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# Review

# Re-creating an RNA world

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**Abstract.** The RNA world hypothesis states that life originated via a system based on RNA genomes and RNA catalysts. Researchers have been trying to develop such a system since catalytic RNAs (ribozymes) were discovered in 1982. This review summarizes the recent progress made in that endeavor and outlines the obstacles that remain to be overcome. After giving a short background on prebiotic chemistry and *in vitro* evolution, the discussion focuses on the generation of three important components of an RNA world: a sufficient polymerase ribozyme, self-replicating membrane compartments and ribozymes that are capable of performing basic metabolic processes.

**Keywords.** Ribozymes, catalytic RNA, RNA world, self-replication, synthetic life, artificial life, origin of life.

## Introduction

Life probably originated as a system of self-replicating, catalytic RNA molecules, an 'RNA world' [1]. The RNA world hypothesis states that at some stage in the early evolution of life, RNA formed both the genome and genome-encoded catalysts [2–5]. The hypothesis could be examined in detail if an experimental model system were available. The utility of such a synthetic system would be threefold. First, it would show that an RNA world can exist, thus raising our confidence that life originated via an RNA world. Second, it might lead us to simpler self-replicating ribozyme systems, which in turn would make the RNA world more plausible. Third, it could be used to find out how an RNA world could evolve into a DNA/protein world, similar to life as we know it.

The RNA world hypothesis is supported by its power to explain [6] a variety of observations in present life forms. First, DNA replication requires protein catalysis, and protein synthesis requires that the amino acid sequence be encoded in a DNA sequence. The RNA world hypothesis explains how this interdependent situation could originate from a much simpler situation, from a world without DNA or proteins: RNA was first, serving both as genome and as catalyst, later passing on its genomic role to DNA and most of its catalytic roles to proteins. Second, the hypothesis explains a number of biochemical observations: the ribosome and RNaseP are ribozymes [7, 8], nucleotide cofactors are conserved throughout biology [9, 10], DNA replication requires RNA primers [11] and DNA is synthesized from RNA precursors [12]. These biochemical findings are explained as molecular fossils [9] of an earlier RNA world. However, only after the identification of catalytic RNAs 24 years ago [8, 13] did scientists start attempting to re-create an RNA world in the laboratory [14, 15; for reviews see refs. 16–18].

For molecular systems to be called 'alive', they must self-replicate, generate heritable variation and have the potential to increase in complexity through evolution [19–21]. Self-replicating ribozyme systems that replicate through polymerization fulfill all these requirements, in contrast to other systems composed of self-replicating hexanucleotides [22], vesicles [23], micelles [24], peptides [25] and non-biological base pairs [26] for a review see ref. 27].

The synthesis of a life-like system similar to the early stages of the evolution of life would not only enable us

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to learn about the likely origin of life, but would also help us understand more about the capabilities of RNA in contemporary organisms and how to manipulate RNA for purposes of molecular bioengineering. Additionally, the relatively simple, self-replicating and evolving system might show us fundamental organizing principles in biology that are currently hidden from view by the complexity of present life forms that have been evolving for billions of years. The known range of RNA functions in modern biology has widened dramatically with the discovery of entire new classes of RNAs like microRNA (miRNAs) [28], riboswitches [29] and the vast number of conserved but non-protein-coding genomic sequences [30]. Together with the usefulness of RNAs like hammerhead ribozymes [31], splicing ribozymes [32] and RNA interference (RNAi) [33] for medical applications and research, these discoveries have sparked increased interest in charting unknown territory in the landscape of the biochemical capabilities of RNA.

## Prebiotic chemistry

The plausibility of an RNA world in origin-of-life scenarios depends crucially on finding probable prebiotic conditions that, in the absence of any biological catalyst, could have produced pools of random RNA molecules, the starting scenario for an RNA world. Prebiotic chemistry has been reviewed in depth [18, 34, 35] and is mostly beyond the scope of this review. However, a brief overview is required, because our choice of experimental conditions must be constrained by the likely chemical environment of an RNA world.

Nucleotides assembled from small, inorganic molecules could have been present on the prebiotic Earth, either as a result of reactions under aqueous conditions on the Earth's surface [36–39] or delivered to Earth by comets after having been generated in space [40, 41]. Ribose can be generated by the formose reaction [42], and its short half-life in aqueous solution [43] can be extended by complexing the ribose 2′,3′-cis diol by borate [44] or by derivatization with cyanamide, which might also facilitate enantiomeric separation [45]. The prebiotic coupling of ribose with nucleic bases is the weakest link in the chain of reactions leading to nucleotides [35]. However, promising approaches use phosphorylated ribose instead of ribose [46] or assemble the base on the ribose [47]. Nucleosides can then react with inorganic phosphate [48] to yield 5′-phosphorylated nucleotides [49].

To polymerize the nucleotides into oligonucleotides, the nucleotides must first be chemically activated. Polyphosphate-activating groups can be synthesized with calcium phosphate and cyanate under prebiotic conditions [50]. Although it is unclear whether imidazolide-activated nucleotides could have occurred in large amounts on the primitive Earth [35] they have been used for most experimental studies because polyphosphate-activated reactions are too slow for many experiments in the laboratory, with $t_{1/2}$ of the order of years [51, 52]. Non-enzymatic RNA polymerization of imidazole-activated nucleotides can generate 3′,5′-linked polymers using divalent cation catalysts [53] during freezing in eutectic phases [54] or by adsorption on clay surfaces [55, 56]. The resulting RNA polymers can serve as templates for further non-enzymatic polymerization, catalyzed by divalent cations [57, 58]. Although all four nucleotides can be incorporated, the templates must contain at least 60% cytosine. Only then can the template strand form a double-helix with the activated mononucleotides (most of them containing guanine) to facilitate polymerization [59]. Therefore, the products of this reaction cannot be used as templates for successive rounds of amplification [59]. However, if strand separation is allowed, a single template molecule could guide the polymerization of multiple RNAs with the same product sequence.

One of the main obstacles to obtaining 3′,5′-linked polymers of $\beta$-D-ribonucleotides in a prebiotic world is the clutter produced by prebiotic chemistry [18], which would be expected to generate molecules that efficiently compete with activated $\beta$-D-ribonucleotides for polymerization. However, reactions such as the synthesis of phosphorylated ribose [60] and the polymerization of imidazolide-activated adenylate [61] showed some tolerance towards heterogeneous reactants. Still, the difficulties in the prebiotic synthesis of RNA and the short half-life of ribose (between hours and years at pH 7 [43]) led to the suggestion that RNA may have been preceded by different informational polymers, such as threose nucleic acid [62], peptide nucleic acids (PNAs) [63, 64], or charge-complementary peptides from racemic mixtures of amino acids [35, 65].

