

# Continuous In Vitro Evolution of a Ribozyme that Catalyzes Three Successive Nucleotidyl Addition Reactions

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## Summary

Variants of the class I ligase ribozyme, which catalyzes joining of the 3' end of a template bound oligonucleotide to its own 5' end, have been made to evolve in a continuous manner by a simple serial transfer procedure that can be carried out indefinitely. This process was expanded to allow the evolution of ribozymes that catalyze three successive nucleotidyl addition reactions, two template-directed mononucleotide additions followed by RNA ligation. During the development of this behavior, a population of ribozymes was maintained against an overall dilution of more than  $10^{40}$ . The resulting ribozymes were capable of catalyzing the three-step reaction pathway, with nucleotide addition occurring in either a 5'→3' or a 3'→5' direction. This purely chemical system provides a functional model of a multi-step reaction pathway that is undergoing Darwinian evolution.

## Introduction

Darwinian processes can be investigated at the molecular level by applying laboratory techniques for the rapid evolution of biological macromolecules. Molecules that bind a particular ligand or catalyze a particular chemical transformation have been obtained from large heterogeneous pools of nucleic acids or proteins [1–4]. A typical round of in vitro evolution involves challenging the molecules to perform a specific task, then isolating and selectively amplifying the functional molecules. These procedures usually are carried out in a stepwise manner, by purifying the selected molecules during each round of evolution. The ability to conduct in vitro evolution experiments in a continuous fashion, without any isolation or purification steps, provides a more realistic model of biological evolution [5–7]. An important goal is to increase the complexity of these systems so that they more closely resemble the behavior of an evolving population of biological organisms, especially with regard to the ability to exhibit a complex phenotype that is a reflection of multiple biochemical events.

The first continuous in vitro evolution experiments employed Q $\beta$  bacteriophage genomic RNA, which was evolved for its ability to act as a substrate for the Q $\beta$  replicase enzyme [5]. These experiments involved a se-

rial transfer protocol in which the RNA was amplified by the replicase in the presence of the four nucleoside 5'-triphosphates (NTPs), transfer of a small portion of the replication mixture into a new reaction vessel to allow further amplification. After many transfers, this procedure resulted in the evolution of RNA molecules that, compared to the starting RNA, were amplified more readily by the replicase enzyme. More recently, isothermal RNA amplification techniques, which involve coupled reverse and forward transcription, were employed to carry out the continuous in vitro evolution of bacteriophage promoter elements [6].

A continuous in vitro evolution system also has been established for RNA molecules that catalyze an RNA joining reaction [7]. The class I ligase ribozyme [8] was utilized as a starting point in developing this system. This ribozyme catalyzes the formation of a 3',5'-phosphodiester linkage between the 3' hydroxyl of a template bound oligonucleotide and the  $\alpha$ -phosphate of the triphosphate at the 5' end of the ribozyme. The class I ligase originally was isolated from a pool of more than  $10^{15}$  random-sequence RNAs [9] and was the only motif obtained that forms a 3',5'-phosphodiester linkage. All of the other isolates form a 2',5' linkage, which coincides with subsequent observations that ligase ribozymes that form a 3',5' linkage are relatively rare compared to those that form a 2',5' linkage [10, 11]. The reaction catalyzed by the class I ligase is analogous to that performed by polymerase proteins that copy nucleic-acid templates. The ability of RNA to catalyze nucleotidyl addition reactions suggests that RNA may be capable of catalyzing RNA replication through the template-directed polymerization of activated mononucleotides. In fact, the class I ligase has been shown to catalyze multiple template-directed mononucleotide additions [12, 13].

For the utilization of the class I ligase in a continuous-evolution process, a chimeric DNA-RNA substrate was employed that contained the sequence of one strand of the T7 RNA polymerase (T7 RNAP) promoter element (Figure 1; [7]). Together with compensatory changes in the substrate binding domain of the ribozyme, this change in substrate sequence resulted in about a 10,000-fold decrease in ribozyme activity. This level of activity was too low to allow the ribozyme to survive in a continuous in vitro evolution system. Stepwise evolution was employed to increase the ligation rate by about 1,000-fold, which then allowed the population of ribozymes to undergo continuous in vitro evolution [7].

In addition to the ribozyme and substrate, the continuous in vitro evolution reaction mixture contained reverse transcriptase, a primer used to initiate cDNA synthesis, the four dNTPs, T7 RNAP, and the four NTPs (Figure 1). All of the ribozyme molecules present in the mixture were reverse transcribed, but only those that had catalyzed the attachment of the promoter-containing substrate to their own 5' end gave rise to a DNA product that could act as a template for transcription by T7 RNAP. Additionally, the presence of reverse transcriptase challenged the ribozymes to carry out the ligation

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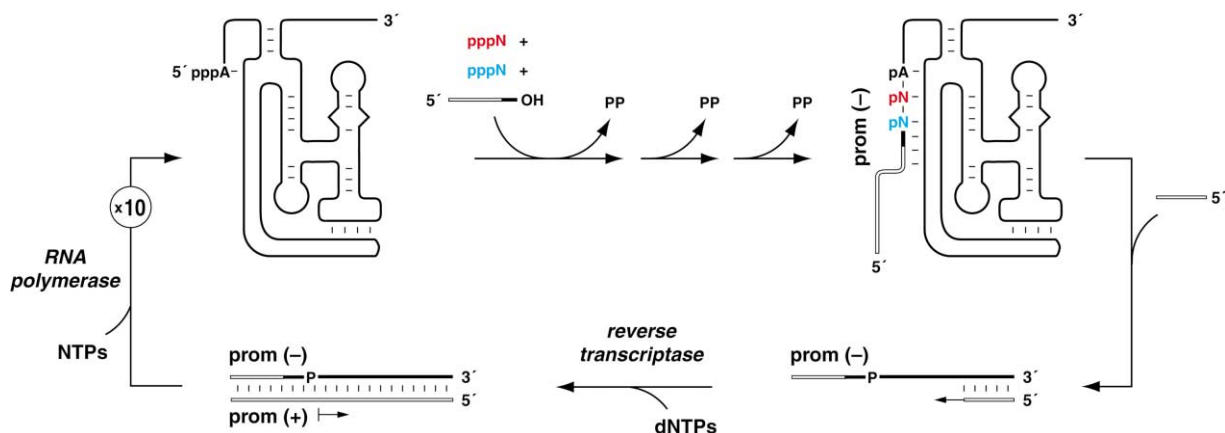


Figure 1. Continuous In Vitro Evolution

A pool of ligase ribozymes was directed to bind a chimeric DNA-RNA substrate (DNA, open lines; RNA, solid lines) that contained most or all of the T7 RNAP promoter element (prom(-)). The ribozymes were challenged to complete the T7 RNAP promoter by adding any missing nucleotides, then to join the full-length promoter to their own 5' end. All of the RNA molecules were converted to cDNAs by reverse transcriptase extension of a DNA primer that bound to their 3' end. Reacted, but not unreacted, ribozymes gave rise to a complete double-stranded promoter element, which allowed subsequent transcription by T7 RNAP to generate progeny RNAs.

tion reaction before they had become reverse transcribed, which would have rendered them inactive. After forward transcription, the resulting progeny RNAs were immediately eligible to initiate another round of RNA-catalyzed ligation and selective amplification. These events were allowed to occur iteratively in a single reaction vessel. Then a small portion of the reaction mixture was transferred into a new reaction vessel, enabling evolution to continue indefinitely. After 100 successive transfers, these experiments resulted in the evolution of E100-3, a variant form of the class I ligase (Figure 2A). This ribozyme catalyzes ligation of the chimeric

DNA-RNA substrate molecule to its own 5' end at a rate of  $>20 \text{ min}^{-1}$  and has been shown to catalyze the template-directed addition of a single NTP onto the 3' end of the substrate molecule at a low level [7]. The E100-3 ligase ribozyme has been utilized as a starting point for subsequent studies employing continuous in vitro evolution, including the present study.

