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Freeze-thaw cycles as drivers of complex ribozyme assembly

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Abstract

The emergence of an RNA catalyst capable of self-replication is considered a key transition in the origin of life. However, how such replicase ribozymes emerged from the pools of short RNA oligomers arising from prebiotic chemistry and non-enzymatic replication is unclear. Here we show that RNA polymerase ribozymes can assemble from simple catalytic networks of RNA oligomers no longer than 30 nucleotides. The entropically disfavoured assembly reaction is driven by iterative freeze-thaw cycles even in the absence of external activation chemistry. The steep temperature and concentration gradients of such cycles result in an RNA chaperone effect that enhances the otherwise only partially realized catalytic potential of the RNA oligomer pool by an order of magnitude. Our work outlines how cyclic physicochemical processes could have driven an expansion of RNA compositional and phenotypic complexity from simple oligomer pools.

There is compelling evidence for a primordial biology in which RNA was the central biomolecule responsible for both molecular heredity and metabolism¹. A cornerstone of this “RNA world” conjecture is the emergence of RNA catalysts capable of self-replication and evolution, through a range of intermediate steps from nucleotide building blocks provided by prebiotic chemistry.

Recent developments have provided plausible avenues for the prebiotic synthesis of some activated ribonucleotides² and for the non-enzymatic polymerization^{3,4} and templated replication^{5–8} of short RNA oligonucleotides even within membranous protocells⁸. Montmorillonite clays³, extended drying⁹ or hydration-dehydration regimes¹⁰ have been shown to promote formation of medium (~40 nts) to long (>300 nts) mono- or dinucleotide RNA polymers. However, polymerisation of RNA from all four natural nucleotides is much less efficient and even the most favourable conditions yield RNA oligomers barely exceeding ~20 nts¹¹.

There are thus plausible mechanisms for the generation of pools of short RNA oligomers from prebiotic precursor molecules. However, how more complex RNA functions such as

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Contributions

H.M., A.W. and P.H. conceived and designed the experiments. A.W. performed in-ice invasion of the stemloop structure and probed structure guided splitting of tC19Z. H. M. performed and analysed all other experiments. All authors discussed the results, and wrote and commented on the manuscript.

Competing financial interests

The authors declare no competing financial interests.

ligation, recombination or templated synthesis (required for self-assembly, self-replication and evolution) could emerge from such pools is far from obvious.

In vitro RNA evolution experiments have yielded several RNA catalysts with ligase, recombinase and / or polymerase activity, and these provide information on the minimal structural and functional complexity required for these processes. Variants of the R3C RNA ligase¹² as well as split constructs of the *Azoarcus* self-splicing intron¹³ can form cross-catalytic self-assembly networks but evolution in both is constrained by the requirement for large pre-synthesized oligomer building blocks with substantial homology to the ribozyme core. To enable a more general capability for self-replication and evolution the use of short activated oligonucleotide substrates together with a long organizing template strand has been explored using the *sunY* self-splicing intron¹⁴, and recently through a cross-chiral RNA ligase system¹⁵. Closer functional analogues of primordial replicases are RNA polymerase ribozymes (RPRs), such as the R18 polymerase ribozyme¹⁶. These are capable of templated synthesis from RNA mononucleotide triphosphates (NTPs) and some variants are able to synthesize another ribozyme¹⁷ or RNA oligomers exceeding their own size (~200 nts)¹⁸, although the synthetic power of these RPRs is confined to favourable template sequences. Replication of long RNAs is complicated by the related problems of inhibitory template secondary structures¹⁸, and the high stability of product RNA duplexes >30 nts^{19,20}.

Thus, efficient RNA assembly and in particular templated RNA synthesis from mononucleotides appears to require catalytic and template RNAs substantially larger than the short oligomers provided from prebiotic chemistry and amenable to non-enzymatic replication. There is thus a compositional as well as a conceptual gap between the primitive pools of short RNA oligomers and the phenotypically complex ribozymes likely to be required for self-replication.

