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Perspective

The Origin of RNA and "My Grandfather's Axe"

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The origin of RNA is one of the most formidable problems facing prebiotic chemists. We consider RNA as a product of evolution, as opposed to the more conventional view of RNA as originally being the product of abiotic processes. We have come to accept that life's informational polymers have changed in chemical structure since their emergence, which presents a quandary similar to the paradox of "My Grandfather's Axe". Here, we discuss reasons why all contemporary components of RNA—the nucleobases, ribose, and phosphate—are not likely the original components of the first informational polymer(s) of life. We also evaluate three distinct models put forth as pathways for how the earliest informational polymers might have assembled. We see the quest to uncover the ancestors of RNA as an exciting scientific journey, one that is already providing additional chemical constraints on the origin of life and one that has the potential to produce self-assembling materials, novel catalysis, and bioactive compounds.

Philosophers have long debated whether an object consisting of multiple parts is still fundamentally the same object if all of its parts have been incrementally replaced over time. This conundrum is captured by the paradox of "My Grandfather's Axe", in which a man proudly proclaims, "This was my grandfather's axe. Of course, it has occasionally required some repairs. My father replaced the handle and I replaced the head." In this perspective, we liken RNA to the heirloom axe, where replacements of both the handle and the head of this metaphorical axe can also coincide with changes in function. Rather than accepting the notion that RNA is identical in chemical structure to the first informational polymer of life, we see persuasive reasons to place RNA as the penultimate member of a continuous series of polymers, with DNA being the most recent member of the series.

Prebiotic chemists have long hypothesized that the polymers of life were first assembled by the spontaneous, nonenzymatic coupling of pre-existing molecular building blocks. This hypothesis remains attractive for the origin of noncoded peptides. Amino acids are abundant in chondritic meteorites and are readily formed in model prebiotic reactions (Miller, 1953; Sephton, 2002). Thus, once a prebiotically plausible mechanism for amide bond formation is discovered, we would have a plausible scenario for the origin of the first noncoded peptides. Indeed, there already exists at least one experimentally tested hypothesis for prebiotic amide bond formation that produces peptides of lengths up to at least tetrapeptides (Leman et al., 2004).

In contrast to peptides, the prebiotic origin of RNA is not at all obvious. Nucleic acid oligomers are produced by the coupling of mononucleotides—monomeric units that are much more complicated and synthetically difficult than amino acids because they are composed of three distinct chemical moieties, each of which present their own distinct challenges. Unlike amino acids, mononucleotides are not found among the products of one-pot model prebiotic reactions and nucleotides will not spontaneously couple together without the aid of synthetic modifications (i.e., chemical activation). Even when chemically activated mononucleotides do couple to each other, various linkages are formed with distinct regiochemistries (e.g., a 3′,5′-linkage phosphodiester versus a 2′,5′-linkage phosphodiester) and different chemical bonds are produced (e.g., phosphodiester versus pyrophosphate).

There are two limiting views among prebiotic chemists regarding the origin of RNA. At one extreme, RNA is seen as the direct product of prebiotic, geochemical processes (Ferris, 1993; Powner et al., 2009; Sanchez and Orgel, 1970). At the other extreme, RNA is considered to be a product of chemical evolution or even a "biological invention". That is, RNA is viewed by some as a molecular descendant of pre-RNAs (Engelhart and Hud, 2010; Joyce et al., 1987), which are descendants of an original polymer that we refer to as proto-RNA.

We see the exquisite functionality of RNA and DNA within life today, juxtaposed against problematic geochemical pathways to the formation of these polymers, as support for the hypothesis that RNA and DNA are both products of a multistep evolutionary process. Throughout this perspective, we discuss experimental results that support the view that RNA evolved from a simpler polymer or polymers. We discuss how specific chemical differences between hypothetical pre-RNAs and contemporary RNA, some chemically subtle and some dramatic, could resolve a number of conceptual problems associated with prebiotic nucleic acid synthesis. In this context, we also discuss three models for how the first informational polymers might have spontaneously assembled. Two models have been previously presented in the literature: what we refer to as the "classic model", in which nucleobases (or alternative recognition units), sugars, and phosphates sequentially couple to form nucleotides and then polymers, and what we refer to as the "ribose-centric model", in which nucleobases form on the ribose sugar. Here, we introduce a third model, which we name the "polymer fusion model", in which the first informational polymers result from the merger of a supramolecular noncovalent assembly of the

