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Perspective

The Emergence of RNA from the Heterogeneous Products of Prebiotic Nucleotide Synthesis

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ABSTRACT: Recent advances in prebiotic chemistry are beginning to outline plausible pathways for the synthesis of the canonical ribonucleotides and their assembly into oligoribonucleotides. However, these reaction pathways suggest that many noncanonical nucleotides are likely to have been generated alongside the standard ribonucleotides. Thus, the oligomerization of prebiotically synthesized nucleotides is likely to have led to a highly heterogeneous collection of oligonucleotides comprised of a wide range of types of nucleotides connected by a variety of backbone linkages. How then did relatively homogeneous RNA emerge from this primordial heterogeneity? Here we focus on nonenzymatic template-directed primer extension as a process that would have strongly enriched for homogeneous RNA over the course of multiple cycles of replication. We review the effects on copying the kinetics of nucleotides with altered nucleobase and sugar moieties, when they are present as activated monomers and when they are incorporated into primer and template oligonucleotides. We also discuss three variations in backbone connectivity, all of which are nonheritable and regenerate native RNA upon being copied. The kinetic superiority of RNA synthesis suggests that nonenzymatic copying served as a chemical selection mechanism that allowed relatively homogeneous RNA to emerge from a complex mixture of prebiotically synthesized nucleotides and oligonucleotides.

1. INTRODUCTION

Given the strong support for an RNA-dominated early stage in the evolution of life, the main problem in understanding the origin of life lies in tracing a plausible pathway from prebiotic chemistry to the RNA world.[1−4] A great deal of progress has been made in the past decade in understanding potentially prebiotic pathways to the canonical ribonucleotides A, G, C, and U.[2,5−7] At a very general level, all such proposals begin with simple feedstocks such as cyanide, cyanamide, formaldehyde, and phosphate. These react under the influence of geochemical constraints to form intermediates such as simple sugars and heterocycles and then depending on the proposed pathway progressively more complex intermediates such as ribose and nucleobases or aminooxazolines and anhydronucleosides, ultimately yielding ribonucleotides accompanied by a range of related types of nucleotides. Finally, phosphate activation allows for the assembly of a highly heterogeneous collection of primordial oligonucleotides (Figure 1A). The nonheritable variation in such oligonucleotides might be expected to interfere with both replication and the reproducible folding of functional sequences such as ribozymes. The question we focus on in this Perspective is how relatively homogeneous RNA oligonucleotides, suitable for the Darwinian evolution of useful functions, could have arisen from a primordial mixture of heterogeneous oligonucleotides.

Nonenzymatic template copying by primer extension was originally considered primarily as a means for the transmission of genetic information from generation to generation of replicating protocells before the advent of highly elaborated ribozyme ligases and RNA polymerases.[8] Much of the early work on nonenzymatic copying chemistry, including the development of imidazolide-activated nucleotides as suitable substrates, was carried out by Orgel and his colleagues from the late 1960s to the early 2000s.[9−11] Recent discoveries suggest that template-copying chemistry could also have played an important role in the emergence of RNA from a mixture of prebiotically synthesized nucleotides. In this model, differences in the reactivity of different types of nucleotides provide a path from primordial heterogeneous genetic polymers to largely homogeneous ribonucleotide based genetic polymers: i.e., RNA (Figure 1B). Here we will review the ways in which noncanonical nucleotides affect the template-directed synthesis of nucleic acids. In all cases examined, the canonical ribose sugar and 3′-5′-phosphodiester backbone chemistry is kinetically favored over the alternatives, suggesting that the standard RNA structure and chemistry would outcompete variant nucleic acids, resulting in the emergence of homogeneous RNA. However, in a few cases, potentially prebiotic variations in nucleobases lead to faster and more accurate template copying, suggesting that primordial RNA may have differed slightly from modern RNA in its nucleobase components.

Before considering alternatives to ribonucleotides and RNA, we briefly review a specific model for ribonucleotide synthesis

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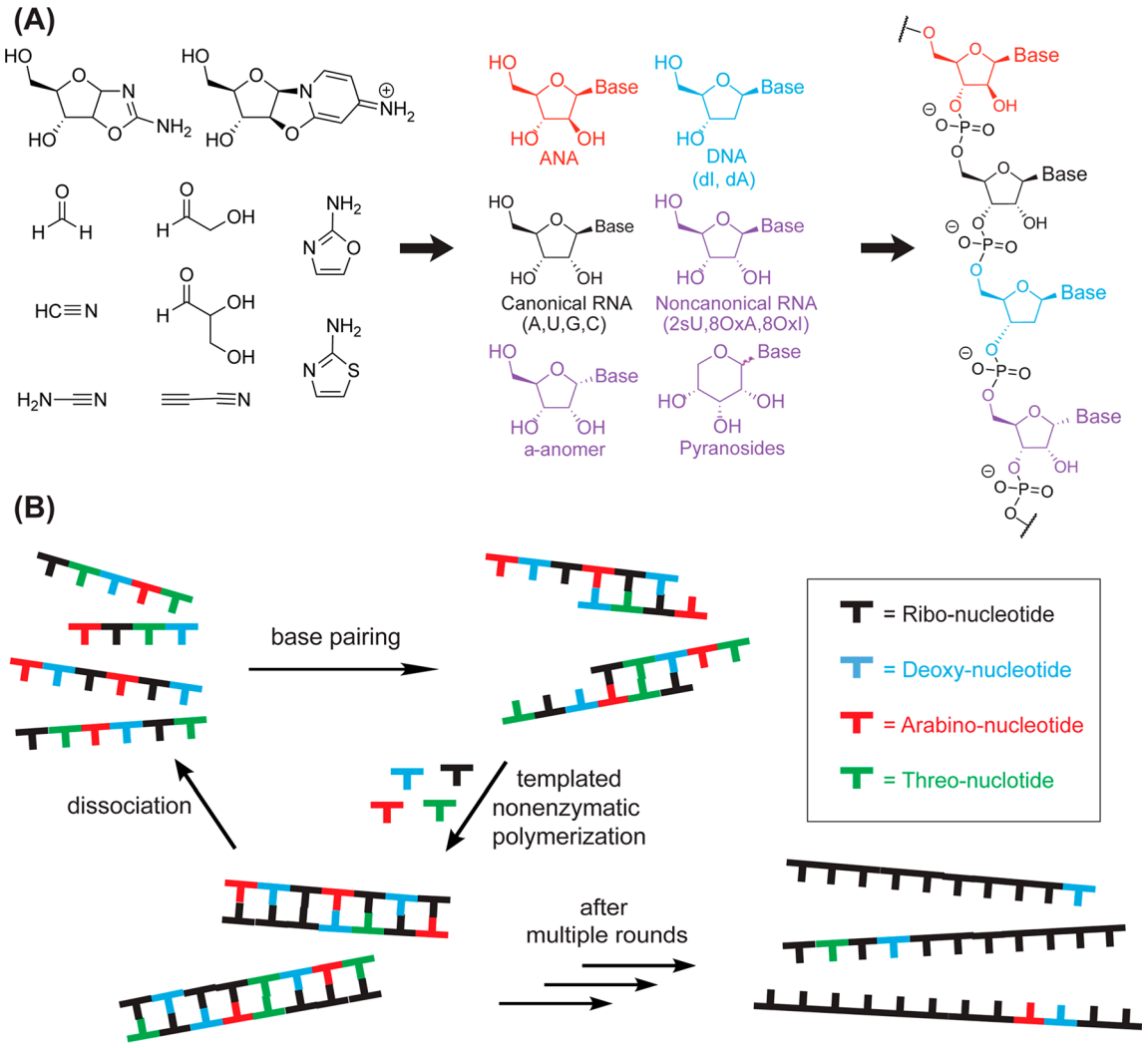

