

Amplification of RNA by an RNA polymerase ribozyme

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In all extant life, genetic information is stored in nucleic acids that are replicated by polymerase proteins. In the hypothesized RNA world, before the evolution of genetically encoded proteins, ancestral organisms contained RNA genes that were replicated by an RNA polymerase ribozyme. In an effort toward reconstructing RNA-based life in the laboratory, *in vitro* evolution was used to improve dramatically the activity and generality of an RNA polymerase ribozyme by selecting variants that can synthesize functional RNA molecules from an RNA template. The improved polymerase ribozyme is able to synthesize a variety of complex structured RNAs, including aptamers, ribozymes, and, in low yield, even tRNA. Furthermore, the polymerase can replicate nucleic acids, amplifying short RNA templates by more than 10,000-fold in an RNA-catalyzed form of the PCR. Thus, the two prerequisites of Darwinian life—the replication of genetic information and its conversion into functional molecules—can now be accomplished with RNA in the complete absence of proteins.

self-replication | ribozyme | RNA enzyme | PCR | *in vitro* evolution

The core informational processes of Darwinian evolution are the replication of genes and their expression as functional molecules, which in modern biology require the action of protein enzymes. Features common to all extant life suggest the existence of an RNA world where the replication and expression of genetic information depended on RNA enzymes rather than genetically encoded proteins (1, 2). Accordingly, substantial efforts have been directed toward reconstructing RNA life via the protein-free replication of RNA (3, 4). RNA-joining ribozymes have been modified to assemble new copies of themselves from smaller RNA substrates (5), in one case achieving self-replication with exponential growth (6). However, these self-replicating ribozymes require complex oligonucleotide substrates, which limits their ability to synthesize functional RNA molecules other than additional copies of themselves, which in turn limits their ability to express and evolve functions beyond self-replication.

A more general solution is offered by the template-directed polymerization of RNA monomers, the mechanism used in modern biology for the synthesis of DNA and RNA. RNA-templated RNA polymerization has been demonstrated both nonenzymatically (7, 8) and with a variety of natural and synthetic ribozymes (9–11). Although none of these systems have been able to replicate RNA exponentially, extensive copying of RNA templates has been achieved with evolved variants of the class I RNA polymerase ribozyme. This ribozyme can synthesize tandem RNA repeats over 100 nt long and even complete a substantial portion of a small endonuclease ribozyme (12–15). However, the polymerase strongly prefers cytidine-rich templates that lack any secondary structure and has much more limited activity in other contexts (15). These limitations preclude the synthesis of most functional RNAs, which often are highly structured, and the replication of RNA, which requires the reciprocal synthesis of both an RNA template and its complement.

Template restrictions might be eased by using *in vitro* evolution to select directly for the synthesis of functional RNAs from “difficult” templates. In the present study, an engineered form of the class I polymerase was selected for its ability to synthesize functional RNA aptamers from complementary templates. In contrast to previous *in vitro* evolution efforts directed at RNA

polymerization, this approach does not select directly for chemical bond formation, but rather for the efficient and accurate transfer of functional information from template to cRNA. A polymerase variant isolated after 24 rounds of evolution exhibited dramatically improved activity and template generality, enabling both the synthesis of structurally complex RNAs and the replication of short RNA sequences in a protein-free form of the PCR. This work demonstrates that the replication and expression of genetic material can be accomplished with RNA alone. The prospect for further improvement of these activities suggests a practical route toward the development of RNA-based life in the laboratory.

Results

Evolution Based on Aptamer Completion. An engineered form of the class I polymerase ribozyme [wild type (WT); Fig. S1] was constructed by combining known advantageous features, including a large deletion within the 3'-terminal domain; a 5'-terminal sequence tag that binds to a complementary region of the template; and a set of activity-enhancing mutations (14, 16). Random mutations then were introduced throughout the molecule at a frequency of 10% per nucleotide position to generate a population of 10¹⁴ distinct variants to initiate the *in vitro* evolution process. During each round of evolution (Fig. 1A), an RNA primer was covalently attached to the 5' end of the polymerase and nucleotide extension reactions were performed using a separate RNA template and the four nucleoside triphosphates (NTPs).