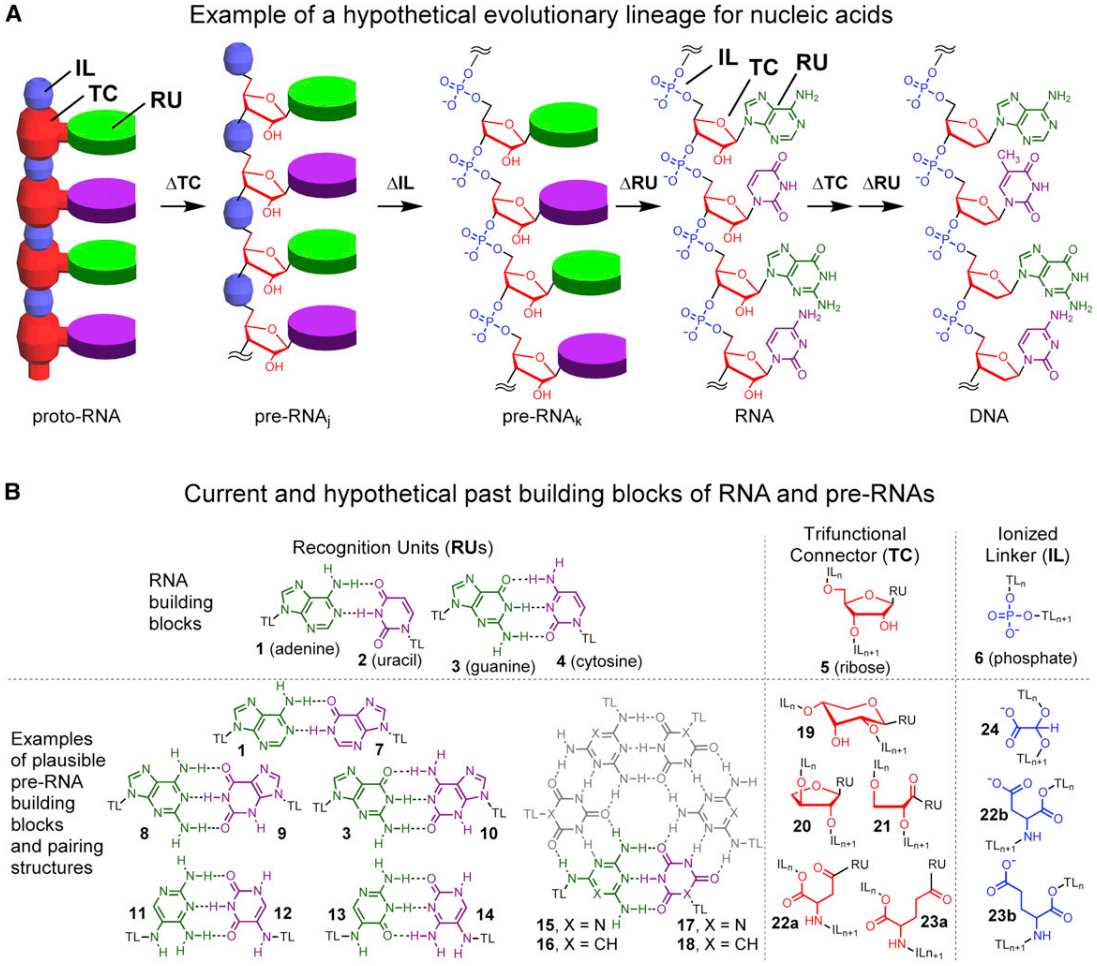

Figure 1. A Proposed Evolutionary Pathway to Contemporary Nucleic Acids with Some Plausible Building Blocks of Pre-RNAs

(A) Schematic representation of a hypothetical evolutionary lineage of nucleic acids from proto-RNA to RNA and DNA. The three components of RNA are the recognition units (RUs), trifunctional connector (TC), and ionized linker (IL). Intermediates between proto-RNA and RNA are shown for illustrative purposes only and are not intended to imply that changes in RUs, TC, or IL proceeded in the particular order or number of steps shown.

(B) Examples of plausible pre-RNA components for RUs, TC, and ILs. Key to structures: 1, adenine; 2, uracil; 3, guanine; 4, cytosine; 5, ribose (furanose form); 6, phosphate; 7, hypoxanthine; 8, 2,6-diaminopurine; 9, xanthine; 10, isoguanine; 11, 2,4,5-triaminopyrimidine; 12, 5-aminouracil; 13, 2,5-diaminopyrimidin-4(3H)-one; 14, 4,5-diaminopyrimidin-2(1H)-one; 15, melamine; 16, 2,4,6-triaminopyrimidine; 17, cyanuric acid; 18, barbituric acid; 19, ribose (pyranose form); 20, threose; 21, glutamine (a as TL; b as IL); 22, aspartate (a as TL; b as IL); 23, glyceric acid; 24, glyoxylate.

nucleobases (or their ancestors) with a pre-existing linear polymer (which became the first backbone).

The Five Components of RNA

A mononucleotide consists of a nucleobase (which we refer to as a recognition unit [RU]), a ribose sugar (a trifunctional connector [TC]), and a phosphate group (an ionized linker [IL]) (Figure 1A). Each mononucleotide can be considered an assemblage of these three distinct molecular units. The view presented here is that each of these units was brought into contemporary RNA, quasi-independently, through the replacement of ancestral RUs, TCs, and ILs during the earliest stages of chemical or biological evolution (Figure 1A). Because nucleic acids have one negative charge per phosphate group, the folding and function of these polymers always requires associated cations to reduce electrostatic charge repulsion. Divalent metals are especially important for complex folds and catalytic activity. The availability of metals in the biosphere has changed radically over long geological timescales, which would have greatly affected life as it transitioned within epochs (Anbar, 2008). Going one step further, water molecules have long been viewed as integral to the stability of nucleic acid secondary and tertiary structures. Thus, cations and solvent molecules can be considered as the fourth and fifth components of nucleic acid structure. We propose that it is important to consider how all five components might have changed over time, how their interdependence would have restricted some changes and promoted others, and how the original constitutional elements of nucleic acids (RU, TL, and IL), if different from today, could have made the emergence of proto-RNA a simple and robust outcome of the physical environments of the early Earth.

