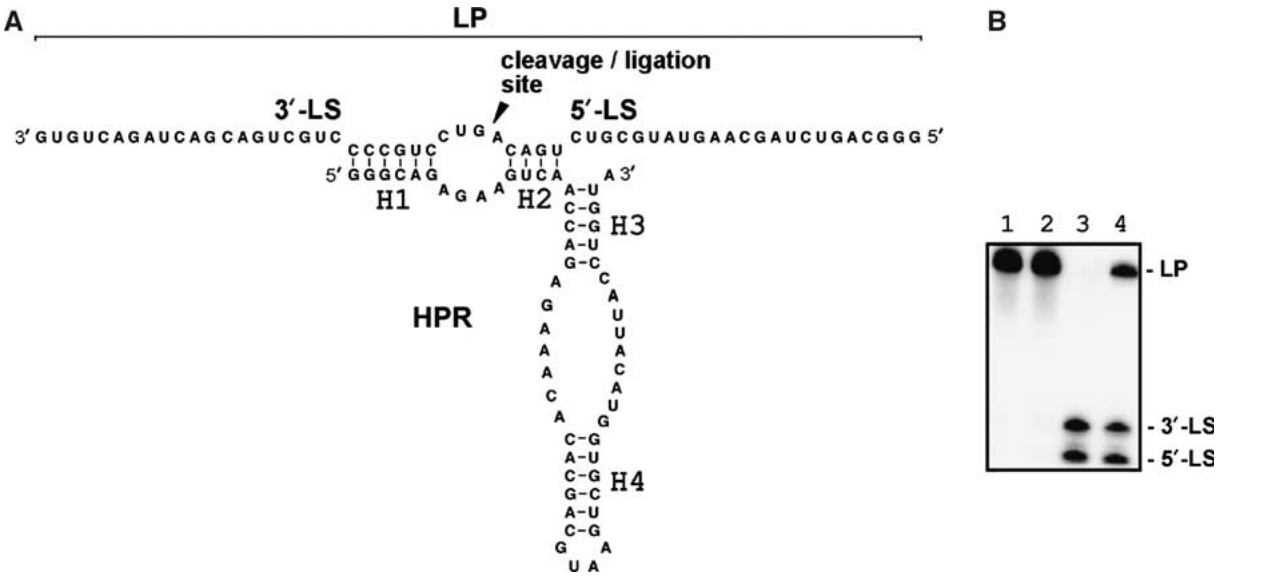

Fig. 3. **A** Structure of the hairpin ribozyme with separated enzyme and ligation substrates. H1–H4, helical segments; 3′-LS and 5′-LS, ligation substrates. **B** HPR-catalyzed ligation in trans under freezing conditions. Lanes 1 and 2, cleavage substrate (identical to ligation product, LP) incubated without and with HPR, respectively. Lanes 3 and 4, ligation substrates (3′-LS and 5′-LS) incubated without and with HPR, respectively. Reactions were performed at a 1:1 ratio of 3′-LS to 5′-LS and a 20:1 ratio of HPR to substrate, in 25 m*M* NaCl, 1 m*M* Tris-HCl (pH 7.5), at –10°C for 14 h.

which remain unfrozen at −10°C (Vlassov et al. 2004; A.V. Vlassov, B.H. Johnston, and S.A. Kazakov, unpublished). This indicates that freezing is absolutely required for the reaction, and high concentrations of salts or low temperatures by themselves are not essential. On the other hand, the reaction is most efficient in the range −4° to −12°C, with rates dropping to near zero for temperatures above −1° or below −20°C. Thus, although the reaction *requires* freezing, the temperature cannot be too cold, presumably due to the need for liquid microinclusions in the ice and sufficient thermal energy to surmount the activation barrier for the reaction. In a prebiotic environment, optimal conditions for the reaction would probably include temperatures slightly below freezing, with fluctuations creating frequent cycles of freezing and melting, as occurs, for example, with day/night cycles in partially frozen pools of water. Melting and warming of frozen solutions would result in dissociation and redistribution of primordial ribozyme and ligation substrates as well as in refolding of the ribozymes. These cycling conditions were mimicked in the laboratory to see if they could affect ligation yields. Samples were frozen at −10°C, melted and incubated at 37°C, then frozen again, with the procedure repeated two to six times. Freeze–thaw cycles indeed increased the ligation yields up to threefold (Vlassov et al. 2004).