Two forms of selection pressure were applied to the population to obtain more active polymerases. First, the polymerase was challenged to extend the attached primer to complete a 3'-truncated RNA aptamer, enabling selection based on binding of the completed aptamer to its cognate ligand. Both the cyanocobalamin (17) and GTP (18) aptamers were used to provide varied sequence contexts that are largely intolerant of mutation (Fig. 1B), thus imposing selection pressure for both sequence generality and accuracy.

Significance

Darwinian life requires the ability to replicate genotypes and express phenotypes. Although all extant life relies on protein enzymes to accomplish these tasks, life in the ancestral RNA world would have used only RNA enzymes. Here, we report the *in vitro* evolution of an improved RNA polymerase ribozyme that is able to synthesize structured functional RNAs, including aptamers and ribozymes, and replicate short RNA sequences in a protein-free form of the PCR. Thus, the replication of RNA and the expression of functional RNA can be accomplished with RNA alone. Combining and improving these activities may enable the self-sustained evolution of RNA and offers a potential route to a synthetic form of RNA life.

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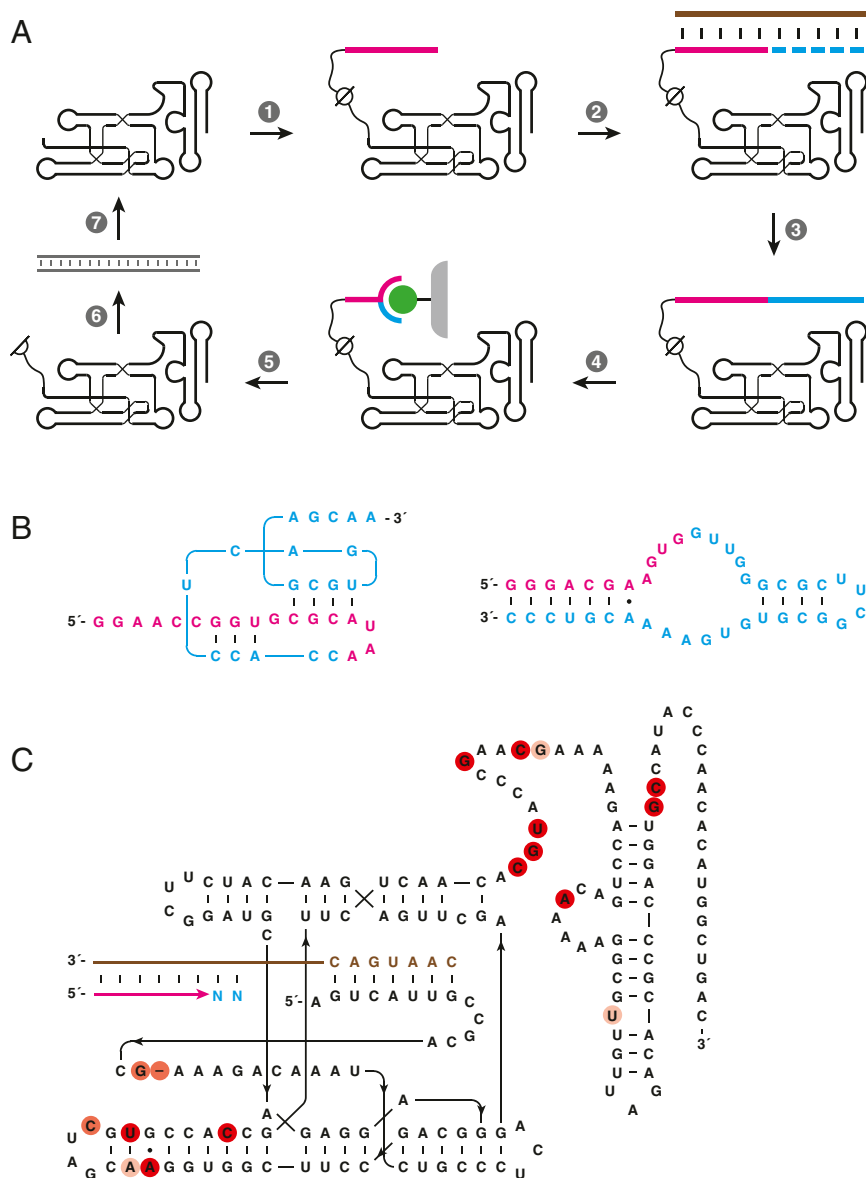


