Title:

Rolling Circle RNA Synthesis Catalysed by RNA

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Abstract

RNA-catalysed RNA replication is widely considered a key step in the emergence of life's first genetic system. However, RNA replication can be impeded by the extraordinary stability of duplex RNA products, which must be dissociated for re-initiation of the next replication cycle. Here we have explored rolling circle synthesis (RCS) as a potential solution to this strand separation problem. We observe sustained RCS by a triplet polymerase ribozyme beyond full-length circle synthesis with strand displacement yielding concatemeric RNA products. Furthermore, we show RCS of a circular Hammerhead ribozyme capable of self-cleavage and re-circularisation. Thus, all steps of a viroid-like RNA replication pathway can be catalysed by RNA alone. Finally, we explore potential RCS mechanisms by molecular dynamics simulations, which indicate a progressive build-up of conformational strain upon RCS with destabilisation of nascent strand 5'- and 3'-ends. Our results have implications for the emergence of RNA replication and for understanding the potential of RNA to support complex genetic processes.

Key words: origin of life, rolling circle, rolling circle synthesis, circular RNA, circular nucleic acids, ribozymes, hammerhead, persistence length, RNA molecular dynamics simulation.

Introduction

The versatility of RNA functions underpins hypotheses regarding the origin and early evolution of life. Such hypotheses of an “RNA world” – a primordial biology centred on RNA as the main biomolecule - are in accord with the essential role of RNA catalysis in present day biology (Cech, 2000; Goldman and Kacar, 2021; Nissen et al., 2000; Wilkinson et al., 2020) and the discovery of multiple prebiotic synthetic pathways to several of the RNA (and DNA) nucleotides (Becker et al., 2019; Kim et al., 2020; Patel et al., 2015; Powner et al., 2009; Xu et al., 2020). In addition, progress in both non-enzymatic (Deck et al., 2011; Hassenkam et al., 2020; Prywes et al., 2016; Rajamani et al., 2008; Sosson et al., 2019; Šponer et al., 2021; Wachowius and Holliger, 2019; Zhang et al., 2020; Zhou et al., 2020) and RNA-catalysed polymerization of RNA and some of its analogues (Attwater et al., 2018, 2013; Cojocaru and Unrau, 2021; Ekland and Bartel, 1996; Horning and Joyce, 2016; Johnston et al., 2001; Mutschler et al., 2018; Shechner et al., 2009; Tagami et al., 2017; Tjhung et al., 2020) is beginning to map out a plausible path to RNA self-replication; a cornerstone of the RNA world hypothesis.

RNA *in vitro* evolution and engineering have led to the discovery of RNA polymerase ribozymes (RPRs) able perform templated RNA synthesis of up to ~200 nucleotides (nt) (Attwater et al., 2013), synthesizing active ribozymes including the catalytic class I ligase core (Horning and Joyce, 2016; Tjhung et al., 2020) at the heart of the most efficient RPRs, as well as initiate processive RNA synthesis using a mechanism with analogies to sigma-dependent transcription initiation (Cojocaru and Unrau, 2021). A RPR capable of utilizing trinucleotide triphosphates (triplets) as substrates (a triplet polymerase ribozyme (TPR)) has been shown to display an enhanced capacity to copy highly structured RNA templates including segments of its own sequence (Attwater et al., 2018).

Nevertheless, there remain a number of fundamental obstacles to be overcome before an autonomous self-replication system can be established. A central problem among these is the so called “strand separation problem”, a form of product inhibition due to the accumulation of highly stable dead-end RNA duplexes, which cannot be dissociated (efficiently) under replication conditions (Le Vay and Mutschler, 2019). The strand separation problem has been overcome by PCR-like thermocycling (or thermophoresis) (Horning and Joyce, 2016; Salditt et al., 2020), but this approach may be limited to short RNA oligomers (even in the presence of high concentrations of denaturing agents) as the melting temperatures of longer RNA duplexes approach or even exceed the boiling point of water (Freier et al., 1986; Szostak, 2012).

While RNA duplexes occur by necessity as intermediates of RNA replication, the extent of the strand separation problem can be modulated by genome topology. Circular rather than linear genomes are widespread in biology including eukaryotes, prokaryotes and viruses (Møller et al., 2018; Moss et al., 2020; Shulman and Davidson, 2017). Circular RNAs (circRNAs) are found as products of RNA splicing (Kristensen et al., 2019) and RNA-based self-circularization is known in multiple ribozymes (Hieronymus and Müller, 2019; Lasda and Parker, 2014; Petkovic and Müller, 2015). Continuous templated RNA synthesis on circular templates (Rolling Circle Synthesis (RCS)) is also widespread and found in the replication of the RNA genomes in some viruses and in viroids. Indeed, viroid RNA replication has been proposed to resemble an ancient mechanisms for replication (Diener, 1989; Flores et al., 2014).

In an idealized RCS mechanism, both strand invasion and displacement processes are isoenergetic and coordinated to nascent strand extension (Blanco et al., 1989; Daubendiek et al., 1995), with rotation of the single-stranded RNA (ssRNA) alleviating the build-up of topological tension (Kuhn et al., 2002). Thus, RCS is a potentially open-ended process leading to the synthesis of single-stranded multiple repeat products (concatemers) with an internally energized strand displacement (Tupper and Higgs, 2021). RCS as a replication mode has therefore potentially unique properties with regards to the strand separation problem. Specifically in the context of triplet-based RNA replication on a circular template, duplex dissociation and strand separation may in principle be driven by trinucleotide (triplet) hybridization and ligation, leading to extension of the nascent strand 3'-end and an equal displacement of the 5'-end in triplet increments (Figure 1A). Triplet binding to the template strand and dissociation of an equal trinucleotide stretch from the 5'-end are both equilibrium processes and nearly isoenergetic. However, extension (i.e. ligation of the bound triplet to the growing 3'-end) is an irreversible step. Thus, in this scenario RCS would be expected to proceed in ratchet-like fashion with strand displacement driven by triphosphate hydrolysis and triplet ligation.

Here we have explored triplet-based RCS of small circular RNA (scRNA) templates as a potential solution to the strand separation problem in RNA-catalysed RNA replication. We show that RCS can be catalysed by the triplet polymerase ribozyme (TPR) (Attwater et al., 2018). We show that the TPR is able to perform continuous templated extension of circular RNA templates beyond full-length circle synthesis with strand displacement yielding concatemeric RNA products. We also investigated the mechanistic basis for RCS and strand displacement by molecular dynamics (MD) simulations of scRNA in explicit solvent. Finally, we consider the potential of a full viroid-like replication cycle catalysed by RNA alone by

123 design and synthesis of a circular Hammerhead ribozyme capable of both product cleavage
124 and self-circularization.

Results

RNA-catalysed primer extension using small circular RNA templates.

We first set out to investigate whether templated RNA synthesis on small circular RNAs (scRNAs) as templates could be catalysed by an RNA catalyst. To extend the RNA nascent strand beyond the full circle and initiate RCS requires duplex invasion and displacement of the original RNA primer and product strand. However, most RPRs are inhibited by duplex RNA both in the form of template secondary structures and as linear duplex RNA. We therefore explored the potential of a recently described TPR (Attwater et al., 2018), which is able to utilize trinucleotide triphosphates (triplets (^{ppp}NNN)) as substrates for polymerization. Due to increased binding of the triplets to the template (compared e.g. to the canonical mononucleotide triphosphates (NTPs)), triplets are able to invade and cooperatively “open up” template secondary structures for replication (Attwater et al., 2018). We hypothesized that this ability might also promote the continuous invasion and displacement of the nascent strand 5'-end and facilitate the RCS mechanism (Figure 1A).

As described previously, RNA synthesis by the TPR is most efficient in the eutectic phase of water ice, due its beneficial reaction conditions for ribozyme catalysis (Attwater et al., 2018). Specifically, eutectic ice phases aid TPR activity by the reduced degree of RNA hydrolysis under low temperature conditions, reduced water activity, and the high concentrations of reactants (ribozyme, scRNA template, triplet substrates and Mg²⁺ ions) present in the eutectic brine phase that arise by excluding solutes from growing ice crystals and remains liquid at subzero temperatures (Attwater et al., 2010). Thus, all RCS experiments were carried out under eutectic conditions.

We prepared scRNA templates (34-58 nt in length) by *in vitro* transcription and ligation. Circularity of the scRNA templates were verified based on gel mobility and resistance to exonuclease (exoT) degradation in contrast to the linear, non-ligated counterparts (Figure 1B, Figure 1 - Figure supplement 1 / sequences for all oligonucleotides in Supplementary file 1). We first investigated primer extensions using a single triplet (^{ppp}GAA) on the scRNA template as this provides an even banding pattern of incorporation. This facilitates analysis and allows primer extension efficiencies of linear and circular templates to be more readily compared (Figure 1C). Primer extension experiments using a purified 36 nt scRNA as template resulted in full-length extension around the circle (Figure 1D), but with reduced efficiency compared to a linear RNA template. Furthermore, we observed a periodic banding pattern of triplet extension efficiency matching the helical pitch of double-stranded RNA (dsRNA) (11.3 base pairs (bp) / turn (Bhattacharyya et al., 1990)) (Figure 1E). Presumably, triplet junctions located on the inside of the scRNA ring are less easily accessible and

therefore less efficiently ligated than in linear RNA, which is freely accessible from all sides (Figure 1F). This may explain both the observed periodicity and reduced synthetic efficiency of RNA synthesis by the TPR on scRNAs.

Figure 1) Primer extension on circular RNA templates. A) Schematic illustration of rolling circle synthesis (RCS). Red product RNA strand is extended by a triplet at the 3'-end while three base pairs dissociate at the 5'-end keeping the total hybridization energy constant. Topological relaxation is allowed by rotation of the single stranded part of the circular template (swivelling arrow). B) Linear or circularized RNA is treated with or without endo- or exonucleases (RNase A / T1 mix or Exonuclease T (ExoT)). Only circular RNA is resistant to ExoT digestion. C) Schematic representation of primer triplet extension on a linear or circular RNA template and of the TPR hetero-dimer comprising the catalytic subunit (5TU (green)) and the non-catalytic subunit (t1 (red)) (Attwater et al., 2018) (TPR sequence: Supplementary file 1). D) PAGE gel of TPR primer extension, P9 (5'-FAM-GAAGAAGAA) is the unextended primer, bands 1 - 9 denote extension of P9 by +1 - +9 triplets (full length). RNA template used was sc12GAA-p (36 nt, 12 repeat UUC template). E) Extension efficiency of formation of bands 1 - 9 in D (see Materials and Methods) is plotted against triplet position. F) Schematic model of the sc12GAA-p illustrating the different accessibility of in- or outside facing triplet junctions on the scRNA template (blue), with P9 primer (red) and the product strand (light grey). Original gel images and numerical values are supplied in Figure 1-source data 1.

Despite the reduced extension efficiency in scRNA, we obtained full circle extension products for multiple templates (34 to 58 nt in size, Figure 2A, B) with a clear trend towards increasing mean extension efficiency for circular templates with increasing size predicting parity with the linear template at around 120 nt (Figure 2C). Note, in these experiments extension beyond full circle was not intended or possible (lane 1 to 6 in Figure 2B) as the specific triplet substrates needed for displacing the primer were not present in the reaction.