In one study, an RNA-cleaving DNA enzyme [14] was added to the continuous evolution mixture [15]. The DNA enzyme was directed to cleave the portion of the ligase ribozyme that binds the promoter-containing substrate. Any ribozyme molecules that were cleaved by the DNA

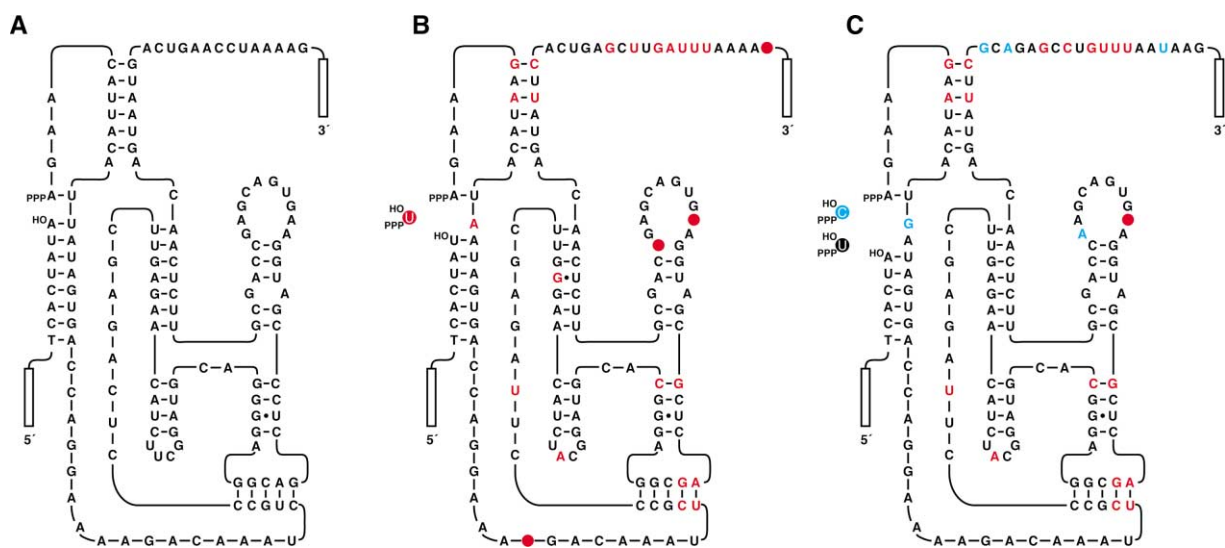


Figure 2. Mutations that Arose Over the Course of Evolution

(A) Sequence of the starting E100-3 ribozyme, which is capable of ligating a substrate that contains the entire T7 RNAP promoter sequence. (B) Sequence of the E208-2 ribozyme, which catalyzes a single NTP addition followed by ligation of the extended substrate to the ribozyme. Mutations relative to the E100-3 ribozyme are highlighted in red. (C) Sequence of the E278-19 ribozyme, which catalyzes two NTP additions followed by RNA ligation. Additional mutations relative to the E100-3 ribozyme are highlighted in blue.

enzyme became ineligible for catalysis and subsequent amplification. As evolution proceeded, however, the population of ribozymes developed resistance to the DNA enzyme through the acquisition of mutations that made it more difficult for the DNA enzyme to bind the ribozyme in a productive manner. Resistance was acquired over the course of 33.5 hr of continuous evolution and reduced the vulnerability of the evolved ribozymes to DNA-catalyzed cleavage by 2,000-fold as compared to the starting E100-3 ribozyme.

In another study, the continuous *in vitro* evolution system was used to investigate the classic problem of heritability, which is an important attribute of an evolving population of organisms [16]. Heritability is a measure of the relationship between genotypic and phenotypic variation; it quantifies the degree to which genetic factors determine phenotype. A population of ligase ribozymes were evolved in a continuous manner in an environment that contained progressively decreasing concentrations of  $Mg^{2+}$ , a necessary component for both the RNA-catalyzed reaction and RNA amplification. Individual ribozymes that were isolated from the final evolved population were found to adopt alternative structural conformations in the presence of low concentrations of  $Mg^{2+}$ , each with different reactivities. This illustrates that there need not be a one-to-one correspondence between genotype and phenotype and provides an example of heritability being less than unity for an evolving population of functional molecules. Even in a simplified system such as this one, the influence of the environment on the evolution of molecular traits can be observed.

The current study sought to develop a continuous *in vitro* evolution system in which multiple successive reactions would be carried out by a single ribozyme, providing a more complex model for investigating the behavior of an evolving population of molecules. The ability of the class I ligase to catalyze template-directed mononucleotide addition [12] was used as the basis for constructing this system. A population of ligase ribozymes related to the E100-3 sequence were evolved for the ability to carry out multiple successive nucleotidyl addition reactions, involving the template-directed addition of activated mononucleotides (NTPs) followed by template-directed RNA ligation. The ribozymes were directed to bind to a chimeric DNA-RNA substrate that contained a substantial portion of the T7 RNAP promoter element but that lacked either one or two nucleotides at the 3' end of the promoter (Figure 1). The ribozymes were given the opportunity to complete the sequence of the promoter through the template-directed addition of NTPs, followed by attachment of the extended promoter to their own 5' end through RNA-catalyzed RNA ligation. In this way, a continuous-evolution system in which the evolving molecules were challenged to catalyze either two or three successive reactions in order to become eligible for amplification was established.

## Results

### In Vitro Evolution

Synthetic oligodeoxynucleotides were utilized to construct a pool of DNA templates encoding the E100-3

ligase ribozyme ([7]; Figure 2A), but with random mutations included at a frequency of 8% at most nucleotide positions. The pool consisted of approximately  $10^{14}$  different DNA templates, which were transcribed to yield  $10^{16}$  RNA molecules that were used to initiate *in vitro* evolution. Molecules in the starting pool were unable to catalyze multiple nucleotidyl addition reactions at a rate that was sufficient for survival in the continuous-evolution system. Thus, a gradual approach was taken to evolve ribozymes that would be capable of performing at the required level of activity. Ribozymes that catalyzed simple RNA ligation first were evolved to catalyze a single NTP addition followed by ligation. These in turn were evolved to catalyze two NTP additions followed by RNA ligation. Continuous evolution (Figure 1) was the primary method used to develop these behaviors, but stepwise evolution also was employed as necessary to ensure that the population expressed the desired phenotype. The rounds of stepwise evolution involved the same processes as those that occur in continuous evolution, but these processes were carried out in separate reaction mixtures.