Who Paired before Watson and Crick?

In contemporary life, the RUs are the four distinct nucleobases of RNA/DNA. The use of four nucleobases provides sufficient information for the triplet genetic code to specify 20 amino acids and start/stop signals. This coding is also sufficiently degenerate for flexibility in codon usage, which provides advantages on multiple levels in biology. Using four RUs is also advantageous for functional RNAs. It is difficult to imagine that the wide range of RNA structures found in nature, such as those that make up the ribosome, could be obtained with only two distinct RUs or with only one class of RU (e.g., only purines or only pyrimidines). Additionally, the asymmetry of the purine-pyrimidine Watson-Crick base pairs increases diversity in the positioning of non-Watson-Crick H-bond donor and acceptor groups, which are recognized by sequence-specific DNA-binding proteins. Reflecting on just this last attribute, distinct base pair edges in the major and minor grooves of DNA/RNA might not have been advantageous and, therefore, not a selective pressure for nucleic acid structure until after the emergence of transcription factors. Overall, our current genetic system of two purine and two pyrimidine bases looks far superior to a genetic system comprised of only one base pair or only one class of heterocycles. However, during the earliest stages of life, it may have been advantageous, or even necessary, to use fewer than four or more than four RUs.

Orgel and Crick were among the first to suggest the possibility of a reduced set of RUs in primitive biological systems (Crick, 1968). They proposed that nucleic acids could have started with purine-purine base pairs, such as adenine paired with hypoxanthine (1 with 7; Figure 1B). Crick argued that adenine was likely abundant on the prebiotic Earth, given its ease of synthesis in model prebiotic reactions, and that hypoxanthine could be obtained from adenine by deamination. Thus, the reactions required to produce the heterocycles would, in principle, be simplified by a purine-only proto-RNA. The feasibility of this hypothesis has recently received support by the demonstration that duplexes with only purine-purine base pairs can be as stable as those with Watson-Crick base pairs (Groebke et al., 1998; Heuberger and Switzer, 2008).

In addition to potentially simplifying the requirements for synthesis, starting with only purine RUs could have facilitated formation of proto- and pre-RNA polymers. Numerous studies have demonstrated the greater propensity for activated purine mononucleotides to polymerize on single-stranded polypyrimidine templates compared to the polymerization of activated pyrimidine mononucleotides on polypurine templates (Stribling and Miller, 1991; van Vliet et al., 1995). This difference has been attributed to the more favorable stacking interactions of purine bases. The pyrimidine bases of RNA and DNA show little tendency to stack in aqueous solution (Ts'o, 1974). Recent experiments with oligonucleotides further illustrate the importance of stacking interactions for nonenzymatic polymerization. Specifically, tetranucleotides that form minihelices with purine-purine base pairs (8 with 9 and 3 with 10; Figure 1B) undergo nonenzymatic ligation with an efficiency that is over 100-fold greater than that of tetranucleotides that form minihelices with purine-pyrimidine base pairs (Kuruvilla et al., 2013).

The possibility that nucleic acids started with only pyrimidine bases or with another class of six-membered rings has also been considered (Schwartz, 1993; Siegel and Tor, 2005). Independently, it was shown that the conjugation of certain pyrimidines and triazenes to a peptide nucleic backbone creates oligonucleotides that form stable duplexes with pyrimidine-triazine or pyrimidine-pyrimidine base pairs (e.g., 11–14; Figure 1B; Mittapalli et al., 2007a, 2007b). Additionally, the difference in the pK_as of complementary pyrimidines and triazines with solvent pH was found to correlate with duplex stability, indicating a potential criterion of prebiotic RU selection (Zhang and Krishnamurthy, 2009). These observations illustrate the necessity for considering the compatibility of changes in the heterocycle and backbone structure as well as the physiochemical properties of these components (Zhang and Krishnamurthy, 2009) when attempting to reconstruct a lineage for RNA evolution.

If we consider the possibility that alternative RUs came before the current nucleobases, we must also consider the possibility that information was not always stored and transferred with base pairs (i.e., a dyad). Perhaps the original mode of RU association was a base tetrad or a base hexad. Although not previously considered in the context of proto- or pre-RNA, base hexads look very promising as an alternative to base pairs. It has been appreciated for some time that 2,4,6-triaminopyrimidine (TAP) and melamine (MA) (a triazine) can form extended sheet-like hydrogen-bonded structures with barbituric acid (BA) (a pyrimidine) or cyanuric acid (CA) (a triazine) (Lehn et al., 1990; Seto and Whitesides, 1990). These molecules can, in principle, form hexads, or "rosettes" (15 and 16 with 17 and 18; Figure 1B), but mixing TAP or MA with BA or CA in water results in the formation of an insoluble complex. Recently, it was demonstrated that modifying one exocyclic amine of TAP allows spontaneous assembly with CA in aqueous solution to form gene-length noncovalent polymers (Cafferty et al., 2013). It is well known that the current nucleobases, as free bases or nucleoside monomers, do not form Watson-Crick base pairs in aqueous solution. Thus, the observation of highly ordered assemblies by TAP and CA recognition units in water, with stacking and hydrogen-bonding interactions akin to those between nucleobases in folded RNA structures, provides a potential resolution to the long-standing problem of how the nucleobases were selected and organized for polymerization in the prepolymer stage of life. In particular, if the first RUs spontaneously organized in this manner, then a coupling chemistry that provided for the formation of covalent linkages between RUs in adjacent, stacked hexads (i.e., along the edges of the long axis of an assembly) would provide a pathway for the de novo synthesis of proto-RNA (see below). The possibility that pyrimidine/triazine hexads could have been the RU assemblies of proto-RNA and early pre-RNAs is also given support by the detection of MA, BA, and CA as products of a single-model prebiotic reaction where urea was the starting material (Menor-Salván et al., 2009).