Having established full-length synthesis on scRNA templates, we next tested if primer extension could proceed beyond full circle requiring duplex invasion and displacement of the primer / product strand. We first tested this using primer P10+3, comprising a 5' extension of three GAA repeats, thus covering the last three UUC triplet binding sites on the circular template (Figure 2B top right). We observed an extension of up to +3 triplets (+9) above the full circle mark (Figure 2B lane 7-12), indicating displacement of the primer 5'-end upon incorporation of three additional ^{ppp}GAA triplets. This indicated that synthesis beyond full circle including strand displacement is possible on scRNA templates, boding well for the implementation of RCS. To that effect, we next optimized buffer and extension conditions for more efficient extension above the full circle mark (Figure 2 - Figure supplement 1). Interestingly, greater dilution of reaction mixtures prior to freezing resulted in more efficient strand displacement. While greater pre-freezing dilution does not alter the final solute concentrations within the eutectic phase (Attwater et al., 2010), it increases the eutectic phase / ice interface area. This suggest that strand invasion may be aided by surface effects, as previously suggested for RNA refolding (Mutschler et al., 2015). Under these optimized buffer and extension conditions, we observed progressive accumulation of progressively longer RCS products, over prolonged reaction times (up to 6 weeks) (Figure 2D) with reaction speed decreasing after ca. 4 weeks incubation, indicating continued RCS over extended periods of time (Figure 2E, F).

Figure 2) Full-length and beyond full-length RNA-catalysed RNA synthesis on circular RNA templates. A) Product strand of primer extension experiments with primer P10 (red) and eight triplet scRNA template strand. Potential beyond full-length circle synthesis triplets are shaded opaque. B) Various scRNA template sizes allow full-length primer extension as indicated (with 8 triplet sites) (grey), GAA triplets (black) and primers P10 (5'-FAM-CUGCCAACCG) or P10 + 3 (5'-FAM-CUGCCAACCG-GAAGAAGAA) (red). PAGE of primer extensions (under standard conditions) with full-length synthesis for different scRNA templates (34 – 58, termed scGAA8-16, supplementary file 1) marked by a black line. C) Mean extension efficiency plotted as a function of scRNA size calculated from extension experiments including B) (Error-bars indicate standard deviation, $n = 5$), with mean extension efficiency for a linear RNA template (red dashed line). D) PAGE of time-course of primer extension of primer P9 on scRNA template, sc12GAA-p (optimized conditions). Full length circle synthesis is marked by a dashed black line (after +9). Bands +10 and beyond (see enhanced contrast section) indicate beyond full-length synthesis. E, F) Mean extension efficiency (from D) plotted against time (E) or triplet position (F), showing the respective amounts of product at full-length (black) and beyond full-length circle (red) synthesis as well as the drop in efficiency at full length, which recovers once beyond full-length synthesis is initiated. V_{ext} and V_{inv} denotes the calculated velocity of formation of bands 9 and 10, respectively. Original gel images and numerical values are supplied in Figure 2-source data 1.

Molecular dynamics simulations of 36 nt scRNA

To better understand the structural and topological constraints of RCS on scRNAs, we performed atomistic molecular dynamics (MD) simulations over 400 ns of the different RCS stages, comprising the starting scRNA template as circular single-stranded RNA (ssRNA) and scRNA with a progressively extended double-stranded RNA (dsRNA) segments (Figure 3). For simplicity, a 36 nt circular RNA sequence of (UUC)₁₂ was chosen as a template strand (similar to the scRNA template (sc12GAA-p) in Figure 1, 2D) for direct comparison with the experimental system. The complementary strand comprising GAA triplets starting from 9 bp dsRNA (corresponding to binding of primer P9) was extended from 18 - 30 bp (after +3 triplet incorporation) in triplet increments of dsRNA (corresponding to extension products of bands +3, +4, +5, +6 and +7 (see gel in Figure 1D), using the most representative structure of the previous simulation as a starting point for the next one.

The simulation trajectories revealed the high energy barrier of dsRNA for bending and accommodating a circular shape (Figure 3A). Instead, we observe that, as dsRNA is elongated, the remaining ssRNA segment of the scRNA becomes increasingly extended. As the dsRNA part reaches 27 bp (corresponding to band 6 in Figure 1D), the ssRNA segment was fully extended and torsional strain was relieved by dissociation (“peeling off”) of the dsRNA 5'- and 3'-ends rather than by bending or the introduction of kinks into the dsRNA segment (Figure 3B). Subsequently, multiple peeling off and rebinding events were observed during the trajectories indicating that the dynamics of this process are fast (Video 1 and 2 and Figure 3 - Figure supplement 1 and 2).

In the experimental data, we also observed an inhibitory effect for insertion of the final triplets (+8, +9, and +10 (beyond full length) / extension to 33, 36 and 39 nt of RNA in Figure

2D) into the corresponding scRNA template. This may indeed reflect the onset of the 3'- and 5'-end destabilization observed in the MD simulations (Figure 3), which would likely attenuate primer extension by the ribozyme. Note however that the extension efficiency recovered beyond full length (+11 / extension to 41 nt, Figure 2F), although at lower speed (Figure 2E).

As a control for the observed dsRNA end destabilization mechanism, we also ran a MD simulation of a linear RNA molecule containing four consecutive triplets with a nick between two of them, but observed neither base opening nor dissociation at either 5'- nor 3'- strand ends (Figure 3 - Figure supplement 3). Groove dimensions and local helical parameters (roll, twist and slide) for the RCS simulations on circular RNA did not show any major adjustment compared with the linear RNA control (Figure 3 - Figure supplement 4). We observed an oscillation of high / low values of bending along the molecule in phase with RNA-turn periodicity in an attempt to create an overall curvature (Velasco-Berrelleza et al., 2020), although with moderate success (~60° on an arc length of 30 bp of dsRNA) and no formation of kinks, internal loops or other disruption of the canonical A-form typical of the RNA duplex (Figure 3 - Figure supplement 4).

To mirror the experimental eutectic phase conditions, simulations were run at relatively high Mg^{2+} concentrations (100 mM) and compared with the presence of monovalent ions like K^+ (200 mM) and high concentration of Mg^{2+} (500 mM), but simulations did not show any major differences in terms of dsRNA strand dissociation or bending (Figure 3 - Figure supplement 3, 4). However, Mg^{2+} ions - compared to K^+ - appear to interact more strongly with different parts of the RNA and, consequently, may increase the probability of distorted conformations facilitating the exposition of nucleobases at the 5'- and 3'-ends. In contrast, K^+ ions are mainly positioned as counterions along the major and minor groove, allowing the bases to orient towards the inside of the dsRNA helix for base-pairing interactions (Figure 3C and Figure 3 - Figure supplement 5). The role of Mg^{2+} in the stabilization of complex RNA folding has been observed repeatedly in several structures (Sponer et al., 2018), like the ribosome (Klein et al., 2004) and the Hepatitis delta virus ribozyme (Nakano et al., 2001). However, increasing $MgCl_2$ concentration to 500 mM does not seem to bring extra stabilization, as the system appears to be saturated already at 100 mM Mg^{2+} (Figure 3 - Figure supplement 5, 6).

In summary, our simulations support the notion that progressive RNA synthesis on a small circular RNA (scRNA) template (in the presence of Mg^{2+} ions) leads to increased dynamics of nucleobase exposure, RNA duplex destabilization and 5'- and 3'-end melting. The simulations also clearly illustrate the implausibility of a small circular fully dsRNA molecule

(as schematically illustrated in Figure 1E), due to the prohibitive energetic cost of dsRNA bending. Instead, the system appears to relieve internal strain by extending the ssRNA segment of the circle (partially shielding the dsRNA segment) and peeling of both dsRNA 5'- and 3'-ends (Figure 3), consistent with the helical period of triplet extension observed (Figure 1, 2) (with ligation junctions facing into the ssRNA centre being less accessible) and the observed reduction in RCS efficiency. Dynamic destabilization of dsRNA 5'-ends clearly has the potential to facilitate strand displacement during RNA replication on a scRNA template (and may aid continuous RCS), but at the same time may reduce the efficiency of primer extension and triplet incorporation by reducing the availability of the primer 3'-end and the downstream template bases. These effects would be predicted to manifest themselves in RNA circles up to 200 bp as suggested by RNA persistence length (Abels et al., 2005).

Figure 3) Molecular dynamics simulation of small circular RNA. A) Main conformations (and zoom-in to relevant regions (squares)) observed from simulations in 100 mM $MgCl_2$ on scRNA exploring consecutive states of primer extension, from 9 to 30 bp dsRNA with pyrimidine (template) strand $(UUC)_{12}$ (khaki), purine (product) strand (GAA) (light blue), 5'-end and unpaired bases (dark blue) and 3'-end unpaired bases (purple) and matching melted bases from the template strand (dark green (5'-end) / light green (3'-end)). B) Percentage of frames from the last 100 ns of the simulations presenting canonical hydrogen bond pairing for each bp. C) Counterion-density maps (in red) around RNA molecules that show an occupancy ~ 10 times or greater than the bulk concentration.

Templated rolling circle RNA synthesis

Having validated RNA synthesis on scRNA templates (Figure 1, 2), we next sought to show RCS beyond a single full-length circle synthesis involving displacement of the primer / nascent strand. To this end, we designed barcoded templates that would allow us to distinguish RNA synthesis products arising from template-instructed RCS from those arising from non-templated terminal transferase (TT) activity of the TPR by sequencing. The barcoded scRNA templates (termed A-D) were prepared either as circular or linear RNAs comprising different internal triplet “barcodes” of variable GC-content (at position 3, 6 and 9) for individual identification (Figure 4A and Figure 4 - Figure supplement 1). We performed one-pot primer extension experiments, in which all four templates (either A-D linear or A-D circular) were mixed in equal proportions. After extension, products were separated by gel electrophoresis and the gel sections above full-length extension products were excised and their RNA content recovered, and sequenced (Figure 4 - Figure supplement 1). Diagram in Figure 4B represents (in %) which triplets were identified at the noted positions. Boxes mark expected triplet according to the template sequence.

Analysis of the sequencing products from the one-pot reaction showed template-dependent high-fidelity RNA synthesis up to full length (position 9) for all templates (linear and circular) (Figure 4B). Further, all templates yielded longer than full-length products indicative of continued RNA synthesis by the TPR beyond full length (positions >9). However, the fidelity dropped after full length was reached, indicative of significant non-templated terminal transferase-like (TT) activity in this regime (Figure 4B). For example, the average fidelity for insertion of the expected triplet (^{ppp}GAA) for position 10 (full length +1) for circular templates was 10.9% whereas for position 9 (full length) it was 89.9%. For linear templates, the fidelity for full length +1 dropped to 0.7% compared to full length 78.8%. Note, that fidelity at full length +1 dropped much more for linear than for circular templates. For this reason, the probability of a product extending to longer than full length (positions 10-12) with the correct sequence was 10-100 fold higher for circular compared to linear RNA templates (Figure 4C). A few events of blunt-end ligation with other template / product strands (see e.g. position 15 for linear template C and D) (Figure 4B) were also observed for linear templates.

