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Functional RNAs exhibit tolerance for non-heritable 2′–5′ versus 3′–5′ backbone heterogeneity

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A plausible process for non-enzymatic RNA replication would greatly simplify models of the transition from prebiotic chemistry to simple biology. However, all known conditions for the chemical copying of an RNA template result in the synthesis of a complementary strand that contains a mixture of 2′–5′ and 3′–5′ linkages, rather than the selective synthesis of only 3′–5′ linkages as found in contemporary RNA. Here we show that such backbone heterogeneity is compatible with RNA folding into defined three-dimensional structures that retain molecular recognition and catalytic properties and, therefore, would not prevent the evolution of functional RNAs such as ribozymes. Moreover, the same backbone heterogeneity lowers the melting temperature of RNA duplexes that would otherwise be too stable for thermal strand separation. By allowing copied strands to dissociate, this heterogeneity may have been one of the essential features that allowed RNA to emerge as the first biopolymer.

The ability of RNA molecules to fold into defined three-dimensional (3D) structures with exquisitely specific molecular recognition and catalytic properties is the conceptual basis of the RNA world hypothesis, an early stage in the evolution of life in which RNA served not only as the polymer of inheritance, but also as the central functional polymer of biochemistry[1–3]. This model is supported most strikingly by the observation that all modern proteins are synthesized by the peptidyl transferase ribozyme at the heart of the ribosome[4,5]. With the RNA world hypothesis supported so strongly by this and other evidence[1], the central question in the origin-of-life field concerns the pathway from the prebiotic chemistry of the early Earth to the emergence of simple forms of cellular life that contain RNA genomes coding for RNA enzymes. Although there has been considerable recent progress towards the elucidation of potentially prebiotic pathways for ribonucleotide synthesis[6–8] and the assembly of activated nucleotides into oligonucleotides[9,10], the non-enzymatic replication of RNA oligonucleotides remains problematic. A series of seemingly intractable difficulties continues to make a robust system for the chemical replication of RNA elusive[11–14]. These problems include the slow rate, poor fidelity and low regioselectivity of non-enzymatic RNA template copying; in addition, activated substrates typically hydrolyse on the same timescale as polymerization. The importance of the latter point is highlighted by the efficient copying that can be attained by flowing fresh substrates over immobilized templates[13]. Other issues are the lack of primers in prebiotic scenarios, the difficulty of strand separation that is a consequence of the high thermal stability of long RNA duplexes and the fast re-annealing of separated strands. Finally, RNA template copying requires high Mg^{2+} concentrations, but prebiotically plausible protocell membranes composed of fatty acids are sensitive to low (10^{-3} M) concentrations of Mg^{2+}, which makes these two key components of primitive cells appear to be mutually incompatible. As a result of these unresolved issues, considerable effort was devoted to the synthesis and characterization of alternative polymers that might have served as progenitors of RNA[15–22]. Nevertheless, the recent advances in understanding potential prebiotic routes to RNA have motivated us to revisit the unsolved problems of RNA replication.

Here we address two of the above eight problems that have so far prevented a demonstration of complete cycles of chemically driven RNA replication. First, we consider the issue of regiospecificity. In contrast to contemporary life, in which the transcription of DNA templates generates exclusively 3′–5′ phosphodiester-linked RNA, prebiotically plausible syntheses of RNA in non-enzymatic template-copying reactions generate a mixture of 2′–5′ and 3′–5′ linkages as a result of the proximity and similar nucleophilicity of the 2′- and 3′-hydroxyls of the ribose moiety (Fig. 1). The regioselectivity of non-enzymatic template-directed RNA synthesis can be improved by the use of oligomer (as opposed to monomer) substrates, by varying the metal ion or leaving group used for monomer activation or by exploiting the differential stability of the 2′–5′ and 3′–5′ linkages in an RNA double helix, but no chemical means of template-directed RNA synthesis reported to date matches the complete regiospecificity achieved in enzymatic RNA synthesis[23–28]. As a result, RNA strands generated by non-enzymatic template-directed copying inevitably contain a random mixture of non-heritable 2′–5′ and 3′–5′ backbone linkages. The presence of 2′–5′ linkages is not a barrier to subsequent rounds of replication, because Switzer and colleagues demonstrated that 2′–5′-linked RNA, as well as mixed 2′–5′/3′–5′ RNA, can template primer extension reactions, albeit more slowly than 3′–5′ RNA can[29]. Based on the reasonable assumption that precise 3D interactions are required for macromolecular folding and catalysis, the presence of randomly distributed 2′–5′ linkages would be expected to interfere with the reproducible formation of the folded 3D structures required for ribozyme catalytic activity, and thus prevent the emergence of primitive life forms based on RNA catalysis. However, recent work from this laboratory shows that aptamers with highly specific molecular recognition properties can be evolved from libraries of polynucleotides that contain randomly interspersed ribo- and deoxyribonucleotides[30]. As nonheritable ribo/deoxyribo backbone heterogeneity did not prevent the evolution of functional RNAs, we decided to investigate whether this tolerance for structural