Figure 1. Synthesis of heterogeneous oligonucleotides from prebiotically synthesized mixtures of nucleotides, and the emergence of increasingly homogeneous RNA after cycles of replication. (A) Small-molecule feedstocks react to form increasingly complex intermediates and eventually a wide range of different types of nucleotides, which then assemble into highly heterogeneous oligonucleotides. (B) Cycles of template-directed primer extension and ligation enrichment for ribonucleotides in the resulting oligonucleotides.

and for template-directed primer extension in an all-RNA system (Figure 2). One proposal for the prebiotic synthesis of the pyrimidine ribonucleotides (Figure 2A) involves the condensation of cyanamide and glycolaldehyde to generate 2-aminooxazole (2AO), followed by a reaction with glyceraldehyde to generate ribose aminooxazoline (RAO),[12] which crystallizes from solution.[13] Three subsequent steps generate the desired β-anomer of cytidine, which can deaminate to provide uridine. Subsequent 5′-phosphorylation and activation with 2-aminoimidazole (2AI) generates activated monomers of the type that we have studied extensively as substrates for template-directed primer extension. Many questions remain concerning this pathway, especially the origin of homochirality. Early work by Joyce et al.[14] showed that template copying was severely inhibited by the presence of activated nucleotides of the opposite chirality, suggesting that homochirality may have emerged at some step on the path to nucleotide synthesis. Indeed, enantiopure RAO can be generated by crystallization of scalemic RAO,[15] and similarly, enantiopure nucleotides can be generated from scalemic mixtures by crystallization.[16] However, the origin of homochirality is a vast field that has been extensively reviewed[17,18] and will not be further considered in this Perspective.

2AI-activated ribonucleotides provide a useful and prebiotically realistic model for the study of template-copying reactions.[19,20] The most efficient pathway for template-directed primer extension begins with the reaction of two 2AI-activated monomers to generate a 5′,5′-imidazolium bridged dinucleotide,[24] which can bind to the template adjacent to the primer, via two Watson−Crick base pairs (Figure 2B−D).[28]

Alternatively, an activated monomer can form an imidazolium bridge with an activated oligonucleotide and then react with the primer.[29] Direct ligation of an activated oligonucleotide to the primer is also possible, although this is much slower.[27,30]

Noncanonical nucleotides affect the rate of template-directed nonenzymatic copying by primer extension in different ways, depending on whether the variant nucleotide is one of the incoming nucleotides, is located at the end of the primer, or is in the template. We will discuss these effects of variant nucleotides, first considering variations in the sugar moiety, followed by nucleobase variations, and concluding with a discussion of the effects of noncanonical backbone linkages.

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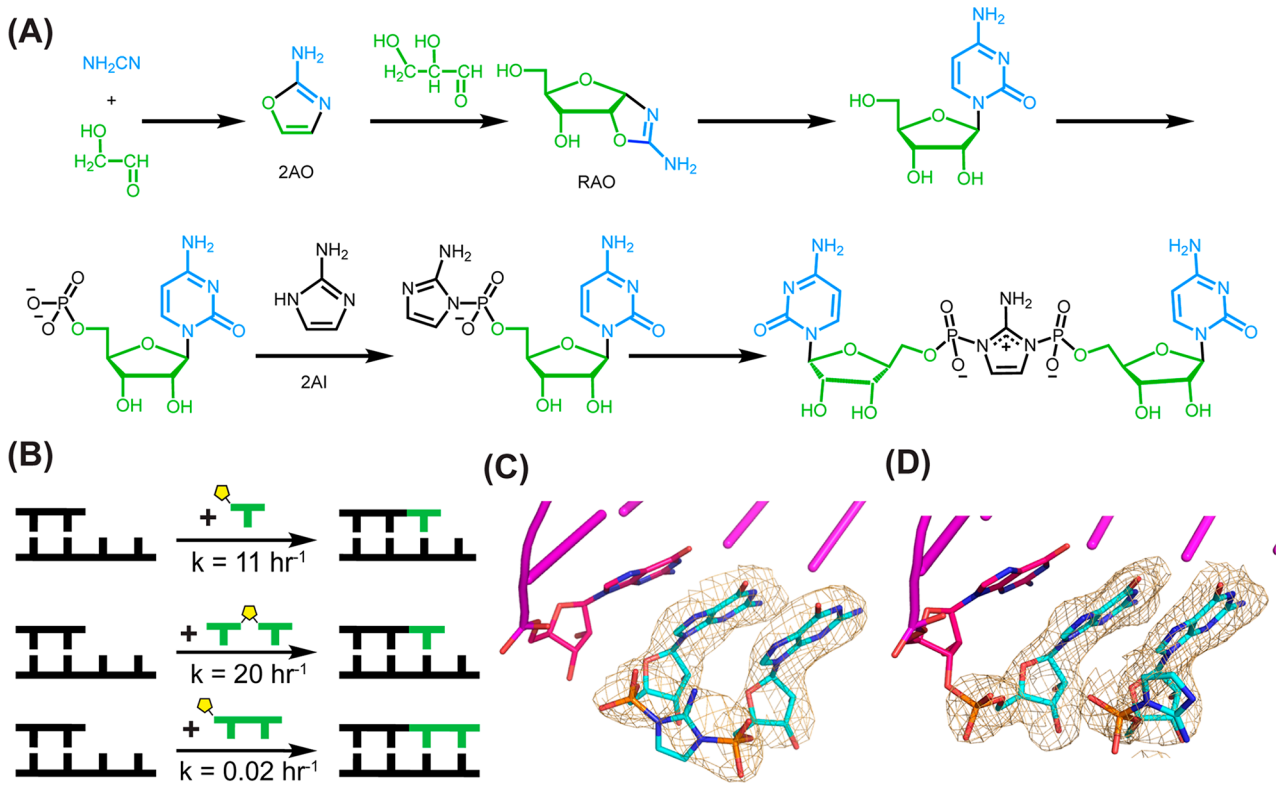

Figure 2. Ribonucleotide synthesis and RNA template copying. (A) Outline of ribonucleotide synthesis via the intermediates 2-aminooxazole (2AO) and ribose aminooxazoline (RAO)[12,55] followed by activation via the isocyanide pathway[21,22] to form an imidazolide. Two activated monomers react with each other to generate an imidazolium-bridged dinucleotide.[23,24] (B) Three aspects of template-directed primer extension that contribute to enrichment for RNA: (top) primer extension in the presence of a 2AI-activated monomer is rapid but occurs via an imidazolium-bridged dinucleotide covalent intermediate; (middle) primer extension with purified imidazolium-bridged dinucleotide is even faster; (bottom) primer ligation with an activated oligonucleotide is comparatively slow (k = 11, 20, and 0.02 h^{-1} for 2-aminoimidazole (2AI)-activated cytidine monomer, 2-aminoimidazolium-bridged cytidine dinucleotide, and 2AI-activated CUGA tetramer, respectively).[25−27] (C) Crystal structure of a partial primer/template duplex, showing a Gp*pG imidazolium-bridged dinucleotide bound to a CC template overhang, adjacent to the 3′-OH at the end of the primer (PDB: 3C8K). (D) Crystal structure of the product of the reaction of the primer and imidazolium-bridged dinucleotide from (C), showing the new phosphodiester bond linking the last base of the primer with the adjacent incoming G residue (PDB: 3C8O). (C) and (D) are reproduced with permission from ref 28. Copyright 2018 eLife Sciences Publications Ltd.

2. SUGAR HETEROGENEITY

Recent investigations of potentially prebiotic pathways leading to the synthesis of the canonical ribonucleotides have revealed that a number of noncanonical nucleotides with alternative sugar constituents are likely to have accompanied ribonucleotide synthesis. These variant sugars include multiple stereoisomers (e.g., L-ribose, arabinose, α-anomeric ribose), constitutional isomers (e.g., pyranosyl-ribose), as well as sugars with different numbers of carbon atoms (e.g., threose) and different degrees of oxygenation (e.g., 2′-deoxyribose). Here, we review three such alternative sugar nucleotides whose behaviors in nonenzymatic copying have recently been examined: arabino-nucleotides, threo-nucleotides, and 2′-deoxyribonucleotides.