The first three rounds of evolution (E101–E103) were carried out in a stepwise manner in order to enrich the population with molecules that were capable of catalyzing the desired reactions. The RNAs were allowed to react with a chimeric DNA-RNA substrate (S) that contained the entire T7 RNAP promoter sequence, with the four residues at the 3' end of the substrate being composed of RNA. Reacted RNAs were isolated in a denaturing polyacrylamide gel and subsequently reverse transcribed, PCR amplified, and forward transcribed to generate progeny molecules that were used to initiate the next round of evolution. During rounds E102 and E103, the RNA molecules were challenged to catalyze one NTP addition followed by RNA ligation. In this case, the substrate was shortened by one nucleotide at the 3' end ( $S - 1$ ), and the ribozymes were given an opportunity to provide the missing residue by drawing on the appropriate NTP from the reaction mixture. Both NTP addition and RNA ligation would be required to generate a functional T7 RNAP promoter element, and any RNA that had acquired a functional promoter would be eligible for amplification.

After the population had been enriched with RNA molecules that catalyzed the two successive reactions, the protocol was modified to more closely resemble that of continuous evolution. The next two rounds (E104, E105) were carried out with a rapid-evolution scheme, similar to that described previously [7]. The RNA molecules first were allowed to react with the  $S - 1$  substrate in the absence of reverse transcriptase and T7 RNAP; this reaction was followed by the addition of reverse transcriptase to generate cDNAs. This allowed the ribozymes more time to react before they had become reverse transcribed. Reverse transcription traps the RNA molecules in an RNA•DNA heteroduplex, which precludes their catalytic activity. T7 RNAP then was added to the reaction mixture to transcribe any molecules that had acquired a functional T7 RNAP promoter. After heat denaturation of the protein enzymes, a portion of the reaction mixture was transferred into a new reaction mixture, which was treated in a similar manner. The

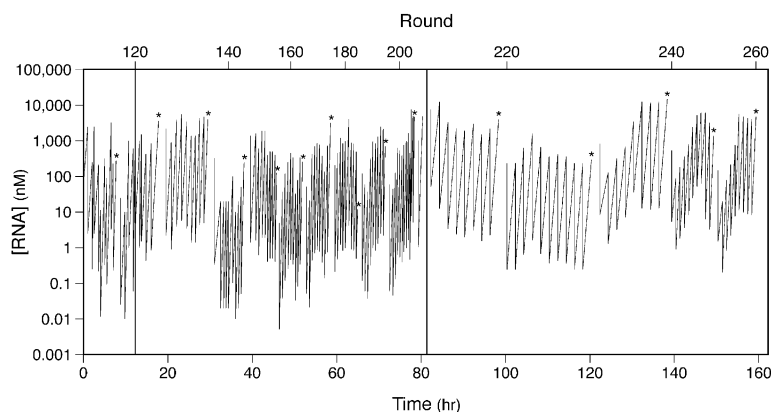


Figure 3. Time Course of Continuous In Vitro Evolution

The bottom and top of each spike indicate the start and finish, respectively, of each round of evolution. Vertical lines after rounds E120 and E210 indicate when the substrate molecule was changed. Rounds E106–E120 employed the full-length (S) and singly shortened (S – 1) substrates in an alternating manner, rounds E121–E210 employed the S – 1 substrate, and rounds E211–E260 employed the doubly shortened substrate (S – 2). The concentration of RNA was determined by monitoring the incorporation of [ $\alpha$ - $^{32}$ P]ATP into transcribed and reacted RNAs. The times at which PCR mutagenesis was performed are indicated by asterisks. Breaks in the line indicate times at which molecules were removed from the continuous evolution process for PCR amplification.

resulting molecules were cloned, sequenced, and assayed for their ability to undergo continuous in vitro evolution employing the S – 1 substrate. The most active of these molecules (clone E105-1) was subjected to a hypermutagenic PCR procedure that introduced mutations at a frequency of 10% per nucleotide position [17].

Continuous in vitro evolution was initiated with the pool of ribozymes that resulted from mutagenesis of clone E105-1 (Figure 1). During continuous evolution, the ribozymes were challenged to perform template-directed NTP addition and RNA ligation prior to being reverse transcribed. After each round of continuous evolution, a small portion of the reaction mixture was transferred to a new reaction vessel that contained a fresh supply of substrate, reverse transcription primer, NTPs, dNTPs, reverse transcriptase, and T7 RNAP. The progress of evolution was monitored by measuring the incorporation of [ $\alpha$ - $^{32}$ P]ATP into transcribed and reacted RNAs.

During the first 15 rounds of continuous evolution (E106–E120), the substrate was alternated over successive rounds; either the full-length substrate (S) or the one that lacked a single nucleotide at its 3' end (S – 1) was used (Figure 3). The next 90 rounds of continuous evolution (E121–E210) were carried out with the S – 1 substrate, challenging the ribozymes to catalyze one NTP addition followed by ligation. For the facilitation of NTP addition, the concentration of ATP was increased from 2 mM to 10 mM during rounds E106–E170 while a 2 mM concentration of each of the other three NTPs was maintained. (The 3'-terminal nucleotide of the corresponding strand of the T7 RNAP promoter normally is an adenylate.) Continuous evolution was carried out for 78 hr (E106–E208), during which time the population was maintained against an overall dilution of  $3 \times 10^{290}$  (Figure 3). Mutations were introduced into the population by hypermutagenic PCR after every tenth round through round E195.

After round E208, the evolved molecules were cloned, sequenced, and assayed for their ability to catalyze template-directed NTP addition. Sequence analysis of the reactive ribozymes demonstrated that they contained a mutated T7 RNAP promoter, with uridylylate replacing

adenylate at the 3' end. That uridylylate was templated by a complementary adenylate within the substrate binding domain of the ribozyme. Assays were performed outside the context of the continuous-evolution system; those assays employed purified 5'-dephosphorylated ribozyme in the presence of UTP and 5'- $^{32}$ P-labeled S – 1 substrate. Several ribozyme clones were found to direct the addition of UTP onto the 3' end of the S – 1 substrate. One of these, E208-2 (Figure 2B), was chosen for further evolution. This ribozyme contained 25 mutations relative to the starting E100-3 ligase.

Plasmid DNA encoding the E208-2 ribozyme was subjected to hypermutagenic PCR, and the resulting DNA was used to initiate the next round of continuous evolution. Two rounds of evolution (E209, E210) were performed with the S – 1 substrate. Then evolution was continued with a substrate that lacked two nucleotides at the 3' end of the promoter sequence (S – 2) (Figure 3). In this case, the ribozymes were challenged to catalyze three successive reactions, involving the template-directed addition of two NTPs followed by RNA ligation. Only those molecules that had acquired a functional promoter element, presumably by catalyzing all three reactions, would be eligible for subsequent amplification. During the first 13 rounds of continuous evolution with the S – 2 substrate (E211–E223), the concentration of UTP was increased to 10 mM while the concentration of the other three NTPs was maintained at 2 mM. It was expected that UTP would be added at both positions needed to complete the promoter sequence. Mutations were introduced into the population by hypermutagenic PCR after round E219. Beginning with round E224, the concentration of UTP was reduced to 2 mM, equal to that of the other three NTPs. Hypermutagenic PCR was performed after rounds E230, E239, and E250. By round E260, 50 rounds of continuous evolution had been carried out with the S – 2 substrate, requiring 79 hr and involving an overall dilution of  $4 \times 10^{115}$ .