Fig. 1. In vitro evolution of RNA polymerase ribozymes. (A) Selective amplification of ribozymes that extend a tethered RNA primer (magenta) on a separate RNA template (brown) to complete a 3'-truncated aptamer. (1) Attachment of the primer to the ribozyme via a photocleavable linker; (2) hybridization of the primer to the template; (3) extension of the primer by polymerization of NTPs (cyan); (4) capture of full-length materials by binding the aptamer portion to its immobilized ligand (green); (5) photocleavage to release the ribozyme portion; (6) reverse transcription and PCR amplification of the released ribozyme; and (7) transcription to generate progeny ribozymes. (B) Sequence and secondary structure of RNA aptamers that bind either cyanocobalamin (*Left*) or GTP (*Right*). (C) Sequence and secondary structure of the 24-3 polymerase. Red circles indicate mutations relative to WT, with color intensity indicating mutations that were universal (dark), common (medium), or sporadic (light) among the clones that were sequenced.

Second, a gel-shift selection procedure was used to ensure the polymerase extension products had reached full length, as reflected by their corresponding mobility in a denaturing polyacrylamide gel.

Twenty-four rounds of in vitro evolution were carried out (Table S1), progressively increasing the selection stringency by increasing the length of RNA to be synthesized and decreasing the time allowed for polymerization. By the 24th round, the population could readily complete a 30-nt truncation of the GTP aptamer and copy purine-rich templates. Analysis of cloned individuals from the evolved population revealed that 11 mutations had swept to fixation and 10 additional mutations were present in many of the clones. A screen of 10 specific individuals showed that all had dramatically improved activity compared with WT, the most active of which (designated 24-3) contained 17 mutations (Fig. 1C).

Properties of the 24-3 Polymerase. The rate of polymerase-catalyzed nucleotide addition to a template-bound primer was measured using a cytidine-rich template of 11 nt, which has been used extensively to demonstrate the prowess of the class I polymerase (12–15). The average rate of primer extension by 24-3 is 1.2 nt/min, which is ~100-fold faster than that of the WT or previously reported tC19Z polymerase (14) (Fig. 2A). The fidelities of the WT and 24-3 polymerases on this template, at comparable yields of product, are 96.6% and 92.0%, respectively (Table S2). The higher error rate of 24-3 is due primarily to an increased tendency for G•U wobble pairing. Excluding these mutations, WT and 24-3 have comparable fidelities. It appears that selection for aptamer completion was tolerant of occasional wobble mutations relative to the demands for increased rate and improved sequence generality.