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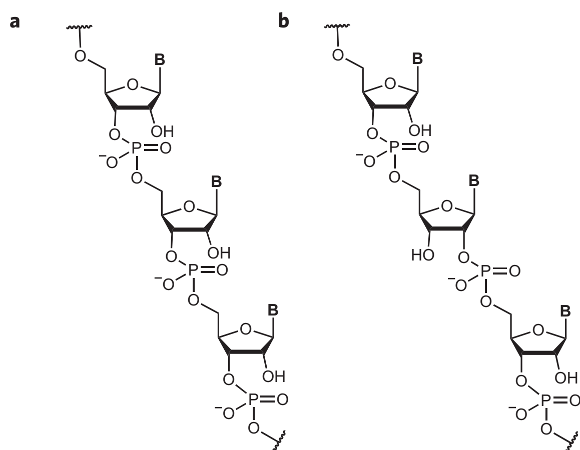

Figure 1 | RNA is enzymatically synthesized with complete regiospecificity to generate a uniform 3′–5′ phosphodiester backbone, but prebiotically plausible non-enzymatic syntheses of RNA result in backbone heterogeneity, and a randomly distributed mixture of 3′–5′ and 2′–5′ linkages. a, Homogeneous 3′–5′-linked RNA; **b**, heterogeneous RNA containing one 2′–5′ phosphodiester linkage. **B** = nucleobase.

heterogeneity might not extend to 2′–5′ versus 3′–5′ backbone heterogeneity. Here, we show that RNAs containing remarkably high proportions of randomly distributed 2′–5′ linkages retain the ability to fold, recognize ligands and catalyse reactions.

A second major problem with the chemical replication of RNA is that RNA duplexes >20–30 nucleotides in length are difficult or impossible to denature thermally under template-copying conditions (most significantly, the presence of high concentrations of Mg^{2+} ions). As strand separation is required to allow for repeated cycles of template copying, the accurate copying of a template strand would generate a dead-end RNA duplex product. However, it has been known for some time that 2′–5′ linkages destabilize RNA duplexes with respect to thermal denaturation[31,32]. Here, we show that 2′–5′ linkages can destabilize long RNA duplexes to the point that thermal strand separation could occur under reasonable geophysical conditions. We therefore propose that 2′–5′ linkages in RNA, far from being problematic, are, in fact, an essential feature that allowed RNA to emerge as the first genetic polymer of life.

Results

We examined the effects of 2′–5′ substitution on two functional RNAs, an aptamer and a ribozyme, both of which had evolved as all 3′–5′-linked RNA (Figs 2a and 3a). The aptamer we chose to study was generated by *in vitro* directed evolution, with selection for binding to flavin mononucleotide (FMN)[33]. The FMN aptamer is formed of two stems that flank a central binding pocket in which its ligand intercalates between a base pair and a base triple, with one residue making a hydrogen-bonding contact with the ligand[34]. The catalytic RNA we studied is the hammerhead ribozyme, which cleaves a substrate strand at a defined site. A minimized *trans*-acting consensus sequence has been described, which we employed here[35].