Another modification of current ideas could make the RNA world more plausible. Instead of Darwin's 'warm little pond' [66], the place of life's origin could be imagined as prebiotic rivers, reducing several problems in prebiotic chemistry. Two merging streams allow the mixing of different prebiotic chemistries such as nitrogenously produced bases with oxygenously produced (and polyphosphorylated) sugars. Low reaction yields in the mixing zone may be tolerated, because the continuous conversion of small fractions of transported chemicals can generate vast deposits of raw material for an RNA world. The sedimentation of less soluble products [44, 45] would lead to the separation of the products, just like a river accumulating fine-grained sand with defined density in protected river bends. Activated nucleotides could adsorb to mineral surfaces and polymerize. Other compounds would drift further downstream, generating fertile grounds for ribo-organisms that acquired metabolic capabilities [see refs. 67, 68]. The river would also create a constant thermodynamic disequilibrium, which is an essential prerequisite for life [69].

In summary, laboratory conditions have been found for most of the reactions needed to convert small, inorganic molecules into activated nucleotides and RNA polymers. However, many of these reactions have low yields and purity, and a single, consecutive prebiotic process has not been experimentally demonstrated. Research in this area is focused on finding a plausible route for prebiotic RNA synthesis or on identifying plausible scenarios for a pre-RNA world that generates the building blocks of an RNA world, with [64, 70] or without [71, 72] a genomic polymer. Whether a pre-RNA world is necessary for the latter or whether a series of biased syntheses, fractionations and other prebiotic enrichment processes is sufficient remains unclear [18].

## Assembling a self-replicating ribozyme system

Whether the original building blocks arose from prebiotic chemistry or the metabolism of a pre-RNA world, the emerging ribozyme system would have had available a pool of activated monomers. In addition, the genetic information from a pre-RNA world would be of little use in the RNA world because pre-RNA nucleic acids and RNA would differ in their structural parameters [73]. Thus, efforts to develop a self-replicating ribozyme system in the laboratory are based on activated ribonucleotides and their polymers.

Which molecules would be required in a self-replicating ribozyme system? The first self-replicating system should be as simple as possible in order to be as likely as possible. The simplest imaginable self-replicating ribozyme system consists of one or two RNA molecules that can replicate themselves. Only two ribozyme activities are candidates for this activity: an oligonucleotide ligase and a nucleotide polymerase. The oligonucleotide ligase approach has fundamental problems in replication fidelity. To enable faithful replication of a template sequence, the oligonucleotide ligase has to choose the correct substrate from all possible substrate oligomers (e.g. 64 different trimers). The small energetic and geometric differences of the possible substrate/template duplexes make such discrimination difficult, limiting faithful copying to only a narrow window of physical conditions [73, 74]. In contrast, a polymerase needs to discriminate between only four different activated nucleotides and their possible pairings to the template and is therefore more likely to achieve sufficient fidelity. For that reason, most studies focus on polymerase ribozymes that use activated mononucleotides.

In the following sections, I will outline the current efforts to generate a simple, self-replicating ribozyme system in the laboratory. After describing the most central technique, *in vitro* evolution, I will cover the promises and problems of developing polymerase ribozymes, a strand-separating activity, a membranous compartmentalization system and a metabolic network.

## *In vitro* evolution of catalytic RNAs

Because the ribozymes in present-day organisms are too limited for the generation of a self-replicating system, new ribozymes must be generated in the laboratory. The most powerful methods to generate new ribozymes are *in vitro* evolution techniques [75, 76; for a review see refs. 77, 78]. These techniques employ large libraries of randomized RNA molecules and select from these pools those RNA molecules that can perform certain tasks, such as binding to a substrate or catalyzing a reaction.

Pools of randomized RNA molecules are typically synthesized in three steps. First, random sequence single-stranded DNA templates are generated by chemical synthesis. Second, a polymerase chain reaction (PCR) transforms the single-stranded DNA into double-stranded DNA, amplifying the polymers and introducing the promoter for T7 RNA polymerase. Third, RNA is transcribed by T7 polymerase from the pool of double-stranded DNA molecules. From this pool of randomized RNA molecules we attempt to isolate ribozymes.

Several considerations guide the construction of a randomized pool. First, how many unique sequences should the pool contain? The probability of finding rare catalysts rises with the size of the pool, so the pool complexities used are typically around $10^{15}$ unique sequences, close to technical limits. Second, how long should the randomized RNA regions be? While there are lower limits for the length of functional sequences [79], the optimum length is between 50–70 nucleotides for an isoleucine-binding RNA [80] but closer to 200 nucleotides when structurally more complex RNAs are desired [81]. Therefore, for each desired activity, the inhibitory effects of additional sequences must be weighed against their resulting increase in combinatorial potential. Third, should random RNA pools contain structural elements to increase the frequency of functional ribozymes? The introduction of helical elements is beneficial [68, 82], although complete structural scaffolds appear to be advantageous only insofar as they provide helical elements [68, 83, 84]. An exception to the latter is if the starting pool already encodes activities that are functionally related to the desired activity; here, recombining the sequences of the starting activities as entire folding domains can be useful [78, 85]. Fourth, should the base composition be biased? Some biases appear promising [86], although the optimal composition has not yet been experimentally determined. Understanding these design principles for partially randomized pools will help us find not only new ribozymes but also conditions under which there was more than a vanishing chance that an RNA world would emerge from RNA polymers [73].

To begin the selection of catalytic molecules from the pool of random RNAs (Fig. 1), the pool is incubated with the substrate molecules, allowing catalytic RNAs to co-

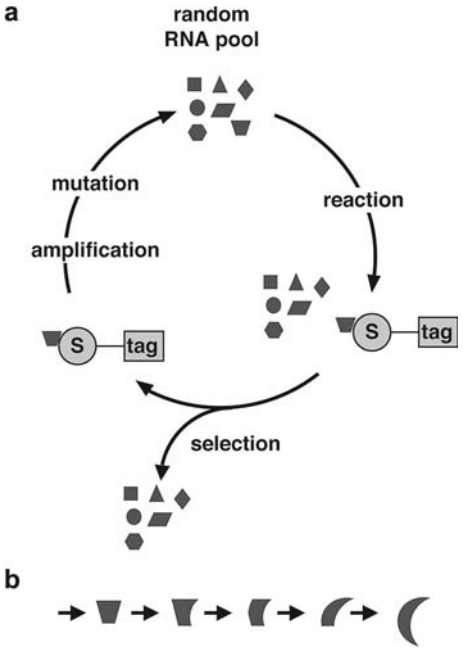


**Figure. 1.** *In vitro* evolution of catalytic RNAs. (*a*) A pool of random RNAs is reacted with a substrate that is covalently linked to a tag. Those RNAs that reacted with the substrate are selected via the tag and amplified by RT-PCR and transcription. Mutagenic PCR generates sequence variants that facilitate the improvement of selected ribozymes. (*b*) Repetitions of this cycle lead to the optimization of initially selected ribozymes.

valently link themselves to the substrate molecules. The substrate molecules are covalently linked to a tag, which is used to isolate the reacted RNA molecules from the unreacted pool molecules. After isolation, the RNA molecules are reverse transcribed and PCR amplified. The resulting pool of double-stranded DNA molecules serves as template for the next pool of RNA molecules, which is enriched in functional sequences. The enrichment increases in successive cycles. The number of cycles required depends on the stringency of the selection step, the size of the initial library and the frequency of molecules with the desired activity in the initial pool. In later rounds of *in vitro* evolution, the PCR amplification step often employs mutagenic conditions so that a larger fraction of possible active sequences can be sampled [87]. After the active sequences are sufficiently enriched, the pool of double-stranded DNA is cloned, and individual clones are sequenced and the corresponding RNAs tested for their catalytic activity.