At lower temperature, fewer base pairs should be required for stable pairing between the ribozyme and the substrate sequences, suggesting that the sequence specificity of the ligation reaction may be reduced for freezing-induced catalysis compared to the normal solution reaction. To test whether this is indeed the case, the 5′ and 3′ ligation substrates (LS), 3′-LS and 5′-LS (Fig. 3A) were partially randomized at residues that should normally form base pairs with the substrate binding sequence of the HPR (helices 1 and 2) and used in the freezing ligation reaction at −10°C. Products of the reaction were reverse transcribed and PCR-amplified using the flanking fixed sequences as primer-binding sites. Cloning and sequencing revealed that 3′-substrate sequences ligated by the HPR all contained a G at the 5′-end where ligation occurred, as is known to be absolutely required for catalysis by the wild-type HPR (Chowrira et al. 1991). However, all other selected positions were variable, with no sequence in common among the clones sequenced. In contrast, for the 5′-substrate most of the successfully ligated sequences matched the original sequence. Thus, under freezing conditions four base pairs are required to bind the 5′-LS but virtually any sequence can be ligated by the HPR–5′-LS complex. These results provide a clear illustration of the feasibility of RNA catalysis under freezing conditions.

Reverse Evolution: In Search of a Ribozyme's Ancestors

To test the hypothesis that freezing temperatures could support RNA synthetic reactions by primitive ribozymes, we used a ''reverse-evolution'' approach (Vlassov et al. 2004). This approach involves progressive sequence deletion, fragmentation, and mutagenesis of the original, naturally existing ribo-