Here we have explored a strategy to bridge this gap. Specifically, we ask if RNA polymerase ribozymes (RPRs) could have emerged from RNA oligomer pools by step-wise modular assembly^{21,22}. Working backwards from tC19Z (one of the most advanced RPRs¹⁷), we show efficient assembly of RPR function from mixtures of RNA oligomers no longer than 30 nts. We discover that a cyclic physicochemical process – freeze-thaw cycling – is a potent driver of RPR assembly and critical to unlocking the full functional potential of the varied short RNA modules through an unanticipated RNA chaperone effect.

Results and discussion

We first sought to establish whether an active RPR might be assembled from RNA oligomer fragments by the action of a small ligase ribozyme that itself could be split and reassembled from such fragments. We chose the naturally occurring hairpin ribozyme (HPz, Fig. 1a), which catalyses both RNA cleavage and ligation reactions through reversible transesterification^{23,24} involving prebiotically plausible 2', 3'-cyclic phosphate (>p) activation chemistry². Based on previously described split HPz variants²⁵, we designed a variant (3frHPz) that self-assembles spontaneously from three short RNA oligomers of 16, 18 and 21 nts (Fig. 1b; Supplementary Tab. 1). 3frHPz retained a high level of activity enabling iterative ligation of a 16-mer oligomer into large RNA molecules (112 nts) in the

eutectic phase of water ice, where HPz catalysed ligation is favoured²⁶⁻²⁹ and RNA stability strongly enhanced^{27,30} (Supplementary Fig. 1). Next, we introduced three canonical HPz ligation sites into the tC19Z polymerase ribozyme (yielding variant RPR4) (Fig. 1c) with only marginal effects on RPR activity (Supplementary Fig. 2). This allowed 3frHPz-directed assembly of full length RPR from four >p activated RNA fragments (1 (47 nts), 2 (55 nts), 3 (38 nts), 4 (58 nts)) by three ligation events. RPR4 assembly was dependent on 3frHPz and eutectic ice phases (Fig. 2a), with no detectable assembly observed under ambient or supercooled conditions.

Freeze-thaw cycling drives covalent ribozyme assembly

However, yields of full-length RPR4 were unexceptional (~3.5%) even after extended incubation (96 h). We hypothesized that the well-known propensity of HPz to fold into a range of kinetically stable but inactive conformational states^{29,31,32} might restrict assembly yields.

The conformational and functional heterogeneity of HPz is common among functional RNAs. In modern biology kinetically-trapped RNA folding intermediates are resolved by (proteinaceous) chaperones and helicases³³. However, such sophisticated molecular machines were not available to primordial catalysts. We chose to investigate the potential of physicochemical gradients as RNA folding ‘engines’ instead. Indeed, thermal variations had previously been used to promote exploration of alternative structures within the RNA folding landscape³⁴ or to release substrates trapped in inactive conformations³⁵. However, in contrast to sub-zero temperatures, high temperatures accelerate RNA degradation³⁶ and >p hydrolysis³⁷ and bias HPz catalysed transesterification towards the cleavage reaction²⁶. The challenge therefore was to identify a plausible physicochemical regime that might exhibit the benefits of thermal cycling without unduly accelerating phosphodiester-bond hydrolysis and RNA fragmentation.

We chose to investigate the potential of the steep thermal and ionic gradients of freeze-thaw (FT) cycles, which had previously been suggested to improve HPz ligation yields²⁷. Harnessing a programmable water-bath we implemented an automated 12h FT-cycling scheme (Fig. 2b) whereby freezing episodes (to -30°C , to ensure homogeneous freezing and avoiding the formation of super-cooled solutions) and thawing episodes (to a peak temperature of 37°C) are interspersed by incubation under frozen eutectic conditions (-9°C). This FT-cycling protocol boosted RNA ligation, enabling the assembly of RNAs of >250 nts from 16-mer model substrates (Supplementary Fig. 3). More importantly it provided a striking increase in the cumulative 3frHPz ligation yields up to ~19% of full length RPR4 after 20 FT-cycles (Fig. 2d) at stoichiometric RNA concentrations (1 μM) with yields monotonically rising with increasing number of cycles (Fig. 2c, d). These yields could be further improved to ~27% (Fig. 2c, d) by optimizing fragment stoichiometries (see Materials & Methods for RNA concentrations).