Continuous *in vitro* evolution [for reviews see refs. 78, 88] is a variation of the discontinuous *in vitro* evolution described above that facilitates hundreds of selection events in a few days [89]. During continuous *in vitro* evolution, successful RNA sequences are not isolated from the reaction mixture but are, instead, amplified in situ using either Q$\beta$ replicase or a combination of reverse transcriptase, RNaseH and T7 RNA polymerase [90]. Amplification is achieved because each cDNA can serve as the template for many RNA copies. Serial dilution steps supply the required substrates and enzymes, and specific primers help to ensure that only the pool sequences are amplified. However, continuous *in vitro* evolution is especially prone to contaminating RNA sequences that can quickly overtake the pool population [91]. To outpace the growth of undesired molecules, the starting activity must allow very rapid amplification of legitimate ribozymes [88, 89]. This difficult requirement has so far only been met in experiments using the class I ligase ribozyme, for which the power of continuous *in vitro* evolution was harnessed to optimize its activity for different reaction conditions [92–94]. Eventually, a self-replicating ribozyme system must facilitate its own continuous *in vitro* evolution, so results from these experiments can help to avoid pitfalls during the design of a self-replicating ribozyme system. For example, one continuous *in vitro* evolution experiment showed that under high selective pressure, high mutation rates are required to escape local fitness optima [88]; this observation may facilitate strategies to meet the challenge of molecular parasites (see 'Compartmentalization' below).

In summary, discontinuous *in vitro* evolution, especially in combination with a stepwise approach and rational design [51, 95–97], appears to be the best choice currently for developing novel ribozyme activities. In contrast, continuous *in vitro* evolution is the most realistic *in vitro* model for biological evolution and is therefore best suited for asking certain evolutionary questions. *In vitro* evolution methods as a whole have proven to be invaluable in the study of the structural, catalytic and evolutionary potential of RNA [79, 98–101].

## The polymerase ribozyme

The most central activity in a self-replicating ribozyme system is a polymerase ribozyme. The most successful polymerase ribozyme so far developed (Fig. 2) is able to bind a primer/template duplex and elongate the 3′-terminus of the primer by 3′,5′-phosphodiester bonds [51] in a template-dependent fashion using nucleoside triphosphates (NTPs) [97].

The average fidelity of polymerization by this ribozyme is about 96.7% [97]. From this value, we can calculate the maximum sustainable genome size, any genome longer than which would slowly lose its information and end up in an error catastrophe [102, 103]. The fidelity of 96.7% is high enough for the sustainable replication of a 230-nucleotide genome [103]. Because the most common error by this ribozyme is caused by the formation of G:U pairs, the fidelity can be increased to 98.5% by reducing the concentration of GTP in the polymerization reaction tenfold (to 0.4 mM) [97]. In fact, skewing all NTP con-

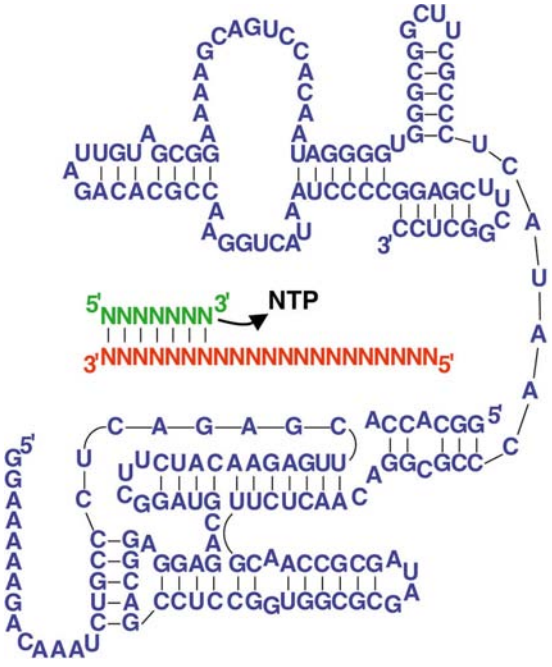


**Figure. 2.** A polymerase ribozyme. A polymerase ribozyme (blue) was developed that binds and extends the 3′-terminus of primers (green) that are base paired to templates (red). The reaction uses nucleoside triphosphates (black) and incorporates the bases complementary to the templating sequence with a fidelity above 96% ([adapted from Johnston et al. [97]).

centrations would lead to an average fidelity of 99.0% at 6 mM CTP, 2.4 mM ATP, 1.5 mM UTP and 0.22 mM GTP (calculated based on the kinetic values in Johnston et al. [97]). This fidelity would be sufficient to allow the sustainable self-replication of a genome with 760 nucleotides [103]. As the polymerase ribozyme is 189 nucleotides long, this genome length could encode a self-replicating set of several ribozymes. Additionally, the described error threshold model [102, 103] assumes growth competition between populations of replicators. If this competition does not exist, a clone would persist indefinitely if each parent produced, on average, at least one viable descendant before disappearing [73]. Lastly, the construction of a multisubunit ribozyme demonstrates that constructing full-length ribozymes may not be necessary if they can assemble from shorter, functional subunits [104]. Therefore, the fidelity of the polymerase ribozyme described appears to be high enough for the first step of constructing a self-replicating ribozyme system. To place the RNA world on solid footing, what remains to be achieved are self-replication and an emergent population of related replicators [73].