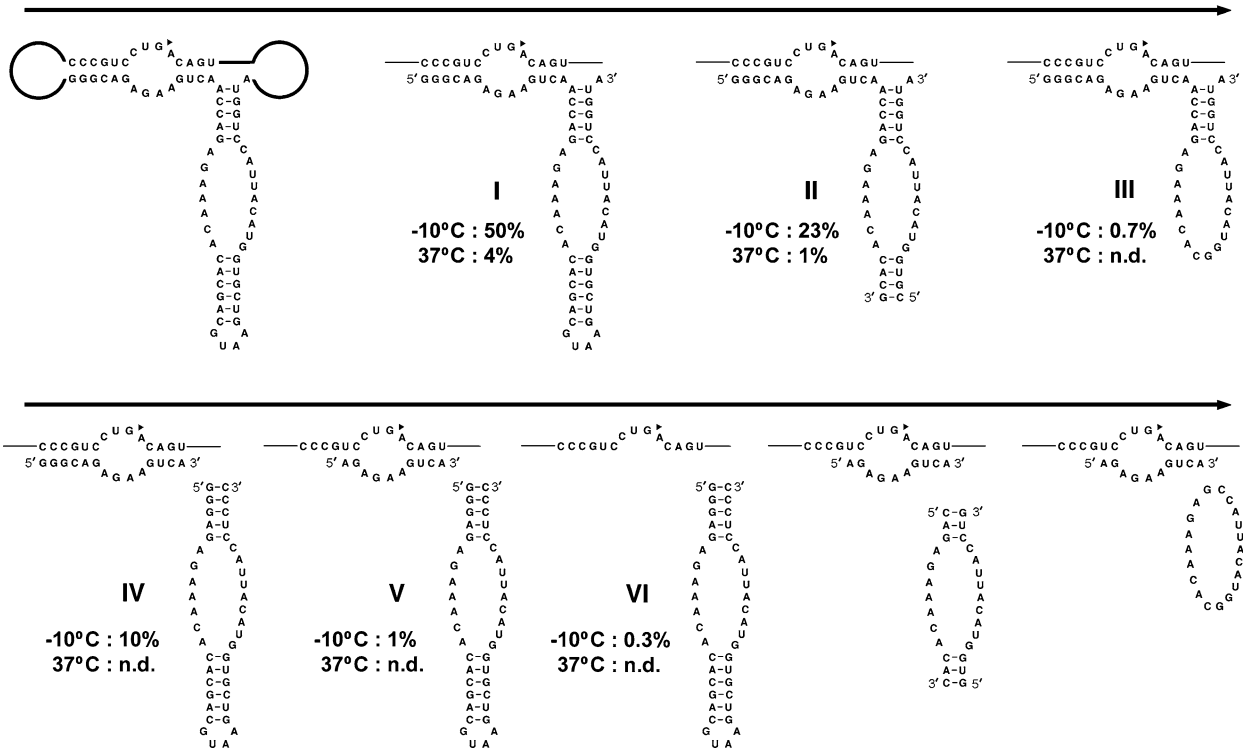

Fig. 4. Scheme of "reverse evolution" of the hairpin ribozyme ligase. The approach involves progressive minimization, fragmentation, and deletion–substitution mutagenesis of the original, naturally existing ribozyme in order to access related sequences that might have included its "ancestors." Following this strategy, the HPR was simplified (along the direction of the arrow) and several constructs were assayed for the ability to ligate pairs of RNA fragments at −10 and 37°C: I, HPR; II, bisected HPR; III, truncated HPR; IV and V, HPRs with separated domains; VI, template-deleted HPR. Ligation yields observed after 14 h of incubation are shown next to the structures. Ligation substrates 3′-LS and 5′-LS were used in equimolar amounts, and the ribozyme-to-substrate ratio was 20:1.

zyme in order to access related sequences, some of which may resemble its "ancestors." We anticipated that at lower temperatures, fewer intermolecular interactions (such as hydrogen bonds) would be required to stabilize complexes from small RNA fragments. Some of these complexes would have catalytic activity and could be selected. Following this strategy and beginning with the HPR consensus structure (Fig. 3), Vlassov et al. progressively eliminated loops, shortened helical segments, eliminated connecting sequences between the catalytic core and the substrate binding domain, and, finally, deleted the substrate binding domain. Each derivative was assayed for the ability to ligate pairs of RNA fragments with defined 5′-LS and 3′-LS sequences. We hoped that this approach would reveal examples of the sort of primitive ribozymes that could have promoted the evolution of RNA complexity in protected, low-temperature environments.

The results are shown in Fig 4. Bisecting the RNA in the catalytic core by cutting the loop at the bottom of helix 4 (structure II) reduced the yield of ligation at −10°C by about half (from 50% to 23%) and reduced the ligation yield at 37°C in the presence of Mg^{2+} from 4% to 1% in standard 14-hr reactions. Eliminating helix 4 and connecting the C and G that would otherwise form its first base pair (structure III) had a severe effect, reducing the yield of the freezing reaction to 0.7%. Separating the catalytic core from the substrate binding domain of I (structure IV) reduced the ligation yield from 50% to 10%, and decreasing the length of the sequence that participates in helix 1 from 6 to 2 nt (structure V) further reduced the yield to 1%. Curiously, the complete elimination of the substrate binding sequence (structure VI) still left detectable ligation activity under freezing conditions (0.3%, 6-fold enhanced over a background of 0.05%); however, in Mg^{2+}-containing solution at 37°C, structure VI (as well as III-V) had no detectable activity ($\leq 0.05\%$). It is unclear whether this untemplated ligation results from nonspecific binding of the substrates to the catalytic core or from substrate sequences providing the role of the substrate-binding domain. In the latter case, with low temperature stabilizing weak interactions, one of the substrates might act as a highly mismatched splint to position a separate pair of 3′-LS and 5′-LS substrates for ligation by the catalytic core.

Finally, we assayed an HPR variant in which G8 is substituted by U for ligase activity under freezing conditions. Substitution of G8 by any other nucleotide is known to completely abolish ribozyme activity under standard solution conditions (37°C/Mg) (Berzal-Herranz et al. 1993). Indeed, in control experiments at

37°C, neither cleavage nor ligation activity were detectable. However, under freezing conditions this mutant was active, with ligation yields of 5.6%.