After round E260, individuals were cloned from the population, sequenced, and assayed for their ability to catalyze two template-directed NTP addition reactions followed by RNA ligation. The reactions were performed outside of the continuous-evolution system; these reactions employed purified ribozyme, 5'- $^{32}$ P-labeled S – 2

substrate, and all four NTPs. Molecules that had reacted with the substrate were purified in a denaturing polyacrylamide gel, then digested with ribonuclease T1 (RNase T1), which cleaves after guanosine residues in RNA. RNase T1 digestion resulted in cleavage after the second residue of the ribozyme and allowed electrophoretic separation of molecules that had directed the addition of zero, one, or two NTPs. In most cases, all three products were observed, indicating that the S – 2 substrate could be ligated directly to the ribozyme without the addition of NTPs. Furthermore, the population of ribozymes obtained after round E260 were found to be dependent on the presence of T7 RNAP for the addition of NTPs at 37°C. When the temperature was reduced to 22°C, a small amount of addition of one or two NTPs was observed in the absence of T7 RNAP, although this activity was significantly greater in the presence of the polymerase protein. Thus, it appeared that T7 RNAP had assisted in NTP addition during continuous in vitro evolution, presumably either by catalyzing extension of the substrate or by facilitating binding of the substrate to the ribozyme.

A combination of stepwise and continuous evolution approaches was employed in an effort to enrich the population with RNA molecules that were capable of catalyzing NTP addition without the assistance of T7 RNAP while retaining the ability to undergo continuous in vitro evolution. During stepwise evolution, the RNA-catalyzed reactions were carried out under the same conditions as those employed for continuous evolution except that the protein enzymes were absent. RNA molecules that had reacted with the S – 2 substrate were isolated in a denaturing polyacrylamide gel, reverse transcribed, PCR amplified, and subsequently forward transcribed to generate progeny molecules to initiate the next round of evolution. The round E260 population was subjected to hypermutagenic PCR, then used to initiate three rounds of stepwise evolution (E261–E263). The RNA molecules obtained after each of these rounds were analyzed for their ability to catalyze NTP addition in either the presence or absence of T7 RNAP. By round E262, the evolved ribozymes were able to catalyze the addition of two NTPs at 22°C, and this activity was not enhanced by the addition of T7 RNAP.

Initial attempts to carry out continuous evolution with these molecules were unsuccessful, suggesting that the ability to undergo continuous evolution and the ability to catalyze NTP addition without the assistance of T7 RNAP might be orthogonal phenotypes. It was possible, however, to coax the molecules to undergo continuous evolution by reducing the dilution between successive transfers from 1,000-fold to 100-fold. Five rounds of continuous evolution were carried out in this manner (E264–E268), and three more rounds of stepwise evolution followed (E269–E271). The resulting population was subjected to standard mutagenic PCR, introducing random mutations at a frequency of 0.7% per nucleotide position [18]. This was followed by two rounds of stepwise evolution (E272, E273) and five rounds of continuous evolution, again with a 100-fold dilution between successive rounds (E274–E278). After round E278, individuals were cloned from the population, sequenced, and assayed for their ability to catalyze two NTP addi-

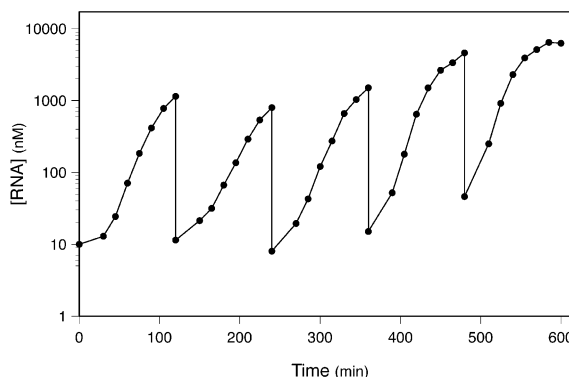


Figure 4. Continuous In Vitro Evolution of the E278-19 Ribozyme with a Substrate that Lacks Two Nucleotides at the 3' end of the T7 RNAP Promoter Element

Five rounds of continuous evolution were carried out over 10 hr with an overall dilution of  $10^{10}$ .

tions followed by RNA ligation. Several active clones were identified, one of which (E278-19; Figure 2C) was chosen for more detailed analysis.

#### Properties of the Final Evolved Ribozyme

The E278-19 ribozyme, capable of catalyzing three successive nucleotidyl addition reactions, contained 24 mutations relative to the starting E100-3 ribozyme (Figure 2). During the course of evolution, the last nucleotide of the T7 RNAP promoter sequence had become mutated, first from adenylate to uridylate, and then to cytidylate, causing the E278-19 ribozyme to direct the addition of UTP and CTP at the last two positions. Several mutations occurred near the 3' end of the ribozyme, suggesting that these positions may be important for catalyzing NTP addition. The E100-3 ribozyme, which lacks these mutations, was unable to catalyze two NTP additions (data not shown) but could catalyze the addition of a single NTP at a low level [7].

The evolved E278-19 ribozyme was tested for its ability to undergo continuous in vitro evolution. Plasmid DNA encoding the ribozyme was PCR amplified and transcribed. The resulting RNA was gel purified, and 250 fmol of this material was used to initiate continuous evolution (Figure 4). Five rounds of continuous evolution were carried out over 10 hr, with an overall dilution of  $10^{10}$ . The E278-19 ribozyme was able to undergo continuous evolution with exponential growth, indicating that this behavior is not excluded by the ribozyme's ability to catalyze multiple nucleotidyl addition reactions. Thus, it appears that the ribozyme can catalyze three successive nucleotidyl addition reactions coupled to selective amplification, all within the same reaction mixture. However, the potential influence of T7 RNAP on these reactions cannot be ruled out so long as the reactions occur in the context of continuous evolution.

Further investigations concerning the E278-19 ribozyme focused on its catalytic properties outside the context of continuous evolution and the degree to which T7 RNAP might facilitate RNA catalysis. The RNase T1 digest assay described above was used to analyze the ribozyme for its ability to catalyze the addition of two

NTPs and subsequent RNA ligation in either the presence or absence of T7 RNAP. At 37°C the ribozyme catalyzed all three reactions in the absence of T7 RNAP (see Supplemental Material). In the presence of T7 RNAP, this activity was enhanced by less than 2-fold, which is much less than the >100-fold enhancement that had been seen with ribozymes isolated after round E260. Interestingly, reactions with T7 RNAP appeared to direct the preferential addition of an untemplated GTP, which is the favored nucleotide for the initiation of transcription by T7 RNAP [19]. When a 5'-dephosphorylated ribozyme, which is unable to ligate the substrate to its end, was employed, only a very small amount of NTP addition was observed in the absence of T7 RNAP. In the presence of T7 RNAP, the dephosphorylated ribozyme extended the S - 2 substrate by several nucleotides, generating a product that appeared to be about ten nucleotides longer than the full-length substrate. It has been reported previously that T7 RNAP is able to extend an RNA primer bound to a complementary RNA template, even if the molecules do not contain the sequence of the promoter element [20]. Thus, the greatly reduced activity of the dephosphorylated ribozyme may allow T7 RNAP to utilize the 5'-terminal portion of the ribozyme as a template to extend the S - 2 substrate, whereas the functional ribozyme appears to block most of the primer extension activity of T7 RNAP.