However, the efficiency of the known polymerase ribozymes is very limited; no more than 14 nucleotides have been added to a primer so far [97], more than an order of magnitude below what is required for self-replication of the 189-nucleotide ribozyme. The limiting factor is not a steric clash between elongated substrates and the ribozyme: primer/template duplexes with longer double-stranded portions, which would be intermediates in a longer polymerization, are extended with similar efficiency [97]. The limiting factor is very low affinity to the primer/template ($K_M$ ~ 3 mM), which leads to non-processive behavior. On average, after one nucleotide is added to the primer, the primer/template duplex is released in 70% of cases. In the remaining 30% of cases, a second nucleotide is added, and again the primer/template is released in most cases [105]. To extend the primer by multiple nucleotides, the substrate needs to go through release-and-rebind cycles, thereby slowing down the polymerization kinetics. This, in turn, limits the total possible extension because the lifetime of the polymerase is limited under optimized reaction conditions, containing 200 mM magnesium cations at a pH of 8.6. Under these conditions, the ribozyme is inactivated by hydrolysis with a half-life around 12 h [106]. Higher substrate concentrations do not improve the weak substrate binding because they inhibit the ribozyme [105]. The recent development of eight more polymerase ribozymes through *in vitro* evolution, based on the same catalytic domain, did not yield ribozymes that promote more efficient polymerization than the first polymerase ribozyme [106]. The tendency toward substrate release is strongly sequence dependent [105]. However, this sequence dependence has also been observed with efficient proteinaceous RNA polymerases and reverse transcriptases [107, 108]. Therefore, to obtain longer polymerization products, one would have to improve the binding of the primer/template duplex or optimize the polymerase ribozyme for reaction conditions in which its lifetime is much longer.

The substrate release-and-rebind mechanism is probably the result of reliance on a few rigid substrate interactions. The polymerase ribozyme contacts seven 2′-hydroxyl groups of the primer-template (Fig. 3), four of which lie in the double-stranded region, very close to the primer 3′-terminus and the active site of the ribozyme [109]. In addition, the ribozyme must precisely recognize the geometry of the Watson-Crick base pair formed between the incoming NTP and the first templating base because the polymerization fidelity is much higher than the 60% predicted by Watson-Crick base pairing alone [97, 110]. These two recognition processes must be coordinated with each other to allow nucleophilic attack of the primer 3′-hydroxyl group on the $\alpha$-phosphate of the correctly paired NTP. Most of these substrate-binding contacts must be rigid to enable precise discrimination between incorrect and correct base pairs, so they must be broken when the primer/template duplex repositions for the next nucleotide addition. The binding energy of the 2′-hydroxyl contacts is about –17 kJ/mol, which is a major portion of total binding energy [109]. Therefore, repositioning for the next nucleotide addition requires

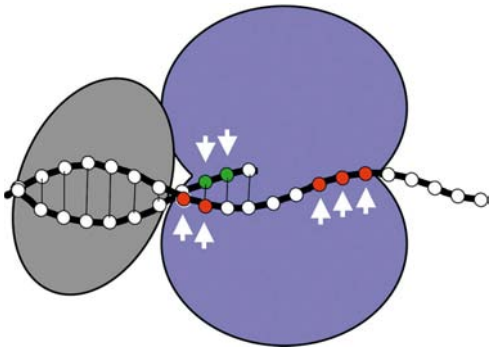

**Figure. 3.** Substrate contacts of the polymerase ribozyme. The polymerase ribozyme (blue) contacts the 2′-hydroxyl groups of primer (green contacts) and template (red contacts) close to the 3′-terminus of the primer. The double-stranded region in the primer/template duplex upstream of the contact site is predestined as binding site for an additional binding domain (gray) in the polymerase ribozyme [109].

the transient loss of a major portion of the total binding energy. While those contacts are broken the primer/template duplex can easily dissociate, causing the observed release-and-rebind mechanism.

## Improving the polymerase ribozyme

If the distributive mechanism of the current polymerase ribozyme indeed stems from its rigid mode of substrate binding, a more processive polymerization will require an additional, more flexible mode of binding that facilitates repositioning of the primer-template without release. Proteinaceous RNA-dependent RNA polymerases (RdRps) use at least three mechanisms for flexible template binding. First, positively charged amino acid residues can establish ionic bonds with the phosphodiester backbone [111, 112]. With the exception of coordinated metal ions, ribozymes lack positive charges, so adopting this strategy for ribozymes might require the use of positively charged cofactors [113, 114]. Second, a clamp that surrounds the template strand to prevent dissociation is characteristic of RdRps [111, 115]. It might be possible to build such binding clamps into the polymerase ribozyme by a combination of design and *in vitro* evolution. Third, RdRps use stacking interactions between aromatic amino acids and bases to recognize single-stranded templates [112, 115]. This approach does not appear promising for the ribozyme because binding of the ribozyme to the single-stranded part of the substrate is prone to cause sequence dependence [109], undesirable because its limits the generality of the polymerase activity.

To develop an additional binding domain on the polymerase ribozyme, one could obtain a double-stranded RNA binding domain by *in vitro* evolution and then link it to the polymerase ribozyme. The binding target for the new domain could be the double-stranded region of the substrate that lies more than three nucleotides upstream of the primer 3′-terminus and that is not known to contact the current ribozyme (Fig. 3) [109]. Such a second domain was not obtained during previous *in vitro* evolution experiments. One reason is that the substrates were covalently coupled to the ribozyme during the selection steps, so that no evolutionary pressure drove the formation of a second substrate-binding domain [97, 106]. Another reason is that, in the *in vitro* evolution that led to the current polymerase ribozyme, for a randomized sequence with a length of 76 nucleotides to evolve into a domain that not only correctly positions the substrate but also forms flexible, sequence-unspecific contacts with the primer/template duplex may be too great a hurdle.

To increase the evolutionary pressure for substrate binding, one would have to perform the reaction in trans, using primer/template duplexes that are not covalently coupled to the ribozyme. However, the isolation of functional molecules from *in vitro* evolution pools requires a physical connection between catalyst and product. A solution to this problem is to compartmentalize members of the random RNA pool with their substrates and then select those compartments in which efficient primer extension proceeds. This principle has been shown to work in connection with fluorescence-activated cell sorting (FACS) for the evolution of trans-acting ribozymes [116]. Those compartments (emulsion droplets or vesicles) in which a non-fluorescent substrate has been converted into a fluorescent substrate by ribozyme catalysis are sorted together to enrich for the desired ribozymes in the pool. Two technical improvements are required to efficiently conduct *in vitro* evolution experiments with this technique. First, the throughput of FACS machines needs to be increased. Currently, FACS instruments can sample around $10^8$ compartments per hour, limiting the maximum number of compartments that can be conveniently sampled to less than $10^{10}$. To increase the number of ribozymes that can be sampled, one can encapsulate multiple ribozymes per compartment; however, this would open the door for molecular parasites because RNAs in the same compartment with efficient ribozymes would be coselected. These technical limitations may be overcome in the near future with the improvement of high-throughput systems [117, 118]. The second challenge for the application of this technique is the design of reactions that generate a fluorescent product upon catalysis. For example, a polymerase ribozyme could generate a hammerhead ribozyme, which in turn cleaves a fluorogenic substrate to yield fluorescent products. As starting material, one could use current polymerase ribozymes and equip them with additional, randomized domains that could evolve toward better primer-template binding. The resulting ribozymes would be bigger than the current polymerase ribozymes, limiting the number and size of other genes the sustainable genome could include, but an increase in

size [79] may be the simplest solution to improve polymerase/substrate interactions.