Taken together, the results show that freezing is uniquely able to relax the sequence and structural requirements for ligation in the case of the hairpin ribozyme. Freezing allows mutated, partially deleted, and fragmented HPR derivatives of minimal size to be efficient catalysts at subzero temperatures, despite having greatly reduced or zero activity in solution. In the prebiotic context, freezing-induced concentration could contribute to ligation efficiency by bringing together different RNA fragments to form catalytically active complexes. Also, freezing provides compartmentalization in the liquid microinclusions trapped in the ice crystal to prevent their dispersal—a function later provided by cells. If lipid-like molecules are present, another method of compartmentalization could come into play: the encapsulation of RNA within lipid vesicles induced by freeze–thaw cycles. And most importantly, low temperatures greatly limit degradation of newly formed RNA molecules. In later stages of molecular evolution, larger, more stable, and efficient catalysts may have survived transport into more "warm and wet" environments by virtue of their synthetic power outpacing degradation.

Conclusion

One of the problems with the RNA world theory is how complex RNAs could evolve and survive on the early Earth, given that the RNA is rapidly degraded under conditions in which it normally functions. The recent discoveries of several very small ribozymes and the finding that short RNAs can catalyze ligation of RNA fragments under conditions that greatly inhibit random degradation present a tantalizing solution to this problem. The existence of such ligases provides a much more efficient path to the formation of more complex RNAs than stepwise polymerization (Schmidt 1999). Importantly, under freezing temperatures, the base pairing required between substrate and catalytic sequences is minimal, to the point that a simple fragment of the HPR could ligate any RNA with a 5′-OH to a given fragment with a 2′,3′-cyclic phosphate (Vlassov et al. 2004). This system comes close to providing a "universal ligase" that can assemble random fragments of RNA into more complex molecules under highly stabilizing conditions. It is not hard to envisage random polymerization of activated mononucleotides creating small fragments with some catalytic activity, followed by assembly of larger RNAs by ligation and the eventual emergence of sufficient catalytic power that the larger molecules could survive under higher-temperature, less protected conditions.

Note Added in Proof: Two Closely related reviews have appeared since this article was accepted for publication: Monnard 2005 and Yarus et al. 2005.

References

Bada JL, Bigham C, Miller SL (1994) Impact melting of frozen oceans on the early Earth: implications for the origin of life. Proc Natl Acad Sci USA 91:1248–1250

Bada JL, Lazcano A (2002) Origin of life. Some like it hot, but not the first biomolecules. Science 296:1982–1983

Bartel DP, Szostak JW (1993) Isolation of new ribozymes from a large pool of random sequences. Science 261:1411–1418

Berzal-Herranz A, Joseph S, Burke JM (1992) *In vitro* selection of active hairpin ribozymes by sequential RNA-catalyzed cleavage and ligation reactions. Genes Dev 6:129–134

Berzal-Herranz A, Joseph S, Chowrira BM, Butche SE, Burke JM (1993) Essential nucleotide sequences and secondary structure elements of the hairpin ribozyme. EMBO J 12:2567–2573

Bruce TC, Butler AR (1964) Catalysis in water and ice. II. The reaction of thiolactones with morpholine in frozen systems. J Am Chem Soc 86:4104–4108

Butcher SE, Heckman JE, Burke JM (1995) Reconstitution of hairpin ribozyme activity following separation of functional domains. J Biol Chem 270:29648–29651

Chowrira BM, Berzal-Herranz A, Burke JM (1991) Novel guanosine requirement for catalysis by the hairpin ribozyme. Nature 354:320–322

Chowrira BM, Burke JM (1992) Extensive phosphorothioate substitution yields highly active and nuclease–resistant hairpin ribozymes. Nucleic Acids Res 20:2835–2840

Clifford SM, Parker TJ (2001) The evolution of the martian hydrosphare: implications for the fate of a primordial ocean and the current state of the northern plains. Icarus 154:40–79