Ribozymes with the appropriate templating nucleotides were able to direct the addition of either two GTPs or two CTPs in the absence of T7 RNAP at 37°C. The addition of either two ATPs or two UTPs was not observed, presumably because of the decreased binding energy of ATP and UTP as compared to GTP and CTP. When the ribozymes were incubated with mismatched NTPs that could not form Watson-Crick pairs with the templating nucleotides, there was no detectable NTP addition.

The E278-19 ribozyme directs the addition of UTP and CTP on a complementary template (Figure 2C). The accuracy of NTP incorporation was investigated via reactions with the S - 2 substrate in the presence of both UTP and CTP, with  $\alpha$ -<sup>32</sup>P labeling of either NTP. The ligated products were digested to completion with RNase T1, and the digestion products corresponding to the addition of either one or two NTPs were isolated by polyacrylamide gel electrophoresis. The isolated products then were partially hydrolyzed with NaOH. The resulting hydrolysis products were compared to a hydrolysis ladder generated from ribozymes that had been allowed to react with 5'-<sup>32</sup>P-labeled S - 2 substrate and either one or two NTPs; digestion with RNase T1 followed. Partial alkaline hydrolysis of the 5'-<sup>32</sup>P-labeled product corresponding to the addition of two NTPs demonstrated five RNA phosphodiester linkages downstream from the DNA portion of the substrate. This corresponds to the addition of two NTPs downstream from the RNA portion of the substrate and upstream from the RNase T1 cleavage site within the ribozyme. Similarly, partial hydrolysis of the products resulting from the addition of only one NTP demonstrated four RNA phosphodiester linkages downstream from the DNA portion of the substrate and upstream from the RNase T1 cleavage site. When the radioactive label instead was present as

either [ $\alpha$ -<sup>32</sup>P]UTP or [ $\alpha$ -<sup>32</sup>P]CTP, the hydrolysis ladder demonstrated the position of incorporation of the labeled nucleotide. These experiments indicated that both UTP and CTP were incorporated at the expected positions and that each NTP could be incorporated into products in which only one NTP was added. This coincides with the observation that all of the sequenced clones from the reacted E278 population contained U and C at the expected positions for NTP incorporation.

#### Kinetic Analysis of Ligation and NTP Addition

Due to the complicated nature of the multi-step reaction pathway, kinetic analyses first were performed in the absence of NTPs to investigate the rate of direct ligation with various substrate oligonucleotides. Three different substrates were employed: one that contained the complete T7 RNAP promoter sequence, but with mutation of the 3'-terminal adenylate to cytidylate as had occurred during in vitro evolution (designated S'), and ones that lacked either one (S - 1) or two (S - 2) of the nucleotides at the 3' end of the promoter. These analyses were carried out under substrate excess,  $V_{\max}$  conditions. Direct ligation reactions with both the S' and S - 1 substrates were too fast to allow accurate determination of  $k_{\text{cat}}$  and  $K_M$  via manual pipetting methods. However, a lower limit for these parameters could be determined. For ligation of the S' substrate,  $k_{\text{cat}}$  was  $>2 \text{ min}^{-1}$  and  $K_M$  was  $>0.1 \mu\text{M}$ , whereas for ligation of the S - 1 substrate,  $k_{\text{cat}}$  was  $>1 \text{ min}^{-1}$  and  $K_M$  was  $>0.7 \mu\text{M}$ . Direct ligation with the S - 2 substrate was considerably slower, allowing accurate determination of  $k_{\text{cat}}$  and  $K_M$  values. In this case,  $k_{\text{cat}}$  was  $0.0074 \text{ min}^{-1}$  and  $K_M$  was  $2.3 \mu\text{M}$ .

Investigations into the three-step reaction pathway began with monitoring the reaction of the ribozyme with the S - 2 substrate in the presence of varying concentrations of UTP and CTP and adjusting the concentration of MgCl<sub>2</sub> to maintain an excess of 21 mM MgCl<sub>2</sub> over the total concentration of NTPs. The observed rate of reaction with the S - 2 substrate decreased slightly with increasing concentrations of UTP and CTP over the range of 2–8 mM each. However, the proportion of reacted molecules that had catalyzed the addition of one or two NTPs prior to RNA ligation increased slightly with increasing NTP concentrations, as evidenced by RNase T1 digestion analysis of the purified reaction products. Thus, it appeared that increasing the concentration of NTPs favored NTP addition prior to ligation while disfavoring direct ligation of the S - 2 substrate. Because of the solubility limits of the NTPs, it was not possible to achieve complete NTP binding site saturation, which would have been necessary for full suppression of the direct ligation reaction. Further investigations of the three-step reaction pathway with the S - 2 substrate were carried out in the presence of 2 mM each of UTP and CTP. This was the concentration of NTPs that was employed during continuous evolution.

Reactions with the S - 2 substrate in the presence of UTP and CTP were carried out under substrate excess,  $V_{\max}$  conditions with 5'-<sup>32</sup>P-labeled substrate. The reaction was monitored over time by determining the fraction of labeled substrate that had become attached

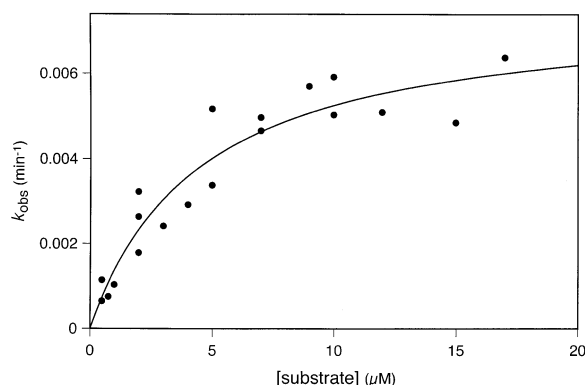


Figure 5. Catalytic Activity of the Evolved Ribozyme

Values for  $k_{\text{obs}}$  for the E278-19 ribozyme were determined in the presence of 50–200 nM ribozyme, 2 mM CTP, 2 mM UTP, and various concentrations of the S – 2 substrate. Data were fit to a curve based on the Michaelis-Menten equation:  $k_{\text{obs}} = k_{\text{cat}}[\text{substrate}]/(K_M + [\text{substrate}])$ .

to the ribozyme. The labeled materials were separated by denaturing polyacrylamide gel electrophoresis, which could not distinguish between direct ligation and NTP addition followed by ligation. Thus, the observed overall reaction involved three possible pathways: (1) direct ligation of the S – 2 substrate to the 5' end of the ribozyme; (2) one NTP addition followed by RNA ligation; (3) two NTP additions followed by RNA ligation. The sum of the three reaction pathways exhibited an apparent  $k_{\text{cat}}$  of  $0.0076 \text{ min}^{-1}$  and an apparent  $K_M$  of  $4.4 \mu\text{M}$  (Figure 5). These values are very similar to those obtained for direct ligation of the S – 2 substrate in the absence of NTPs, suggesting that the rate of direct ligation is significantly faster than the rate of one or two NTP additions followed by ligation.