## Genomes and helicase ribozymes

Even at the level of a minimal self-replicating system, it is trivially necessary to have a genome because a single polymerase ribozyme cannot replicate itself: for a ribozyme to transcribe its own sequence, it would have to unfold its own catalytic site and thereby inactivate itself. Although one can imagine scenarios in which the catalytic site is synthesized separately and then spliced into the correct site, the existence of a second, genomic RNA is a simpler solution to this problem. This second RNA would be complementary to the polymerase ribozyme and serve as template for the generation of more polymerase ribozymes.

All currently existing polymerase ribozymes require a single-stranded template [97, 106]. However, a single-stranded genome poses several problems for self-replicating ribozyme systems. First, single-stranded RNAs hydrolyze up to four orders of magnitude faster than double-stranded RNAs [119] and are therefore less suited for the storage of genomic information. Second, single-stranded RNAs of almost any sequence form secondary structures [120, 121] which slow down polymerization by proteinaceous RNA polymerases [122] and probably present formidable barriers for polymerase ribozymes with slow polymerization kinetics and weak substrate binding [105]. Third, the polymerization products would have to be displaced to regenerate the single-stranded genome. The tendency of complementary genome and transcript strands to anneal with each other would require a constant, energy-expensive strand-separating activity. The latter problem is reduced if both transcript and single-stranded genome immediately form stable self-structures [110].

A double-stranded genome would avoid these three problems, but a strand-separating activity is still required: a double-stranded genome has to be locally melted to allow base pairing with the newly forming strand. After the polymerase has passed, the two genomic strands can either re-anneal and release the newly synthesized strand (conservative mechanism), or the newly synthesized strand can stay base paired with the complementary genomic double strand, releasing the previously base paired strand for catalytic function (semi-conservative mechanism; Fig. 4) [110, 123]. The separation of double-stranded RNAs is not an easy task because RNA double strands are thermodynamically very stable [110, 124]. The necessary energy would have to be provided through coupling to a second reaction, catalyzed either by the polymerase itself or by a separate helicase [16].

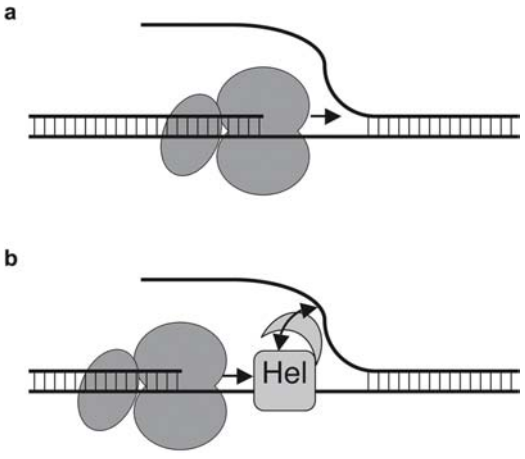


**Figure. 4.** Models for strand separation during ribozyme-catalyzed RNA polymerization. (*a*) The strand separation is mediated by the polymerase ribozyme (dark gray). The combination of Brownian motion and irreversible primer elongation is sufficient to plough through the double strand, provided that the polymerase remains bound [108]. (*b*) The strand separation is mediated by a separate helicase activity (light gray). ATP hydrolysis drives conformational changes that mediate strand separation, while polymerization fixes the directionality (see text).

If the polymerase ribozyme harbors the strand separation activity (Fig. 4a), it could first use its substrate-binding energy to melt a promoter region with low thermodynamic stability, as appears to be the case with some proteinaceous RNA polymerases [111, 125]. After initiating polymerization, the combination of Brownian motion and irreversible primer elongation is sufficient to plow through the double helix, provided that the polymerase remains bound [108]. To use this approach for the polymerase ribozyme, future experiments would have to modify an existing polymerase ribozyme so that it can (i) melt the promoter region of the double strand upon binding, (ii) initiate polymerization in this bubble and (iii) remain bound to the primer/template duplex until a full strand is synthesized. These tasks will probably require additional *in vitro* evolution experiments to identify and optimize the required functional motifs from random sequences added to a polymerase ribozyme.

If the double-stranded RNA genome is melted by a helicase ribozyme (Fig. 4b), this ribozyme would have to couple an exergonic reaction, such as ATP hydrolysis, to the endergonic reaction of strand separation. This task is extremely challenging. One approach is to couple an RNA with high ATP affinity and low AMP affinity [126] to an ATPase activity, which should be easy to obtain from existing ribozymes [101, 127] or from random sequences. If binding to ATP changes the conformation of the ribozyme, the cleavage of ATP and release of low-affinity AMP would restore the old conformation. This flipping back and forth would create a mechanical force that might be sufficient to mechanically separate the double strand, similar to the ratchet mechanism of some

proteinaceous RdRps [128, 129]. To target the machinery to the double-stranded genome, a domain that binds to the forking double-stranded RNA would have to be developed (compare with 'Improving the polymerase ribozyme' above). Coordinating an ATP aptamer, an ATPase and a genome-fork-binding domain to form a functional helicase would rely on *in vitro* evolution methods, using RNA pools with recombined elements and randomized sequences. The resulting helicase activity would also have to be coordinated with polymerization activity to allow templated polymerization before the separated RNA strands re-anneal.

To prevent the genome from shortening in successive replication cycles, at least three approaches are possible. First, a circular genome would avoid this genome shortening but requires at least one additional ribozyme that circularizes the products after polymerization. Second, the termini of a linear genome could be regenerated by a telomerase ribozyme. Third, the chromosome termini could form 'genomic tags', self-structures that function as simple telomeres and as initiation sites for replication [130].

## Compartmentalization

Lipid membrane compartments [reviewed in ref. 131] meet two needs of self-replicating and evolving ribozyme systems. First, evolution requires that a genome be rewarded with amplification if it encodes efficient catalysts that help replication. To ensure the coamplification of genome and catalyst, they have to be physically linked to each other, and separated from unrelated RNAs. This exclusion of other RNAs draws the border of the evolutionary unit, defining the 'self' in self-replication. The physical link can be achieved by many different mechanisms: RNA polymers can be held together on charged mineral particles, on organic colloids or coacervates [35], as colonies on mineral surfaces or in gel-like structures [132], by transient and non-covalent interactions [133], or in membrane-enclosed compartments as found in present-day cells. Although any of these methods might suffice for the link between genome and catalysts, most of them are not well suited for the exclusion of external, possibly parasitic RNA molecules. Parasitic RNA molecules are abundant sequences that have no activity beneficial to the self-replicating system but are excellent templates, quickly overtaking continuously replicating systems [88, 134, 135]. Although such parasitic sequences can also arise from copying errors within a self-replicating system [134], the compartmentalization drastically reduces the problem of molecular parasites because it excludes parasites from outside.