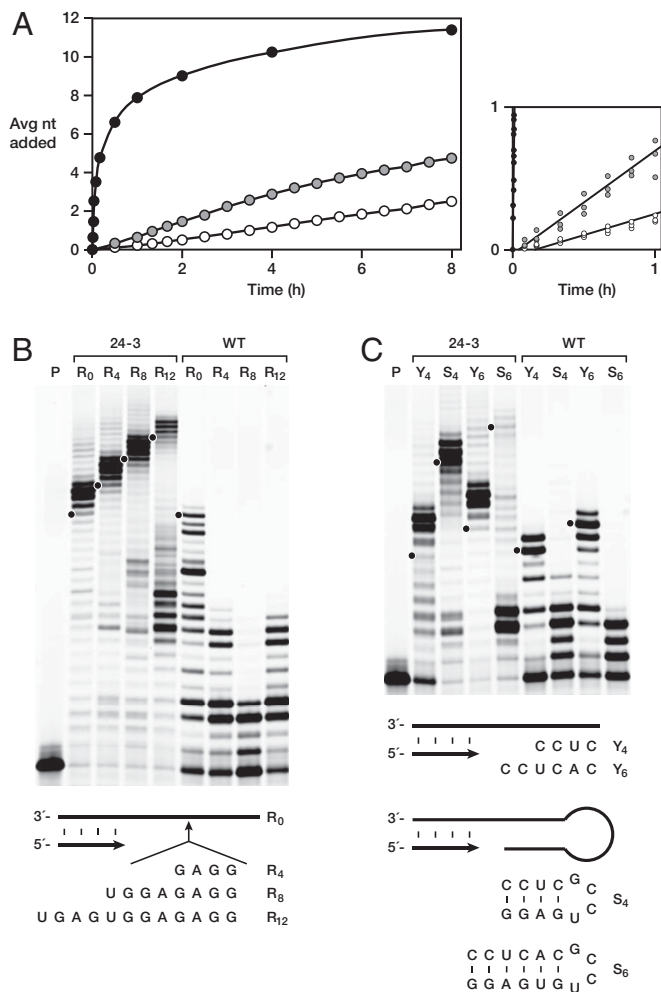


Fig. 2. Improved activity of the 24-3 polymerase. (A) Primer extension on an 11-nt C-rich template catalyzed by the 24-3 (black), WT (gray), or tC19Z (white) polymerase. Data represent the average of three replicates, with SDs in the range of 0.03–0.09 nt/min and not visible in the figure. Initial rates based on a linear fit of the data (at right, with replicates shown) were 1.2, 0.012, and 0.005 nt/min, respectively. Reaction conditions: 1 μ M polymerase, 0.4 μ M primer, 0.5 μ M template, 4 mM each NTP, 200 mM MgCl₂, and 50 mM Tris (pH 8.3) at 17 °C. (B) Primer extension on templates containing a 20-nt C-rich sequence interrupted by a purine-rich sequence of 0, 4, 8, or 12 nt (R₀, R₄, R₈, or R₁₂, respectively). (C) Primer extension on templates containing stem-loops of either 4 (S₄) or 6 bp (S₆), in comparison with the 5' portion of the stem alone (Y₄ or Y₆, respectively). Reaction conditions for B and C as above, but using 100 nM polymerase, 40 nM primer, and 50 nM template and incubating for 24 h. P, unextended primer. Dots indicate full-length extension products, with extralength material corresponding to continued extension through the spacer and portion of the template that is complementary to the sequence tag of the ribozyme.

The WT and 24-3 polymerases were challenged to extend a primer through various difficult sequence features over 24 h. Both WT and 24-3 can extend readily through a 20-nt C-rich sequence, but extension by WT halts at four consecutive purines, whereas 24-3 extends readily through purine-rich sequences of 12 nt (Fig. 2B). Similarly, when presented with a 4-bp stem closed by a stable “tetraloop” structure, WT halts at the start of the stem, even though it can extend through the same sequence in single-stranded form (Fig. 2C). In contrast, 24-3 reads through the entire stem-loop in high yield. Longer stems pose a barrier to 24-3, although detectable full-length extension products are seen for stems of up to 8 bp (Fig. S2).

Synthesis of Functional RNAs. The improved properties of 24-3 enable it to synthesize structured, functional RNAs that previously were inaccessible for any polymerase ribozyme. The cyanocobalamin and GTP aptamers can be synthesized from a separate primer in 47% and 18% yield, respectively, after 24 h (Fig. 3A and B). The 24-3 polymerase also can synthesize ribozymes that have heterogeneous sequence and substantial secondary structure, such as the entire F1 ligase ribozyme (19), which is obtained in 2% yield after 24 h (Fig. 3C). Following their RNA-catalyzed synthesis, the full-length aptamers and ribozyme were gel purified and tested for their respective activities (Fig. 3E and F). Compared with chemically synthesized controls, the aptamers synthesized by the polymerase are captured 3- to 6-fold less effectively by the corresponding immobilized ligand and the ligase has 10-fold reduced initial rate of reaction. This reduction is presumably due to mutational load, consistent with the observation that when the polymerase is required to synthesize only 12 (rather than 18) nucleotides to complete the cyanocobalamin aptamer, ligand capture is reduced by only 20%.

As a final test of polymerase generality, 24-3 was used to synthesize yeast phenylalanyl tRNA from a 15-nt primer (Fig. 3D). Despite the stable and complex structure of the template, full-length tRNA was obtained in 0.07% yield after 72 h. This RNA product is close to the limit of what can be achieved with the polymerase, but is likely the first time a tRNA molecule has been synthesized by a ribozyme since the end of the RNA world, nearly four billion years ago.