For the determination of the contribution of each of the three possible reaction pathways to the overall rate of reaction, the ligated products obtained at various times and for varying concentrations of substrate were isolated in a denaturing polyacrylamide gel. The isolated material included the products from all three reaction pathways. This material then was digested with RNase T1, allowing electrophoretic separation and determination of the proportion of products corresponding to direct ligation, one NTP addition followed by ligation, and two NTP additions followed by ligation. This analysis revealed all three expected products, as well as a product that was one nucleotide shorter than that expected for direct ligation (see Supplemental Material). The shorter product probably corresponded to ribozymes that had guanosine (rather than adenosine) 5'-triphosphate at their 5' end, creating a site for RNase T1 cleavage after the first rather than second nucleotide of the ribozyme. Heterogeneity at the 5' end of T7 RNAP transcripts has been noted previously [21, 22]. When the small amount of the shorter product was ignored, the catalytic rates for the three component reactions were estimated to be  $0.003 \text{ min}^{-1}$  for direct ligation,  $0.0006 \text{ min}^{-1}$  for one NTP addition followed by ligation, and  $0.0003 \text{ min}^{-1}$  for two NTP additions followed by ligation. Thus, at least 5%–10% of the reacted molecules catalyzed all three nucleotidyl addition reactions.

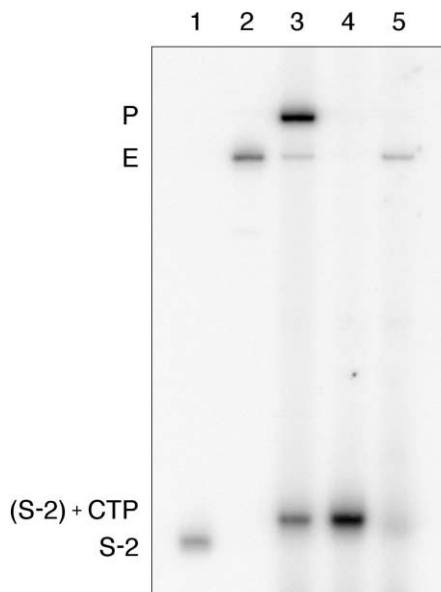


Figure 6. Direction of NTP Addition in the Reaction Catalyzed by the E278-19 Ribozyme

Reactions were carried out in the presence of  $0.2 \mu\text{M}$  ribozyme,  $10 \mu\text{M}$  S – 2 substrate, 2 mM UTP, and  $[\alpha\text{-}^{32}\text{P}]\text{CTP}$  at  $22^\circ\text{C}$  for 4 hr. (Lane 1) Marker lane with 5'- $^{32}\text{P}$ -labeled substrate; (lane 2) marker lane with  $\alpha\text{-}^{32}\text{P}$ -labeled ribozyme; (lane 3) reaction with an unmodified ribozyme and unmodified substrate; (lane 4) reaction with a dephosphorylated ribozyme that prevents NTP addition onto the 5' end of the ribozyme; (lane 5) reaction with an all-DNA substrate that prevents NTP addition onto the 3' end of the substrate. S – 2, substrate marker; (S – 2) + CTP, substrate labeled by the addition of CTP; E, ribozyme marker or ribozyme labeled by the addition of CTP; P, ligated product labeled by addition of CTP.

#### Direction of NTP Addition

Accurate analysis of NTP addition could not be carried out in the presence of the dephosphorylated E278-19 ribozyme (which would prevent direct ligation) because it resulted in very little NTP addition when 5'- $^{32}\text{P}$ -labeled S – 2 substrate and unlabeled UTP and CTP were employed at  $37^\circ\text{C}$ . When an ATP analog that contained a methylene group in place of the  $\alpha\text{-}\beta$  bridging oxygen was incorporated at the 5' end of the ribozyme, ligation of the substrate was prevented, but a significant amount of NTP addition onto the 3' end of the substrate still was observed. However, it was substantially less than that which was observed with the 5'-triphosphorylated ribozyme. This suggests that the presence of a triphosphate at the 5' end of the ribozyme is important for efficient NTP addition.

Reaction conditions that made it possible to monitor the addition of NTPs onto either the 3' end of the substrate or the 5' end of the ribozyme were found. This required the use of unlabeled ribozyme, unlabeled S – 2 substrate, 2 mM UTP, and highly radioactive  $[\alpha\text{-}^{32}\text{P}]\text{CTP}$  in a reaction that was performed at  $22^\circ\text{C}$  rather than  $37^\circ\text{C}$ . Under these conditions, both the unligated substrate molecule and the unligated ribozyme became labeled (Figure 6). When addition onto the 3' end of the substrate was prevented with an all-DNA substrate, only the unligated ribozyme was labeled. In the presence

of dephosphorylated ribozyme, which prevented NTP addition onto the 5' end of the ribozyme, only the unligated substrate was labeled. Thus, the unmodified E278-19 ribozyme is capable of catalyzing NTP addition in both a 5'→3' and a 3'→5' direction with the S - 2 substrate.

The ribozyme also was capable of adding NTPs in both a 5'→3' and a 3'→5' direction with the S - 1 substrate. In this case, however, addition onto the 5' end of the ribozyme was observed only when addition onto the 3' end of the substrate was prevented with an all-DNA substrate. Thus, the selection pressure imposed when the ribozymes were challenged to catalyze two NTP addition reactions may have enhanced their ability to operate in a 3'→5' direction.

## Discussion

### Continuous Evolution as a Model of Biological Evolution

A continuous in vitro evolution system was employed to develop ribozymes that catalyze three successive nucleotidyl addition reactions. The evolved ribozymes catalyze the template-directed addition of two NTPs followed by ligation of an oligonucleotide onto their own 5' end. The reactions can be carried out in the context of continuous evolution, with catalysis and selective amplification occurring in the same reaction mixture (Figure 1). Continuous in vitro evolution provides a simplified model of biological evolution in which the phenotypic advantage resulting from a multi-step biochemical pathway is linked to selective amplification of the corresponding genotype. Previous examples of the continuous in vitro evolution of catalytic function have been limited to ribozymes that catalyze a single reaction [7, 15, 16].

Continuous evolution allows a large number of rounds of selective amplification to be carried out in a short period of time and enables one to study the behavior of evolving populations over hundreds of generations. Unlike stepwise evolution, which typically requires 1–3 days to perform each round of catalysis and selective amplification, continuous evolution allows several successive rounds to occur in 1 hr. In this study, for example, the evolving population was maintained against an overall dilution of  $3 \times 10^{406}$  between rounds E106 and E260, equivalent to several hundred rounds of stepwise evolution. Over the course of the entire evolutionary lineage, starting with the experiments that generated the E100-3 ribozyme [7], the evolving population was maintained against an overall dilution of more than  $10^{700}$ . Furthermore, continuous evolution allows functional molecules to become eligible for amplification immediately after they have performed the required task. Thus, the fittest individuals are reproduced more quickly than the less fit, favoring rapid enrichment of the most advantageous phenotypes.