The second benefit of membrane compartmentalization is that a small-molecule metabolism can be established. The synthesis of small molecules (metabolites) is of evolutionary advantage to the respective catalysts and genome only if the metabolites are prevented from diffusing away. This requires the encapsulation of catalysts and genome within a membrane that is impermeable to macromolecules and some metabolites, but permeable to metabolic substrates that must be replenished from the outside. Because certain lipid bilayer encapsulation systems fulfill all requirements for compartmentalization, and because they are most relevant to biology, most studies have focused on lipid membranes as a means of compartmentalization.

Membrane encapsulations can be formed from fatty acids when the pH of the solution is close to the $pK_A$ of the acid [136]. Fatty acids are plausible prebiotic molecules because they can be synthesized in a methane environment with electric discharge [137] or in hydrothermal vents [138, 139], or delivered by meteorites [140] or comets [141]. In contrast to the phospholipids that are ubiquitous in biology, vesicles consisting of fatty acids can multiply autocatalytically [142] and can go through repeated cycles of growth and division in the laboratory [143]. Interestingly, this autocatalytic growth can be triggered by the same clay minerals that also catalyze the non-templated, non-enzymatic polymerization of activated nucleotides [143]. Vesicles can grow either by incorporating fatty acids from micelles [142] (Fig. 5a) or by incorporating fatty acids from neighboring vesicles, if the growing vesicles contain osmotically active substances such as RNA polymers complexed with counterions [144] (Fig. 5b).

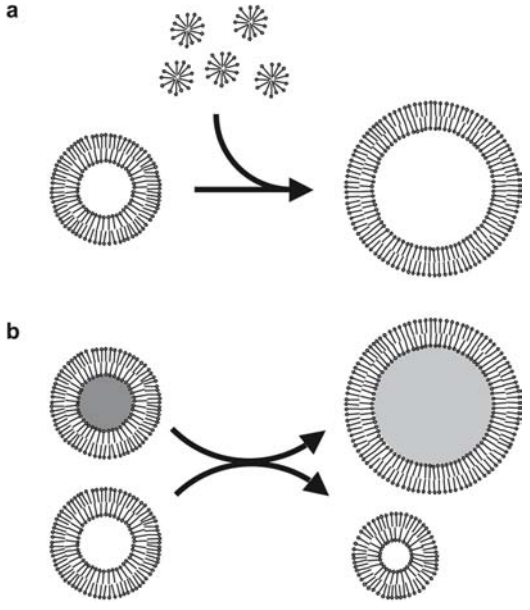


**Figure. 5.** Two methods for growth of fatty acid vesicles. (*a*) Addition of micelles at high pH to a solution of vesicles buffered at the $pK_A$ of the fatty acid [142]. (*b*) Osmotically swollen vesicles grow at the expense of vesicles with lower osmotic pressure [144].

However, pure fatty acid vesicles are not stable under conditions that allow ribozyme catalysis: divalent cations in millimolar concentrations are essential for the activity of almost all ribozymes [145], but lead to the precipitation of fatty acids [146]. This problem was solved with the finding that incorporation of fatty acid alcohols stabilizes fatty acid vesicles against divalent cation precipitation [146]. Glycerol esters of fatty acids form in high yields from glycerol and fatty acids under aqueous conditions, making them prebiotically plausible compounds [147]. In addition, vesicles from myristoleic acid (C14:1) and its glycerol ester are not only stable in the presence of 4 mM $Mg^{2+}$, but are also permeable to $Mg^{2+}$ and uridine monophosphate, while being impermeable to an RNA decamer. These characteristics have allowed ribozyme catalysis inside vesicles, with $Mg^{2+}$ added from outside [148].

In summary, these results suggest that the following scenario might be possible (Fig. 6): an efficient polymerase ribozyme, a helicase activity and their genome are compartmentalized into fatty acid/fatty acid alcohol vesicles. The addition of $Mg^{2+}$ allows the ribozymes to acquire their active conformations, and the addition of activated nucleotides allows the ribozymes to self-replicate inside the vesicles. Because the influx of activated nucleoside diphosphates into lipid vesicles is possible after temperature adjustment [149], the influx of NTPs might also be possible with different membrane compositions or at higher temperatures [150]. After RNA synthesis, the increased osmotic pressure inside the vesicles leads to vesicle growth through incorporation of fatty acids from neighboring vesicles [144], coordinating RNA replication with vesicle growth. At a certain size, the vesicles are big enough so that high shearing forces lead to vesicle division [143]. More prebiotic duplication mechanisms remain to be found. At this point, self-replication of the ribozyme system will have been achieved. The error rate of the polymerase ribozyme (currently 1–3.3% per nucleotide) would quickly lead to the generation of ribozyme sequence variants, a good start for continuous evolution of the system. Small RNA populations in each vesicle would facilitate genetic drift, allowing the fitness landscape to be explored [88]. As soon as an efficient polymerase ribozyme and a helicase activity are developed in the laboratory, enclosing them in a growing and dividing membrane system such that a self-replicating and evolving ribozyme system results should be possible.

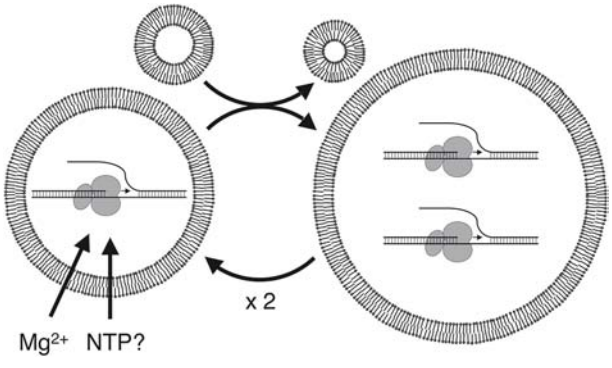


**Figure. 6. A simple self-replicating ribozyme system.** Fatty acid/fatty acid glyceride vesicles allow the uptake of magnesium ions and ribozyme catalysis in their interior [148]. Whether NTPs can permeate double lipid layers is unclear, but this is possible for nucleotides [148] and nucleoside diphosphates [149]. Fatty acid vesicles can grow through interior osmotic pressure, incorporating lipids from 'empty' vesicles [144]. Grown vesicles can divide by physical shearing forces [143].

## A simple metabolism

Everything that follows is in the realm of natural evolution [35]. However, attempts to recapitulate the origin of life require more than sustaining a minimal system, they also require an evolutionary pressure for establishing a metabolism. If the ribozyme system is successively challenged to obtain its substrates from a less and less supportive environment, it must develop or integrate catalysts for more and more synthetic steps (Fig. 7). When this is attempted in the laboratory, care has to be taken that each step is evolutionarily accessible to the system. The development of a ribozyme-catalyzed metabolism has been reviewed before [18]; I will summarize here the central themes and recent developments.