Ribose: Not so Sweet in the RNA-First Story

There are ample reasons to question ribose as the TC of proto-RNA. Robust prebiotic routes for ribose synthesis have not been established. The formose reaction, long speculated as a possible source of prebiotic carbohydrates, converts formaldehyde (via glycoaldehyde) into a complex mixture of linear and

branched sugars, with ribose as a minor product (~1%) (Kim et al., 2011). The problem of low ribose production is exacerbated by instability of ribose compared to most other sugars (Larralde et al., 1995). Several scenarios have been put forth for increasing prebiotic ribose concentrations. Examples include using glycoaldehyde phosphate in the formose reaction instead of glycolaldehyde, a substitution that restricts reaction products to linear aldose phosphates (including ribose-2′-phosphate) (Müller et al., 1990); complexing ribose with borate, which alters product distribution in the formose reaction and slows ribose degradation (Benner et al., 2012); and crystallizing ribose from solution after derivatization with cyanamide (Springsteen and Joyce, 2004). The added complication of requiring such "fixes" to counter the problems of ribose synthesis and stability must be weighed against the possibility that ribose was not the original TC. Ribose-later scenarios are attractive, given the steadily increasing number of alternative TCs (e.g., 19–23; Figure 1B) that have been shown to replace ribose in the current nucleic acid backbone while retaining the capacity for duplex formation (Eschenmoser, 2007). Moreover, ribose appears optimal for its structural role in RNA (Eschenmoser, 2007), and therefore, ribose seems more likely a product of evolution as opposed to a "frozen accident" (i.e., a sugar that was initially incorporated because it was available at the time proto-RNA formed).

The questionable status of ribose as the original TC inspired some researchers to consider threose (20; Figure 1B), a four-carbon sugar, as a possible preribose TC. Threose is simpler to form by abiotic reactions, at least conceptually, and is attractive on structural grounds, since nucleic acids containing the threose sugar (TNA) form stable duplexes (Schöning et al., 2000). One should also consider the possibility that the TC of proto-RNA might not have been a cyclic sugar (Joyce et al., 1987). In support of this possibility, oligonucleotides containing a simple glycerol moiety in place of ribose (Meggers and Zhang, 2010) and, more recently, those containing a glyceric acid (21; Figure 1B) substitution have demonstrated that acyclic nucleic acids can form stable duplexes with RNA (Hernández-Rodríguez et al., 2011), a property often considered a prerequisite for any pre-RNA candidate.

The most radical proposal for a pre-RNA backbone is, arguably, the peptide nucleic acid (PNA) (Nielsen, 2007). In support of this proposal, albeit with a structure distinct from the classic PNA, glutamate and aspartate (22 and 23; Figure 1B) have been used to replace both ribose and phosphate to form a different type of peptide nucleic acid backbone (Mittapalli et al., 2007a, 2007b). Specifically, a peptide of repeating aspartic acid (or glutamic acid) residues can serve as a nucleic acid backbone with alternating residues that act as either an IL or a TC, with the latter moiety-possessing RUs conjugated to amino acid side chains. A particularly attractive feature of this polymer as a pre- or proto-RNA is that it requires only one molecular species to make the backbone.

Why Nature Probably Waited to Choose Phosphate

"Why Nature Chose Phosphates" by Westheimer provides an elegant analysis of the suitability of phosphate for its various roles in extant biology (Westheimer, 1987). The phosphate group is the IL of contemporary nucleic acids, providing solubility in water and resistance to spontaneous hydrolysis while being readily hydrolyzed by enzymes; nucleic acids utilizing this IL are thermodynamically unstable but kinetically trapped. The positive free energy of formation associated with the phosphodiester linkage in water necessitates the input of energy for polymerization, which is one reason to consider phosphate as a latter addition to nucleic acids. In model prebiotic reactions, chemists often activate phosphates of nucleotides by transient modification with high-energy chemical agents, such as carbodiimide- and cyanide-containing compounds. While such reagents are common and useful in synthetic organic chemistry, their prebiotic relevance is questionable. Furthermore, the rate of hydrolysis of an activated phosphate group is comparable to the rate of uncatalyzed phosphodiester bond formation, which can result in inefficient polymerization. It is reasonable to propose that pre-RNAs would have benefitted from backbone linkages that were thermodynamically favored (in at least some accessible environment) with low kinetic barriers to hydrolysis—properties that would facilitate activation-free polymerization and would allow environmental switching between conditions that alternately promote polymerization and hydrolysis. Facile thermodynamically driven switching of this type would promote rapid recycling of materials between polymers and monomers, a property that could have greatly accelerated sequence evolution and emergence of enzymatic activity (Walker et al., 2012). Furthermore, low-energy, reversible backbone linkages allow for selection of thermodynamically favored products, thereby providing an enzyme-free mechanism for accurate templating and error correction (Hud et al., 2007; Li et al., 2002).