2.1. Arabinonucleic Acid (ANA). Arabinose is the 2′-epimer of ribose (Figure 3A), and arabino-nucleotides are the building blocks of arabino-nucleic acid (ANA). ANA oligomers can form a moderately stable duplex with a complementary RNA strand, but not with a complementary ANA strand.[31,32] This property renders ANA self-replication implausible but opens the question of whether mixed RNA/ANA oligonucleotides could replicate. A pathway for the synthesis of β-arabino-cytidine from arabinose was first proposed by Orgel et al. in 1970.[33] Almost 20 years later, the Sutherland group devised a more plausible pathway, beginning with an efficient synthesis of the key intermediate 2-aminooxazole (2AO). The reaction of glyceraldehyde with 2AO generates two stereoisomers, ribose aminooxazoline (RAO), which is a precursor of α-ribo-cytidine, and arabinose aminooxazoline (AAO), a precursor of β-arabino-cytidine (this route to ara-C is outlined in Figure 3A),[9,12] suggesting that arabino- and ribo-nucleotides may have coexisted on the early earth. Recently, the Powner group has demonstrated a potentially prebiotic synthesis of all four arabino-nucleosides that diverges from the common synthetic intermediate oxazolidinone-2-thione.[34]

Given the potential for multiple routes for the prebiotic synthesis of arabino-nucleotides, we investigated the kinetics of nonenzymatic primer extension on an RNA template (Figure 3B) with the purified imidazolium-bridged ara-A dinucleotide, which we found to be ∼100-fold slower than that with the imidazolium-bridged ribo-A dinucleotide.[35] In solution, the formation of the imidazolium-bridged dinucleotide intermediate occurs at roughly the same rate for both ribo- and arabino-nucleotides. In a competition experiment with equal concentrations of ribo- and arabino-mononucleotides, only primer extension with ribonucleotides was detectable (Figure 3C). However, activated RNA downstream helper oligonucleotides facilitate the incorporation of activated arabino-monomers (Figure 3B), suggesting that, in a more complex

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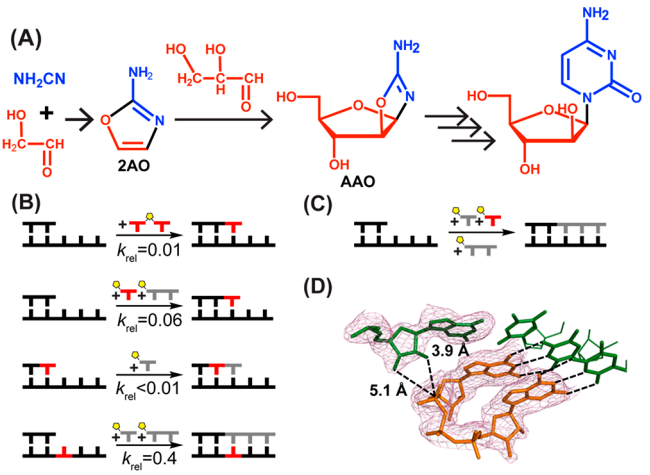

Figure 3. Relative rates of template directed primer extension with ribo- vs arabino-nucleotides in different contexts. (A) Outline of a potential pathway to arabino-nucleotides, via the intermediates 2-aminooxazole (2AO) and arabino-aminooxazoline (AAO). (B) Schematic representation of primer extension reactions: (top) all-RNA primer/template complex (black) reacts very slowly with incoming arabino-nucleotides (red) shown as imidazolium-bridged dinucleotides; (second from top) primer extension with an incoming arabino-nucleotide (red) is facilitated by the presence of a downstream helper oligoribonucleotide (gray); (third from top) a primer ending in an arabino-nucleotide (red) is extended very slowly by reaction with an incoming ribonucleotide (gray); (bottom) primer extension across an arabino-nucleotide in the template is only slightly inhibited. (C) Schematic representation of competition between arabino- and ribo-nucleotides, showing preferential incorporation of ribo-nucleotides. (D) Crystal structure of a primer ending in an ara-G next to the intermediate analogue GpppG, showing the much greater O3′−P distance in comparison to that for the corresponding all-RNA structure (PDB: 6OWL). (D) is reproduced with permission from ref 35. Copyright 2020 American Chemical Society.

scenario where activated monomers and oligomers are present, some primer extension with arabino-nucleotides could take place. To our surprise, we found that once an arabino-nucleotide is incorporated, the extended primer is virtually incapable of continued elongation, indicating that arabino-nucleotides act as chain terminators. This observation is at least partially explained by our crystallographic study showing that the O3′−P distance is much greater for an arabino-terminated than for a ribo-terminated primer (Figure 3D), as a consequence of the 2′-*endo* conformation of the arabinose sugar vs the 3′-*endo* conformation of ribose.[28,35] Thus, 2AI-activated arabino-nucleotides cannot participate in productive nonenzymatic copying by RNA-templated primer extension, although other as yet unknown activation chemistries or reaction conditions could in principle circumvent this limitation. The decreased incorporation rates and the results of competition experiments suggest that arabino-nucleotides, even if they are produced alongside ribonucleotides, would to a large extent be innocent bystanders to RNA copying chemistry, neither helping nor hurting RNA primer extension. Even the occasionally generated oligonucleotides ending with a 3′-terminal arabino-nucleotide could serve a number of auxiliary catalytic roles in RNA replication, including functioning as downstream helpers,[36] as invaders for strand displacement synthesis,[37] or as templates for primer extension or splinted ligations.[27] Interestingly, RNA strands containing an internal arabino-nucleotide, which might be formed by nontemplated polymerization, are good templates for RNA polymerization. Thus, primer extension on chimeric RNA-ANA strands would generate predominantly RNA products, suggesting that multiple rounds of copying chemistry would gradually dilute out the arabino-nucleotides and generate a population of oligonucleotides substantially enriched in RNA.

2.2. Threo-Nucleic Acid (TNA). Replacement of the ribose sugar of RNA with the four-carbon sugar threose generates threose nucleic acid (TNA). TNA oligomers were first synthesized by Eschenmoser, who showed that, despite a backbone repeat that is one atom shorter than that of RNA, TNA forms a stable Watson−Crick base-paired antiparallel duplex with itself, RNA, and DNA.[38] Furthermore, TNA oligonucleotides can act as aptamers,[39] suggesting the potential for functionality in protocells. TNA has been considered as a plausible RNA progenitor[38,40] because the simpler chemical structure of threo-nucleotides (Figure 4A) suggests a prebiotically plausible synthesis[41] from two sequential reactions of glycolaldehyde[42] as opposed to the sequential reaction of glycolaldehyde and glyceraldehyde required for ribose synthesis.

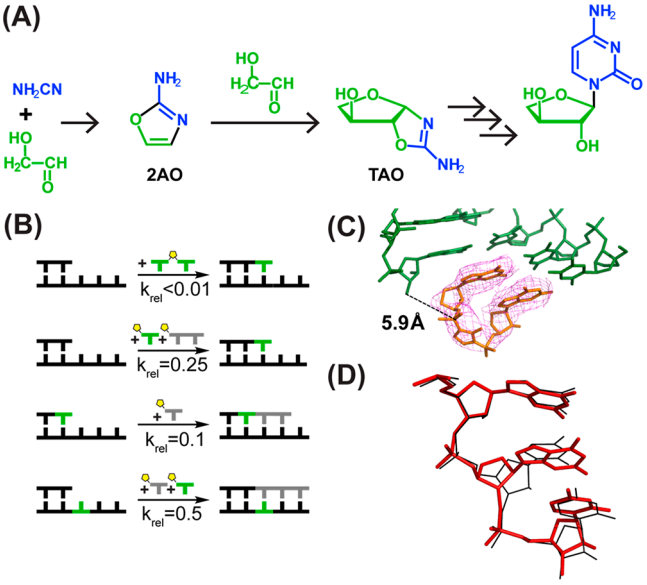

Figure 4. Relative rates of template-directed primer extension with threo- vs ribo-nucleotides in different contexts. (A) Outline of a potential pathway to threo-cytidine, via the intermediates 2-aminooxazole (2AO) and threose-aminooxazoline (TAO). (B) Schematic representation of primer extension reactions: (top) all-RNA primer/template complex (black) reacts very slowly with incoming threo-nucleotides (green) shown as imidazolium-bridged dinucleotide); (second from top) primer extension with an incoming threo-nucleotide (green) is facilitated by the presence of a downstream helper oligoribonucleotide (gray); (third from top) a primer ending in a threo-nucleotide (green) is extended slowly by reaction with incoming ribonucleotides (gray); (bottom) primer extension across a threo-nucleotide in the template is only slightly inhibited. (C) Part of a crystal structure of an RNA primer/template complex (green) with bound imidazolium-bridged TNA dinucleotide. The long O3′−P distance likely contributes to the slow rate of primer extension with threo-nucleotides. (D) Portion of a crystal structure of an RNA duplex with one threo-nucleotide in one strand, showing only local structural perturbations. (C) and (D) are reproduced with permission from ref 25. Copyright 2021 Oxford University Press.