On all circular templates (with the exception of template B, where too few reads were obtained) extension beyond full length (while containing a significant TT component) continued to insert the barcode triplets correctly, indicating continuous RCS at least up to position 18 (63 nt, more than 1.5 times full length-circle synthesis on the scRNA template). Control experiments, with individually incubated templates (in contrast to the one-pot experiments) mixed after gel purification, showed essentially identical results (Figure 4 -

Figure supplement 2A). Interestingly, non-diluted samples had a decreased fidelity at position 10 (the point of strand invasion) compared to diluted samples (Figure 4 - Figure supplement 2B) suggesting that dilution appears to aid not only extension efficiency (Figure 2 - Figure supplement 1D), but also strand invasion fidelity and continued templated synthesis. In summary, these results are consistent with RNA-catalysed RCS on scRNA templates beyond the full circle.

Figure 4) RNA-catalysed RNA synthesis beyond full length for circular templates. A) Product strands of primer extension experiments with linear and scRNA templates A-D with primer P9₁. Opaque sequence illustrate potential beyond full-length synthesis on scRNA. Barcode triplets at positions 3 (A/U rich) (cyan), 6 (mix) (blue), 9 (G/C rich) (purple) allow identification of product RNAs. Barcode triplet at position 12 is the final triplet of primer P9₁ and at position 15 is the same as that of position 3 but after beyond full-length circle synthesis on the scRNA template. B) Fidelity heat-map of the sequences derived from the one-pot experiments with linear (left) or circular (right) templates. Red colour indicates high prevalence of a given triplet (vertical axis) at the position noted (3-18). *n*: denotes the number of sequence reads (3×10^5) used to calculate the fidelity for each triplet at the given position. Transparent grey boxes cover positions with $n \leq 5$. C) Plot shows ratio (fold difference) of the probability of a product of reaching positions 4-12 on circular compared to linear templates. Fold difference was calculated based on fidelity data presented in B). D) Model illustrating (1.) beyond full-length extension on a circular template (templated RCS) and (2.) on a linear template (non-templated). Full analysis of the data in Figure 4B is supplied in Figure 4-source data 1.

Multiple repeat rolling circle products

Next, we sought to test if RCS efficiency could be increased by double priming on the circular RNA template, an approach known as branched RCS (Berr and Schubert, 2006). Indeed, we observed a higher degree of RCS with strand displacement with the 36 nt scRNA template termed sc8211 having two identical primer sites leading to two different products being formed (product I or II) (Figure 5A, B). In order to test the primer site functionality individually, we used different triplet combinations (Figure 5B). When only the ^{ppp}GAA triplet was present, primers were extended only by two triplets as expected (lane 1 in Figure 5B) (with a small amount of non-templated TT incorporation of a 3rd triplet). When ^{ppp}GAA and ^{ppp}AUA or ^{ppp}GCG triplets were added, respectively (lane 2 or 3 in Figure 5B), products extended up to 5 triplet-incorporations with extension stopping at triplet 6 (coding for CUG) showing that both primer sites were functioning. Finally, when all triplets (^{ppp}GAA, ^{ppp}AUA, ^{ppp}GCG, ^{ppp}CUG) were present, extension continued to beyond full circle (positions ≥ 10) (Figure 5B) and bands corresponding to extension products exceeding two times full-length circle synthesis ($>$ triplet 21 (63nt)) of replication were observed (Figure 5C).

Figure 5) RNA-catalysed branched RCS. A) Product strand of primer extension experiments with scRNA template containing two priming sites (I and II) for primer P9₁. Depending on the priming site two different products will be made (I or II). B, C) Scheme and PAGE of primer extension experiments with only the noted triplets added with C) long electrophoretic separation to achieve optimal resolution of long products. Cycling (Cyc.) indicates that the samples had been exposed to four thermal and freeze-thaw cycles (80°C 2 min, 17°C 10 min, -70°C 5-15 min, -7°C 7 days) leading to increased efficiency. D) Sequencing of longer than full length branched RCS products on the double primer site scRNA (without cycling). Products I and II both reaching almost three full rounds of replication of the circular RNA template (up to 96 nt, 32 triplet incorporations). Original gel images and full analysis of the data in Figure 5D are supplied in Figure 5-source data 1.

Sequencing of the long, branched RCS RNA products (excised from gel band corresponding to ≥ 15 triplets, Figure 5 - Figure supplement 1) identified a range of long reads (from both products I and II) including many reads of the product with 15 correct triplet incorporations (Figure 5E) representing ~ 1.5 times full-length circle synthesis ($n: 7 \times 10^3$ and 1×10^5 reads of Product I and II, respectively). However, much longer sequences were present in decreasing numbers of reads, with the longest products comprising 29 correct triplet incorporations (96 nt) ($n=2$) representing RCS of >2.5 times full-length circle synthesis and the longest reported product synthesised by the TPR. Thus, RNA-catalysed RCS has the potential to yield extended RNA concatemer products under isothermal conditions. Freeze-thaw (FT) cycles have been previously shown to enhance ribozyme activity by effecting RNA refolding (Mutschler et al., 2015) and indeed inclusion of 4 FT cycles lead to more efficient production of longer RCS RNA products (Figure 5B and C). In summary, isothermal branched RCS yields long concatemeric products comprising >2.5 tandem repeats of the scRNA template with RCS efficiency further enhanced by FT cycling.

Proto-viroid like self-circularizing ribozyme

A number of biological systems including viroids use an RCS strategy for genome replication. However, RCS synthesis of RNA concatemers is only one part of the viroid replication cycle, which also involves resection (i.e. cleavage) of the concatemer into individual units and circularisation of unit length RNAs by ligation to recreate the original circular RNA genome. As both RNA cleavage and RNA ligation can be efficiently catalysed by RNA, we sought to investigate, if a viroid-like replication cycle might be catalysed by RNA alone. To this end, we designed a proto-viroid RNA comprising a 39 nt scRNA template encoding a designed micro-hammerhead ribozyme (μHHz) as well as its substrate for cleavage (Figure 6A). We envisaged a viroid-like replication cycle (schematically illustrated in Figure 6B) comprising primer extension on a circular template (step 1) reaching full-length circle synthesis and beyond (RCA, step 2) resulting in formation of the μHHz and its 3'-end substrate. When separated from its template, the μHHz exists in (at least) two conformations (step 4) of which one is the active Hammerhead ribozyme cleaving the 3'-end substrate yielding a 2',3'-cyclic phosphate ($>p$) (step 5). Finally, the ribozyme re-ligates (chemical mechanism illustrated in

Figure 6B) to form a circular product strand (step 6 and 7). The μ HHz could be synthesized by the TPR (Figure 6C). Furthermore, the μ HHz could catalyse both self-cleavage (forming a 2',3'-cyclic phosphate (>p)) and re-ligation leading to circularization (under RCS reaction conditions at -7°C in eutectic ice) (Figure 6D, lane 2). A similar equilibrium between cleavage and ligation in eutectic ice had previously been observed for the unrelated hairpin ribozyme (Mutschler et al., 2015).

Figure 6) Steps of a viroid-like replication cycle catalysed by RNA alone. A) Illustration of the μ HHz (-) and (+) strand. B) Schematic illustration of the RNA-catalysed viroid-like replication with steps comprising RNA-catalysed combined RCS (steps 1-3), resection (step 4, 5) and self-circularization (steps 6, 7). Panel between step 5 and 6 illustrates the presumed chemical structures of the transition state of cleavage and re-ligation between the μ HHz 3'- and 5'-ends. Blue arrow denotes the re-ligation reaction by bond formation with the 5'-nucleotide (forming the circle) and transesterification of the 2,3-cyclic phosphate bond (>p). Red arrows denote cleavage of the phosphate diester backbone (cleaving the circle) and formation of the 2',3'-cyclic phosphate (>p). X and Y denotes acids/bases involved in the transesterification reaction C) PAGE gel showing primer extension of RCS synthesis of the μ HHz with substrate overhang to allow self-cleavage. scHHz temp was used as template and PHHz was used as primer (Pri) D) PAGE gel showing cleavage and circularization of a μ HHz, but only when incubated at -7 °C, allowing eutectic phase to form, and with a free 5'-OH, needed for circularization, but not for cleavage. Original gel images are supplied in Figure 6-source data 1.

When the μ HHz had been 5'-phosphorylated (Figure 6D, lane 3) only cleavage but no circularization was seen, as phosphorylation blocks the 5'-hydroxyl required as the nucleophile for re-ligation (see steps 5 and 6, Figure 6B). To the best of our knowledge, the μ HHz is the smallest (39 nt) self-cleaving and -circularizing RNA system reported to date and the first-time self-circularization has been shown in a Hammerhead ribozyme. Kinetic analysis of the cleavage and circularization reaction show a slow but accumulating amount of cleavage product as a function of time (black points in Figure 6 - Figure supplement 1A). Analysing the ratio between linear (cleaved) and circular (ligated) products (Figure 6 - Figure supplement 1) indicated that the proportion of circle was initially very high (approx. 40% after 12 h). Based on this, it is likely that all or most μ HHz molecules are transiently circular at some point immediately after cleavage, but become progressively trapped in a state unable to re-ligate, most likely due to hydrolysis of the >p or misfolding. While these steps only validate half of a full replication cycle (formation of a (+)-strand scRNA from a (-)-strand scRNA template), these results outline the potential for a full viroid-like rolling circle RNA replication cycle based on RNA-catalysis alone.

Discussion

Viroids are transcriptional parasites composed entirely of a circular RNA genome and are considered the simplest infectious pathogens known in nature. They lack protein coding regions in their genome, and can be completely replicated in ribosome-free conditions (Daròs et al., 1994; Diener, 2003; Fadda et al., 2003; Flores et al., 2009). They can comprise a circular RNA genome of as little as ~300 nt in e.g. *Avsunviroidae* encoding a Hammerhead ribozyme responsible for maturation by resecting the replicated RNA genome (Flores et al., 2000). The resected viroid genome is then ligated (circularized) by a host protein (e.g. tRNA ligase) (Nohales et al., 2012). Due to the simplicity of this replication strategy, viroids have been suggested to represent possible “relics” from a primordial RNA-based biology (Diener, 1989; Flores et al., 2014). Indeed, circular RNA genomes would present a number of potential advantages for prebiotic RNA replication, including increased stability by end protection (Litke and Jaffrey, 2019) and a reduced requirement for specific primer oligonucleotides to sustain replication (Attwater et al., 2018; Szostak, 2012). A circular RNA genome also resolves the end replication problem, i.e. the potential loss of genetic information from incomplete replication in linear genomes. Circular RNA structures can self-assemble from RNA mononucleotides through wet-dry cycling (Hassenkam et al., 2020) providing potential initiation points for primordial RNA replication. Thus, Viroid-like replication systems are plausible candidates to have emerged as the simplest genetic systems.

Here we have explored to what extent such a potentially prebiotic replication strategy can be carried out by RNA alone. Our data shows that RNA can indeed perform rolling circle synthesis (RCS) on small circular RNA (scRNA) templates yielding concatemeric RNA products, which can be processed (i.e. resected) and re-circularized by an encoded ribozyme in a scheme reminiscent of viroid replication. Thus, one half of full a viroid replication cycle ((-)-strand replication leading to a self-circularizing (+)-strand) can be carried out by just two ribozymes. Completing a full viroid replication cycle would then require the reverse (+)-strand replication leading to a self-circularizing (-)-strand product. This may require a second ribozyme (e.g. a second μ HHz) encoded in the (-)-strand akin to the mechanism used by natural viroids (Flores et al., 2000).