The limited availability of phosphate on the prebiotic Earth is another reason to consider alternative ILs for pre-RNAs. Specifically, phosphate would have been sequestered in minerals on the early Earth due to its limited solubility in the presence of divalent metal ions (Keefe and Miller, 1995). Recent investigations of phosphorylation by phosphite (HPO_3), a less oxidized form of phosphorus, and the presence of phosphite in some meteorites suggests that phosphate could have initially entered life through a more soluble form (Pasek, 2008). Nevertheless, the pre-enzymatic incorporation of phosphate would have presented problems for monomer recycling in pre-RNA, which was always necessary, due to the limits of finite resources (Engelhart and Hud, 2010; Walker et al., 2012).

In considering possible ancestral ILs, electrostatic charge seems indispensable, as it greatly enhances polymer solubility in water and is arguably essential for nucleic acids to function as a genetic material (Benner et al., 2004). As mentioned above, peptide nucleic acids with ionized residues (glutamate or aspartate) at every other position along the polymer backbone look quite promising as early pre-RNA polymers. Plausible prebiotic ILs that are closer in size and structure to phosphate include glyoxylate (24; Figure 1B). Glyoxylate has been shown to form an acetal linkage between two OH groups of two free nucleosides in a drying-heating reaction (Bean et al., 2006). Glyoxylate is also attractive as a possible pre-RNA IL, because it is used in life today and is a product of model prebiotic reactions (Weber, 2001).

Connecting the Pieces to Make Proto-RNA

In addition to deciphering the building blocks of proto-RNA, another grand challenge for prebiotic chemists is determining

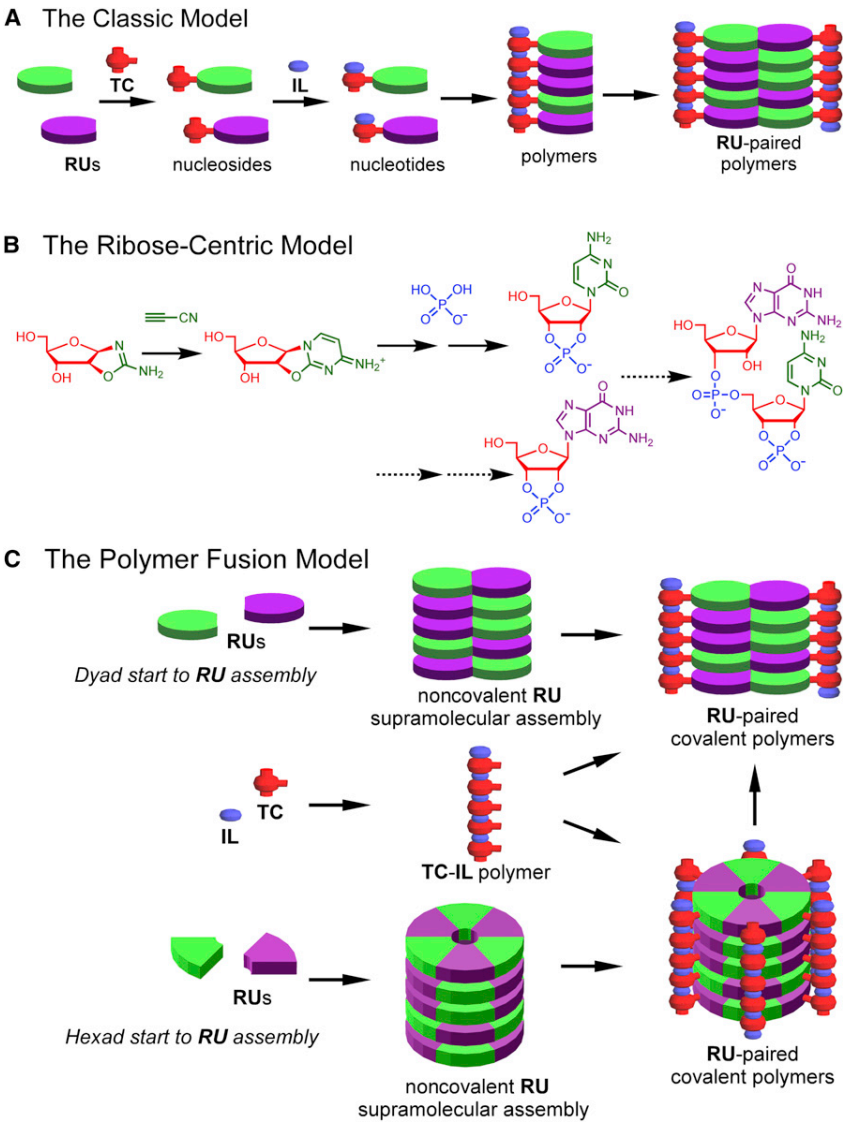

Figure 2. Three Models for the Prebiotic Assembly of the First Informational Polymers

(A) The classic model. The recognition unit (RU), trifunctional connector (TC), and ionic linker (IL) assemble sequentially to produce nucleotides (or protonucleotides) before becoming polymerized to form RNA (or proto-RNA) polymers. Base pairing is not expected until polymers of a critical length are synthesized.