Collins RA (2002) The neurospora varkud satellite ribozyme. Biochem Soc Trans 30:1122–1126

Dange V, Van Atta RB, Hecht SM (1990) A Mn2(+)–dependent ribozyme. Science 248:585–588

Ding PZ, Kawamura K, Ferris JP (1996) Oligomerization of uridine phosphorimidazolides on montmorillonite: a model for the prebiotic synthesis of RNA on minerals. Orig Life Evol Biosph 26:151–171

Fedor MJ (2002) The role of metal ions in RNA catalysis. Curr Opin Struct Biol 12:289–295

Fedorova O, Su LJ, Pyle AM (2002) Group II introns: highly specific endonucleases with modular structures and diverse functions. Methods 28:323–335

Feig (2000) The use of manganese as a probe for elucidating the role of magnesium ions in ribozymes. Met Ions Biol Syst 37:157–182

Ferris JP, Hill JAR, Liu R, Orgel LE (1996) Synthesis of long prebiotic oligomers on mineral surfaces. Nature 381:59–61

Gilbert W (1986) The RNA World. Nature 319:618

Grant NH, Alburn HE (1965) Fast reactions of ascorbic acid and hydrogen peroxide in ice, a presumably early environment. Science 150:1589–1590

Grant NH, Alburn HE (1966) Acceleration of enzyme reactions in ice. Nature 212:194

Grant NH, Clark DE, Alburn HE (1961) Imidazole and base-catalyzed hydrolysis of penicillin in frozen systems. J Am Chem Soc 83:4476–4477

Gryaznov SM, Letsinger RL (1993) Chemical ligation of oligonucleotides in the presence and absence of a template. J Am Chem Soc 115:3808–3809

Harris RJ, Elder D (2000) Ribozyme relationships: the hammerhead, hepatitis delta, and hairpin ribozymes have a common origin. J Mol Evol 51:182–184

Holland HD (1984) Chemical evolution of the atmosphere and oceans. Princeton University Press, Princeton, NJ

Jadhav VR, Yarus M (2002) Coenzymes as coribozymes. Biochimie 84:877–888

Johnston WK, Unrau PJ, Lawrence MS, Glasner ME, Bartel DP (2001) RNA-catalyzed RNA polymerization: accurate and general RNA-templated primer extension. Science 292:1319–1325

Joseph S, Burke JM (1993) Optimization of an anti-HIV hairpin ribozyme by in vitro selection. J Biol Chem 268:24515–24518

Joyce G (2002) The antiquity of RNA-based evolution. Nature 418:214–221

Joyce GF (2004) Directed evolution of nucleic acid enzymes. Annu Rev Biochem 73:791–836

Kanavarioti A, Monnard P-A, Deamer DW (2001) Eutectic phases in ice facilitate nonenzymatic nucleic acid synthesis. Astrobiology 1:271–281

Kazakov SA (1996) Nucleic acid binding and catalysis by metal ions. In: Hecht SM (ed) Bioorganic chemistry: Nucleic acids. Oxford University Press, New York, pp 244–287, 467–476

Kazakov S, Altman S (1992) A trinucleotide can promote metal ion-dependent specific cleavage of RNA. Proc Natl Acad Sci USA 89:7939–7943

Kazakov SA, Balatskaya SV, Johnston BH (1998) Freezing-induced self-ligation of the hairpin ribozyme: cationic effects. In: Sarma RH, Sarma MH (eds) Structure, motion, interaction and expression of biological macromolecules. Adenine Press, Albany, New York, pp 155–161

Kiovsky TE, Pincock RE (1966) The mutarotation of glucose in frozen aqueous solutions. J Am Chem Soc 88:4704–4710

Kirsebom LA (2002) RNase P RNA-mediated catalysis. Biochem Soc Trans 30:1153–1158

Kumar R, Yarus M (2001) RNA-catalyzed amino acid activation. Biochemistry 40:6998–7004

Kuntz ID, Brassfield TS, Law GD, Purcell GV (1969) Hydration of macromolecules. Science 163:1329–1331

Lacey JC Jr, Staves MP, Thomas RD (1990) Ribonucleic acids may be catalysts for the preferential synthesis of L-amino acid peptides: a minireview. J Mol Evol 31:244–248.