MD simulations indicate that the RCS process may be aided by accumulating strain in the nascent dsRNA segment leading to increased displacement (“peeling off”) of dsRNA 5'- and 3'-ends (i.e. strand displacement). In turn, this peeling off creates a more dynamic environment potentially aiding 5'-end invasion by extending the 3'-end. This topological strain induced strand displacement may be general and independent of the precise RCS

mechanism on scRNA templates and thus should also apply to non-enzymatic polymerization of RNA. Our observation that RCS can be enhanced by the use of branched extension, freeze-thaw thermocycling and pre-freezing dilution may also be related to this. Indeed, while the precise mechanistic basis for these enhancements is currently unknown, it seems plausible that all of these enhance 5'-end displacement on the product strand by accelerating conformational equilibration through RNA un- and refolding as observed previously (Mutschler et al., 2015).

In biology, both viroids and Hepatitis D virus (HDV) replication proceeds through RCS on circular RNA genomes mediated by proteinaceous RNA polymerases, but RCS has also been reported for circular DNA templates and proteinaceous DNA polymerases in nature (Wawrzyniak et al., 2017) and in biotechnology (Daubendiek et al., 1995; Givskov et al., 2016; Kristoffersen et al., 2017; Mohsen and Kool, 2016). dsDNA persistence length is somewhat shorter than dsRNA (dsDNA: 45-50 nm (140-50 nt) vs. dsRNA 60 nm (200 nt)) and stacking interactions in dsDNA are weaker than in dsRNA (Kebbekus et al., 1995; Svozil et al., 2010)). Therefore, dsDNA may more readily adopt a circular shape or allow internal kinks to alleviate build-up of strain or to adopt strong bends (Wolters and Wittig, 1989)). Nevertheless, we would expect a similar strand displacement effect would play part in RCS on small circular DNA templates. Indeed, in both cases RCS proceeds efficiently for circular genomes ranging from a few hundred nt to over 1.5kb (Mohsen and Kool, 2016). In contrast, RNA-catalysed polymerization (current maximum approx. 200 nt products (Attwater et al., 2013)) is currently limited to RCS on small circular RNAs. A more efficient RNA-catalysed RCS-based replication strategy will likely require improvements to the ribozyme polymerase catalytic activity, speed and processivity as well as the design of the template. Improvements to ribozyme polymerase processivity, which is known to be poor (Johnston et al., 2001; Lawrence and Bartel, 2003), might have the greatest impact and might be realized either through e.g. tethering or other topological linkages to the circular template (Cojocaru and Unrau, 2021). A more processive polymerase ribozyme should also result in less non-templated triplet terminal transferase activity, which appears to be a consequence of slow RCS extension and is likely aggravated by dissociation of the 3' end. Thus, more efficient RCS may also require the stabilization of the 3'-end triplet junction in the ribozyme active site in the same way as primer / nascent strand termini are stabilized within the active sites of proteinaceous RNA- and DNA polymerase (Chim et al., 2018; Houlihan et al., 2020). Finally, introduction of secondary structure motifs in the RNA nascent strand might drive increased 5'- end dissociation (e.g. through formation of stable hairpin structures) relieving strain at the 3'-end.

Larger circular RNA templates might provide advantages for the RCS as they are less strained. They would also provide increased access to the internal face of the circle and might be able to encode the whole ribozyme itself. On the other hand, reduced torsional strain on the dsRNA would be expected to reduce strand invasion and “peeling off” of the product strand. All of these factors merit detailed investigation.

Our motivation for investigating RNA-catalysed RCS was as a potential solution towards the so-called “strand separation problem”, the inhibition of RNA replication by exceedingly stable RNA duplex products. This form of product inhibition has both a thermodynamic component, i.e. the high amount of energy required to dissociate RNA duplexes, and a kinetic component as RNA replication must outpace duplex reannealing, which is rapid unless duplex concentrations are low or reactions take place in a highly viscous medium (He et al., 2017; Tupper and Higgs, 2021). In this context, we reasoned that RCS might provide favourable properties: synthesis and strand displacement on a circular template can in principle proceed essentially iso-energetically as base-pairing (H-bonding / stacking) interactions broken at the 5'-end during strand displacement are continuously compensated for by new base-pairing interactions formed at the nascent strand 3'-end. In turn, this enables an open-ended formation of template-coupled stoichiometric excess of single stranded RNA product strand to encode functions to further aid replication as we show here with resection and recircularization by an encoded ribozyme.

In the course of this work, we discovered another property of RNA synthesis on circular RNA templates that might contribute towards overcoming the strand separation problem. MD simulations indicate that - at least - on small circular RNA templates – the build-up of strain in the nascent dsRNA segment could aid strand displacement (Figure 3). However, the MD simulations also suggest that strain is non-directional destabilizing both nascent strand 5'- as well as 3'-ends equally. Thus, the potential advantages of scRNA RCS seems to be tempered by opposing effects such as strain as well as reduced template accessibility due to circular RNA ring geometry (Figure 1). Nevertheless, we find that a viroid-like replication strategy can be accomplished by RNA catalysis alone, with one ribozyme performing RCS on circular RNA templates yielding concatemeric RNA products, which can be processed (i.e. resected) and recircularized by a second ribozyme. Future improvements in polymerase ribozyme activity and processivity may allow all necessary components of such a replication cycle to be encoded on a circular RNA “genome” and propagated by self-replication and - processing reactions.

Materials and methods

Oligonucleotides

Base sequences of all oligonucleotides used throughout this work can be found in Supplementary file 1.

In vitro transcription

dsDNA templates (containing T7 promotor sequence at the 5' end upstream of the region to transcribe) for *in vitro* transcription was generated by "fill-in" using three cycles of mutual extension using GoTaq HotStart, (Promega, Madison, Wisconsin) between the relevant oligonucleotide and primers: 5T7 or HDVrt (the latter for defined 3' terminus formation (Schürer et al., 2002)). The T7 transcription protocol used is based on Megascript. Briefly explained, transcriptions of RNA requiring a triphosphate at the 5'-end (termed GTP Transcription) reaction were carried out under the following conditions: 40 mM Tris-Cl pH 8, 10 mM DTT, 2 mM spermidine, 20 mM MgCl₂, 7.5 mM each NTP (Thermo Fisher Scientific), dsDNA templates (varying amount, preferably >5 µM), 0.01 units/µL of inorganic pyrophosphatase (Thermo Fisher Scientific, Waltham, Massachusetts), ~50 µg/µL of T7 RNA polymerase (home made by Isaac Gallego). Reactions were left overnight (~16 hours) at 37°C. Then 0.5x volume EDTA (0.5 M) was added together with (at least) 2.5x volume of loading buffer (final conditions >50% formamide or >8M Urea and 5 mM EDTA). For transcription of RNA with a monophosphate 5'-end (termed GMP Transcription) the same procedure is followed as for NTP Transcription, however 10 mM GMP and 2.5 mM of each NTP instead of the higher amount of NTP used for GTP transcription.

Gel electroporation for analysis or purification

The sample in appropriate loading buffer were separated on 10-20% 8 M Urea denaturing PAGE gel using an EV400 DNA Sequencing Unit (Cambridge Electrophoresis). The product band was visualised by UV shadowing (for non-labelled RNA) or fluorescence scanning (Typhoon scanner, Amersham Typhoon) (for labelled RNA). When needed the identified product based on relative migration was excised. The excised gel fragment was then thoroughly crushed using a pipette tip and suspended in 10 mM Tris-Cl pH 7.4 to form a slurry. For freeze and squeeze extraction, the slurry was frozen in dry ice, then heated to 50°C (~5 min) and finally left rotating at room temperature (from 2 hours to overnight) to elute the product from the gel material. The eluate was then filtered using a Spin-X column (0.22 µm pore size, Costar), ethanol precipitated, (100 mM Acidic acid and 80% ethanol (10 µg glycogen carrier was present when noted)). UV absorbance was measured with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) to determine yield of redissolved purified RNA.

Calculation of extension efficiency

Gel Images from the Typhoon scanner were analysed in ImageQuant software (Cytiva life science) for quantifying band-intensity. Quantified band intensities were exported to Excel (Microsoft, Redmond, Washington) for further analysis. Extension efficiency (E) for a given band (b) was calculated as the sum of the intensities (I) of all the bands from b to n , (n being the highest detectable band), divided by the sum of I of all bands from $b-1$ to n :

$$E_b = \frac{\sum_b^n I_b}{\sum_b^n I_{b-1}} (1)$$

Thus, E represents the efficiency of the given ligation junction (L_b) to allow production of the extension product in band b , i.e. the extension efficiency.

Triplet transcription

Triplets were prepared via run-off *in vitro* transcription with T7 RNA polymerase. More details on the method can be found in (Attwater et al., 2018). Reaction conditions were as follows: 100 pmols of DNA template for each triplet was mixed with equimolar DNA oligo 5T7 to form the template for transcription. For triplets starting with purines, the NTP transcription protocol was used as described above with a total NTP concentration of 30 mM but only adding the nucleotides necessary for the triplet (e.g. AUA was transcribed with only ATP and UTP). For triplets starting in pyrimidines a lower total NTP concentration was used (4.32 mM) as this yielded better defined bands for purification. 50 μ L transcription reactions were stopped with 2 μ L EDTA (0.5M) and 5 μ L of 100% glycerol was added to facilitate gel loading. The samples were separated by 30% 3 M Urea denaturing PAGE as described above. Correct sequence composition was confirmed by A260/280 absorbance ratio, measured with the Nanodrop.

Circularization of RNA

300 μ L reaction volume) or ordered directly as chemically synthesized RNA (Integrated DNA Technologies (IDT), Iowa, United States). Linear RNA was gel purified as described above. When needed purified RNA was treated with T4 polynucleotide kinase (PNK) (New England Biolabs (NEB), Ipswich, Massachusetts) to remove 3'-end cyclic phosphate then RNA was phenol/chloroform extracted, ethanol precipitated and redissolved in ddH₂O. For splinted ligation, 3 pmol purified RNA was mixed with equimolar splint RNA in 262.5 μ L ddH₂O. The sample was heated to 80°C (2 min.) followed by cooling to 17°C (10 min.) and finally incubated on ice (5-30 min.). Then reaction conditions were adjusted to 50 mM Tris-HCl pH 7.5, 2 mM MgCl₂, 1 mM DTT, 400 μ M ATP (1x T4 RNA ligase 2 reaction buffer (NEB)) including 0.25 units/ μ L T4 RNA ligase 2 (Neb) (final volume 300 μ L) and samples left over night (~16 hours) at 4°C. For non-splinted ligation, 0 pmol gel purified RNA was mixed in

237 μ L ddH₂O followed by heating to 95°C and then quickly moved to ice. Then reaction conditions were adjusted to 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT (1x T4 RNA ligase reaction buffer (Neb)), 100 μ M ATP including 1 unit/ μ L T4 RNA ligase 1 (NEB) (final volume 300 μ L) and samples left over night (~16 hours) at 16°C. Circularized RNA was electrophorated by 10% 8M Urea denaturing PAGE for analysis and purification as described above.