(B) The ribose-centric model. The cytosine base is built on a pre-existing sugar. Like the classic model, nucleosides are formed before being coupled into polymers and before base pairing. Unlike the classic model, the chemistry of the ribose-centric model is dependent on the exact structures used in the assembly pathway and, therefore, implies that RNA has not evolved from an earlier polymer.

(C) The polymer fusion model. Recognition units (RUs) form supramolecular assemblies that involve pairings, either as dyads or hexads, that are the same as those that will hold strands together in the informational polymers. Trifunctional connector (TCs) and ionized linkers (ILs) form covalent polymers, among the many other polymers that exist in the prebiotic chemical inventory. The match in the spacing of functional groups of the TCs in the TC-IL polymers with the RUs in their supramolecular assembly promotes the fusion of these polymers through the covalent linking of TCs and RUs. Note that only in this model is there a mechanism that guarantees that the RUs incorporated into polymers will be able to actually act as recognition units through their ability to form pairing structures prior to being linked by a backbone.

how these building blocks became joined into protonucleotides, oligomers, and polymers. In water, polymeric RNA is an unstable, far-from-equilibrium polymer; hydrolysis of phosphodiester and glycosidic bonds is thermodynamically favored over bond formation. The simplest way to favor the formation of these bonds and to drive oligomerization and polymerization is to remove water from the vicinity of nucleic acid building blocks. A long-speculated scenario for promoting prebiotic polymer formation is the "drying pool" or "drying lagoon", in which regular cycles of dehydration-rehydration coincided with day-night, tidal, or seasonal environmental cycles. In what we refer to as the classic model, pre-existing nucleobases and ribose are coupled to give nucleosides, which are then phosphorylated to give nucleotides. The phosphorylated nucleosides are then joined to give oligonucleotides (Figure 2A). Within this framework, Orgel and coworkers explored the potential for glycosidic bond formation between the current nucleobases and ribose by drying and heating with general acid catalysts. Despite considerable effort, only adenine was found to couple with ribose in appreciable yield (~2%), forming adenosine (β-furanosyl nucleoside) (Fuller et al., 1972). The low solubility of guanine proved to be a severe limitation to guanosine nucleoside formation. Attempts to form pyrimidine nucleosides with uracil and cytosine in heating-drying reactions were unsuccessful.

Difficulties encountered in early attempts at abiotic glycosidic bond formation gave rise to the term "the nucleoside problem". Orgel sought to circumvent this problem by "building" the cytosine base on a pre-existing sugar. Specifically, cyanamide will react with D-ribose-5-phosphate, D-ribose, and D-arabinose to give isomers of cytosine that can be converted (anaomerized or epimerized) to cytosine by irradiation with UV light (Sanchez and Orgel, 1970). Following this lead, Sutherland and coworkers subsequently presented a complete abiotic synthesis of cytosine starting with simpler precursor molecules (Powner et al., 2009). We refer to this approach to prebiotic RNA formation as the ribose-centric model, as the chemistry of this model is inseparable from the structure of ribose (Figure 2B). Implicit in the ribose-centric model is the assumption that RNA is not a product of evolution. As detailed by Sutherland and coworkers, abiotic nucleotide synthesis presents nine formidable chemical challenges (Powner et al., 2011), some of which have been

introduced above (e.g., selection of a specific sugar, specific glycosylation site, regiospecific phosphorylation, etc.). While Sutherland's synthetic protocol for abiotic cytidine synthesis is elegant as a synthetic organic achievement, its prebiotic legitimacy has been questioned (Benner et al., 2012; Eschenmoser, 2011). For example, the synthesis involves the sequential addition of two reactive carbohydrates: glycolaldehyde and glyceraldehyde. The synthesis fails if these precursor molecules are added at the same time or in a different order. Large quantities of each molecule are required, and since both would be present through known reaction schemes, it is challenging to rationalize how high-yielding separation and ordered timely addition could be realized (i.e., through geophysical means).

As with other problems that face prebiotic RNA formation, nucleoside formation is not such a problem if we accept that proto-RNA started with different RUs and a different TC. The potential merits of alternative RUs in addressing the nucleoside problem was demonstrated by Miller and coworkers, who reported that urazole (a five-membered triazine ring that resembles uracil) can form nucleosides in excellent yield when dried and heated with ribose or other sugars (Dworkin and Miller, 2000; Kolb et al., 1994). Subsequently, 2-pyrimidinone (which only differs from uracil and cytosine by having a hydrogen atom at the C4 of the pyrimidine ring) was also shown to form the β-furanosyl nucleoside with ribose in excellent yield under the same conditions used by Orgel for adenosine formation (Bean et al., 2007). It should be noted that nucleosides formed by urazole and 2-pyrimidinone are more labile than the nucleosides of contemporary RNA. Consistent with this trend that heterocycles, which easily form nucleosides, also produce more labile nucleosides, it has been recently recognized that modifications to the current nucleobases generally result in decreased stability of the glycosidic bond, an observation that has been suggested as evidence that the current nucleobases were refined by evolution to have increased stability against deglycosylation (Rios and Tor, 2012). Given the large number of heterocycles closely related to the current nucleobases as well as possible alternatives to ribose, we no longer see prebiotic nucleoside formation as an insurmountable challenge but as a potential test of plausible RUs and TCs as building blocks for protonucleosides.