Freezing enhances formation of intermolecular RNA complexes

Next we sought to disentangle the contributions of the individual steps of the freeze-thaw cycles on HPz catalysis. We explored a model system comprising an RNA duplex with a

central loop akin to 3frHPz (Fig. 3a), with one strand labelled with a fluorophore (F) the other with a quencher (Q) thus suppressing fluorescence in the duplex when F and Q are in close proximity. Conversely, strand dissociation increases the distance between F and Q and activates fluorescence. The duplex is kinetically stable during thawing as well as the peak temperature of 37°C and is only slowly invaded by a competing RNA oligonucleotide during prolonged incubation at 37°C (~70% after 60 h, Fig. 3b, c). In sharp contrast, freezing at -9°C strongly accelerates strand exchange by two orders of magnitude (~65% after 40 min, Supplementary Fig. 4) and invasion of the internal loop by the competing RNA strand is essentially complete after just one FT-cycle (Fig. 3b, c). In analogy, we observed rapid invasion of a RNA stem-loop structure by competing oligonucleotides down to temperatures of -25°C, with no strand exchange observed at ambient temperatures (Supplementary Fig. 5).

Based on these observations we propose a model for the FT-driven enhancement of HPz ligation activity (Fig. 3d): at ambient temperatures, both 3frHPz domains (loop B and loop A, Fig. 1b) are likely to be associated but ligation activity is attenuated due to their inability to form a catalytically proficient docking complex in absence of Mg^{2+} ions or high levels of monovalent cations³⁸, which are omitted from our experimental setup. Freezing triggers formation of a sub-population of active complexes, presumably due to the steep increase in RNA and solute concentrations^{29,30} (in analogy to effects described for various crowding agents³⁹) while others form alternative inactive complexes⁴⁰. Thawing disrupts both active as well as unproductive complexes due to the steep drop in RNA and solute concentrations. Thus, FT cycling allows a “reboot” of HPz catalytic complex formation (effectively giving unproductive complexes a “second chance” at catalysis) and iteratively drives the HPz reaction towards thermodynamic equilibrium. Indeed, when we analysed the transesterification reaction of RPR4 fragments 1 and 2 from both the substrate (unligated) and product (fully ligated) side, we found step-wise convergence towards equilibrium proceeding through multiple FT-cycles (Fig. 3e). In contrast no such convergence was observed when the reaction was incubated under constant freezing conditions, indicating that without an intermittent thawing step a substantial amount of ribozyme remains trapped in inactive complexes.

Based on this model FT-driven assembly of RPR4 by 3frHPz can be described by a simple set of recurrence relations that assumes independent ligation events in which the order of fragment ligation is insignificant (see Supplementary Methods and Supplementary Fig. 6). According to this model between 38% - 65% of the RPR4 junctions remain unproductive during each cycle but repeated FT-cycling allows each individual 3frHPz ligase to approach equilibrium ligation yields between 50% and 75% (Supplementary Fig. 6).

Emergence of RNA polymerase activity

Having established a strategy for efficient RPR assembly we next investigated if this provided a concomitant rise in RNA polymerase activity or if unligated starting material and partially ligated intermediates (Fig. 4a) might inhibit polymerase activity by sequestering primer-template duplexes or forming inhibitory complexes with assembled RPRs. However, despite a highly heterogeneous pool of ligase and RPR components (17 RNA oligomers), we

observed RPR activity equivalent to 37% full-length RPR activity after just two FT cycles (Fig. 4b). At this point, only ~9% of the 5' fragments had been incorporated into full-length RPR4 (as judged by gel electrophoresis). After 8 FT cycles activity increased to >60% (with only 15% - 19% full-length assembled RPR4). While consistent with the fact that some split variants of RPR4 display polymerase activity (Supplementary Fig. 7) and may therefore contribute to pool activity, the observed primer extension exceeds the expected activity for an additive model of all sub-activities. This suggests that (some) RPR4 assembly intermediates cooperate in primer extension and contribute synergistically to global polymerase activity of the heterogeneous RNA pool (Supplementary Fig. 7).