The above-described ribozyme system is built on NTPs. So far, no known ribozyme can transfer polyphosphates to nucleosides (Fig. 7d), but many ribozymes can catalyze a single 5′-phosphorylation of oligonucleotides, corresponding to the conversion from nucleoside to nucleotide [101, 127], and at least one ribozyme can convert GDP to GTP using other NTP molecules [101]. Because ribozymes are adept at catalyzing phosphoryl transfers [77], it should be possible to find ribozymes that use phosphate donors with high phosphate donor potential to generate nucleoside oligophosphates. In the RNA world, these phosphate donors were probably polyphosphates, which are still ubiquitous in biology [151]. The ancestors of hexose kinases probably used polyphosphate [152]. Polyphosphates are also likely to be prebiotic because they are readily synthesized in volcanic condensates [153] or by heating ammonium phosphate with urea [154]. An alternative to 5′-oligophosphate as the activating group is a 5′,5′-pyrophosphate-linked nucleotide. This activating group can be generated in a ribozyme-catalyzed reaction [155] and another *in-vitro*-evolved ribozyme has been demonstrated to use this activation group to catalyze oligonucleotide ligation [156]. Although it may be possible to base a metabolism on these 5′,5′-pyrophosphate linkages, it would not avoid the necessity of finding high-energy phosphate donors such as polyphosphates for their synthesis.

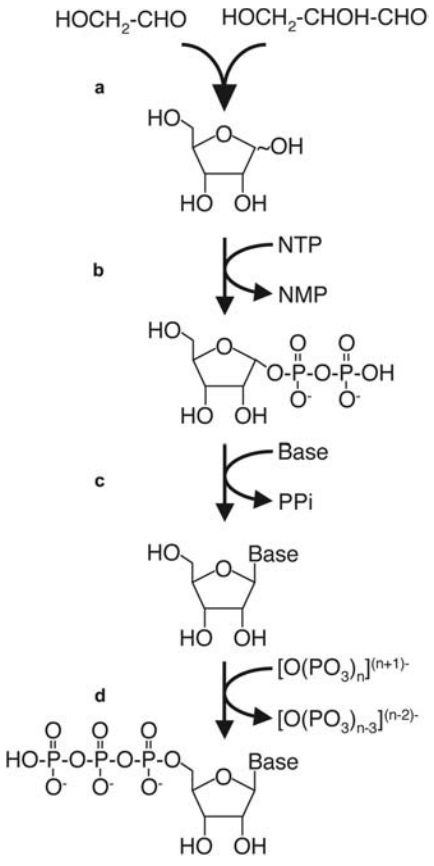


**Figure. 7.** Synthesis of NTPs by ribozymes. (*a*) Aldol condensation, the chemistry for ribose synthesis, can be catalyzed by ribozymes [158]. (*b*) No known ribozymes transfer polyphosphate groups, but phosphorylation of ribose hydroxyl groups and nucleoside diphosphates has been achieved [101, 127]. (*c*) Ribozymes can catalyze the formation of nucleoside N-glycosidic bonds [67, 68]. (*d*) same as (*b*).

The synthesis of nucleosides from phosphorylated ribose and purines or pyrimidines has been demonstrated by two *in-vitro*-selected ribozymes [67, 68] (Fig. 7c). These ribozymes use 1-pyrophosphoryl ribose attached to their 3′-terminus to react with the respective base, generating the proper N-glycosidic linkage. The catalysis is nucleobase specific [67, 68], allowing discrimination against the presumed clutter of nucleobases after their prebiotic synthesis [18]. This is a significant evolutionary advantage for the ribozyme-catalyzed reaction with regards to reducing the frequency of replication errors caused by non-canonical bases. Because both known nucleotide synthase ribozymes catalyze a cis reaction at their 3′-terminus, they would need to be modified for the trans reaction in order to generate free nucleosides or nucleotides. Interestingly, the pyrimidine nucleotide synthase ribozyme seems to use an oxocarbenium ion transition state [157], which was thought to be too reactive for a ribozyme to shield from side reactions with water.

Ribozyme-catalyzed ribose synthesis would also be of evolutionary benefit because it could generate a source of pure ribose instead of the heterogenic mixture produced by prebiotic chemistry [18] (Fig. 7a). A $Zn^{2+}$-dependent ribozyme was recently shown to be able to catalyze aldol condensations in trans [158], the chemistry to form ribose. To perform such a ribozyme-catalyzed ribose synthesis, glycolaldehyde and glyceraldehyde could be used, whose phosphorylated forms react to form ribose 2,4-diphosphates under mild conditions [159]. Interestingly, a $[Zn(proline)_2]^{2+}$ complex is able to catalyze the reaction of glycolaldehyde and glyceraldehyde [160]. Thus, perhaps with the use of similar cofactors, generating ribozymes catalyzing ribose synthesis should be possible. Without these ribozymes being available to the system, ribose could be enriched from heterogenic sugars outside the vesicles because the membrane permeability for ribose is greater than that for the other aldopentoses or hexoses [161]. In an ancestral RNA world, this selective membrane permeability could have been the factor that decided that ribose be the sugar of the nucleic acid backbone [161]. In biological systems, ribose is pyrophosphorylated using ATP [162] (Fig. 7b). In an RNA world, this pyrophosphorylation should have been accessible to ribozymes because ribozymes readily catalyze phosphoryl transfer reactions [77] (see above).

No ribozyme yet known can catalyze nucleobase synthesis. This synthesis would be of advantage by reducing the occurrence of non-canonical bases that could interfere with replication fidelity. However, because the nucleotide synthases discussed above have good specificity [67, 68], this feature may not be very important for an early RNA world. Which precursors should be used for a ribozyme-catalyzed synthesis [18] and if the bases should be assembled on the ribose or in free form is unclear.

The ribozyme-catalyzed synthesis of membranes in the expected prebiotic environment is also difficult: intermediates in prebiotic fatty acid synthesis can probably not be used by ribozymes because efficient fatty acid synthesis appears to work only under hydrothermal conditions [137, 139] where RNAs have half-lives up to hours at neutral pH [163, 164]. To synthesize membrane components from other precursors, carbon-carbon bonds have to be formed. Currently, only two carbon-carbon bond-forming reactions are catalyzed by ribozymes: Diels-Alder reactions [165, 166] and aldol condensations [158]. However, the products of Diels-Alder reactions cannot pack well into a membrane, and the products of aldol condensations would need to be reduced twice by a redox ribozyme [167, 168] before being able to assemble into hydrophobic layers. A route to fatty acid synthesis similar to that in living organisms might have been chosen using cofactors like acetyl-CoA [114], and molecular fos-

sils suggest that this biosynthesis had a very early origin in evolution [10]. Alternative encapsulations that might be accessible to ribozymes are bilayers from $\beta$ sheets of hydrophobic peptides [169] or terpenoid encapsulations [170]. To regulate metabolic synthesis, the metabolites could be sensed by aptamers or aptazymes [171] to increase or decrease RNA polymerization, as occurs in present-day organisms [29].