Exponential Amplification of RNA. The most commonly practiced method for amplifying nucleic acids is the PCR, which entails repeated cycles of heat denaturation and reciprocal primer extension and depends on the activity of a polymerase protein. The 24-3 RNA polymerase was used to carry out PCR-like amplification, but in an all-RNA system (riboPCR) using a starting RNA template, RNA primers, and the four NTPs. The concentration of Mg²⁺ was reduced to minimize spontaneous RNA cleavage, PEG8000 was used as a molecular crowding agent to improve ribozyme activity at the reduced Mg²⁺ concentration (20, 21), and tetrapropylammonium chloride was added to lower the melting temperature of the duplex RNA (22, 23). Under these conditions, 1 nM of a 24-nt RNA template, composed of two 10-nt primer-binding sites flanking the sequence AGAG, was driven through repeated thermal cycles, resulting in 98 nM newly synthesized template and 106 nM of its complement, corresponding to 100-fold amplification (Fig. 4A). Sequencing of the amplified products revealed that the central AGAG sequence was largely preserved, albeit with a propensity to mutate the third position from A to G, reflecting the low barrier to wobble pairing (Table S3).

Amplification of a 20-nt template (without central insert) was monitored in real time, exploring input template concentrations ranging from 10 nM to 1 pM. The resulting amplification profiles are typical for real-time PCR, shifted by a constant number of cycles per log-change in starting template concentration (Fig. 4B). A plot of cycle-to-threshold vs. logarithm of template concentration is linear across the entire range of dilutions (Fig. 4C), indicating exponential amplification of the template RNA with a per-cycle amplification efficiency of 1.3-fold. This result matches the value obtained from direct measurements made after each thermal cycle starting at low template concentrations (Fig. S3A). As with conventional PCR, product inhibition at high template concentrations slows amplification, which for riboPCR occurs starting at ~10 nM template. Below this concentration, exponential amplification is robust, causing 1 pM starting RNA to be amplified to ~40 nM product after 24 h, which corresponds to 40,000-fold amplification (Fig. S3B). In comparison, over the same time, riboPCR without any input template yielded only