In its initial form, the continuous evolution system challenged the ribozymes to catalyze a single RNA ligation reaction prior to reverse transcription [7]. In the present study, the complexity of continuous evolution was increased by challenging the ribozymes to catalyze two or three reactions, involving two or three different

substrates, prior to reverse transcription. This does not begin to approach the complexity observed in biological systems, in which even a simple metabolic pathway may involve the coordination of multiple, often chemically unrelated catalytic events. In order to achieve the same level of complexity in an in vitro evolution system as occurs in biological systems, one would have to carry out a tremendous amount of evolutionary optimization, presumably requiring cellular organization or some other form of compartmentalization. This is the pathway that has proven so successful in biology.

### Evolution of a Complex Phenotype

The biochemical phenotype of a nucleic acid enzyme typically is described in terms of its catalytic parameters, such as  $k_{cat}$  and  $K_M$ . Even for a simple enzyme, however, these parameters involve a complex set of contributing factors, such as the ability of the molecule to fold into an active conformation, recognize a substrate molecule, and carry out a reaction under a particular set of environmental conditions. When one considers the in vitro evolutionary development of a nucleic-acid enzyme, one must also consider selection pressures related to reverse transcription, PCR amplification, and forward transcription. In the continuous evolution system, the number of extraneous factors contributing to the observed phenotype is even greater. For example, it is necessary that RNA catalysis occur prior to reverse transcription, that the ribozymes adopt a structure that supports catalysis yet does not impede reverse transcription, and that the ribozymes gain access to the nucleic acid substrate(s) in the presence of polymerase proteins that themselves have a propensity to bind these substrates [7].

As evidenced by the behavior of the E278-19 ribozyme, direct ligation of the oligonucleotide substrate without NTP addition is a significant competing reaction. Thus, the ribozymes were under selection pressure to increase the likelihood of NTP addition prior to ligation. The E208-2 ribozyme (Figure 2B), which directs the addition of a single NTP, had mutated the templating uridylylate to adenylate in order to direct the addition of UTP rather than ATP. The 5'-terminal nucleotide of the ribozyme is an adenylate, which could form a Watson-Crick pair with a templating uridylylate and thus facilitate direct ligation. By mutation of the templating residue to an adenylate, this pairing was prevented, and the opportunity for UTP to bind to the template was increased. In the subsequent E278-19 ribozyme (Figure 2C), the templating nucleotide was mutated to a guanylate, directing the addition of CTP and taking advantage of the greater stability of a G•C pair compared to an A•U pair.

After round E260, the ribozymes were dependent on the presence of T7 RNAP for their ability to catalyze NTP addition at 37°C. It was only when the population was forced, via stepwise evolution, to operate in the absence of T7 RNAP that the ribozymes evolved to function without the assistance of the protein enzyme. This illustrates both the power and potential weakness of in vitro evolution. Desired phenotypes can be obtained under a controlled environment, but understanding and controlling the selection pressures imposed by that environment may not be straightforward.



Attempts were made to further evolve variants of the E278 population. Variants were selected for their ability to catalyze the addition of three NTPs and subsequent RNA ligation. This effort employed a substrate lacking three nucleotides at the 3' end of the T7 RNAP promoter (S – 3) and utilized a combination of stepwise and continuous evolution. All of the ribozymes that were obtained evaded the intended selection pressure by evolving mutant T7 RNAP promoter sequences that did not contain all three added nucleotides and that allowed transcription to occur at a reduced level. Most of the mutant promoters have not been described previously (see Supplemental Material). The overriding selection pressure during continuous evolution is for the ribozymes to acquire the ability to support transcription. In this case, it apparently was more difficult to evolve ribozymes that catalyzed the addition of three NTPs to generate a full-length promoter than to discover an alternative means of supporting transcription, even though the transcription yield was significantly reduced.

#### Catalytic Strategies of the Evolved Ribozyme

Analysis of the direct ligation reaction involving the S', S – 1, and S – 2 substrates provides insight into the way that the E278-19 ribozyme recognizes its substrate. There was a progressive increase in  $K_M$  as the length of the substrate decreased, which may be attributed to the progressive loss of base-pairing interactions. However, based on the predicted  $\Delta G$  values for Watson-Crick pairing of these substrates [23], a more dramatic increase in  $K_M$  would have been expected. Thus, the ribozyme appears to use strategies in addition to simple Watson-Crick pairing to recognize its substrate.

Direct ligation occurs rapidly with the S – 1 and S' substrates. These reactions are equivalent to ligation of the S – 2 substrate after the addition of either one or two nucleotides, respectively, onto its 3' end. Direct ligation is more than 100-fold slower for the S – 2 substrate, allowing time for the first NTP addition to occur. If one assumes that the S – 1 and S' substrates represent intermediates in the three-step reaction pathway, there are two competing pathways after the first NTP addition: either direct ligation of the singly extended substrate or addition of a second NTP followed by rapid ligation. The first of these two pathways is at least 10- to 20-fold faster than the second, based on the fact that a complete promoter sequence is generated only 5%–10% of the time when the E278-19 ribozyme is allowed to react with the S – 2 substrate in the presence of NTPs. Nonetheless, this appears to be sufficient to support continuous evolution, with only a fraction of the copies of the ribozyme giving rise to viable progeny.

The ability of T7 RNAP to promote NTP addition also may contribute to the survival of ribozyme molecules in the continuous evolution system. However, because the activity of the E278-19 ribozyme is enhanced by less than 2-fold in the presence compared to the absence of T7 RNAP, at least half of the ribozyme molecules that obtained a functional promoter element within the context of continuous evolution would have done so as a consequence of their intrinsic catalytic activity. Furthermore, the ribozyme also must have the ability to catalyze nucleotidyl addition reactions in the absence

of T7 RNAP in order to survive the rounds of stepwise evolution. Molecules that directly ligate the S – 2 substrate or add only one NTP prior to ligation are able to support transcription by “borrowing” one or two nucleotides from the 5' end of the ribozyme to complete the promoter element. The resulting transcripts, however, would be missing one or two nucleotides from their 5' end, making them incapable of supporting further catalysis.

The E278-19 ribozyme catalyzes NTP addition reactions in both a 5'→3' and 3'→5' direction (Figure 6). This dual mechanism may have been selectively advantageous for the reaction with the S – 2 substrate because it allowed the first NTP addition to occur onto the 5' end of the ribozyme. It may be more difficult for the competing direct ligation reaction to occur when the ribozyme has been extended by one nucleotide at its 5' end as opposed to when the substrate has been extended by one nucleotide at its 3' end.

#### RNA Catalysis in an RNA World

RNA-catalyzed template-directed addition of NTPs demonstrates a potential mechanism for information transfer in an RNA world. The RNA world hypothesis proposes that there was a time in evolutionary history when RNA was responsible for both genotype and phenotype [24]. In order for this type of genetic system to have existed, there must have been some mechanism by which genetic information could be copied, presumably through the action of a ribozyme with polymerase-type activity [25].