We examined the effect of 2′–5′ linkages on FMN aptamer functionality by chemically synthesizing a series of variants of the aptamer. We first prepared two fully 3′–5′-linked RNAs by chemical synthesis, which corresponded to the two halves of the FMN aptamer. On mixing in buffer, the two RNA oligonucleotides spontaneously assembled to generate the aptamer structure. We measured the affinity of the two-piece aptamer for FMN by titrating increasing amounts of this RNA into a solution that contained 1 μM FMN, and monitored the resulting quenching of FMN fluorescence. The two-piece FMN aptamer exhibited a K_D for FMN of 560 nM, in line with previously reported values for this complex (Fig. 2b)[33]. We then synthesized versions of both oligonucleotides with 10, 25 and 50% of randomly distributed 2′–5′ linkages, which were introduced by mixing 3′-TBDMS (*tert*-butyldimethylsilyl)-protected 2′-phosphoramidites at the specified ratios with the standard 2′-TBDMS-protected 3′-phosphoramidites. In every case, the replacement of one strand with a variant of the same sequence but with a higher extent of 2′–5′ substitution resulted in a functional aptamer with a moderately higher K_D (Fig. 2b). When the aptamer was assembled with both strands containing 10% 2′–5′ linkages (a plausible prebiotic scenario given the previously achieved regioselectivity described by Orgel and co-workers[24,25,36]), the apparent (population average) K_D for FMN was 5.7 μM, or an average penalty of only +1.3 kcal mol^{-1}. Even the aptamer in which both strands contained an equal number of randomly distributed 2′–5′ and 3′–5′ linkages exhibited clear FMN binding, with an apparent K_D of 90 μM, which corresponds to a penalty of +2.8 kcal mol^{-1} to the energy of ligand binding. Thus, this aptamer, which was evolved to function as an entirely 3′–5′-linked RNA, exhibited a remarkable tolerance to 2′–5′/3′–5′ backbone heterogeneity.

We then examined the effect of increasing levels of 2′–5′ linkages on the self-cleavage activity of the hammerhead ribozyme. We began by chemically synthesizing two RNA oligonucleotides that would self-assemble into the hammerhead structure, such that one of the oligonucleotides would self-cleave at a specific site. By synthesizing the substrate oligonucleotide with a 5′-fluorescent dye, we were able to monitor the self-cleavage activity of the two-component ribozyme by polyacrylamide gel electrophoresis (PAGE) separation of intact oligonucleotide from cleaved oligonucleotide, with fluorescence detection of the substrate and product oligonucleotides. The two-stranded hammerhead ribozyme with all 3′–5′ linkages self-cleaved to 50% of the maximal extent in 30 minutes, with a maximal extent of cleavage of 81% at 96 hours (Fig. 3b). As before, we chemically synthesized both strands with 10, 25 and 50% of randomly distributed 2′–5′ linkages. Oligonucleotides that contain increasing levels of 2′–5′ linkages resulted in slower self-cleavage and a lower maximal extent of

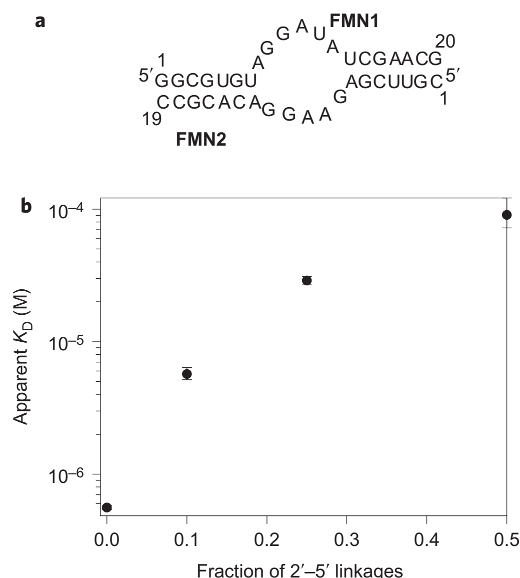

Figure 2 | A pool of FMN aptamers that contains moderate levels of 2′–5′ linkages is observed to retain FMN-binding activity. a, Schematic representation of the FMN aptamer. **b**, Apparent K_D of the FMN aptamer versus fraction of 2′–5′ linkages present in the aptamer pool. Apparent K_D represents the average value for a heterogeneous population of RNAs, in which 2′–5′ linkages are positioned randomly. Error bars are ±1 s.d. from the curve fit.