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Nonenzymatic primer extension on an RNA template using only 2-AI-activated threo-nucleotide monomers is more than 100-fold slower than that for identically activated RNA monomers[25] (Figure 4B, top). This dramatic defect in primer extension appears to be due to a combination of two effects. First, direct measurements of the rate of reaction between 2AI-activated TNA monomers in solution shows that the second-order rate constant for formation of the imidazolium-bridged dinucleotide intermediate is ∼4-fold slower than that for RNA monomers. However, even when the imidazolium-bridged intermediate has formed, its reaction with the primer seems to be very slow. A structural reason for this effect is apparent from the crystal structure of an RNA duplex with the TNA imidazolium-bridged intermediate bound to a template overhang.[25] In this structure the distance between the RNA primer 3′-OH and the adjacent threo-nucleotide phosphate is much longer than in the corresponding all-RNA case (5.9 Å vs 4.2 Å) (Figure 4C). This is expected, since the reactive phosphate of the threo-nucleotide is held more closely to the sugar than in the case of a ribo-nucleotide, due to the absence of the 5′-methylene carbon. The sterically crowded environment of the threo-nucleotide phosphate may contribute both to the slower rate of formation of the imidazolium-bridged intermediate and to the slower rate of primer extension. Slow primer extension with 2AI-activated threo-nucleotide monomers is not restricted to the context of an otherwise all-RNA primer/template complex. Indeed, primer extension with threo-nucleotides is even slower with an all-TNA primer or template or a primer/template complex.

The slow rate of primer extension with 2AI-activated threo-nucleotides can be partially overcome if the incoming threo-monomer can form an imidazolium bridge to an activated downstream RNA helper oligonucleotide (Figure 4B, second from top), in which case the rate defect drops to only ∼4-fold. Additional structural work will be required to address the possibility that this rescue effect results from the downstream ribonucleotide "pushing" the upstream threo-nucleotide closer to the primer 3′-OH. The rescue effect suggests that, if TNA monomers were generated alongside RNA monomers or oligomers, the formation of mixed TNA-RNA imidazolium-bridged species would result in the occasional incorporation of threo-nucleotides into a growing chain by primer extension. The frequency of internal threo-nucleotides would depend upon the ratio of 2AI-activated threo- to ribo-nucleotides, since only mixed threo-ribo imidazolium-bridged intermediates lead to significant incorporation of threo-nucleotides. Alternatively, a different activation chemistry that compensated for the shorter threo-nucleotide backbone could potentially favor the polymerization of TNA over RNA.

Continued primer extension from a primer ending in a threo-nucleotide is about 10-fold slower than that for the all-RNA system but does proceed, suggesting that oligonucleotides containing scattered internal threo-nucleotides could be formed. Templates containing single or multiple TNA residues can be fully copied with activated RNA monomers, at rates that are only 2−4-fold lower than in the corresponding all-RNA systems, consistent with the minor and local structural distortions induced by the presence of a threo-nucleotide in an RNA template (Figure 4D). Thus, in a competitive situation where both ribo- and threo-nucleotides were present, template copying and replication could proceed, with predominantly RNA oligonucleotides expected to emerge. Additional competition experiments at the stages of nucleotide activation, imidazolium-bridged dimer formation, and primer extension should provide further insight into the effects of threo-nucleotides on prebiotic RNA composition and replication.

2.3. 2′-Deoxyribonucleic Acid (DNA). As the genetic material of modern cells, DNA and its 2′-deoxyribo-nucleotide building blocks (Figure 5A) are important to consider in a prebiotic context. Early versions of the RNA world model proposed that RNA preceded DNA, which emerged later via enzymatic reduction of ribonucleotides.[43] However, it is difficult to imagine why ribonucleotide reductases would have evolved unless deoxynucleotides were already functionally important: e.g., as substrates for DNA polymerases. In turn it is difficult to imagine how DNA polymerases could have evolved unless deoxynucleotides were already present in the environment. In fact, several recent studies suggest potential prebiotic pathways for deoxynucleotide synthesis, suggesting that deoxynucleotides and ribonucleotides could have coexisted.[44,45] However, the potential coexistence of ribo- and 2′-deoxyribo-nucleotides raises the question of whether the primordial genetic polymer was a chimera of these two types of building blocks, and if so, how homogeneous polymers could have arisen from a mixture of monomers.

The Krishnamurthy and Sutherland groups first showed that 2-thiouridine could be converted to 2,2′-thioanhydrouridine and thence by photoreduction in the presence of hydrosulfide to 2′-deoxy-2-thiouridine.[45] Furthermore, the 2′-deoxy-2-thiouridine could be converted by reaction with adenine into

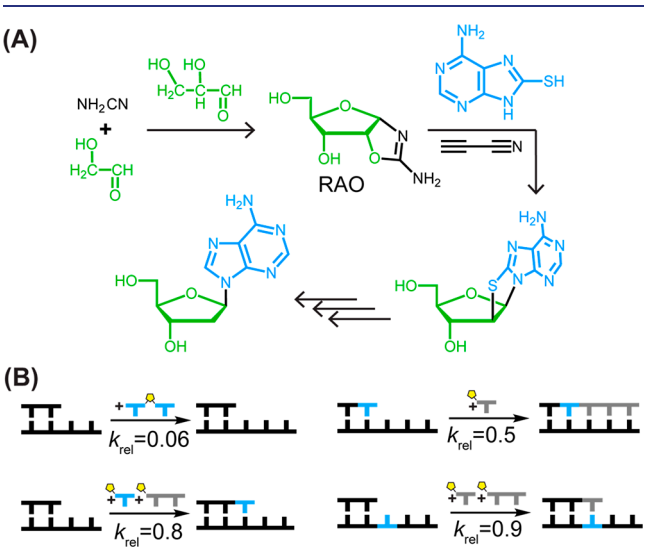

Figure 5. Potentially prebiotic synthesis of 2′-deoxy-nucleotides and comparison of nonenzymatic copying reactions with 2′-deoxy- and ribo-nucleotides. (A) 2′-Deoxy-purine nucleotide synthesis begins with RAO, which reacts with cyanoacetylene to generate α-2,2′-anhydropyrimidines, which in turn react with 8-thio-adenine to generate 8,2′-thio-anhydropurines. Photochemical reduction and desulfurization generate dA, which can be converted to dI in the presence of nitrous acid. (B) Schematic representation of non-enzymatic primer extension reactions: (left, top) primer extension with 2-aminoimidazolium-bridged 2′-deoxy dinucleotides (blue) is quite slow in comparison to the all-RNA reaction, but (left, bottom) primer extension with a 2AI-activated 2′-deoxy-nucleotide is almost fully rescued by the presence of an activated downstream oligoribonucleotide; (right, top) the extension of a primer ending in a 2′-deoxy-nucleotide is moderately slower than that from an all-RNA primer, but (right, bottom) primer extension across a single 2′-deoxy-nucleotide in the template is barely affected.