Assembly from prebiotically plausible RNA oligomer sizes

The above experiments involved RNA oligomers ranging from 38 to 58 nts, but these are not accessible by known prebiotic processes of mixed RNA oligomer formation. We therefore sought to achieve RPR assembly from shorter RNA oligomers, but this presents a range of technical challenges. Fragmentation of the RPR into oligomers of 30 nts (or shorter) requires the definition of no less than six mutually exclusive (orthogonal) ligation sites in the RPR sequence with a concomitant steep rise in the potential for misassembly. To aid design of such orthogonal sites and relieve functionally problematic sequence constraints, we performed *in vitro* selections to expand the sequence spectrum of HPz. This yielded 64 functional ligation sites (LG) (Supplementary Fig. 8), which allowed the design of another active RPR variant (RPR7, Supplementary Fig. 9) comprising one canonical LG (as used in RPR4) together with five novel LGs identified by selection. These define seven RNA fragments ranging from 18 to 30 nts, which are recognized by six substrate binding strands (SBS, Fig. 1b) ranging from 18 to 21 nts (Supplementary Tab. 1).

As with RPR4, assembly of RPR7 was monitored using a fluorescently labelled 5'-fragment, expected to visualize five of the 20 cognate concatenation intermediates as well as the full-length product (Fig. 5a). Although potential non-cognate side-reactions increase steeply with the number of ligation sites, we observed rapid accumulation of the cognate ligation products again driven by FT cycles (Fig. 5b). The newly selected LGs displayed lower fractional activities (8%-29%) per FT cycle compared to the wild type LGs used for RPR4 assembly (35%-62%) (Supplementary Fig. 10). However, at near-stoichiometric fragment concentrations (see Methods section) we observed putative full-length RPR7 (as confirmed by reverse transcription and DNA-sequencing) as early as at 8 FT cycles, reaching ~3% yield after 34 FT-cycles (Fig. 5b).

Assembly without >p preactivation

RNA oligomer pools created by non-enzymatic (or enzymatic) polymerization of 5'-activated nucleotides retain a free 3'-OH and are therefore not activated for ligation. However, we reasoned that the reversible nature of the transesterification reaction might be exploited to activate RNA oligomers through the *in situ* generation of terminal >p groups, by cleavage of short (7 – 8 nts) 3'-overhangs (Supplementary Tab. 1). Thus, full-length RNA assembly proceeds through iterative steps of cleavage, strand exchange, and ligation (Fig. 6a, b) reminiscent of modern-day recombination or processing of viroids and viroid-like satellite RNAs⁴¹. Starting from RNA oligonucleotides with a cleavable 3' sequence tag, we

observed iterative assembly of up to 10% full-length RPR4 proceeding through the predicted cleavage intermediates after 48 FT-cycles (Fig. 6c; Supplementary Fig. 11). Thus, some pools of short RNA oligomers exposed to the RNA chaperone activity of freeze-thaw cycles not only have a capacity for self-assembly but also an inherent potential for self-activation and recombination as seen in more complex ribozyme systems^{13,42}.