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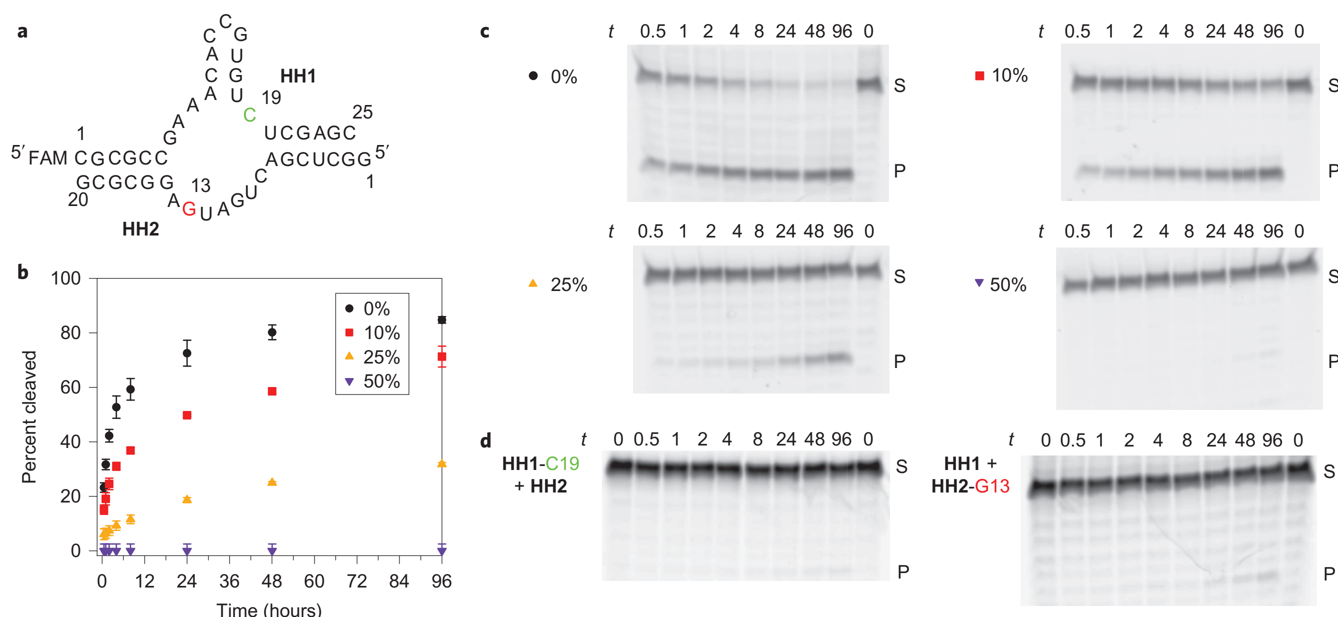

Figure 3 | A pool of hammerhead ribozymes that contains up to 25% randomly distributed 2′-5′ linkages is observed to retain catalytic activity. **a**, Schematic representation of the hammerhead ribozyme construct used in these studies. The ribozyme is assembled from the two oligonucleotides **HH1** and **HH2**. **HH1** contains the self-cleavage site, C19, coloured green, and is labelled with a 5′-fluorophore. **b**, Fraction of hammerhead cleavage versus time for fully 3′-5′-linked and 2′-5′-doped hammerhead ribozymes. Error bars are ± s.e.m. from triplicate measurements, except the 50% 2′-5′-doped strand, which is given as 0% + 2.5%/−0% yield, which represents cleavage yields below the limit of detection. **c**, Representative electropherograms from (**b**). **d**, Electropherograms of point-2′-5′-substituted hammerhead ribozyme reactions. 2′-5′ linkages were incorporated at C19, the site of cleavage in **HH1**, coloured green in Fig. 3a, or at G13 in **HH2**, coloured red in Fig. 3a. S = substrate; P = product.

self-cleavage (Fig. 3b,c). With both strands containing 10, 25 or 50% 2′–5′ linkages, the maximal extent of self-cleavage fell to 69, 34 and <2%, respectively, at 96 hours (Fig. 3b). At the prebiotically plausible 10% level of 2′–5′ linkages, the ribozyme retained 85% of the maximal self-cleavage activity, but the rate of self-cleavage slowed from a $t_{1/2}$ of about 0.5 hours to about two hours. The decreasing maximal extent of self-cleavage with increasing fraction of 2′–5′ linkages suggests that some specific linkages (or combinations of linkages) are critical for catalytic activity, although the slower rate of self-cleavage suggests that other linkages slow, but do not prevent, self-cleavage. Consistent with this, we found that two specific point substitutions of 2′–5′ linkages within the catalytic core of the enzyme result in substantial or total diminution of hammerhead activity (Fig. 3d).