Supramolecular Assemblies: Facilitating the Formation of Proto-RNA

The nucleobases found in life today and their corresponding free nucleosides/nucleotides do not self-assemble through Watson-Crick hydrogen bonding in aqueous solvents (Ts'o, 1974). This inability to pair at the monomer level presents a logical conundrum for the selection of functional (i.e., base-pairing) RUs. If molecules coupled at the positions occupied by RUs in proto- and pre-RNA polymers were not selected for their pairing capacity before being incorporated into these polymers, then these polymers would have been riddled with monomeric units that could not function in base pairing. It is expected that the prebiotic pool of molecules would have contained many molecules similar in structure and reactivity to the original building blocks of proto-RNA, but most of these could not have functioned as RUs. This conundrum, termed "the paradox of base pairing" (Engelhart and Hud, 2010), previously inspired Hud and Anet to propose that the original RUs formed supramolecular assemblies that selected and organized RUs into stacked arrays before they were connected by a common backbone to form proto-RNA (Hud and Anet, 2000). This proposal does not define the prebiotic process by which the first nucleosides or nucleic acid polymers were formed, only that proto-RNA polymer formation would have been restricted to RUs that can form supramolecular assemblies that include base pairing (Figure 2C), thereby ensuring that only pairing, stacking molecules were incorporated as RUs.

As mentioned above, two pyrimidine and triazine nucleobase analogs have recently been shown to self-assemble in water through Watson-Crick-like pairing and stacking interactions to form long supramolecular polymers (Cafferty et al., 2013), demonstrating that some potentially prebiotic RUs possess an intrinsic mechanism for their mutual selection. Additionally, experimental studies have demonstrated the ability for nucleic acid intercalators (acting as "midwife" molecules) to preorganize short oligonucleotides and thereby promote nonenzymatic polymerization (Horowitz et al., 2010; Jain et al., 2004). Encouraged by the benefits of supramolecular self-assembly to enable RU selection and to promote RU preorganization to facilitate nonenzymatic polymerization, we present a third model for the formation of proto-RNA as an alternative to the classic model and the ribose-centric model. In what we call the polymer fusion model, we propose that proto-RNA was the result of a covalent merger of two distinct polymers. One of these polymers would have been a supramolecular assembly of RUs, or a noncovalent polymer, (i.e., stacks of RUs or RUs interspaced with intercalators). These assemblies could have coexisted with covalent prebiotic polymers, such as polypeptides, polysaccharides, and polyesters. We propose that the spacing of RUs within their supramolecular assemblies matched the spacing of functional groups on one or more species of covalent polymer in the prebiotic polymer pool. The reaction of TC functional groups along covalent polymers with RUs in supramolecular assemblies would have created proto-RNA (Figure 2C). An advantage of this polymer fusion model over the classic model and the ribose-centric model is that functionality is operative for the selection of RU, TC, and IL units from the first appearance of proto-RNA polymers. That is, only RUs that form specific base pairs would be incorporated into the RU noncovalent polymers, only TC and IL species that can react to form a stable, soluble polymer would be candidates for the proto-RNA backbone, and finally, only TC-IL polymers with functional groups matching the spacing of RUs along the long axis of the RU assemblies would be able to merge to form proto-RNA.

The formation of RNA-like polymers by the fusion of two polymers has yet to be demonstrated. However, experiments by Ghadiri and coworkers with a peptide composed of a glutamate-cysteine repeating sequence and thioester-modified nucleobases illustrate some of the promising characteristics of informational polymers built on preformed backbones (Ura et al., 2009). In this system, the RUs spontaneously attach to cysteine residues of the peptide through transthioesterification, resulting in what they called tPNA. Upon addition of a DNA oligonucleotide to a solution containing tPNA, it was found that the thioester-linked nucleobases rearranged to form a sequence that is the Watson-Crick complement of the DNA strand, thereby forming a complementary tPNA-DNA duplex. This observation

illustrates how a kinetically labile informational polymer (under thermodynamic control) can adapt to its environment by changing the sequence of its recognition elements. Such a property could be seen as a rudimentary form of rapid and noncatalytic sequence evolution, which, as mentioned above, would have been advantageous for early pre-RNAs.

Did Proto-RNA Arise in a Nonaqueous Solvent?