Despite the considerable heterogeneity of the assembly components in this system (comprising up to 29 RNAs including 16 different RPR species), RPR activity again rose rapidly during FT cycling (Fig. 6d) although requiring more cycles due to the multi-step nature of the reaction trajectory (Fig. 6b). Detectable RPR activity manifested itself early (3 FT-cycles), even preceding the detection of a full-length RPR4 band by gel electrophoresis. After 12 FT cycles, RPR activity of the heterogeneous assembly mix matched the activity of *in vitro* transcribed RPR4 despite an apparent assembly of only ~5% full-length RPR4. Despite the much larger number of intermediate species, the steep rise in polymerase activity parallels that seen earlier in RPR4 assembly by direct fragment ligation (Fig. 4b) and is again consistent with synergy among assembly intermediates. Notably the activity of this heterogeneous pool of assembly intermediates with RPR and HPz components and cleaved sequence tags is superior to a homogeneous mixtures of rationally designed RPR fragments that assemble through non-covalent hybridisation (Supplementary Fig. 7).

Discussion

We have explored prebiotically plausible processes by which structurally and phenotypically complex RNAs such as RNA polymerase ribozymes (RPR) can emerge from simple mixtures of short RNA oligomers. We show that such oligomers need not be longer than 30 nts, need not be individually activated or be catalytically active and even as an ensemble need possess only meagre ligase and no detectable polymerase activity. However, subjected to iterative cycling between ambient and sub-zero temperatures, between a liquid and frozen eutectic phase, a pool of such RNA oligomers is able to rapidly organize itself into a catalytic network with potent emergent polymerase activity.

Repeated FT cycles, likely to have occurred on the early earth diurnally, seasonally, or as a result of periodic geothermal and meteoritic activity, emerge here as critical drivers of RNA assembly. Unlike other physicochemical cycles like wet-dry or thermocycles at higher temperatures, freezing supports RNA integrity as well as RNA ligation over RNA cleavage in the HPz reaction. FT cycles provide mild conditions of moderate temperature changes together with steep gradients in solute concentration, water activity and surface area. These are well known to cause protein denaturation^{43,44}, but appear to act on RNA akin to a RNA chaperone, effecting a re-equilibrating kinetically trapped RNA conformers (Fig. 3d, e). The mechanistic basis of this effect is currently unknown and its elucidation complicated by the difficulty of direct biophysical measurements within the eutectic phase. However, our model experiments suggest that a putative mechanism involves the local and transient unfolding of RNA duplex structures and tertiary interaction most likely driven by changes in RNA and solute concentrations. However, at this point, we cannot rule out contributions of surface effects such as localized pH changes and ionic potentials^{45,46} at the ice-brine interface.

FT cycles thus underline the potential importance of cyclic phenomena, surface and gradient effects in RNA biogenesis. Such phenomena may form part of both “hot” and “cold” scenarios^{47,48} for the origin of life. High temperatures are thought to be required for RNA strand separation in different scenarios of RNA replication¹⁹ and cyclic hydration–dehydration in hot environments can promote nucleotide polymerisation into long RNA-like chains¹⁰. Furthermore, spatial temperature gradients have recently been shown to promote replication, feeding and positive length selection of genetic polymers through thermophoretic effects⁴⁹. However, prolonged exposure of RNA to high temperatures also accelerates RNA decomposition thereby complicating scenarios of continuous RNA replication and evolution. Conversely, cold and in particular frozen environments reduce water activity, enhance RNA stability while also promoting RNA biogenesis¹¹ and replication^{5,27,50} but may lack the energy required for RNA strand separation. Our results indicate that cyclic temporal gradients of solute concentration and temperature can drive formation of long RNA molecules and hint at a potentially crucial role of the interfaces between “hot” and “cold” environments in abiogenesis.