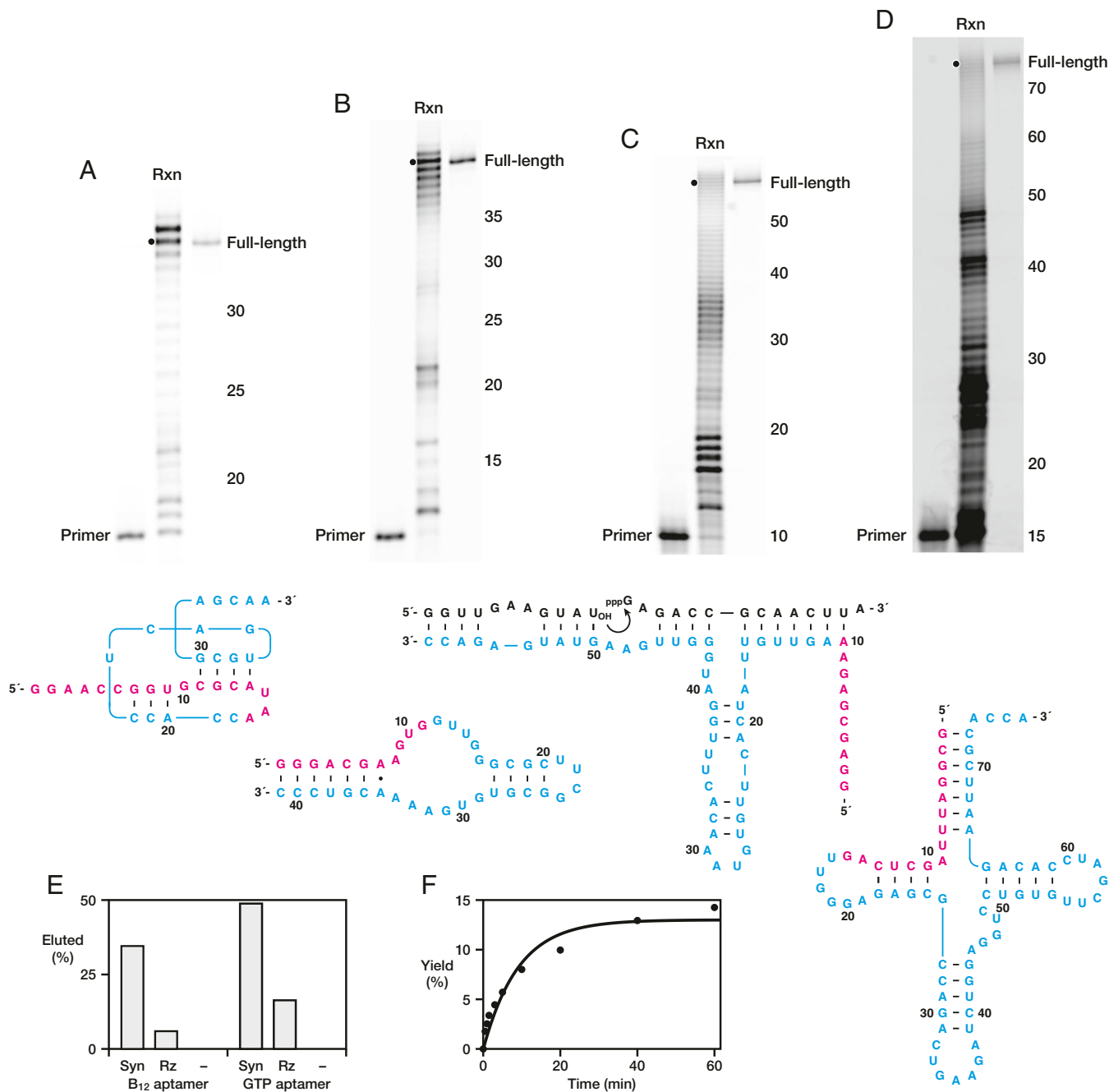


Fig. 3. Synthesis of functional RNAs by the 24-3 polymerase. Synthesis of (A) the cyanocobalamin aptamer after 24 h, (B) the GTP aptamer after 24 h, (C) the F1 ligase ribozyme after 24 h, and (D) yeast phenylalanyl-tRNA after 72 h. Polymerization conditions as in Fig. 2B, but using 50 nM polymerase. Dots indicate full-length extension products. The slightly reduced mobility of full-length aptamers generated by the polymerase compared with those from chemical synthesis is due to a small difference (29 Da) in the composition of the 5'-fluorescein label. Sequence and secondary structure of the primer (magenta) and polymerized portion (cyan) of each RNA are shown below the respective gel images; substrates for the F1 ligase are shown in black. (E) Fraction of cyanocobalamin (B₁₂) or GTP aptamers bound and eluted from corresponding ligand-derived resins, comparing synthetic (Syn) and ribozyme-synthesized (Rz) aptamers and a nonbinding control RNA (-). (F) Catalytic activity of the full-length, ribozyme-synthesized F1 ligase in a reaction mixture containing 1 μM ligase, 0.2 μM 5' substrate, 10 μM 3' substrate, 25 mM MgCl₂, and 50 mM EPPS (pH 8.5) at 20 °C. Data were fit to the following equation: yield = yield_{max} (1 - e^{-k_{obs}t}), where yield_{max} = 13% and k_{obs} = 0.11 min⁻¹.

~5 nM of similarly sized, nonspecific product RNAs that likely correspond to “primer-dimers” and materials generated by other mispriming events.

Discussion

The RNA-templated synthesis of RNA, as catalyzed by a ribozyme, has been known for 20 y (10). All examples to date, however, have

suffered from slow rates and a strong preference for unstructured, C-rich templates (12–16). In vitro evolution was used to select polymerase variants that can synthesize functional RNA aptamers, resulting in dramatically improved activity and sequence generality. The evolved 24-3 polymerase can extend through regions of secondary structure, enabling the synthesis of a variety of functional RNAs, including aptamers and ribozymes.