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a mixture of the α- and β-anomers of 2′-deoxy-adenosine, albeit in low yield. An alternative route to the 2′-deoxy-nucleosides has been proposed by the Trapp group.[44] Recently, the Sutherland group reported a more efficient and stereoselective synthesis of the 2′-deoxy-purine nucleosides dA and dI, based on the reaction of 8-thio-adenine with the α-2,2′-anhydro-pyrimidines[46] (Figure 5A). Together, these reports imply that 2′-deoxyribo nucleotides are likely to have been synthesized alongside the ribonucleotides, raising the possibility that the primordial genetic material was an RNA/DNA chimera. It seems quite likely that chimeric oligonucleotides would be generated by the nontemplated copolymerization of 2′-deoxy- and ribo-nucleotides, making it important to study replication-like reactions in such mixed systems.

There is a long history of studies of nonenzymatic primer extension comparing RNA and DNA primers and templates and 2′-deoxy- vs ribo-nucleotides. Pioneering work from Orgel and his co-workers showed that an A-form duplex structure is important for the efficient template-directed polymerization of 5′-phosphoroimidazolide nucleosides.[47] This early work indicated that RNA synthesis from activated ribonucleotides was more efficient than DNA synthesis with activated 2′-deoxyribo-nucleotides, in nonenzymatic template-copying processes on both RNA and DNA templates.

We have recently compared the rates of primer extension using 2AI-activated 2′-deoxyribo- and ribo-nucleotides[35] (Figure 5B). With an all-RNA primer/template complex, and using purified 2-aminoimidazolium-bridged 2′-deoxyribo-nucleotide dimers, primer extension was almost 20-fold slower than that with the corresponding ribonucleotide-bridged dimers. However, 2′-deoxy- and ribo-nucleotides were incorporated at almost the same rates in the presence of an activated downstream helper oligoribonucleotide.[35] The mechanism responsible for the suppression of the defect in 2′-deoxynucleotide incorporation remains unclear. Competition experiments will have to be done to assess the relative rates of incorporation of 2′-deoxy- vs ribo-nucleotides in more prebiotically realistic mixed systems. Once a 2′-deoxy-nucleotide is added to a growing primer, subsequent addition of a ribonucleotide is about 2-fold slower than that from an all-RNA primer, providing an additional factor that would slow down DNA synthesis. Finally, as seen with other variant nucleotides, a 2′-deoxy-nucleotide in the template strand has little effect on RNA primer extension, suggesting that 2′-deoxy-nucleotides could be copied over and gradually replaced with ribonucleotides over the course of multiple rounds of replication.

However, our results suggest that 2′-deoxy-nucleotides would be retained in chimeric oligonucleotides to a extent greater than that for either arabino- or threo-nucleotides.

Recently, the Krishnamurthy group reported that heterogeneous backbone chimeric RNA/DNA duplexes are significantly less stable than either homogeneous RNA/RNA or DNA/DNA duplexes.[48] Building on this surprising observation, Krishnamurthy et al. have shown that nonenzymatic ligation favors the production of all-RNA and all-DNA products because of the preferential binding of homogeneous backbone oligomers (either all-RNA or all-DNA) to chimeric TNA/RNA or DNA/RNA template strands.[49] Thus, both nonenzymatic ligation and nonenzymatic polymerization seem to promote the formation of homogeneous backbone oligoribonucleotides, although through quite different mechanisms.

3. NUCLEOBASE HETEROGENEITY

Recent studies of prebiotic nucleotide synthesis have suggested that a number of noncanonical nucleobases may have been present during the origin of life.[5,6] An important question is whether ribonucleotides with nonstandard nucleobases would have been helpful, harmful, or neutral for early genetic systems. In order to maintain a functional genome, a high fidelity of information copying is essential, as the maximum error rate cannot exceed the reciprocal of the number of functionally significant bases in the genome.[50] Nonenzymatic copying is plagued by low fidelity, as it relies on the weak noncovalent interactions of base pairing, without the steric restrictions imposed by a polymerase ribozyme or protein enzyme. In addition, the rate of nonenzymatic copying needs to be faster than the rate of RNA or activated monomer degradation.[8] Using fidelity and the rate of nonenzymatic copying as two key parameters, we discuss the potential roles of prebiotically plausible alternative nucleobases in nonenzymatic replication, revealing an expanded view of plausible early genetic systems.

3.1. 2-Thiopyrimidines and Inosine. The 2-thio-pyrimidines have long been of interest because of their potential to enhance the rate and fidelity of nonenzymatic template copying (Figure 6). The thermodynamic stability of

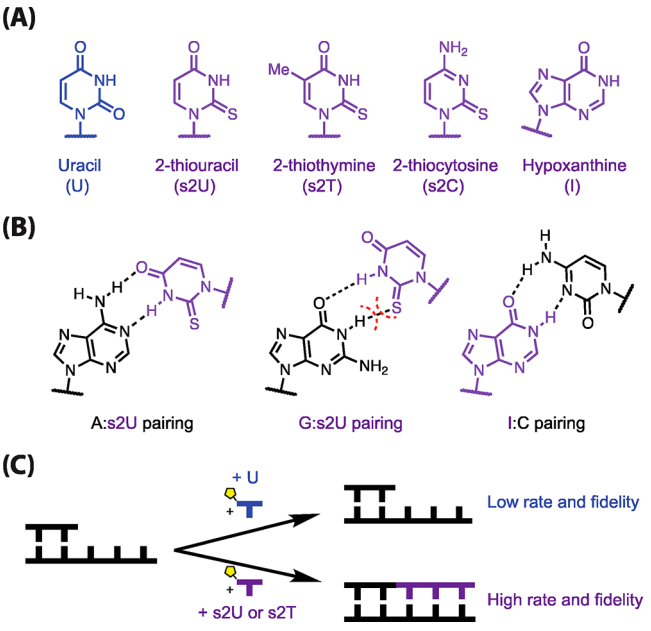

Figure 6. 2-Thio-pyrimidines and inosine allow for superior nonenzymatic copying. (A) Chemical structures of the nucleobase components of uridine, 2-thiouridine, 2-thioribothymidine, 2-thio-cytosine and inosine. (B) Chemical structures of the A:s2U, G:s2U, and I:C base pairs. (C) Replacing the activated uridine monomer with an activated 2-thiouridine monomer improves both the rate and fidelity of nonenzymatic primer extension.

the canonical A:U base pair is comparable to that of the G:U wobble base pair, which leads to high error rates during template copying. In contrast 2-thio-uridine (s2U) forms a more stable Watson−Crick s2U:A base pair, and a less stable s2U:G wobble pair.[51] As a result, 2-thio-substitution either in the activated U (or T) monomer or in the template improves both the kinetics and the fidelity of chemical copying chemistry (Figure 7C).[52] Interestingly, the stabilizing effect appears to result from the 2-thio-induced preorganization of the