Furthermore, we find that the emergence of robust polymerase activity is not dependent on (and indeed precedes) the assembly of the full-length RPR, due to an unanticipated synergy among RPR assembly intermediates (Supplementary Fig. 7). This observation again underlines the potential benefits of compositional heterogeneity⁵¹, and suggests that emergence of RPR activity does not require complex feedback controls to regulate levels and stoichiometry of the individual components. This bodes well for RNA replication schemes that involve cycling between fragmented genotypes and assembled phenotypes^{52,53}. Indeed, it might be argued that functional RNA phenotypes might arise with higher frequency from pools of fragmented genotypes (as e.g. a 2 μ mol pool of random 30-mers contains all (10^{18}) sequence blocks needed to assemble any larger ribozyme). Furthermore, such fragmented genotypes are likely to be more amenable to replication due to reductions in duplex stability and template secondary structure propensity in RNA oligomers < 35 nts¹⁹ while at the same time allowing an expansion of ribozyme size and complexity beyond pool oligomer lengths as we demonstrate. Our work thus outlines how cyclic physicochemical processes (such as freeze-thaw cycles) can drive an expansion in both compositional and phenotypic complexity of short RNA oligomer pools, and begin to bridge the conceptual gap from the prebiotic chemistry of short RNA oligomers to the quasi-biological activity of complex replicating ribozymes.

Methods

Oligonucleotides

Ribozyme, RNA template and DNA oligonucleotide sequences, sources and purification methods are described in Supplementary Methods and listed in Supplementary Tab. 1 and 2.

Ligation experiments

Ligation experiments without FT-cycling were performed similar as described previously^{27,29}. Briefly, 3frHPz fragments and substrate strands were mixed in ligation buffer (1 mM Tris•HCl pH 8.3/25°C, 25 mM NaCl) and incubated for 10 minutes at 37°C.

Samples were frozen using dry ice and tubes transferred to a TXF200-R3 refrigerated water-bath (Grant) filled with 50% (v/v) Blue Star Antifreeze (Carplan) cooled to -9°C . Ligation under influence of FT-cycling was performed in the same buffer using a second water bath. The temperature profile shown in Fig. 2C was created using the following automated protocol: 1: Set target temperature to 37°C for 10 min; 2: Set target temperature to -30°C for 2 h 30 min; 3: Set target temperature to -9°C for 9 h 20 min 4: Return to step 1.

Shorter freezing to -30°C (step 2) was prevented by the limiting cooling rate of the refrigerator unit. All samples were resolved by urea-PAGE of 0.5 pmol fluorescently labelled RNA and analysed using a Typhoon Trio scanner (GE Healthcare). Fractional intensities of starting material and ligation products were determined using ImageQuant TL. Gel image brightness and contrast were adjusted to illustrate ligation product banding patterns. Quantitative analyses and fitting of ligation experiments are described in the Supplementary Methods section.

Strand invasion experiments

Strand invasion was measured using a 24 nts duplex with a 4 nts internal loop formed from two RNA strands labelled with either a 5'-TEX 615 fluorophore (F24MM_fw) or a 3'-Iowa Black RQ quencher (24Q_rev, for sequences see Tab. S1). Preformed F24MM_fw / 24Q_rev duplex (0.25 μM) was mixed with a 4-fold excess of unlabelled RNA oligonucleotide (24_fw, 1 μM) with perfect complementarity to Q24_rev in transparent and sealable 96-well PCR plates (StarLab) and either incubated at a constant temperature of 37°C or in the programmed refrigerated water bath described in the previous section. Plates were removed periodically during the thawing episodes of the FT-protocol and quickly scanned using a Typhoon Trio scanner (GE Healthcare, 3 mm focal plane, 610-nm band-pass filter). Fluorescence signals were measured in triplicates and quantified using ImageQuant TL. Experiments probing invasion into a stem-loop structure are described in Supplementary Fig. 5. Normalized $\text{FQ}/\text{F}_{\text{free}}$ values were obtained by dividing the observed intensities by the fluorescence intensity of unquenched F24MM_fw.