Templated RNA-catalysed RNA synthesis (the primer extension assay)

Ribozyme activity assay was performed essentially as described in (Attwater et al., 2018). In a standard reaction (modified where specified), ribozyme heterodimer (5TU/t1), template, primer (5 pmol of each) and triplets (50 pmol of each) were annealed in 7.5 μ L water (80 °C 2 min, 17 °C 10 min). Then 2.5 μ L 4x reaction buffer was added (final volume 10 μ L) and samples were left on ice for ~5 min to ensure folding. Final pre-frozen conditions were (unless otherwise noted) either (Tris buffer system) 50 mM Tris (pH 8.3 at 25 °C), 100 mM MgCl₂ and 0.01% Tween20, or (CHES buffer system) 50 mM CHES (pH 9 at 25 °C), 150 mM KCl, 10 mM MgCl₂ and 0.01% Tween20. At this point some samples (noted in the text) were diluted by adding ddH₂O (e.g. 50x dilution corresponds to adding 490 μ L ddH₂O to the 10 μ L samples). Finally, samples were frozen on dry ice and incubated at -7 °C in a R4 series TC120 refrigerated cooling bath (Grant,Shepreth, UK) to allow eutectic phase formation and reaction, respectively. To end the incubations, samples that had been diluted were thawed, moved to 2mL tubes, ethanol precipitated (with glycogen carrier) and redissolved in 10 μ L ddH₂O. This step was avoided for undiluted samples that were already 10 μ L. Finally, 0.5 μ L EDTA (0.5M) was added to all samples to a final volume of 15 μ L. (In experiments where the effect of dilution was investigated, e.g. as experiment presented in Supporting Figure 5, ddH₂O was added to all the thawed samples to reach the same volume before precipitation). To prepare for separation of extension products, 3 μ L of the reacted samples after addition of EDTA (corresponding to 1 pmol template RNA) was diluted to reach the final loading conditions: 166 mM EDTA, 6M Urea (+ Bromophenol blue) and 10-20 pmol competing RNA (to prevent long product/template reannealing) (final volume 10 μ L). Finally, samples were denatured (95°C for 5 min) and RNA separated by 8M Urea denaturing PAGE.

Sequencing of extension products

In the primer extension reactions used for sequencing, the primer extension was performed as described above except for the following changes: 5 pmol ribozyme heterodimer/template, 20 pmol primer (with a 5'adapter sequence) and 100 pmol of each triplet was used. In the cases where multiple templates were mixed in the same reaction (one-pot), the final

template concentration remained 5 pmol in total. All reactions were done in the CHES buffer and were diluted 50x as standard.

Adapter ligation and RT-PCR: After Urea PAGE separation of the extension products, the noted region of the gel was dissected out, and carefully recovered as described above. The RNA was ethanol precipitated (80% ethanol with 10 ug glycogen carrier) resulting in a dry RNA pellet. To append an adaptor sequence to the 3'-end of the purified RNA products the dry RNA was redissolved in conditions allowing adenylated adapter ligation by T4 RNA Ligase 2 truncated K227Q (Neb) following manufacturers descriptions. Final adapter ligation conditions were: 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT (1x T4 RNA ligase reaction buffer (NEB)), 15 % PEG8000, 0.04% Tween 20, 5 pmol adenylated DNA primer (Adap1, for base sequences see Supplementary file 1) and 20 U/μL T4 RNA Ligase 2 truncated K227Q (Neb) (final volume 10 μL). The samples were then ligated at 16°C for two hours. Pre-adenylation of Adap1 using Mth RNA Ligase (Neb) was performed following manufacturers descriptions. After adapter ligation, samples were diluted 10-fold to achieve conditions for performing RT-PCR (25 cycles) using 0.5 μM forward (PCRp3) and reverse primer (RTp1) and the SuperScript III One-Step RT-PCR system with Platinum Taq DNA polymerase (Thermo Fisher Scientific). Finally, RT-PCR products were gel purified in 3% agarose gel and cleaned up using QIAGEN gel extraction kit (QiaGen, Hilden, Germany).

Sanger sequencing: Purified RT-PCR products were cloned in to pGEM vector using pGEM-T easy vector Systems (Promega) as described by manufacturer and transformed into heat-competent 10-Beta cells (NEB). Inserts from single colonies were PCR amplified (using primers pGEM_T7_Fo, pGEM_SP6_Ba) and send in for Sanger sequencing (Source bioscience) (using pGEM_T7_Fo as sequencing primer).

Illumina sequencing: Illumina adaptors were added to purified RT-PCR products by PCR (15 cycles) using 0.5 μM forward (Illx_Fo, x denotes different barcodes 1-15, see oligo sequences in supplementary file 1) and reverse primers (Ill_Ba) and Q5 Hot-Start High-Fidelity 2X Master Mix (Neb). PCR products were gel purified in 3% agarose gel and qPCRred (using NEBNext Library Quant Kit for Illumina) to quantify concentration. Finally, the DNA (consisting of Illumina adapters, barcodes and RT-PCRred sequence from the RNA extension) were prepared following manufactures protocol for MiSeq Illumina sequencing (Illumina, San Diego, California) (see e.g. MiSeq System Guide).

Sequencing data analysis

Illumina Sequencing data were acquired and processed as FASTQ files using Terminal (and available software packages such as FASTX-toolkit). Prior to analysis the whole output file from Illumina sequencing runs (containing also unrelated sequences) was split based on barcodes identifying the individual samples and trimmed starting with the original (P9₁) primer sequence (GAAGAACTG). After the P9₁ sequence, the triplets at positions 1, 2, 3 etc. would be identifiable representing extension products made by the ribozyme. The presence for the 3' adapter sequence (GTCGAATAT...) in the aligned sequences marked the end of the original RNA extension product. Sequencing data can be found as described below under section data availability: File 1 include sequence data for circular and linear one-pot analysis (C1 and L1, respectively), File 2 include sequence data for branched RCS analysis (B3)).

Analysis of the one-pot experiments: By counting the number of times a given triplet was present at a given position, we were able to calculate the fidelity for each triplet at this position. Identifying and counting the sequencing reads (*n*) for each position was done using *grep* (in Terminal) with a list of all relevant sequences (positions 3 to 18) and the sequencing files. The triplet at position 3, the first barcode position, was used to classify the sequences into coming from template A to D and thus has 100% fidelity for the correct triplet (Figure 4B).

In example, for analysing the fidelity (*F*) of position 4, the following list was used: GAAGAACTG_(primer)GAA_(pos1)GAA_(pos2)YYY_(pos3)XXX_(pos4). Here YYY was either of the first barcode triplets for templates A-D, (ATA_(template A), AAA_(template B), TTA_(template C) or ATC_(template D)) and XXX was either of the 14 possible triplets (CTG, ATA, CCA, CCC, AAA, CAC, GGG, TTA, TCC, GGC, ATC, GAT, CGC, GAA). *F* at position 4 was then calculated for template A-D as the number of occurrences of a triplet in position 4 (e.g. CCA) divided by the sum of occurrences of all the triplets multiplied by 100%. A generalized term for calculating the *F* at all positions (3-18) and for all templates (A-D) is:

$$F_{(a,Y)} = \frac{n_{(xxx,a,Y)}}{\text{Sum}(n_{(XXX,a,Y)})} \times 100\%^{(2)}$$

Here *F* is the fidelity, *a* is the position of the triplet, *Y* is the template A-D. *n* is the number of sequencing reads for a given triplet (xxx) for position *a* on template *Y* or for all the 14 triplets (XXX), for *a* on *Y*. Eventually, the fidelity for positions 3-15 in the context of template A-D for all triplets was plotted in Figure 4B. Accumulated chance for a product of reaching positions *X* (shown in plot in Figure 4C) was calculated by multiplying all fidelities for moving from position 3 to position *X* with correct triplets (fidelities found in Figure 4C). Data for this

analysis can be found as described in data availability section below (File 1). Numerical data and calculation is supplied in Figure 4-source data 1.

Analysis of the branched RCS: By counting the number (n) of correct sequences with a specific length ending in the 3'adapter sequence, we identified long RCS products (Figure 5D). This was done using *grep* (in Terminal) with a list of all relevant sequences (positions 9 to 30, both product I and II), and the sequencing file. Data for this analysis can be found as described in data availability section below (File 2) Numerical data and calculation is supplied in Figure 5-source data 1.

Self-circularizing Micro Hammerhead ribozyme (μ HHz) assay

RNA catalysed synthesis of fluorophore labelled self-circularizing micro-Hammerhead ribozyme (μ HHz) was prepared in 2x large (500 pmol) reactions set up and incubated as described above. Specifically, 500pmol ribozyme heterodimer (5TU/t1) and circular template (scHHz_temp), 2000 pmol primer (HHrzP12) and 50 μ mol of each of the triplets were annealed followed by adding buffer to 50 mM CHES, pH 9; 150 mM KCl; 10 mM MgCl₂, 0.05% Tween 20 (1mL). Then the sample was diluted 50 times to a final volume of 50 mL. After 4 weeks incubation at -7 °C, EDTA was added (5mM final concentration), reactions were thawed and concentrated to a final volume of ~300 μ L using a centrifugation filter (Amicon Ultra, 3 kDa cut off) retaining long RNA products. μ HHz RNA (marked in Figure 6C) was purified by gel electrophoresis and excised product was dissolved to 10 μ M in H₂O with 0.5 mM EDTA.

Chemically synthesized fluorophore labelled self-circularizing μ HHz RNA (IDT) was gel purified as described above and excised product was dissolved to 10 μ M in H₂O with 0.5mM EDTA.

Micro-Hammerhead ribozyme (μ HHz) cleavage / circularization assay

Self-circularization assays of chemically synthesized fluorophore labelled μ HHz comprise 10 pmol μ HHz annealed (80 °C 2 min, 17 °C 10 min) in 4 μ L water with 1 μ L 5x reaction buffer, final reaction conditions: 50 mM CHES, pH 9; 150 mM KCl; 10 mM MgCl₂ (same as for the Templated RNA-catalysed RNA synthesis). Then incubated in ice for 5 min to ensure folding. This was then frozen on dry ice and either moved to -7 °C for eutectic phase formation (reaction) or -80 °C (control). After incubation, 10 μ L loading buffer (95% Formamide, 25 mM EDTA, Bromophenol blue) was added directly to the cold samples to stop the reaction and mixed while thawing. Finally, reactions were analysed by 20% denaturing PAGE like described above. 5'-phosphorylation of μ HHz RNA with polynucleotide kinase (NEB) was

carried out following manufacturer's directions. RNA was then phenol/chloroform extracted, precipitated and dissolved in ddH₂O with 0.5mM EDTA to 10 µM (determined by Nanodrop).

Molecular Dynamics simulations.

All simulations were set up with the AMBER 18 suite of programs and performed using the CUDA implementation of AMBER's pmemd program (Case, n.d.). A linear ssRNA of 36 nt with the sequence (UUC)₁₂ was built using the NAB utility, which was then circularised using an in-house programme (Pyne et al., 2021). From there, the complementary strand containing GAA triplets was progressively grown representing the different stages of the rolling circle replication, containing 9, 18, 21, 24, 27 till 30 nt of dsRNA keeping the rest single-stranded. For each stage, a representative structure was used as a scaffold to grow the dsRNA part and thus build the structure to model next stage. A linear dsRNA fragment containing 4 GAA triplets with a nick between the first and second was run as a control. This molecule had a total length of 16 bp as it was capped by a CG dimer on each end.