Landweber L (1999) Experimental RNA Evolution. TREE 14:353–358

Landweber LF, Pokrovskaya ID (1999) Emergence of a dual-catalytic RNA with metal-specific cleavage and ligase activities: the spandrels of RNA evolution. Proc Natl Acad Sci USA 96:173–178

Landweber LF, Simon PJ, Wagner TA (1998) Ribozyme design and early evolution. BioScience 48:94–103

Larralde R, Robertson MP, Miller SL (1995) Rates of decomposition of ribose and other sugars: implications for chemical evolution. Proc Natl Acad Sci USA 92:8158–8160

Lazcano A, Miller SL (1996) The origin and early evolution of life: prebiotic chemistry, the pre-RNA world, and time. Cell 85:793–798

Lee DH, Granja JR, Martinez JA, Severin K, Ghadiri MR (1996) A self-replicating peptide. Nature 382(6591):525–528

Leman L, Orgel L, Ghadiri MR (2004) Carbonyl sulfide-mediated prebiotic formation of peptides. Science 306(5694):283–286

Levy M, Miller SL (1998) The stability of the RNA bases: implications for the origin of life. Proc Natl Acad Sci USA 95:7933–7938

Levy M, Miller SL, Oró J (1999) Production of guanine from NH(4)CN polymerizations. J Mol Evol 49:165–168

Lilley DM (2003) The origins of RNA catalysis in ribozymes. Trends Biochem Sci 28:495–501

Liu R, Orgel LE (1997) Efficient oligomerization of negatively-charged beta-amino acids at –20 degrees C. J Am Chem Soc 119(20):4791–4792

Lundbäck T, Härd T (1996) Sequence-specific DNA-binding dominated by dehydration. Proc Natl Acad Sci USA 93:4754–4759

McGinness KE, Joyce GF (2003) In search of an RNA replicase ribozyme. Chem Biol 10:5–14

Meierhenrich UJ, Munoz Caro GM, Bredehoft JH, Jessberger EK, Thiemann WH (2004) Identification of diamino acids in the Murchison meteorite. Proc Natl Acad Sci USA 101(25):9182–9186

Miller SL (1953) A production of amino acids under possible primitive earth conditions. Science 117:528–529

Miyakawa S, Cleaves HJ, Miller SL (2002) The cold origin of life: B. Implications based on pyrimidines and purines produced from frozen ammonium cyanide solutions. Orig Life Evol Biosph 32:209–218

Monnard P-A, Kanavarioti A, Deamer D (2003) Eutectic phase polymerization of activated ribonucleotide mixtures yields quasi-equimolar incorporation of purine and pyrimidine nucleobases. J Am Chem Soc 125:13734–13740

Monnard P-A (2005) Catalysis in abiotic structured media: an approach to selective synthesis of biopolymers. Cell Mol Life Sci 62:520–534

Murray JB, Seyhan AA, Walter NG, Burke JM, Scott WG (1998) The hammerhead, hairpin and VS ribozymes are catalytically proficient in monovalent cations alone. Chem Biol 5:587–595

Nesbitt SM, Erlacher HA, Fedor MJ (1999) The internal equilibrium of the hairpin ribozyme: temperature, ion and pH effects. J Mol Biol 286:1009–1024

Orgel LE, Lohrmann R (1974) Prebiotic chemistry and nucleic acid replication. Acc Chem Res 7:368–377

Pace NR (1991) Origin of life—Facing up to the physical setting. Cell 65:531–533

Prusoff WH (1963) Low-temperature reversal of the ultraviolet photochemical reaction product of 2′-deoxyuridive. Biochim Biophys Acta 68:302–310

Psenner R, Sattler B (1998) Life at the freezing point. Science 280:2073–2074

Pyle AM (1993) Ribozymes: a distinct class of metalloenzymes. Science 261:709–714