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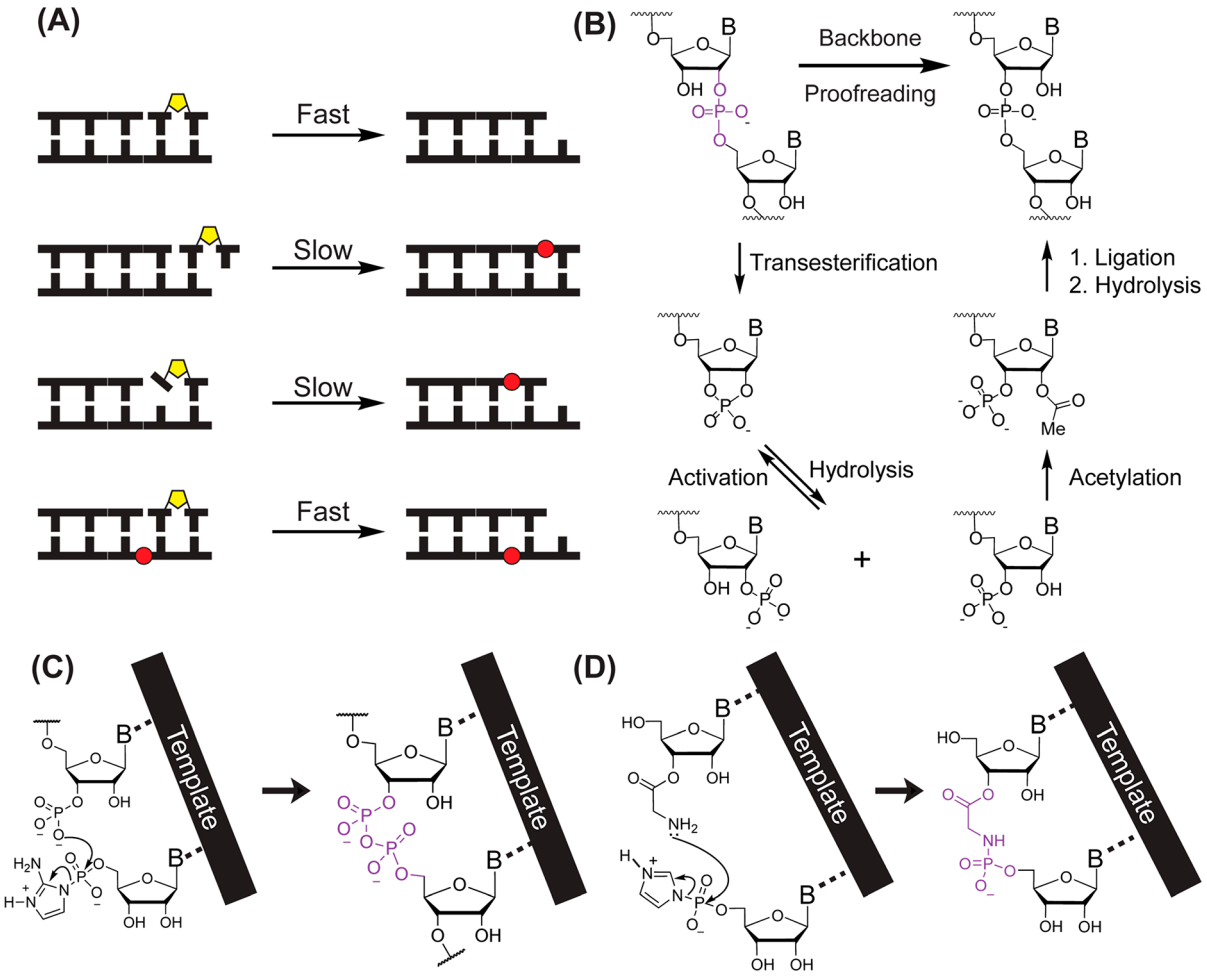

Figure 7. Potentially prebiotic noncanonical backbone linkages in RNA. (A) Nonenzymatic RNA copying generates 2′-5′-linkages when the imidazolium-bridged intermediate is unable to bind to the template via two Watson−Crick base pairs: (red circles) 2′-5′-linkages; (yellow pentagons) imidazolium bridges. (B) Correction of a 2′-5′-linkage by sequential transesterification, hydrolysis, 2′-O-acetylation of 3′-phosphate monoesters, 3′-phosphate activation, and finally, ligation. (C) A primer ending in a 3′-phosphate is efficiently extended by reaction with 2AI-activated ribonucleotides, generating a 3′-5′-pyrophosphate backbone linkage. (D) A 3′(2′)-aminoacylated primer reacts with incoming activated ribonucleotides to generate an amino acid bridged backbone linkage.

nucleotide into the C3′-*endo* conformation, which is optimal for nonenzymatic template copying.[51,53,54] The increased thermodynamic stability resulting from the presence of the methyl group at the 5-position of thymidine also increases the rate of chemical copying.

A potentially prebiotic pathway to 2-thio-uridine was not elaborated until after the favorable effects of this nucleotide on template copying were established. The prebiotic synthetic pathway to s2U emerged as a surprising solution to a longstanding enigma, which is that the key intermediate RAO, with its attractive property of self-purification by crystallization, leads to the synthesis of the α-anomer of ribo-C. Conversion to the β-anomer, for example by photoanomerization, is quite poor with a yield of ∼4%.[55] Remarkably, α-2-thio-C, formed by thiolysis of α-anhydro-C, photoanomerizes to give the β-anomer of 2-thio-C in ∼80% yield. While conversion of 2-thio-C to 2-thio-U by hydrolysis or UV-induced deamination is relatively slow and inefficient, treatment with nitrous acid (derived from atmospheric NO) leads to efficient conversion of C to U,[56] as well as the efficient conversion of adenosine to inosine.[46] Although incomplete, these tantalizing results hint at the possibility of a primordial version of RNA based on the two 2-thio-pyrimidines and the purines A and I, in which the two base pairs would be of similar energy and in which the preorganization of the pyrimidines in the C3′-*endo* conformation would lead to faster and more accurate copying.[54]

Inosine exhibits surprisingly rapid and accurate chemical copying.[57] Despite relatively weak base pairing with C, most likely due to the loss of one H-bond relative to a G:C pair,[58] inosine performed well as an activated mononucleotide with chemical copying rates only slightly lower than those of guanosine and with slight improvements in the error frequency. The higher fidelity observed with inosine appears to be a consequence of the lower thermodynamic stability of I:U wobble pairs in comparison to G:U wobble pairs.[59] It is not entirely clear why inosine should be incorporated with such high fidelity, since previous work suggests that stacking interactions rather than H-bonds are more energetically significant for terminal base pairs.[60,61] One possible interpretation is that, since primer extension proceeds predominantly through reaction of the primer with an imidazolium-bridged dinucleotide, the nucleotide being incorporated is sandwiched between the primer and the downstream nucleotide and may thus behave more like an internal than a terminal nucleotide. Primer extension with inosine at the 3′-

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end of a primer also showed rates comparable to those of guanosine. Recently, Sutherland and his co-workers reported a potentially prebiotic synthesis of deoxy-adenosine (dA), with deoxy-inosine (dI) subsequently generated from dA by deamination.[46] It is likely that rI could be similarly derived from rA, suggesting that inosine may have acted as a surrogate for guanosine in early copying chemistry. Further improvement in the rate and fidelity of template copying may be achieved by taking advantage of the 2-thio-C:I base pair. Although this has not yet been explored experimentally, thermodynamic data[54] show that this base pair is stronger than the C:I base pair, while 2-thio-C substitution slightly weakens the G:C base pair.

3.2. Other Noncanonical Nucleobases. A recent study from the Powner laboratory reported potentially prebiotic routes to the 8-oxo-purine nucleotides[62] that diverge from a common precursor to the pyrimidine ribonucleotides. These findings suggest that 8-oxo-purine ribonucleotides could have coexisted with the standard pyrimidine ribonucleotides, raising the question of whether they could have been components of a primordial genetic system. We have shown that the 8-oxo-purines (8-oxo-adenosine, 8-oxo-guanosine, and 8-oxo-inosine) all perform poorly as substrates for nonenzymatic RNA copying chemistry, regardless of whether they are present as activated mononucleotides, in the template, or at the end of a primer.[57] Activated 8-oxo-purine monomers are incorporated very poorly in primer extension reactions, at rates comparable to those observed with mismatched canonical nucleotides, consistent with the strongly destabilizing effects of these modified nucleotides on duplex thermodynamic stability.[63−65]

Similarly, an 8-oxo-purine nucleotide at the 3′-end of a primer leads to very slow subsequent primer extension. In contrast, 8-oxo-purines in templates engage in Hoogsteen type pairing with incoming monomers (e.g., 8-oxoG:A), leading to very poor fidelity. These results suggest that the incorporation of 8-oxo-purines into oligonucleotides by template copying chemistry would be quite rare, while 8-oxo-purines incorporated by nontemplated oligomerization would be rapidly replaced by standard nucleotides during subsequent copying reactions.