In our pools of RNA with randomly distributed 2′–5′ linkages, a binomial distribution of linkages is probably present in proportion to the level of 2′–5′ monomer employed in the synthesis. Not surprisingly, such a distribution predicts that, even at the 10% doping level, only about 2% of our 39-nucleotide FMN aptamer and only about 1% of the 45-nucleotide hammerhead would have no 2′–5′ linkages. Essentially, no strands at higher doping levels would have no 2′–5′ linkages (see Supplementary Fig. S1a). Clearly, the observed aptamer and ribozyme activities cannot result from complexes that lack, or even contain very few, 2′–5′ linkages. However, given the ability of 2′–5′-linked RNAs to form a duplex, it is more probable that 2′–5′ substitution is only fatal to ribozyme or aptamer function at a few critical sites. Even at highly conserved positions, a 2′–5′ linkage may have little effect if the RNA conformation is determined largely by hydrogen-bonding and stacking interactions between the bases, and the backbone plays a relatively passive role. Given a critical core of five linkages in a hypothetical ribozyme or aptamer, 59% of RNAs at the 10% doping level would have no 2′–5′ linkages, and 24% at the 25% doping level and 3% at the 50% doping level would have no 2′–5′ linkages (see Supplementary Fig. S1b). These numbers are in qualitative agreement with the fraction of maximum cleavage obtained with hammerhead ribozymes at these doping levels (Fig. 3b). In a library of functional RNAs with a low-to-moderate level of randomly distributed 2′–5′ linkages, only a fraction would be inactivated fatally by 2′–5′ linkages at critical residues, consistent with our observations and with previous work on ribozymes that contain point substitutions of these linkages[37,38].

It has been known for over a decade that 2′–5′ linkages destabilize RNA duplexes, based on melting studies of short duplexes that contain 2′–5′ linkages at defined sites[31]. To test the idea that low-to-moderate (10–25%) levels of 2′–5′ linkages would lower the melting temperature of an RNA duplex of sufficient length to exhibit function enough to allow for thermal strand separation and, thus, repeated cycles of non-enzymatic replication, we examined the melting behaviour of longer duplexes that contained 2′–5′ linkages in the presence of 50 mM Mg^{2+} as representative of typical RNA template-copying conditions. A 30-nucleotide duplex that contained four 2′–5′ linkages in each strand exhibited a T_m of 79 °C, although the same sequence in fully 3′–5′-linked strands was not denatured, even at 95 °C (Fig. 4). The duplex containing 2′–5′ linkages exhibits diminished hyperchromism on heating, consistent with the possibility that diminished stacking interactions contribute to the decreased thermal stability of duplexes that contain 2′–5′ linkages.

Discussion

The most important implication of our finding that functional RNAs can tolerate 2′–5′ versus 3′–5′ backbone heterogeneity is that this heterogeneity is not necessarily an undesirable consequence of non-enzymatic template-copying chemistry. Therefore, it is not required that backbone heterogeneity must be eliminated through improvements to the chemistry. Indeed, when combined with the long-standing observation that 2′–5′ linkages lower the melting temperature of RNA duplexes, our observations suggest that the generation of 2′–5′ versus 3′–5′ heterogeneity may have

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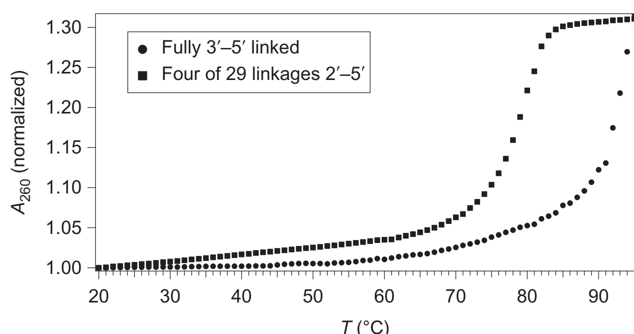

Figure 4 | An RNA duplex containing 14% 2′–5′ linkages is observed to denature at a temperature at least 15 °C lower than that for the corresponding homogeneous 3′–5′-linked duplex, which facilitates thermal strand separation under geochemically plausible conditions. 30-mer RNA duplexes were formed by annealing oligonucleotides **Duplex30-1** and **Duplex30-2**. Strands were fully 3′–5′ linked (filled circles) or contained four 2′–5′ linkages on each strand (filled squares).