Pyle AM (2002) Metal ions in the structure and function of RNA. J Biol Inorg Chem 7:679–690

Rau DC, Le B, Parsegian VA (1984) Measurement of the repulsive force between polyelectrolyte molecules in ionic solution: hydration forces between parallel DNA double helices. Proc Natl Acad Sci USA 81:2621–2625

Renz M, Lohrmann R, Orgel LE (1971) Catalysts for the polymerization of adenosine cyclic 2′, 3′–phosphate on a poly(U) template. Biochim Biophys Acta 240:463–471

Ricardo A, Carrigan MA, Olcott AN, Benner SA (2004) Borate minerals stabilize ribose. Science 9:196

Sanchez R, Ferris J, Orgel LE (1966) Condition for purine synthesis: Did prebiotic synthesis occur at low temperatures? Science 153:72–73

Schmidt F (1999) Ribozymes—Why so many, why so few? Mol Cells 9:459–463

Schuster M, Aaviksaar A, Haga M, Ullmann U, Jakubke HD (1991) Protease-catalyzed peptide synthesis in frozen aqueous systems: the ''freeze-concentration model.'' Biomed Biochim Acta 50:84–89

Siwkowski A, Humphrey M, De-Young MB, Hampel A (1998) Screening for important base identities in the hairpin ribozyme by in vitro selection for cleavage. Biotechniques 24:278–284

Squyres SW, Grotzinger JP, Arvidson RE, Bell JF III, Calvin W, Christensen PR, Clark BC, Crisp JA, Farrand WH, Herkenhoff KE, Johnson JR, Klingelhofer G, Knoll AH, McLennan SM, McSween HY Jr, Morris RV, Rice JW Jr, Rieder R, Soderblom LA (2004) *In situ* evidence for an ancient aqueous environment at Meridiani Planum, Mars. Science 306:1709–1714

Szathmary E (1999) The origin of the genetic code: amino acids as cofactors in an RNA world. Trends Genet 15:223–229

Tamura K, Alexander RW (2004) Peptide synthesis through evolution. Cell Mol Life Sci 61:1317–1330

Tõugu V, Talts P, Meos H, Haga M, Aaviksaar A (1995) Aminolysis of acyl-chymotrypsins by amino acids. Kinetic appearance of concentration effect in peptide yield enhancement by freezing. Biochim Biophys Acta 1247:272–276

Usher DA (1977) Early chemical evolution of nucleic acids: a theoretical model. Science 196:311–313

Van Atta RB, Hecht SM (1994) A ribozyme model: site-specific cleavage of an RNA substrate by Mn2 + . Adv Inorg Biochem 9:1–40

Vlassov A, Khvorova A, Yarus M (2001) Binding and disruption of phospholipid bilayers by supramolecular RNA complexes. Proc Natl Acad Sci USA 98:7706–7711

Vlassov AV, Johnston BH, Landweber LF, Kazakov SA (2004) Ligation activity of fragmented ribozymes in frozen solution: implications for the RNA world. Nucleic Acids Res 32:2966–2974

Walter NG, Burke JM (1998) The hairpin ribozyme: structure, assembly and catalysis. Curr Opin Chem Biol 2:24–30

Walter NG, Engelke DR (2002) Ribozymes: catalytic RNAs that cut things, make things, and do odd and useful jobs. Biologist 49:199–203

Weber AL, Miller SL (1981) Reasons for the occurrence of the twenty coded protein amino acids. J Mol Evol 17:273–284

Westhof E (2002) Group I introns and RNA folding. Biochem Soc Trans 30:1149–1152

Yarus M (1999) Boundaries for an RNA world. Curr Opin Chem Biol 3:260–267

Yarus M, Caporase JG, Knight R (2005) ORIGINS OF THE GENETIC CODE: The Escaped Triplet Theory. Annu Rev Biochem 74:179–198

Zhang B, Cech TR (1997) Peptide bond formation by in vitro selected ribozymes. Nature 390:96–100