Assembly of RPR4 and RPR7

Ligation of RPR4 under stoichiometric conditions was carried using 1 μM >p activated fragments (RPR4.1 - RPR4.3) or 1 μM tailed fragments (RPR4.1t - RPR4.3t), 1 μM RPR4.4, 1.1 μM SBS (SBS.RPR4.1 - SBS.RPR4.4), and 4 μM of loopB5 and loopB3. Direct ligation under more optimized conditions was performed by adding every 3'-strand in 1.25-fold excess over its 5' binding partner therefore driving the reaction further towards formation of full-length RPR. This resulted in the following aqueous RNA concentrations: 0.5 μM RPR4.1, 0.63 μM SBS.RPR4.1, 0.79 μM RPR4.2, 1 μM SBS.RPR4.2, 1.24 μM RPR4.3, 1.55 μM SBS.RPR4.3, 1.94 μM RPR4.4, and 4 μM of loopB5 and loopB3. The identity of RPR4 was confirmed by gel-extraction of the putative full-length product followed by RT-PCR (SuperScript III One-Step RT-PCR System, Life Technologies), cloning (pGEM-T Easy Vector System, Promega) of the PCR product into NEB 10-beta cells (NEB), and colony sequencing (Beckman Coulter). Assembly of RPR7 was performed by adding every 3'-strand in 1.05-fold excess over its 5' binding partner resulting in the following aqueous RNA concentrations: 0.5 μM RPR7.1, 0.525 μM SBS.RPR7.1, 0.55 μM RPR7.2, 0.58 μM

SBS.RPR7.2, 0.61 μ M RPR7.3, 0.64 μ M SBS.RPR7.3, 0.67 μ M RPR7.4, 0.7 μ M SBS.RPR7.4, 0.74 μ M RPR7.5, 0.76 μ M SBS.RPR7.5, 0.81 μ M RPR7.6, 0.86 μ M SBS.RPR7.6, 0.9 μ M RPR7.7, and 4 μ M of loopB5 and loopB3. Under these conditions, loop B remained in excess over the maximal amount of complete SBS-substrate ligation junctions (3.88 μ M). The identity of PRP7 was confirmed by gel-extraction of the putative full-length product followed by RT-PCR and sequencing as described for RPR4.

RPR primer extension assays

Primer extension assays using full-length RPRs or RPR fragments were carried out as described previously¹⁷ and are briefly described in the Supplementary Methods section.

Selection and deep sequencing of novel HPz variants

For a detailed description see the Supplementary Methods section.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. The hairpin ribozyme and RPR4

a, Secondary structure and conserved residues of a typical 2-way junction variant of the hairpin ribozyme (~50 nts). An RNA substrate (green) is reversibly ligated at a specific site (red arrow) from a 2', 3'-cyclic phosphate and a 5'OH. This activity requires docking of the loop A and loop B domains. **b**, Illustration of the minimal 3frHPz-substrate complex in which the hairpin ribozyme is minimized and fragmented into three short oligonucleotides: A substrate binding strand (SBS, black, 16 - 22 nts in this study) that binds to the substrate strands (variable length, green) thereby forming loop A, which can assemble

into a catalytically active *trans*-complex with loop B (18 nts and 21 nts, blue) through tertiary interactions. **c**, Predicted secondary structure of the tC19Z derivative RPR4. Different colours indicate fragments defined by canonical HPz ligation junctions. Nucleotides forming the ligation junction with the SBS of wild type HPz are shown in bold.

Figure 2. Freeze-thaw cycling drives RPR4 assembly

a, Time course of covalent RPR4 assembly by 3frHPz from four fragments under eutectic conditions (-9°C). No assembly is observed after 96 h when either loop B of 3frHPz ($-B$) is omitted or the reaction mix remains super-cooled (sc) (right panel). **b**, Recorded temperature profile of two automated FT cycles (24 h) used to promote HPz catalysis. After thawing at 37°C , samples are frozen by cooling to -30°C . Increasing the temperature to -9°C induces eutectic conditions and restores HPz catalysis. **c**, FT-driven RPR4 assembly by 3frHPz at optimized fragment stoichiometries (see Material and Methods for concentrations). Full-length RPR4 was gel-extracted and tested for primer extension activity (right panel). **d**, Yields of full-length RPR4 (stoichiometric fragment concentrations) at continuous incubation at -9°C (grey) or incubation interrupted by the FT-cycles shown in (**b**) (cyan). The further enhancement of the reaction to $\sim 27\%$ full-length RPR at optimized fragment stoichiometries is shown in green.