The AMBER99 forcefield (Cheatham et al., 1999) with different corrections for backbone dihedral angles including the parmBSC0 for α and γ (Pérez et al., 2007) and the parmOL3 for χ (glycosidic bond) (Zgarbová et al., 2011) were used to describe the RNA. All initial structures were explicitly solvated using a truncated octahedral TIP3P box with a 14 Å buffer. They were neutralized by two different types of salt, KCl and MgCl₂, described by the 'scaled charged' Empirical Continuum Correction (ECC) set of ion parameters (Duboué-Dijon et al., 2018), which substantially reduce the overestimation of ion-ion interactions with respect to water-mediated interactions typical of empirical forcefield calculations (Fingerhut et al., 2021; Kirby and Jungwirth, 2019) (see Figure 3 - Figure supplement 6). The necessary ion pairs (Machado and Pantano, 2020) were added for matching 0.2 M in the case of KCl, and 0.1 and 0.5 M in the case of MgCl₂. Simulations were performed at constant T and P (300 K and 1 atm) following standard protocols (Noy and Golestanian, 2010) for 400 ns.

The last 100 ns sampled every 10 ps were used for the subsequent analysis. AMBER program CPPTRAJ (Roe and Cheatham, 2013) was used to determine base-pair step parameters, radial distribution functions of ions around RNA and distances between atoms, including groove width and hydrogen bonds. The latter were defined with a distance cutoff of 3.5 Å and an angle cutoff of 120°. Counterion-density maps were obtained using Canion (Lavery et al., 2014) and were subsequently visualized with Chimera (Pettersen et al., 2004). SerraNA software was used to calculate curvatures at different sub-fragment lengths (Velasco-Berrelleza et al., 2020).

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Competing interests

The authors declare no competing interest.

Additional files

Supplementary file 1. Oligonucleotide sequences.

Transparent reporting form.

Data availability

Simulations are available at the University of York Data Repository ([10.15124/b92977bd-f016-4740-8b4a-f86c68d5eb2c](https://doi.org/10.15124/b92977bd-f016-4740-8b4a-f86c68d5eb2c)). Sequencing data used for analysis presented in Figure 4 (File 1) and 5 (File 2) are available on Dryad (<https://doi.org/10.5061/dryad.tht76hf10>).

Figure supplements and Videos

Figure 1 - Figure supplement 1. Purification of circularized RNA (scGAA8-16). Identification and dissection of circularized RNA were performed by denaturing PAGE. Representative SyBr Gold stained 10% Urea PAGE gel is shown here for illustrating the circularization process of circular RNA templates used in main text Figure 2B. In the gel, RNA before (odd lanes) and after (even lanes) ligation with T4RNA ligase 2 was analysed. A 10 nucleotide (nt) RNA splint (covering 5 nt of the 5'-end and 3'-end of the linear RNA strand) was used for circularization as required by T4 RNA ligase 2. In the ligated samples, multiple bands (A-D) appeared representing various combinations of ligated RNA strands. By migration analysis (right panel), we identified A and C as linear constructs and B and D as circularized constructs (illustrations of the identified structure of A-D can be seen to the right of the gel). Monomeric circularized RNA (corresponding to band B) was dissected out and used throughout this work. In general, we observe that circularized RNA is retained in PAGE compared to the linear strand of the same sequence. All circles used in this work were purified based on the approach outlined above. Bands A-D discussed here should not be confused with templates A-D used in main text figure 4. Original gel is supplied in Figure 1- Figure supplement 1-source data 1.

Figure 2 - Figure supplement 1. Optimization of Rolling Circle Synthesis. A) Comparison between linear and circular primer extension using the CHES reaction buffer system (similar to main text Figure 1D that is in the Tris buffer system). Extensions performed at -7 °C for 2 weeks. Template sc12GAA-p and primer P9 were used. B) The periodic oscillations were observed with various repeat sequence templates (CGG, GAC and GAA, termed sc12CGG-p, sc12GAC-p and sc12GAA-p, respectively) in both CHES and Tris buffer systems. Primers P9(CGG), P9(GAC) or P9 was used respectively. Extensions performed at -7 °C for 4 weeks. C) Dilution of samples increased the efficiency. The plot in C) shows the difference in extension efficiency (Δ -extension efficiency) between the undiluted (Un.) and the 2-50 fold diluted (x2-x50) samples. The Δ -extension efficiency of the ligations before invasion (mean of bands 1-9) were unaffected by the dilution (giving a Δ -extension efficiency of ~1). However, the Δ -extension efficiency of band 10 (full length +1 triplet, invasion) increased strongly with dilution. Extensions performed at -7 °C for 1 week. D) The same effect of dilution (improving invasion) was seen over a range of MgCl₂ concentrations (50-200 mM). Extensions performed at -7 °C for 1 week. All extension reactions presented here were run at standard reaction conditions described in main text Materials and Methods except when specified otherwise for dilution, salt or buffer system. In C and D, template sc12GAA-p and primer P9 were used. Original gels and numeric values are supplied in Figure 2-Figure supplement 1-source data 1.

Figure 3 - Figure supplement 1. Time sequence of snapshots from the simulation of scRNA with 27 bp dsRNA in 100 mM MgCl₂ with pyrimidine (template) strand (UUC)₁₂ (khaki), purine (product) strand (GAA) (light blue), We observe a series of melting and reannealing events in the 3' end (purple and light green) during the first half of the simulation and in the 5' end (dark blue and dark green) during the second half.

Figure 3 - Figure supplement 2. Time sequence of snapshots from the simulation of scRNA with 30 bp dsRNA in 100 mM MgCl₂, We observe a series of melting and reannealing events in the 5' end (dark blue and dark green). The rest of the colour code is the same as Figure 3 - Figure supplement 1.

Figure 3 - Figure supplement 3. Percentage of frames from the last 100 ns of the simulations presenting canonical hydrogen bond pairing for each base pair (bp): A) Linear RNAs solvated with the three buffers (100 mM KCl, 200 mM MgCl₂ and 500 mM MgCl₂); B) Rolling circle RNA synthesis (RCS) simulations solvated with 100 mM KCl; C) RCS simulations solvated with 500 mM MgCl₂.

Figure 3 - Figure supplement 4. A-E) Averages and standard deviations (as error bars) of bp-step parameters (roll, slide and twist) together with major and minor groove widths (MajW and MinW, respectively) calculated over the last 100 ns of the simulations. The trajectory of the 16 bp linear RNA is labelled as 16L. F) Bending profile for all the sub-fragments 4 bp-long along 30 bp of dsRNA embedded in a 36-bp circular ssRNA.

Figure 3 - Figure supplement 5. Counterion-density maps around RNA molecules that show an occupancy ~10 times or greater the bulk concentration (in red) as seen in simulations. These areas are the molecular regions where cations localize preferentially. In the case of 200 mM KCl, these align along the grooves, whereas, in the case of MgCl₂, they tend to be closer to the backbone and bridge distant backbone points, making the bases more exposed. Extremely high Mg²⁺ concentrations provide similar interacting profiles to moderate levels indicating saturation on the preferred binding sites in both cases.

Figure 3 - Figure supplement 6. (Top) Averages and standard deviations (as error bars) of radial distribution functions (rdf) of cations around RNA backbone phosphates. The rdf indicate the probability of finding an ion within a certain distance of a particular RNA atom in relation to its bulk concentration (set at 1). Magnesium ions make more direct interactions with RNA backbone (first peak) and mediated by water molecules (subsequent peaks) than

potassium. The smaller rdf peaks observed on 500 mM compared to 100 mM indicate a relatively lower ion condensation around RNA with respect to bulk concentration due to saturation. (Bottom) Cumulated ion populations as function of simulation time within the first and the second solvation shells around RNA backbone phosphates, which corresponds to the first and second peak of the rdf, respectively.

Figure 4 - Figure supplement 1. Deep sequencing of extension products. A) Representative SyBr Gold stained 10% Urea PAGE gel showing linear and circularized templates A-D. Circularized RNA was retained in the gel as expected. A small degree of hydrolysed RNA is seen as a lower band in the gel indicating that circles that a low fraction of circles has opened. B) 10% Urea PAGE separation of one-pot extension reaction used for deep sequencing. Dashed box denotes excised region (above the full-length product (band 9)) used for RNA recovery and Deep-sequencing. C) Illustration of the protocol for sequencing of extension products. The initial extension products gets gel purified, then 3'-adaptor ligated with a 5'-adynalated DNA adapter strand, and finally RT-PCR amplified (adding additional adapter sequences) and submitted for sequencing. Original gels are supplied in Figure 4-Figure supplement 1-source data 1.

Figure 4 - Figure supplement 2. Controls for deep-sequencing data. A) Plot shows the fidelity ratio at the noted triplet positions between extension reactions where the templates were incubated either in one-pot (Mix) or in individual tubes (Ind). This is shown for the circular templates (blue lines) and linear templates (orange line). B) The effect of dilution with water leads to increased fidelity at the point of invasion (position 10). Bar chart show the fidelity for insertion of the expected triplet at position 10 (making invasion) calculated from deep-sequenced samples that were either not diluted (Un.) or diluted 50 fold (x50).

Figure 5 - Figure supplement 1. 10% Urea PAGE separation of circular template extension reaction used for deep sequencing. Excised gel piece is marked with green.

Figure 6 - Figure supplement 1. Kinetic analysis of the μ HRz. A) Quantification of band intensities as a function of time. Here we see that cut RNA accumulate while the amount of circular RNA seems to reach an equilibrium. B) Fraction of circular RNA relative to cut as a function of time. This plot shows that a very high amount of circle is formed at short time points slowly dropping in relation to non-circular cut RNA.

Video 1. Movie of the RCS simulation where dsRNA is 27 bp long. We observe fraying and annealing of 5' and 3' ends demonstrating the quick timescales of these transitions.

968

969 **Video 2.** Movie of the RCS simulation where dsRNA is 30 bp long. We observe again
970 fraying and annealing of 5' and 3' ends demonstrating the quick timescales of these
971 transitions.