## Cofactors and peptides

Considering the range of biological cofactors such as CoA, NAD and FAD that can be synthesized by ribozymes [114], it appears feasible that an early RNA world made use of a broad repertoire of cofactors. Indeed, cofactors for ribozymes appear to be essential for a number of functions, supplementing the limited chemical diversity of RNA [113, 114]. One limitation of RNA is its lack of hydrophobic groups, preventing it from efficiently inserting into lipid membranes to form a membrane channel [172]. Although *in-vitro*-evolved RNA heterotrimers are able to transiently destabilize a lipid membrane and increase permeability for GTP [173], and although membrane permeability for nucleoside phosphates and diphosphates can be increased under narrow physical and chemical conditions [148, 149], hydrophobic structures are necessary to generate more efficient membrane channels [172]. If an array of RNA 2′-hydroxyl groups were decorated with hydrophobic amino acids, this RNA might be able to insert through the membrane and establish a channel (Fig. 8a). The aminoacylations required for such modifications can be catalyzed by ribozymes, in cis [174] as well as in trans [175], so that the generation of membrane channels from covalently modified RNAs appears possible. A second limitation of RNA is its lack of functional groups with a $pK_A$ in the neutral range [113]. The cofactor histidine has been used to provide proton transfer capacity at neutral pH, as a ligand bound at the catalytic site [176] (Fig. 8b).

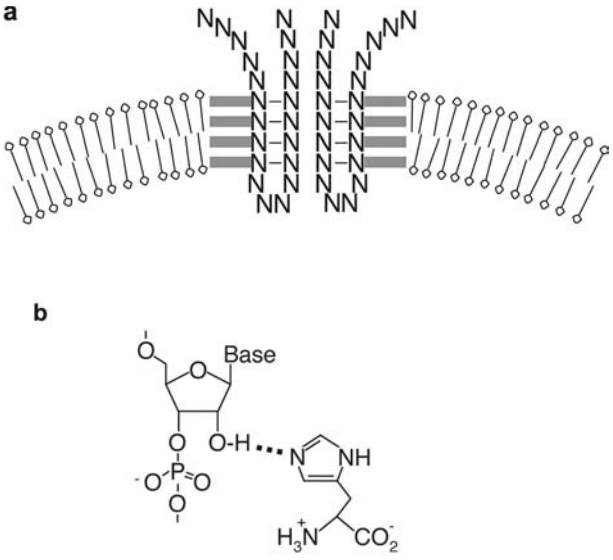


**Figure. 8.** Cofactors for RNAs. (*a*) If RNAs are modified with hydrophobic residues (gray rectangles), they could create membrane channels. (*b*) Histidine can deprotonate with a $pK_A$ that supplements RNA catalytic function [176].

The synthesis of peptides in an RNA world appears accessible because every step of biological peptide synthesis can be catalyzed by ribozymes [18] (Fig. 9): ribozymes can aminoacylate their own 5′-phosphate [177], similar to the biosynthesis of 5′-aminoacyl-AMP (Fig. 9a), and can use 5′-aminoacyl-AMP to aminoacylate the 2′(3′)-terminus of tRNAs [178] (Fig. 9b). At this step, dipeptide formation has been observed, which might serve as an evolutionary transition from an RNA world to an RNA/protein world [179]. Peptide bond formation is catalyzed by ribozymes using either 2′(3′)-aminoacylated AMP [180] or, in the ribosome, 2′(3′)-aminoacylated tRNA [7] (Fig. 9c). The evolutionary origin of this process might have been simplified by prebiotic synthesis of 5′-aminoacyl-AMP [181]. Each step in this chain of reactions would be of evolutionary benefit because covalently linked amino acids and short peptides can be considered as cofactors in an RNA world, increasing the catalytic capabilities of ribozymes. The specificity of RNA/ligand interactions [182] could then control the amino acid diversity of the resulting peptide cofactors until a more sophisticated translation apparatus evolved.

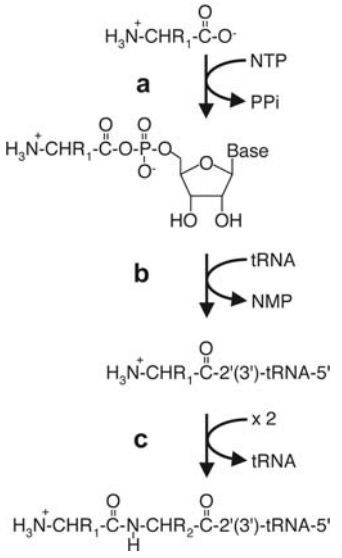


**Figure. 9.** Peptide synthesis catalyzed by ribozymes. (*a*) The nucleotidyl activation of amino acids can be catalyzed by a ribozyme [177]. (*b*) The aminoacylation of tRNA can be catalyzed by ribozymes in trans [175] (*c*) Peptide bond formation can be catalyzed by ribozymes [7, 180]. A more direct approach can synthesize 2′(3′) dipeptides using 5′-aminoacyl-AMP (not shown) [179].

From our biological perspective, the rise of a DNA/protein world over the RNA world may seem inevitable. A number of arguments are ready to explain this transition. First, proteins are better than RNA at binding small, nonplanar compounds with negative charge or hydrophobic character, and protein catalysts are superior to RNA catalysts at stabilizing transition states, enhancing electrostatic interactions in hydrophobic pockets and forming more tightly packed cores and solvent boundaries [77, 79]. Second, DNA is a more stable genome [163]. Although the chemical conversion from RNA to DNA is difficult, ribozymes might have the potential to catalyze the required reduction of the 2′-carbon [157, 183]. However, other chemistries may carry the same benefits, and only the experiment of creating and analyzing other RNA worlds will tell us if our DNA/protein world is a necessary consequence of or one out of many choices for an RNA world.

## Conclusions

The synthesis of a self-replicating system from catalytic RNAs would be a milestone in the search for the origins of life and for our understanding of life itself. Although a polymerase ribozyme and many other ribozymes have been generated in the laboratory together with vesicles that could encapsulate a self-replicating ribozyme system, there remains much work to be done. Current polymerase ribozymes have to acquire a sufficient processivity, and a strand separation activity has to be developed. To establish a system that has a small metabolism, several existing ribozymes would have to be modified, a few ribozymes would have to be newly developed, and all ribozymes would have to be compartmentalized by lipid vesicles. Since the requirements of a self-replicating and evolving molecular system can be clearly delineated, laboratory work toward generating the requisite system compounds can be highly focused. Thus, the synthesis of a model system for life from defined components may soon be achievable.

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