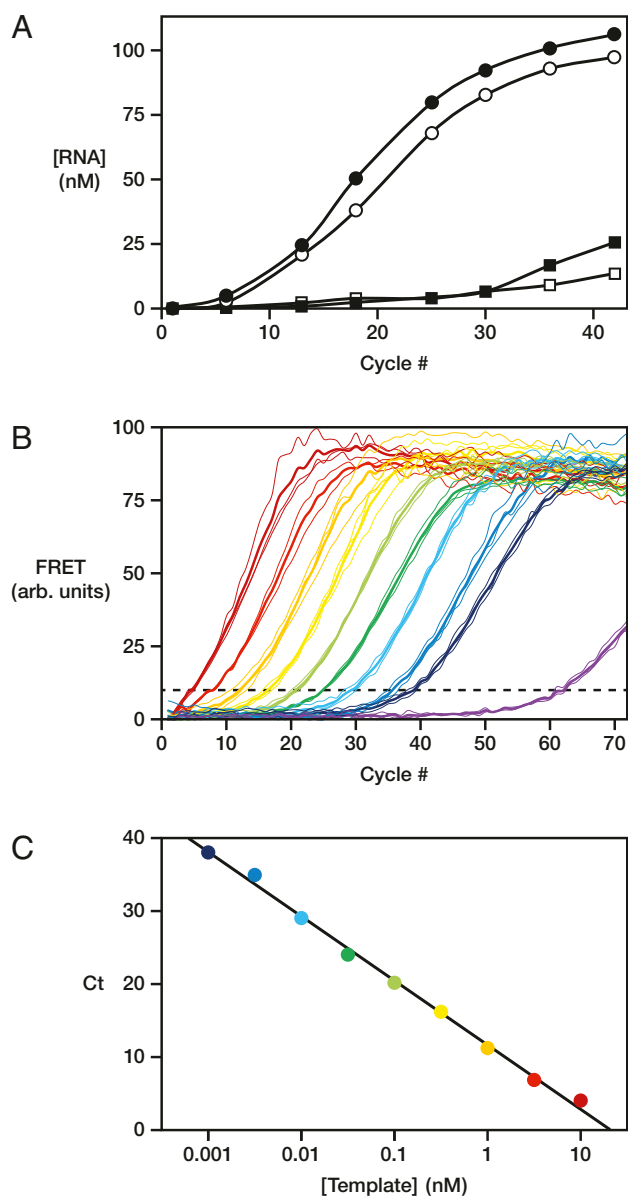


Fig. 4. RNA-catalyzed exponential amplification of RNA (riboPCR). (A) Amplification of a 24-nt template, tracking either the template (white circles) or complement (black circles) compared with the reaction with no input template (white and black squares, respectively). Reaction conditions: 0.4 μ M polymerase, 0.2 μ M each primer, ± 1 nM starting template, 4 mM each NTP, 50 mM $MgCl_2$, 0.9 M TPA, 6% (wt/vol) PEG8000, and 50 mM Tris (pH 8.3), with thermal cycles of 72 $^{\circ}C$ for 2 s and then 17 $^{\circ}C$ for 4 h. (B) Real-time riboPCR of a 20-nt template, monitored by FRET between two fluorescently labeled primers. Reaction conditions as above, but with starting template concentrations of 10 nM (dark red), 3.2 nM (red), 1 nM (orange), 0.32 nM (yellow), 0.1 nM (light green), 32 pM (green), 10 pM (cyan), 3.2 pM (blue), 1 pM (indigo), or 0 (violet) and with thermal cycles of 68 $^{\circ}C$ for 1 s and then 17 $^{\circ}C$ for 30 min. Dashed line indicates a threshold FRET value of 10% of maximum. For each starting template concentration, three replicates were obtained (thin lines) and averaged (thick line) to determine a cycle-to-threshold (Ct) value. (C) Semilog plot of Ct vs. starting template concentration, demonstrating a linear relationship with slope of -8.8 , corresponding to per-cycle amplification of 1.3-fold.

Previously, the strong limitations on template sequence in protein-free RNA polymerization systems excluded the possibility of carrying out cycles of replication because both the template and its complement could not conform to these limitations. Furthermore, to

sustain life, RNA replication must proceed with sufficient yield to support exponential amplification. The improved properties of the 24-3 polymerase enable the residue-by-residue copying and exponential amplification of short RNAs, with a per-cycle amplification efficiency of 1.3-fold. Analogous processes have been proposed for the replication of RNA on the early Earth, with hot-cold cycles potentially driven by diurnal variation or convection in a hydrothermal vent (24).

Inefficient strand displacement and modest fidelity limit the yield and specific activity of RNAs synthesized by the 24-3 polymerase and similarly limit riboPCR amplification to templates of only 20–25 nt. Both of these properties might be improved by selecting polymerase ribozymes that complete the synthesis of more demanding functional motifs, such as other ribozymes. Alternatively, selection methods might be used that take advantage of riboPCR, selecting directly for the amplification of functional RNAs. Such methods would be powerful because differences in fitness would be reflected as an exponential rather than linear effect, which is a key feature of Darwinian evolution.