Many other nonstandard nucleobases have been investigated due to their potential synthesis in prebiotic reactions.[66] However, nucleobase analogues such as urazole and 2-pyrimidinone, which are attractive because of their ability to spontaneously form β-nucleosides upon drying or heating with ribose, do not base-pair well with A, since only a single H-bond can form and stacking interactions may also be weaker. On the other hand, alternative nucleobases such as 2,6-diaminopurine (2,6DAP) and 2-aminopurine (2AP) do base-pair well with U, and DAP has been shown to lead to enhanced template copying in a non-RNA model system.[67] DAP in particular deserves more study in an RNA copying context, although it must be noted that DAP and 2AP suffer from the twin disadvantages of lower tautomeric specificity in comparison to A (alternative tautomers can participate in nonstandard base pairing, leading to error-prone copying), as well as likely photoinstability due to the long lifetimes of their UV excited states.[68]

4. BACKBONE HETEROGENEITY

Unconstrained by enzymatic control, the prebiotic polymerization of activated monomers has the potential to generate noncanonical backbone structures in both templated and nontemplated reactions. Here we discuss the regioselectivity of template-directed primer extension and the formation of both pyrophosphate and amino acid bridged internucleotide linkages during primer extension reactions.

4.1. Regioselectivity of RNA Copying. The regioselectivity of the nonenzymatic copying of RNA has been questioned since the first published example of nonenzymatic template copying.[69] In early template-copying experiments using imidazole activated G, mixtures of 2′-5′- and 3′-5′-linkages were obtained, but activation with 2-methylimidazole led to improved 3′-5′-specificity.[70,71] Recently, we have found that nonenzymatic RNA polymerization produces predominantly 3′-5′-linkages when the primer extension reaction proceeds through reaction with a 2-aminoimidazolium 5′,5′-bridged dinucleotide intermediate that forms two Watson−Crick base pairs with the template.[72] However, detectable levels of 2′-5′-linkages are formed when the primer is extended by a mismatched base or when the last nucleotide of the template is copied. In addition, the copying of stretches of A or U residues in the template with activated U or A monomers leads to high levels of 2′-5′-linkages. Remarkably, this effect is suppressed by the use of 2AI-activated 2-thio-U as the incoming monomer, possibly due to the stronger base pairing, which could help to maintain both base pairs of the imidazolium-bridged intermediate with the template. Alternatively the preorganization of the 2-thio-U monomer in the 3′-*endo* conformation could contribute to the enhanced regiospecificity.

Switzer et al. had previously shown that copying of a 2′-5′-linked template with 2-methylimidazole activated monomers was possible, at a moderately reduced rate but with almost no regiospecificity.[73] In experiments with 2AI-activated monomers, we have found that a single 2′-5′-linkage in the template slightly reduces the rate of primer extension but that only 3′-5′-linkages are formed[72] (Figure 7A). Thus, 2′-5′-linkages are not heritable, and RNA copying with 2AI-activated monomers enriches 3′-5′-linkages (although this need not be the case with alternative activation chemistries).

While the copying of an all-RNA template with RNA 2-aminoimidazolium-bridged dinucleotides appears to be reasonably regioselective, it is still important to consider the effects of low levels of 2′-5′-linkages on RNA replication and function. Our laboratory has shown that functional RNAs such as aptamers and ribozymes tolerate a surprising extent of backbone heterogeneity,[74] suggesting that low levels of 2′-5′-linkages will still allow for the evolution of functional RNAs. Moreover, the presence of 2′-5′-linkages lowers the melting point of an RNA duplex,[75,76] a feature which might be useful in RNA replication by facilitating the strand separation required for copying chemistry to proceed. When they are taken together, these phenomena suggest that a low fraction of 2′-5′-linkages resulting from incomplete regiospecificity of template copying would not be harmful and might actually be helpful.

2′-5′-Linkages are, however, much more labile than 3′-5′-linkages in the context of an RNA duplex,[76] leading to interest in pathways for the correction of 2′-5′-linkages. One such pathway, described by Mariani and Sutherland,[77] begins with the 2′-3′-cyclic phosphate product of strand cleavage, which hydrolyses to a mixture of 2′- and 3′-phosphates (Figure 7B). The two regioisomeric phosphates can be differentiated by acetylation,[78] which occurs preferentially on the 3′-phosphate, followed by transfer of the acetyl group to the 2′-hydroxyl. When a phosphate activating agent is added, the acetylated species can be ligated to generate a 3′-5′-phosphodiester bond.

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In contrast, 2′-phosphate activation regenerates the cyclic phosphate. Multiple cycles of hydrolysis, acetylation, and ligation thus transform a substantial fraction of 2′-5′-phosphodiester bonds into the natural 3′-5′-isomer.

4.2. Pyrophosphate Linked RNA. Internucleotide pyrophosphate linkages can be generated by primer extension or ligation when the primer ends in a 3′-phosphate (Figure 7C). Such primers can be generated by the internal cleavage of an RNA strand followed by hydrolysis of the 2′,3′-cyclic phosphate product. Although a 2′-phosphate acts as a chain terminator, primers ending in a 3′-phosphate extend surprisingly well, generating a 3′-5′-pyrophosphate linkage.[79] Subsequent monomer additions occur at rates similar to those seen for normal primer extension. A crystal structure of the product of primer extension shows that the RNA duplex architecture easily accommodates the unnatural pyrophosphate linkage with only local distortion, explaining the minimal effect of the pyrophosphate linkage on primer extension. For the same reason, a 3′-5′-pyrophosphate linkage in the template can be copied over by primer extension, albeit at a slower rate.

However, the 3′-5′-RNA pyrophosphate linkage is unstable in single-stranded RNA in the presence of Mg^{2+}. Thus, a template strand containing a pyrophosphate linkage can only serve as a temporary patch for "repair" of a cleaved RNA strand, because either it may be copied to generate a normal RNA complementary strand or it may be cleaved before copying can be completed. If the pyrophosphate linkage is cleaved and the resulting 2′,3′-cyclic phosphate product is hydrolyzed, 2′- and 3′-phosphate products can enter the acetylation−ligation repair pathway described above.[77]

4.3. Amino Acid Linked RNA. Protein synthesis in biology uses aminoacylated tRNAs as substrates, suggesting that aminoacylation chemistry predated the subsequent evolution of ribosomal peptide synthesis. If so, primordial RNA aminoacylation could have led to the generation of chimeric amino acid−nucleotide biopolymers.[79] The Orgel group first investigated this possibility by showing that aminoacylated adenosine reacts with adenosine 5′-phosphorimidazolide in the presence of a poly(U) template.[80] The free amino group of the amino acid displaces the imidazole leaving group, leading to phosphoramidate bond formation (Figure 7D). The high yield and fast rate of the reaction, together with its strong dependence on the template, suggest that an amino acid bridge is compatible with Watson−Crick base pairing of the flanking nucleotides.

Our laboratory has recently reinvestigated the formation of amino acid bridged RNAs, making use of the flexizyme ribozyme[81] to generate aminoacylated oligonucleotides. We find that both primer extension and ligation can lead to the rapid template-directed extension of an aminoacylated primer, even in the absence of the catalytic divalent metal ions that are essential for normal primer extension chemistry.[82] In addition, amino acid bridged oligonucleotides are good templates for primer extension: i.e., the amino acid bridge can be copied over to generate a normal RNA complementary strand.

However, aminoacylated nucleotides are unstable to hydrolysis, and no efficient nonenzymatic aminoacylation chemistry is known. Amino acid imidazolides can aminoacylate RNA, but the yield is low.[82] The Sutherland laboratory has recently developed an amino acid activation chemistry that converts a 3′-phosphate to a phospho-carboxy anhydride, which then intramolecularly acylates the adjacent 2′-hydroxyl.[83] Dipeptides and N-acylated amino acids provide high yields, but free amino acids do not work well in this system, leaving open the question of whether an efficient prebiotic pathway to RNA aminoacylation is possible and thus whether aminoacylation could play a significant role in nonenzymatic RNA replication or assembly.