References

- Abels JA, Moreno-Herrero F, Van Der Heijden T, Dekker C, Dekker NH. 2005. Single-molecule measurements of the persistence length of double-stranded RNA. *Biophys J* **88**:2737–2744. doi:10.1529/biophysj.104.052811
- Attwater J, Raguram A, Morgunov AS, Gianni E, Holliger P. 2018. Ribozyme-catalysed RNA synthesis using triplet building blocks. *Elife* **7**. doi:10.7554/eLife.35255
- Attwater J, Wochner A, Holliger P. 2013. In-ice evolution of RNA polymerase ribozyme activity. *Nat Chem* **5**:1011–1018. doi:10.1038/nchem.1781
- Attwater J, Wochner A, Pinheiro VB, Coulson A, Holliger P. 2010. Ice as a protocellular medium for RNA replication. *Nat Commun* **1**. doi:10.1038/ncomms1076
- Becker S, Feldmann J, Wiedemann S, Okamura H, Schneider C, Iwan K, Crisp A, Rossa M, Amatov T, Carell T. 2019. Unified prebiotically plausible synthesis of pyrimidine and purine RNA ribonucleotides. *Science (80-)* **366**:76–82. doi:10.1126/science.aax2747
- Berr A, Schubert I. 2006. Direct labelling of BAC-DNA by rolling-circle amplification. *Plant J* **45**:857–862. doi:10.1111/j.1365-313X.2005.02637.x
- Bhattacharyya A, Murchie AIH, Lilley DMJ. 1990. RNA bulges and the helical periodicity of double-stranded RNA. *Nature* **343**:484–487. doi:10.1038/343484a0
- Blanco L, Bernad A, Lazaro JM, Martin G, Garmendia C, Salas M. 1989. Highly efficient DNA synthesis by the phage Φ 29 DNA polymerase. Symmetrical mode of DNA replication. *J Biol Chem* **264**:8935–8940.
- Case DA et al. n.d. Amber 2018 Reference Manual. <http://ambermd.org/contributors.html>.
- Cech TR. 2000. The ribosome is a ribozyme. *Science (80-)*. doi:10.1126/science.289.5481.878
- Cheatham TE, Cieplak P, Kollman PA. 1999. A modified version of the cornell et al. force field with improved sugar pucker phases and helical repeat. *J Biomol Struct Dyn* **16**:845–862. doi:10.1080/07391102.1999.10508297
- Chim N, Jackson LN, Trinh AM, Chaput JC. 2018. Crystal structures of DNA polymerase I capture novel intermediates in the DNA synthesis pathway. *Elife* **7**. doi:10.7554/eLife.40444
- Cojocar R, Unrau PJ. 2021. Processive RNA polymerization and promoter recognition in an RNA World. *Science (80-)* **371**:1225–1232. doi:10.1126/science.abd9191
- Daròs JA, Marcos JF, Hernández C, Flores R. 1994. Replication of avocado sunblotch viroid: Evidence for a symmetric pathway with two rolling circles and hammerhead ribozyme processing. *Proc Natl Acad Sci U S A* **91**:12813–12817. doi:10.1073/pnas.91.26.12813
- Daubendiek SL, Ryan K, Kool ET. 1995. Rolling-Circle RNA Synthesis: Circular Oligonucleotides as Efficient Substrates for T7 RNA Polymerase. *J Am Chem Soc* **117**:7818–7819. doi:10.1021/ja00134a032

1009 Deguzman V, Vercoutere W, Shenasa H, Deamer D. 2014. Generation of oligonucleotides
1010 under hydrothermal conditions by non-enzymatic polymerization. *J Mol Evol* **78**:251–
1011 262. doi:10.1007/s00239-014-9623-2

1012 Diener TO. 2003. Discovering viroids — a personal perspective. *Nat Rev Microbiol* **1**:75–80.
1013 doi:10.1038/nrmicro736

1014 Diener TO. 1989. Circular RNAs: Relics of precellular evolution? *Proc Natl Acad Sci U S A*
1015 **86**:9370–9374. doi:10.1073/pnas.86.23.9370

1016 Duboué-Dijon E, Mason PE, Fischer HE, Jungwirth P. 2018. Hydration and Ion Pairing in
1017 Aqueous Mg²⁺ and Zn²⁺ Solutions: Force-Field Description Aided by Neutron
1018 Scattering Experiments and Ab Initio Molecular Dynamics Simulations. *J Phys Chem B*
1019 **122**:3296–3306. doi:10.1021/acs.jpcc.7b09612

1020 Ekland EH, Bartel DP. 1996. RNA-catalysed RNA polymerization using nucleoside
1021 triphosphates. *Nature* **382**:373–376. doi:10.1038/382373a0

1022 Fadda Z, Daròs JA, Fagoaga C, Flores R, Duran-Vila N. 2003. Eggplant Latent Viroid, the
1023 Candidate Type Species for a New Genus within the Family Avsunviroidae
1024 (Hammerhead Viroids). *J Virol* **77**:6528–6532. doi:10.1128/jvi.77.11.6528-6532.2003

1025 Fingerhut BP, Schauss J, Kundu A, Elsaesser T. 2021. Contact pairs of RNA with
1026 magnesium ions-electrostatics beyond the Poisson-Boltzmann equation. *Biophysj*.
1027 doi:10.1016/j.bpj.2021.10.029

1028 Flores R, Daròs JA, Hernández C. 2000. Avsunviroidae family: Viroids containing
1029 hammerhead ribozymes. *Adv Virus Res*. doi:10.1016/s0065-3527(00)55006-4

1030 Flores R, Gago-Zachert S, Serra P, Sanjuán R, Elena SF. 2014. Viroids: Survivors from the
1031 RNA world? *Annu Rev Microbiol*. doi:10.1146/annurev-micro-091313-103416

1032 Flores R, Gas ME, Molina-Serrano D, Nohales MÁ, Carbonell A, Gago S, De la Peña M,
1033 Daròs JA. 2009. Viroid replication: Rolling-circles, enzymes and ribozymes. *Viruses*.
1034 doi:10.3390/v1020317

1035 Freier SM, Kierzek R, Jaeger JA, Sugimoto N, Caruthers MH, Neilson T, Turner DH. 1986.
1036 Improved free-energy parameters for predictions of RNA duplex stability. *Proc Natl*
1037 *Acad Sci U S A* **83**:9373–9377. doi:10.1073/pnas.83.24.9373

1038 Givskov A, Kristoffersen EL, Vandsø K, Ho YP, Stougaard M, Knudsen BR. 2016. Optimized
1039 detection of Plasmodium falciparum topoisomerase I enzyme activity in a complex
1040 biological sample by the use of molecular beacons. *Sensors (Switzerland)* **16**:1916.
1041 doi:10.3390/s16111916

1042 Goldman AD, Kacar B. 2021. Cofactors are Remnants of Life's Origin and Early Evolution. *J*
1043 *Mol Evol*. doi:10.1007/s00239-020-09988-4

1044 Hassenkam T, Damer B, Mednick G, Deamer D. 2020. AFM images of viroid-sized rings that
1045 self-assemble from mononucleotides through wet–dry cycling: Implications for the origin

of life. *Life* **10**:1–11. doi:10.3390/life10120321

He C, Gállego I, Laughlin B, Grover MA, Hud N V. 2017. A viscous solvent enables information transfer from gene-length nucleic acids in a model prebiotic replication cycle. *Nat Chem* **9**:318–324. doi:10.1038/nchem.2628

Hieronymus R, Müller S. 2019. Engineering of hairpin ribozyme variants for RNA recombination and splicing. *Ann N Y Acad Sci*. doi:10.1111/nyas.14052

Horning DP, Joyce GF. 2016. Amplification of RNA by an RNA polymerase ribozyme. *Proc Natl Acad Sci U S A* **113**:9786–9791. doi:10.1073/pnas.1610103113

Houlihan G, Arangundy-Franklin S, Porebski BT, Subramanian N, Taylor AI, Holliger P. 2020. Discovery and evolution of RNA and XNA reverse transcriptase function and fidelity. *Nat Chem* **12**:683–690. doi:10.1038/s41557-020-0502-8

Johnston WK, Unrau PJ, Lawrence MS, Glasner ME, Bartel DP. 2001. RNA-catalyzed RNA polymerization: Accurate and general RNA-templated primer extension. *Science (80-)* **292**:1319–1325. doi:10.1126/science.1060786

Kebbekus P, Draper DE, Hagerman P. 1995. Persistence Length of RNA. *Biochemistry* **34**:4354–4357. doi:10.1021/bi00013a026

Kim SC, Zhou L, Zhang W, O’Flaherty DK, Rondo-Brovetto V, Szostak JW. 2020. A Model for the Emergence of RNA from a Prebiotically Plausible Mixture of Ribonucleotides, Arabinonucleotides, and 2’-Deoxynucleotides. *J Am Chem Soc* **142**:2317–2326. doi:10.1021/jacs.9b11239

Kirby BJ, Jungwirth P. 2019. Charge Scaling Manifesto: A Way of Reconciling the Inherently Macroscopic and Microscopic Natures of Molecular Simulations. *J Phys Chem Lett* **10**:7531–7536. doi:10.1021/acs.jpcllett.9b02652

Klein DJ, Moore PB, Steitz TA. 2004. The contribution of metal ions to the structural stability of the large ribosomal subunit. *RNA* **10**:1366–1379. doi:10.1261/rna.7390804

Kristensen LS, Andersen MS, Stagsted LVW, Ebbesen KK, Hansen TB, Kjems J. 2019. The biogenesis, biology and characterization of circular RNAs. *Nat Rev Genet*. doi:10.1038/s41576-019-0158-7

Kristoffersen EL, Givskov A, Jørgensen LA, Jensen PW, W. Byl JA, Osheroff N, Andersen AH, Stougaard M, Ho Y-P, Knudsen BR. 2017. Interlinked DNA nano-circles for measuring topoisomerase II activity at the level of single decatenation events. *Nucleic Acids Res* **45**:7855–7869. doi:10.1093/nar/gkx480

Kuhn H, Demidov V V., Frank-Kamenetskii MD. 2002. Rolling-circle amplification under topological constraints. *Nucleic Acids Res* **30**:574–580. doi:10.1093/nar/30.2.574

Lasda E, Parker R. 2014. Circular RNAs: Diversity of form and function. *RNA*. doi:10.1261/rna.047126.114

1082 Lavery R, Maddocks JH, Pasi M, Zakrzewska K. 2014. Analyzing ion distributions around
 1083 DNA. *Nucleic Acids Res* **42**:8138–8149. doi:10.1093/nar/gku504

1084 Lawrence MS, Bartel DP. 2003. Processivity of ribozyme-catalyzed RNA polymerization.
 1085 *Biochemistry* **42**:8748–8755. doi:10.1021/bi034228l

1086 Le Vay K, Mutschler H. 2019. The difficult case of an RNA-only origin of life. *Emerg Top Life*
 1087 *Sci*. doi:10.1042/ETLS20190024

1088 Litke JL, Jaffrey SR. 2019. Highly efficient expression of circular RNA aptamers in cells
 1089 using autocatalytic transcripts. *Nat Biotechnol* **37**:667–675. doi:10.1038/s41587-019-
 1090 0090-6

1091 Machado MR, Pantano S. 2020. Split the Charge Difference in Two! A Rule of Thumb for
 1092 Adding Proper Amounts of Ions in MD Simulations. *J Chem Theory Comput* **16**:1367–
 1093 1372. doi:10.1021/acs.jctc.9b00953

1094 Mohsen MG, Kool ET. 2016. The Discovery of Rolling Circle Amplification and Rolling Circle
 1095 Transcription. *Acc Chem Res* **49**:2540–2550. doi:10.1021/acs.accounts.6b00417

1096 Møller HD, Mohiyuddin M, Prada-Luengo I, Sailani MR, Halling JF, Plomgaard P, Maretty L,
 1097 Hansen AJ, Snyder MP, Pilegaard H, Lam HYK, Regenberg B. 2018. Circular DNA
 1098 elements of chromosomal origin are common in healthy human somatic tissue. *Nat*
 1099 *Commun* **9**:1–12. doi:10.1038/s41467-018-03369-8