facilitated an essential aspect of primordial RNA replication, because it would allow repeated cycles of replication to occur as a result of thermal strand separation. Although a number of environmental factors, such as high pH, low salt or the presence of denaturants, such as urea or formamide, would lower the T_m of long RNA duplexes, these conditions might also interfere with either non-enzymatic RNA replication or with the function of folded RNAs such as ribozymes. Our results suggest that the presence of 10–25% 2′–5′ linkages is a very effective means of lowering the melting temperature of RNA duplexes that is also compatible with both non-enzymatic RNA copying[23] and ribozyme activity. A more speculative extension of this idea derives from the randomness of the incorporation of 2′–5′ linkages during non-enzymatic RNA copying, which implies that different copies of the same sequence would contain 2′–5′ linkages in different locations. Some copies might, by chance, contain most or all 2′–5′ linkages in positions with minimal effects on, for example, ribozyme function. As well-folded structures, however, these copies would be poor templates for subsequent rounds of replication. Other copies would have some 2′–5′ linkages at positions that interfered with the formation of well-folded structures, and these copies would be poor ribozymes but much better replication templates. Thus, the generation of 2′–5′ versus 3′–5′ linkage heterogeneity could result in the formation of a heterogeneous set of copies of every sequence in a protocell, with some being better for function (phenotype) and others being better for replication (genotype). In effect, this structural heterogeneity would result in a primitive and incomplete separation of genotype from phenotype within one polymer system. This new way of looking at chemical RNA replication raises a number of questions and priorities for experimental directions, which we discuss below.

The aptamer and the ribozyme we examined evolved to function as all 3′–5′-linked RNA; therefore, it is unsurprising that they exhibited diminished aptamer affinity and ribozyme activity with increasing incorporation of 2′–5′ linkages in the RNA backbone. A better mechanistic understanding of how functional RNAs that contain 2′–5′ linkages retain function or, conversely, lose function when 2′–5′ linkages are present at critical locations, will emerge ultimately from the comparison of high-resolution structures of native RNAs and RNAs that contain 2′–5′ linkages. It is possible that structures that are even more tolerant of 2′–5′ versus 3′–5′ heterogeneity would be found if evolutionary optimization were to occur within a mixed backbone system. At present, no enzymes are known that provide a means to transcribe, reverse-transcribe or replicate mixed sequence 2′–5′ RNA backbone linkages, although reverse transcriptase can read through isolated 2′–5′ linkages[39]. Recent advances in directed evolution may provide the means to generate enzymes that would enable the *in vitro* directed evolution experiments with mixed backbone RNA populations that would be required to test the above hypothesis[40].

If primordial RNA was, indeed, characterized by considerable 2′–5′ versus 3′–5′ heterogeneity, what selective forces might have contributed to the subsequent evolution of the machinery that enables replication of fully 3′–5′-linked RNA? We proposed previously that the energy required for strand separation could come from transient exposure to high temperatures, possibly as a result of entrainment in the streams of hot water that emanate from hydrothermal vents in surface ponds in geothermally active areas[41,42]. Such an environmental scenario implies a strong selective advantage for the evolution of strand-separating ribozymes that would enable continued replication under more isothermal conditions, and thus enable the colonization of new environmental niches. Once strand-separating ribozymes (for example, helicases or strand-displacing polymerases) had evolved, the requirement for 2′–5′ linkages to lower the duplex melting point could be eliminated. The gradual nature of the effects of changing the regioisomer composition of the RNA backbone on functional RNAs (as measured by ribozyme activity or aptamer K_D) and duplex RNAs (as measured by T_m) suggests that the transition from mixed to homogeneous backbone RNAs could also have occurred gradually, in similar fashion to the gradually increasing incorporation of phospholipids into (proto)cell membranes[43]. Thus, as helicases or polymerases with increasing strand-displacement activity emerge, the advantage of being able to optimize function in structurally homogeneous RNA transcripts could be realized. Additional advantages of an all 3′–5′-linked backbone might include greater stability to hydrolysis in the duplex state[22], and faster and/or more accurate replication[23,37].

The results reported in this paper, along with other recent advances[13], are encouraging with respect to the search for a non-enzymatic RNA replication process. Future effort must focus on removing the remaining barriers to chemical RNA replication, including the slow rate and poor fidelity of non-enzymatic template copying, the difficulty of maintaining substrates in a chemically activated state, replication without added primers, the fast re-annealing of separated strands and the incompatibility of RNA-copying chemistry with protocell membranes. If these problems can be solved, it should be possible to assemble and study self-replicating protocells in a laboratory recapitulation of the emergence of cellular life on the early Earth.