Figure 3. Effect of freezing and thawing on RNA and 3frHPz dynamics

a, Model system to probe RNA-RNA dynamics. Hybridisation of an internal loop formed was measured using a fluorophore/quencher FRET pair and the increase in fluorescence caused by invasion of an unlabelled oligonucleotide. **b**, Fluorescence intensity of the fluorophore-labelled strand (F) at either continuous incubation at 37°C or repeated FT-cycling and in either in absence or presence of quencher-labelled strand (Q) and competing oligonucleotide (C). **c**, Kinetics of the normalized fluorescence increase (FQ/F_{free} , $n = 3$) of the fluorophore-labelled strand (F) bound to the quencher labelled strand at either continuous incubation at 37°C (orange) or repeated FT-cycling (cyan) and in either in absence or presence of competing oligonucleotide (C). **d**, Model of the RNA-chaperone effect of FT-cycling on 3frHPz catalysis. In absence of bivalent metal ions 3frHPz remains inactive in unfrozen solutions. Freezing to eutectic conditions allows a sub-population of the ribozyme to associate and fold and perform catalysis, while the remaining RNAs become trapped in kinetically stable but poorly active or inactive conformations. FT cycling increases the probability for substrates to encounter an active ribozyme through iterative re-equilibration. **e**, Transesterification of the first RPR4 ligation site at continuous incubation at -9°C (grey) or incubation interrupted by FT-cycles (cyan). The reaction was either started from the substrate side of the reaction (triangles, unligated fragments) or product side of the reaction (squares, fully ligated fragments).

Figure 4. RPR4 assembly intermediates pathways and emergence of polymerase activity

a, Network diagram of all cognate ligation reactions of RPR4 assembly. Corners represent the input fragments. Lines are drawn between ligatable fragments and nodes represent ligation products. Only products containing fragment 1 are visible in Fig. 2. **b**, Primer extension activity of ligation mixtures as a function of FT-cycling. Ligation conditions were the same as in Fig. 2d. Primer extension reactions were started after quenching of the 3frHPz SBS using antisense-oligonucleotides (Supplementary Tab. S1). Primer only control (–), and the activity of *in vitro* transcribed full-length RPR4 in presence of 3fHPz and antisense oligos (FL) are shown for comparison.

Figure 5. Assembly of a polymerase ribozyme from fragments 30 nucleotides

a. Network diagram of all possible cognate ligation reactions of RPR7 assembly as in Fig. 4a. The colour of the nodes indicates the number of incorporated fragments (from blue (2) to red (7)). Numbered nodes highlight products that are visualized in Supplementary Fig. 8. **b.** FT assisted ligation of RPR7. Fragment concentrations ranged from 0.5 μM (fragment 1) to 0.9 μM (fragment 7). Products verified by sequencing are coloured according to (a).

Figure 6. Assembly of RPR4 from oligonucleotides devoid of >p pre-activation

a, Schematic representation of the assembly trajectory involving (anti-clockwise from top left), cleavage of a short 3' tail (red) generating a 2', 3' cyclic phosphate (>p) (red dot), dissociation of the cleaved tail and strand exchange to cognate substrate (orange) followed by ligation of substrate 5' OH with >p. **b**, Network diagram of RPR4 assembly from 4 tailed fragments. Tailed input fragments can ligate to their cognate 5' fragments but have to be cleaved (red lines) before ligation to 3' fragments. **c**, FT-cycle driven assembly of RPR4 by 3frHPz from four inert fragments (1 μ M) proceeding from the fluorescently labelled 5'.

fragment (1t) through several intermediates to full-length RPR (1234). **d**, Primer extension activity of the reaction mixtures shown in (c). Primer only control (–), and the activity of full-length RPR4 from *in vitro* transcription (FL) are shown for comparison. Reaction conditions were the same as in Fig. 4b.