1100 Moss EL, Maghini DG, Bhatt AS. 2020. Complete, closed bacterial genomes from
 1101 microbiomes using nanopore sequencing. *Nat Biotechnol* **38**:701–707.
 1102 doi:10.1038/s41587-020-0422-6

1103 Mutschler H, Taylor AI, Porebski BT, Lightowlers A, Houlihan G, Abramov M, Herdewijn P,
 1104 Holliger P. 2018. Random-sequence genetic oligomer pools display an innate potential
 1105 for ligation and recombination. *Elife* **7**. doi:10.7554/eLife.43022

1106 Mutschler H, Wochner A, Holliger P. 2015. Freeze-thaw cycles as drivers of complex
 1107 ribozyme assembly. *Nat Chem* **7**:502–508. doi:10.1038/nchem.2251

1108 Nakano S, Proctor DJ, Bevilacqua PC. 2001. Mechanistic characterization of the HDV
 1109 genomic ribozyme: Assessing the catalytic and structural contributions of divalent metal
 1110 ions within a multichannel reaction mechanism. *Biochemistry* **40**:12022–12038.
 1111 doi:10.1021/bi011253n

1112 Nissen P, Hansen J, Ban N, Moore PB, Steitz TA. 2000. The structural basis of ribosome
 1113 activity in peptide bond synthesis. *Science (80-)* **289**:920–930.
 1114 doi:10.1126/science.289.5481.920

1115 Nohales M-A, Molina-Serrano D, Flores R, Daros J-A. 2012. Involvement of the
 1116 Chloroplastic Isoform of tRNA Ligase in the Replication of Viroids Belonging to the
 1117 Family Avsunviroidae. *J Virol* **86**:8269–8276. doi:10.1128/jvi.00629-12

1118 Noy A, Golestanian R. 2010. The Chirality of DNA: Elasticity Cross-Terms at Base-Pair

1119 Level Including A-Tracts and the Influence of Ionic Strength. *J Phys Chem B* **114**:8022–
 1120 8031. doi:10.1021/jp104133j
 1121 Patel BH, Percivalle C, Ritson DJ, Duffy CD, Sutherland JD. 2015. Common origins of RNA,
 1122 protein and lipid precursors in a cyanosulfidic protometabolism. *Nat Chem* **7**:301–307.
 1123 doi:10.1038/nchem.2202
 1124 Pérez A, Marchán I, Svozil D, Sponer J, Cheatham TE, Lughton CA, Orozco M. 2007.
 1125 Refinement of the AMBER force field for nucleic acids: Improving the description of α/γ
 1126 conformers. *Biophys J* **92**:3817–3829. doi:10.1529/biophysj.106.097782
 1127 Petkovic S, Müller S. 2015. RNA circularization strategies in vivo and in vitro. *Nucleic Acids*
 1128 *Res* **43**:2454–2465. doi:10.1093/nar/gkv045
 1129 Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE.
 1130 2004. UCSF Chimera - A visualization system for exploratory research and analysis. *J*
 1131 *Comput Chem* **25**:1605–1612. doi:10.1002/jcc.20084
 1132 Powner MW, Gerland B, Sutherland JD. 2009. Synthesis of activated pyrimidine
 1133 ribonucleotides in prebiotically plausible conditions. *Nature* **459**:239–242.
 1134 doi:10.1038/nature08013
 1135 Prywes N, Blain JC, Del Frate F, Szostak JW. 2016. Nonenzymatic copying of RNA
 1136 templates containing all four letters is catalyzed by activated oligonucleotides. *Elife* **5**.
 1137 doi:10.7554/eLife.17756
 1138 Pyne ALB, Noy A, Main KHS, Velasco-Berrelleza V, Piperakis MM, Mitchenall LA,
 1139 Cugliandolo FM, Beton JG, Stevenson CEM, Hoogenboom BW, Bates AD, Maxwell A,
 1140 Harris SA. 2021. Base-pair resolution analysis of the effect of supercoiling on DNA
 1141 flexibility and major groove recognition by triplex-forming oligonucleotides. *Nat*
 1142 *Commun* **12**:1–12. doi:10.1038/s41467-021-21243-y
 1143 Rajamani S, Vlassov A, Benner S, Coombs A, Olasagasti F, Deamer D. 2008. Lipid-assisted
 1144 synthesis of RNA-like polymers from mononucleotides. *Orig Life Evol Biosph* **38**:57–74.
 1145 doi:10.1007/s11084-007-9113-2
 1146 Roe DR, Cheatham TE. 2013. PTRAJ and CPPTRAJ: Software for processing and analysis
 1147 of molecular dynamics trajectory data. *J Chem Theory Comput* **9**:3084–3095.
 1148 doi:10.1021/ct400341p
 1149 Salditt A, Keil LMR, Horning DP, Mast CB, Joyce GF, Braun D. 2020. Thermal Habitat for
 1150 RNA Amplification and Accumulation. *Phys Rev Lett* **125**:048104.
 1151 doi:10.1103/PhysRevLett.125.048104
 1152 Schürer H, Lang K, Schuster J, Mörl M. 2002. A universal method to produce in vitro
 1153 transcripts with homogeneous 3' ends. *Nucleic Acids Res* **30**. doi:10.1093/nar/gnf055
 1154 Shechner DM, Grant RA, Bagby SC, Koldobskaya Y, Piccirilli JA, Bartel DP. 2009. Crystal
 1155 structure of the catalytic core of an RNA-Polymerase ribozyme. *Science* (80-)

1156 **326**:1271–1275. doi:10.1126/science.1174676

1157 Shulman LM, Davidson I. 2017. Viruses with Circular Single-Stranded DNA Genomes Are
 1158 Everywhere! *Annu Rev Virol* **4**:159–180. doi:10.1146/annurev-virology-101416-041953

1159 Sosson M, Pfeffer D, Richert C. 2019. Enzyme-free ligation of dimers and trimers to RNA
 1160 primers. *Nucleic Acids Res* **47**:3836–3845. doi:10.1093/nar/gkz160

1161 Sponer JE, Šponer J, Výřavský J, Šedo O, Zdráhal Z, Costanzo G, Di Mauro E, Wunnavu S,
 1162 Braun D, Matyášek R, Kovařík A. 2021. Nonenzymatic, Template-Free Polymerization
 1163 of 3',5' Cyclic Guanosine Monophosphate on Mineral Surfaces. *ChemSystemsChem*
 1164 **3**:e2100017. doi:10.1002/syst.202100017

1165 Sponer J, Bussi G, Krepl M, Banas P, Bottaro S, Cunha RA, Gil-Ley A, Pinamonti G, Poblete
 1166 S, Jurečka P, Walter NG, Otyepka M. 2018. RNA structural dynamics as captured by
 1167 molecular simulations: A comprehensive overview. *Chem Rev*.
 1168 doi:10.1021/acs.chemrev.7b00427

1169 Svozil D, Hobza P, Šponer J. 2010. Comparison of intrinsic stacking energies of ten unique
 1170 dinucleotide steps in A-RNA and B-DNA duplexes. Can we determine correct order of
 1171 stability by quantum-chemical calculations? *J Phys Chem B* **114**:1191–1203.
 1172 doi:10.1021/jp910788e

1173 Szostak JW. 2012. The eightfold path to non-enzymatic RNA replication. *J Syst Chem*.
 1174 doi:10.1186/1759-2208-3-2

1175 Tagami S, Attwater J, Holliger P. 2017. Simple peptides derived from the ribosomal core
 1176 potentiate RNA polymerase ribozyme function. *Nat Chem* **9**:325–332.
 1177 doi:10.1038/nchem.2739

1178 Tjhung KF, Shokhirev MN, Horning DP, Joyce GF. 2020. An RNA polymerase ribozyme that
 1179 synthesizes its own ancestor. *Proc Natl Acad Sci U S A* **117**:2906–2913.
 1180 doi:10.1073/pnas.1914282117

1181 Tupper AS, Higgs PG. 2021. Rolling-circle and strand-displacement mechanisms for non-
 1182 enzymatic RNA replication at the time of the origin of life. *J Theor Biol* **527**.
 1183 doi:10.1016/j.jtbi.2021.110822

1184 Velasco-Berrelleza V, Burman M, Shepherd JW, Leake MC, Golestanian R, Noy A. 2020.
 1185 SerraNA: A program to determine nucleic acids elasticity from simulation data. *Phys*
 1186 *Chem Chem Phys* **22**:19254–19266. doi:10.1039/d0cp02713h

1187 Wachowius F, Holliger P. 2019. Non-Enzymatic Assembly of a Minimized RNA Polymerase
 1188 Ribozyme. *ChemSystemsChem* **1**:12–15. doi:10.1002/syst.201900004

1189 Wawrzyniak P, Plucienniczak G, Bartosik D. 2017. The different faces of rolling-circle
 1190 replication and its multifunctional initiator proteins. *Front Microbiol*.
 1191 doi:10.3389/fmicb.2017.02353

1192 Wilkinson ME, Charenton C, Nagai K. 2020. RNA Splicing by the Spliceosome. *Annu Rev*

1193 *Biochem.* doi:10.1146/annurev-biochem-091719-064225

1194 Wolters M, Wittig B. 1989. Construction of a 42 base pair double stranded DNA microcircle.

1195 *Nucleic Acids Res* **17**:5163–72.

1196 Xu J, Chmela V, Green NJJ, Russell DAA, Janicki MJJ, Góra RWW, Szabla R, Bond ADD,

1197 Sutherland JDD. 2020. Selective prebiotic formation of RNA pyrimidine and DNA purine

1198 nucleosides. *Nature* **582**:60–66. doi:10.1038/s41586-020-2330-9

1199 Zgarbová M, Otyepka M, Šponer J, Mládek A, Banáš P, Cheatham TE, Jurečka P. 2011.

1200 Refinement of the Cornell et al. Nucleic acids force field based on reference quantum

1201 chemical calculations of glycosidic torsion profiles. *J Chem Theory Comput* **7**:2886–

1202 2902. doi:10.1021/ct200162x

1203 Zhang SJ, Duzdevich D, Szostak JW. 2020. Potentially prebiotic activation chemistry

1204 compatible with nonenzymatic RNA copying. *J Am Chem Soc* **142**:14810–14813.

1205 doi:10.1021/jacs.0c05300

1206 Zhou L, Ding D, Szostak JW. 2021. The virtual circular genome model for primordial RNA

1207 replication. *RNA* **27**:1–11. doi:10.1261/rna.077693.120

1208 Zhou L, O’Flaherty DK, Szostak JW. 2020. Template-Directed Copying of RNA by Non-

1209 enzymatic Ligation. *Angew Chemie - Int Ed* **59**:15682–15687.

1210 doi:10.1002/anie.202004934

A

B

C

D

27 bp

A) Linear RNA

B) KCl 100 mM

C) $MgCl_2$ 500 mM

A **B** **C** **D** **E** **F**

200 mM KCl

100 mM MgCl₂

500 mM MgCl₂

A

B

Gel electroporation of
One-pot extension of A-D

C

A

B

Effect of dilution, Pos. 10 (Invasion)

8211

Enhanced contrast

A

Kinetics of micro HHrz

B

Fraction of circle