The vestiges of the late RNA world appear to be shared by all extant life on Earth, most notably in the catalytic center of the ribosome (25), but most features of RNA-based life likely were lost in the Archaean era (2). Whatever forms of RNA life existed, they must have had the ability to replicate genetic information and express it as functional molecules. The 24-3 polymerase is the first known ribozyme that is able to amplify RNA and to synthesize complex functional RNAs. To achieve fully autonomous RNA replication, these two activities must be combined and further improved to provide a polymerase ribozyme that can replicate itself and other ribozymes of similar complexity. Such a system could, under appropriate conditions, be capable of self-sustained Darwinian evolution and would constitute a synthetic form of RNA life.

Materials and Methods

Materials. All oligonucleotides used in this study are listed in Table S4. Synthetic oligonucleotides were either purchased from Integrated DNA Technologies or prepared by solid-phase synthesis using an Expedite 8909 DNA/RNA synthesizer, with reagents and phosphoramidites purchased from Glen Research. All RNA templates used in RNA polymerization reactions contained a 5'-terminal region complementary to the 5' end of the polymerase (14). RNA templates were prepared either synthetically or by in vitro transcription from synthetic DNA templates, as described in SI Materials and Methods. RNA polymerase ribozymes were prepared by in vitro transcription, with dsDNA templates generated by PCR from corresponding plasmid DNA using the primers listed in Table S4. All RNA templates and ribozymes were purified by denaturing (8 M urea) PAGE and ethanol precipitation before use.

In Vitro Evolution. A pool of synthetic oligodeoxynucleotides was prepared, encoding the WT polymerase ribozyme (Fig. S1) and randomizing all nucleotide positions between the two primer regions at a frequency of 10% per position. The DNA was made double-stranded by primer extension using SuperScript II reverse transcriptase (ThermoFisher), except that 1.5 mM $MnCl_2$ was added to enhance extension through DNA lesions that occurred during synthesis (26). The yield of full-length dsDNA was 165 pmol (55% extension efficiency), corresponding to 10^{14} distinct sequences. The dsDNA was amplified by five cycles of PCR and then purified using the Qiagen PCR purification kit.

Detailed procedures for each step of selection are provided in SI Materials and Methods, and the reaction conditions used in each round of in vitro evolution are listed in Table S1. In outline, each round began with in vitro transcription of the population of ribozymes, followed by tethering of an RNA primer to the 5' end of the ribozymes. The resulting ribozyme-primer conjugates were annealed to a template RNA, and the primer was extended by the ribozyme in the presence of the four NTPs. The extended materials were captured on streptavidin, the template RNA was removed, and the desired products were selected by either a gel-shift method or capture of a ribozyme-synthesized aptamer. For gel-shift selection, the products were separated by PAGE and the fully extended materials were eluted from the gel and reverse transcribed. For aptamer-based selection, the ribozyme portion of the products was reverse transcribed to form an RNA-cDNA

heteroduplex, and then extended materials that contained a functional aptamer were captured by binding to the corresponding ligand (cyanocobalamin or GTP). In both cases, the isolated cDNA was amplified by PCR, followed by *in vitro* transcription to generate the progeny population of ribozymes.

RNA-Catalyzed RNA Polymerization. RNA polymerization reactions were performed with separate ribozyme, RNA template, and 5'-biotinylated RNA primer under two different concentration regimes. For kinetic analyses and fidelity studies, the reaction mixtures contained 1 μ M ribozyme, 0.5 μ M template, and 0.4 μ M primer; for all other studies they contained either 50 or 100 nM ribozyme, 50 nM template, and 40 nM primer. In all cases, the RNAs first were heated at 80 °C for 1 min, cooled to 17 °C over 5 min, and then added to the final mixture, which also contained 4 mM each NTP, 200 mM MgCl₂, 0.05% TWEEN20, and 50 mM Tris (pH 8.3, prepared at 19 °C). Polymerization was carried out at 17 °C and quenched by adding 0.4 volumes of 500 mM EDTA. The biotinylated primers and extended products were captured on streptavidin C1 Dynabeads (ThermoFisher), washed four times with alkali (25 mM NaOH, 1 mM EDTA, and 0.05% TWEEN20) and twice with TE (1 mM EDTA, 0.05% TWEEN20, and 10 mM Tris, pH 8.0) plus 8 M urea, and then eluted with 98% formamide and 10 mM EDTA (pH 8.0) at 95 °C for 10 min. The reaction products were analyzed by PAGE to determine the average number of nucleotides added to the primer at various times. Sequencing of the extension products for fidelity studies and analysis of their aptamer binding or ligase activity are described in *SI Materials and Methods*.

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