Returning to the challenge of creating reversible chemical bonds that are thermodynamically unstable in water (e.g., the glycosidic bond), a significant limitation of hydration-dehydration reactions as the prebiotic driving force of nucleic acid formation is that many molecules (including nucleic acid building blocks) precipitate from solution before water activity is low enough to favor condensation-dehydration bond formation. In a solvent-free state, bond formation is kinetically impaired, due to limited molecular motions. Once again, we must consider that other molecules, not necessarily present in the environment today, might have played an important role in the origin of nucleic acids. There are increasing discussions about whether life could initiate in nonaqueous solvents, including formamide and even liquid ammonia (Benner et al., 2004; Saladino et al., 2007). Such ideas once seemed highly speculative, but the burgeoning field of ionic liquids and deep eutectic solvents (DES) continues to demonstrate the advantages of polar organic solvents. There are now some formulations for these bi- and tricomponent solvents that merit consideration as a possible milieu for early life, particularly those that are composed of molecules that have high solubility in water. Specifically, if such molecules were present in aqueous pools on the prebiotic Earth, regular evaporation of water would have left behind a nonaqueous liquid, rather than only dry precipitate. Some ionic liquids and DES are excellent solvents for uncharged molecules and salts. Thus, the removal of water by evaporation could produce a concentrated pool of biological building blocks and salts. Such conditions would be ideal for a hydration-dehydration cycle to drive polymerization, as water activity would be low, but molecular motions would not be hindered.

Thus far, formamide has received much attention as a prebiotic solvent due to the formation of nucleobases from this reagent upon heating with mineral catalysts or exposure to UV light (Barks et al., 2010; Saladino et al., 2007). Formamide is also intriguing because nucleosides have been shown to become phosphorylated when heated in formamide with KH_2PO_4 (Schoffstall and Laing, 1985). However, the same study did not detect phosphodiester bond formation. Furthermore, formamide destabilizes nucleic acid base pairs. If prebiotic polymerization took place in an alternative solvent, then we might also expect this solvent to support base pairing so that template-directed synthesis would have been possible in the solvent. An example of one such candidate solvent is the DES formed by one part choline chloride and two parts urea. This DES has been shown to support several nucleic acid-folded structures, with some structures being even more stable in this solvent than in water (Mamajanov et al., 2010).

Fe^{2+} as a Possible RNA Cofactor before Mg^{2+}

As noted above, nucleic acid folding and function requires cations (Bowman et al., 2012). Divalent metals are especially important for complex folds and catalytic activity. While we can only speculate at this point about possible alternative solvents, one aspect of the prebiotic milieu is clear: the soluble metal ions of the prebiotic Earth were dramatically different from those of today (Hazen et al., 2008). Before life invented photosynthesis, O_2 levels in the atmosphere were extremely low (Anbar, 2008). The rise of molecular oxygen coincided with the precipitation of Fe^{2+} into the banded iron formations that are found across the Earth (Klein, 2005). In our current O_2-rich environment, free Fe^{2+} is toxic to living cells due to hydroxyl radical chemistry (Prousek, 2007). However, before the rise of O_2, Fe^{2+} would have been a powerful cofactor of RNA and pre-RNAs. Many extant RNA molecules, including the ribosome, depend upon Mg^{2+} for folding and catalytic activity. The coordination chemistry of Fe^{2+} and Mg^{2+} are sufficiently close that iron and magnesium form near-ideal solid solutions in some minerals (Birle et al., 1968). Fe^{2+} has recently been shown to substitute for Mg^{2+} for the folding and catalysis of some ribozymes (Athavale et al., 2012). In fact, catalytic activity can be enhanced with Fe^{2+}, even for ribozymes selected in the presence of Mg^{2+}. The rich redox chemistry possible with Fe^{2+}/Fe^{3+} that is used extensively in extant life could have been provided by Fe-ribozymes before the rise of free O_2. In support of this hypothesis, it has recently been observed that RNA can catalyze an electron transfer reaction when provided Fe^{2+} (C. Hsiao, I.-C. Chou, C.D. Okafor, J.C. Bowman, E.B. O'Neill, S.S. Athavale, A.S. Petrov, N.V.H., R.M. Wartell, S.C. Harvey, et al., unpublished data). If pre-RNA/RNA molecules once made use of iron for redox chemistry, then the chemistry, carried out today by proteins, could have been accomplished by nucleic acids to a larger extent than previously imagined.

Conclusions

The possibility that RNA is a product of evolution was once considered a "gloomy prospect" (Orgel, 1998), but identifying candidates of pre- and proto-RNA now seems less intractable and less daunting through a combination of empiricism and a clear focus on discovering robust, prebiotically relevant pathways to RNA-like polymers. We recognize that there are still many challenges to address before we can construct a complete and chemically sound evolutionary framework from proto-RNA to RNA. As with the metaphorical grandfather's axe, we imagine that RNA evolution occurred in stages, with different chemical pieces being substituted multiple times and for different reasons. Some steps are easy to imagine and would have coincided with changes in the environment (e.g., the substitution of Mg^{2+} for Fe^{2+}). Changes in pre-RNA covalent structure could have coincided with altered or increased functions of nucleic acids. Chemical differences between the extant nucleic acids provide insight into how and why such transitions between genetic molecules would occur. In particular, the 2′-OH of ribose is important for RNA structure and catalysis, but this chemical group drastically enhances RNA susceptibility to strand cleavage. Removal of the 2′-OH, to create DNA, provides an obvious advantage for supporting a more stable genome. Likewise, substituting T for U in DNA provides a mechanism to prevent mutations by the spontaneous conversion of C to U by deamination. With this in mind, most would agree that nucleic acid optimization must have occurred between the emergence of RNA

and the biological invention of DNA. Given all the arguments presented above for the optimal chemical structures of RNA and the dearth of evidence in support of a plausible abiotic origin for RNA, we are compelled to conclude that nucleic acid evolution must have also occurred in the more distant past.

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