An alternative approach to the synthesis of amino acid bridged oligonucleotides has been recently reported by the Richert laboratory.[84] In this strategy, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) is first used to generate a phosphoramidate linkage between the 5′-phosphate group of a nucleotide or oligonucleotide and the α-amino group of a peptide or amino acid. When amino acid conjugated dinucleotides are added to a primer−template duplex in the presence of EDC, the free carboxyl group of the amino acid or peptide connects to the 3′-end of the primer (in this case a 3′-amino-, 2′,3′-dideoxy-terminated primer was used), forming a peptide or amino acid bridged RNA product (Figure 7). Remarkably, the reaction is most efficient with dipeptides but also works well with a single amino acid or a tripeptide.[85] Thus, if appropriate prebiotic activation chemistry existed, amino acid bridged RNAs may have been common in the RNA world.

5. CONCLUSIONS

Studies of the nonenzymatic copying of oligonucleotides suggest that this process could have served as a chemical selection mechanism for the emergence of RNA from a variety of prebiotically plausible nucleotides. The three examples of nucleotides with altered sugar chemistry (arabino-, threo-, and 2′-deoxyribo-nucleotides)[25,35] that have been studied so far are all inferior to ribonucleotides in nonenzymatic copying kinetics, and in many cases structural studies provide a rationale for the decreased reaction rates. Nevertheless, all three variants, when they are present in the template, can be copied over to generate a standard RNA product strand. Similarly, certain nucleobase variations, such as the 8-oxo-purine ribonucleotides,[57] are essentially excluded from primer extension reactions, but when they are present in the template can be copied over to generate a normal RNA complementary strand. Finally, we have recently shown that three classes of nonstandard backbone linkages (2′,5′, pyrophosphate, and amino acid bridged linkages)[72,79,82] in the template strand can also be copied over to generate a canonical RNA complementary strand. In addition, Kozlov and Orgel[86] showed that even one or two L-nucleotides in the template can be copied over to generate normal RNA. These observations provide strong support for the idea that repeated cycles of copying chemistry would lead to the synthesis of oligonucleotides that are enriched in ribonucleotides.

To date, the only prebiotically plausible variant nucleotides that are superior to the canonical ribonucleotides in the rate and accuracy of nonenzymatic template copying are inosine and the 2-thio-pyrimidine ribonucleotides. The stronger 2-thio-U:A and weaker 2-thio-C:I base pairs, relative to the standard A:U and G:C base pairs, suggest that a primordial RNA based on these modified nucleotides might exhibit a more uniform rate of copying in comparison to modern RNA, which exhibits a strong bias in favor of the incorporation of C and G over U and A. In addition, the weaker 2-thio-U:I wobble base pair could lead to enhanced fidelity. These observations suggest that the properties and provenance of a potentially primordial version of RNA containing 2-thio-pyrimidines and inosine deserve further investigation.

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An additional potential selection mechanism is based upon the surprising observation of the Krishnamurthy group of the stronger annealing of DNA and RNA oligonucleotides of homogeneous composition, in comparison to chimeric oligonucleotides composed of mixtures of ribo-, threo-, and deoxy-nucleotides.[48,49] Because primer extension requires the primer to anneal to a template strand, primer extension will be more favorable if the primer is all-RNA (or all-DNA), and not a mixed RNA/DNA sequence. Because RNA/RNA pairing is the strongest, homogeneous RNA primers may be the most likely to be further extended, and these will tend to be elongated with ribonucleotides for the reasons noted above. The effects of heterogeneity on oligonucleotide annealing should be investigated for other nucleotide variations such as arabino-nucleotides, to assess the generality of this effect. Krishnamuthy et al. have also noted that the same effect would lead to the enhanced ligation of homogeneous oligonucleotides.[49] Thus, repeated cycles of template directed ligation could also contribute to the emergence of increasingly homogeneous oligonucleotides.

In this Perspective we have focused on the potential role of nonenzymatic template copying as a selective filter that would act to progressively enrich for RNA over multiple cycles of copying. However, it is clear that other physical and chemical processes are also likely to have contributed to the emergence of RNA from the heterogeneous products of prebiotic chemistry. For example, the canonical ribonucleotides may have been selected because of their resistance to UV photodegradation. The short excited-state lifetimes of the canonical nucleobases and nucleosides lead to an expectation of photostability.[87] In addition, Sutherland et al. have noted the greater photostability of β-ribonucleotides to UV, in comparison to certain byproducts of prebiotic synthesis, such as α-ribocytidine and β-arabinocytidine.[12]

Ribonucleotides may also have been selected over some alternative types of nucleotides on the basis of their inherent chemical reactivity. For example, phosphorylated acyclic nucleotides are typically removed from the pool of reactive monomers by immediate cyclization after activation.[88] Moreover, it is highly likely that different classes of nucleotides will exhibit differential rates of phosphate activation and of incorporation into oligonucleotides by nontemplated oligomerization. For example, threo-nucleotides are sterically crowded, likely impeding both phosphate activation and oligomerization.[25] Clearly, there is much to be done in order to delineate the unique sets of chemical and physical properties that resulted in the elimination of each class of alternative nucleotides and the consequent ascendency of the ribonucleotides as the basis of biological inheritance.

Despite the combined effects of synthetic specificity and the multiple filters that favor ribonucleotides, a degree of residual heterogeneity in oligonucleotide composition would remain, reflecting the relative concentrations and rates of incorporation of alternative nucleotides. If oligonucleotide replication were occurring in a closed environment, with no input or loss of nucleotides, then continued template copying would decrease ribonucleotide concentrations and increase the concentrations of competing nucleotides. However, in an open system analogous to a flow reactor, a steady-state ratio of ribonucleotides to alternative nucleotides might be reached, leading to a steady-state level of heterogeneity in the oligonucleotide population. Resistance to degradation would also favor the accumulation of ANA, TNA, and DNA over RNA and would also select for the accumulation of double-stranded RNA segments.

Because RNA would only be enriched to a relatively homogeneous state, it remains important to consider the likely nature of and effects of such residual heterogeneity. The results we have reviewed strongly suggest that arabino- and threo-nucleotides would be efficiently filtered out but that deoxy-nucleotides would be subject to a weaker degree of selection bias and would therefore be more likely to remain as scattered substitutions in otherwise homogeneous RNA oligonucleotides. Functional RNAs including aptamers and ribozymes have been shown to tolerate surprisingly high levels of deoxy-nucleotide substitution; thus, the effects of residual deoxy-nucleotides should be relatively mild.[74] Early ribozymes may even have evolved so as to tolerate deoxy-nucleotide substitutions especially well. In this regard it is interesting to note that, in modern eukaryotic organisms, ribonucleotides are incorporated into genomic DNA not only by primase but also by DNA polymerases during replication. These ribonucleotides may serve to distinguish the newly synthesized DNA strand from the preexisting strand, thereby facilitating mismatch repair.[89] It is also notable that engineered strains of *E. coli* are able to tolerate the presence of remarkably high levels of genomic ribonucleotides, suggesting that early ribo-organisms may have evolved into modern DNA organisms through a transitional stage involving organisms with a mixed RNA/DNA genome.[90] In contrast to deoxy-nucleotides, the incorporation of arabino-nucleotides blocks further primer extension. However, oligonucleotides terminating in an arabino-nucleotide could still serve auxiliary roles in replication as downstream helpers, splint templates, and catalysts of strand displacement.[35]

We hope that the continuing exploration of novel prebiotic pathways and of the effects of nonstandard nucleotides on nonenzymatic copying will lead to a more complete picture of the constraints that shaped the primordial genetic polymers of life. The emergence of RNA from the heterogeneous set of prebiotically synthesized nucleotides once seemed like an intractable puzzle. Now, thanks to advances in our understanding of prebiotic chemistry, the emergence of RNA is beginning to seem more like an inevitable and perhaps deterministic outcome of its underlying chemistry.

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Notes

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