Methods

RNAs were synthesized in-house on an ABI Expedite 8909, or by IDT, Oligos, Etc. or by the Keck Biotechnology Resource Laboratory, using 3′- or 2′-TBDMS phosphoramidites obtained from ChemGenes. Libraries of RNA strands that contained randomly interspersed 2′–5′ linkages were synthesized using mixtures of 3′- or 2′-TBDMS phosphoramidites at the specified stoichiometry. We synthesized a GpG dinucleotide using a 1:1 mixture of these phosphoramidites, and used NMR analysis of the resulting mixture of products to show that the 3′- and the 2′-TBDMS phosphoramidites exhibited essentially identical coupling efficiencies (see Supplementary Fig. S2). Oligonucleotides were purified either chromatographically (cartridge or HPLC) or by PAGE. Oligonucleotides synthesized in-house, by Oligos, Etc. or by Keck were analysed by high-resolution LC/MS; in all cases, the deconvoluted monoisotopic mass was found to be within $<$20 ppm of the nominal value (see Supplementary Table S1).

Functional RNAs comprised the following sequences: FMN aptamer, **FMN1** (r(GGC GUG UAG GAU AUC GAA CG)) and **FMN2** (r(CGU UCG UGA AGA AGG ACA CGC C)); hammerhead ribozymes, **HH1** (r(FAM-CG CGC CGA AAC ACC GUG UCU CGA GC)) and **HH2** (r(GGC UCG ACU GAU GAG GCG CG)). The hammerhead strands with point substitutions, **HH1-C19** (r(FAM-CG CGC CGA AAC ACC GUG UC*U CGA GC)) and **HH2-G13** (r(GGC UCG ACU GAU G*AG GCG CG)), contained a single 2′–5′ linkage, denoted by an asterisk. The 30mer duplex used in thermal denaturation comprised the following sequences: **Duplex30-1** (r(GAA G*UC AGU AC*G CCA UUC* GAG AUC CUC* AUG)) and **Duplex30-2** (r(CAU G*AG GAU C*UC GAA UGG* CGU ACU GAC* UUC)). These sequences were synthesized in two versions: an all 3′–5′-linked form and a version in which the four linkages denoted with asterisks were 2′–5′.

Binding-constant studies for the FMN aptamer–FMN complex were performed on a Molecular Devices SpectraMax EM in a 384-well plate by preparing 16 samples

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in 100 mM sodium HEPES, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, with a fixed 1 μM concentration of ligand and 0–50 μM **FMN1–FMN2** aptamer complex. The sample was held at 8 °C, excited from the bottom at 444 nm and the emission was read at 538 nm. The fluorescence emission was background corrected by subtracting the average value of neighbouring empty wells, which were essentially the same as non-neighbouring cells; this indicates neighbouring wells did not interact significantly (see Supplementary Fig. S3). The resulting fluorescence values were fit to a nonlinear single binding site model in Igor Pro (Wavemetrics), by which they were well described[44].

For hammerhead ribozyme reactions, ribozymes composed of oligonucleotides **HH1** and **HH2** that contained the specified fraction of 2′–5′ linkages were incubated at room temperature in 50 mM sodium HEPES, pH 7.5, 10 mM $MgCl_2$ and 100 μM EDTA. Reactions were quenched by addition to 8 M urea and 250 mM EDTA, separated by denaturing PAGE, imaged by fluorescence scanning (Typhoon) and quantitated by integration (ImageQuant).

Thermal denaturation was performed using a 1 mm quartz Starna cell in an Agilent Cary 60 ultraviolet spectrophotometer with a Quantum Northwest LC 600 temperature controller. The temperature reported is the block temperature. The second heating trace after an initial annealing heat/cool cycle is shown in Fig. 4. The 30-mer melts were 5 μM in **Duplex30-1** and **Duplex30-2** in 100 mM sodium HEPES, pH 7.5, 50 mM $MgCl_2$, 100 mM NaCl, 200 mM sodium citrate and 1 mM sodium EDTA. For both melts, at each 1 °C temperature step the system was allowed to equilibrate at the setpoint until it was within 0.25 °C of the setpoint for 30 seconds, at which point a spectrum was collected. This resulted in a temperature increase of about 0.45 °C min^{-1}.

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Author contributions

All authors contributed to the design of the experiments and to writing the paper. Experiments were conducted by A.E.E. and M.W.P.

Additional information

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Competing financial interests

The authors declare no competing financial interests.

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