In vitro evolution experiments have been utilized to demonstrate the ability of RNA to catalyze polymerase-like reactions, specifically the attack of the 3' hydroxyl group of a template bound oligonucleotide onto the  $\alpha$ -phosphate of a nucleoside- or oligonucleotide-5'-triphosphate. These studies have resulted in the generation of several ligase ribozymes [11, 26, 27], including the class I ligase [8]. The class I ligase also has been shown to catalyze the template-directed addition of NTPs onto the 3' end of an RNA primer [12] and has been utilized as a core catalytic domain to generate a ribozyme with true polymerase activity that is capable of generalized primer extension on an external template [13]. The hc ligase also has been evolved to catalyze ligation on an external template and is capable of primer extension in this format [28]. Both the class I and hc ribozymes catalyze NTP addition reactions in a 5'→3' direction, according to the model of all known protein polymerases. In the present study, the direction of NTP addition was not constrained; the ribozymes were required only to fill the gap between the 3' end of the substrate and the 5' end of the ribozyme. The resulting E278-19 ribozyme is able to catalyze polymerase-like reactions in either a 5'→3' or a 3'→5' direction, expanding the view of how an RNA replicase ribozyme might have operated in an RNA world.

#### Significance

The complexity of biological Darwinian evolution makes it difficult to study the changing biochemical properties of evolving populations, pointing out the

need for simplified model systems. Laboratory systems have been developed for the evolution of biological macromolecules. These systems involve either stepwise evolution with segregation of functional molecules or continuous evolution with expression of function and selective amplification occurring in a common reaction vessel. In this study, continuous evolution was used to evolve a ligase ribozyme that catalyzes three successive nucleotidyl addition reactions. In order to be selectively amplified, the RNA molecules were challenged to carry out two mononucleotide addition reactions followed by RNA ligation. The evolved ribozymes catalyze the same chemistry as RNA polymerization, supporting the idea that an RNA replicase ribozyme could have carried out information transfer in an RNA world. Unlike previously described polymerase-like ribozymes that operate in a 5'→3' direction, the ribozymes obtained in this study add mononucleotides in either a 5'→3' or a 3'→5' direction. In the consideration of RNA-catalyzed RNA replication in an RNA world, it remains an open question whether polymerization occurred in a 5'→3' or a 3'→5' direction, or perhaps both.

## Experimental Procedures

### Materials

Synthetic DNA-RNA chimeric oligonucleotides were prepared with a PerSeptive Biosystems automated DNA/RNA synthesizer. Expedite DNA amidites and solid supports were purchased from Applied Biosystems, and TOM RNA amidites and solid supports were from Glen Research. The oligonucleotides were deprotected according to the manufacturers' protocols. DNA oligonucleotides were purchased from Operon Technologies and Integrated DNA Technologies. All oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis and quantified by UV spectroscopy.

Histidine-tagged T7 RNA polymerase (T7 RNAP) was purified from *E. coli* strain BL21 containing plasmid pBH161 (kindly provided by William McAllister) by the use of His-Bind resin (Novagen) according to the manufacturer's protocol. *Thermus aquaticus* DNA polymerase was cloned from total genomic DNA and purified as previously described [29]. Superscript II RNase H<sup>-</sup> reverse transcriptase was obtained from Gibco-BRL, T4 polynucleotide kinase from New England Biolabs, Sequenase 2.0 modified T7 DNA polymerase from U.S. Biochemical, Eam1104 I restriction endonuclease from Stratagene, inorganic pyrophosphatase from Sigma Chemical, and calf intestinal phosphatase and RNase-free DNase I from Boehringer Mannheim. Nucleoside 5'-triphosphates (NTPs) and deoxynucleoside 5'-triphosphates (dNTPs) were purchased from Pharmacia, dideoxynucleoside 5'-triphosphates (ddNTPs) from U.S. Biochemical,  $\alpha$ - $\beta$ -methylene-ATP (AMP-CPP) from Sigma Chemical, adenosine-5'-O-[3-thiotriphosphate] (ATP- $\gamma$ -S) from Roche Molecular Biochemicals, and [ $\gamma$ -<sup>32</sup>P]ATP, [ $\alpha$ -<sup>32</sup>P]ATP, [ $\alpha$ -<sup>32</sup>P]CTP, [ $\alpha$ -<sup>32</sup>P]UTP, and [ $\alpha$ -<sup>35</sup>S]dATP from ICN Radiochemicals. Thiopropyl-activated agarose was obtained from Sigma Chemical, the TA cloning kit from Invitrogen, and PCR purification kits from Qiagen. All in vitro-transcribed RNAs were treated with RNase-free DNase I, then purified by denaturing polyacrylamide gel electrophoresis. All cloning procedures were carried out with the TA cloning kit. Individual clones were sequenced by the dideoxynucleotide method with [ $\alpha$ -<sup>35</sup>S]dATP and Sequenase 2.0 modified T7 DNA polymerase.

### Construction of the Initial Pool

Template DNAs for construction of the initial pool of RNAs were prepared by ligation of PCR-amplified synthetic randomized oligonucleotides that were based on the E100-3 sequence [7]. The 5' half of the ribozyme was encoded by the sequence 5'-GGACTAA TACGACTCACTATARGAACATTACATTATAGTGACCAGGAAAGA CAAATCTGCCCTCAGAGCTTGAGAACATCTTCGGGAAGAGTCC

AGGCTGACGTCAAG-3' (the T7 RNAP promoter sequence is underlined, nucleotides randomized at 8% degeneracy are in bold, the Eam1104 I restriction site is in italics, and R is either A or G), which was PCR amplified with upstream primer 5'-GGACTAATACGACT CACTATA-3' and downstream primer 5'-AGGTCCGACTGCAGTTC-3'. The 3' half of the ribozyme was encoded by the sequence 5'-CTT GACGTCAGCCTGGACTCTTCCTCGGATGCAGGGGAGGCAGCTCC CGATGGAAGTGACGAGCCAGCGTTCTCAACAGTAATGACTGAA CCTAAAAGCCAATCGCAGGCTCAGC-3' (nucleotides randomized at 8% degeneracy are in bold; the Eam1104 I restriction site is in italics), which was PCR amplified with upstream primer 5'-CTTGAC GTCAGCCTGGA-3' and downstream primer 5'-GCTGAGCCTGC GATTGG-3'. The products of both PCR amplifications were combined and then digested in a 2 ml volume containing 10 mM magnesium acetate, 100 mM potassium acetate, 25 mM Tris-acetate (pH 7.6), 0.5 mM  $\beta$ -mercaptoethanol, 10  $\mu$ g/ml bovine serum albumin (BSA), and 0.48 U/ $\mu$ l Eam1104 I; this mixture was incubated at 37°C for 4.5 hr. The digested products were ligated in a 3 ml volume containing 1 mM ATP, 10 mM MgCl<sub>2</sub>, 50 mM Tris-HCl (pH 7.8), 10 mM dithiothreitol (DTT), 50  $\mu$ g/ml BSA, and 0.05 U/ $\mu$ l T4 DNA ligase; this mixture was incubated at 16°C for 16 hr. Half of the resulting ligation products were transcribed as previously described [30] in a 2 ml volume, followed by treatment with RNase-free DNase I to generate the pool of RNAs used to initiate the first round of in vitro evolution.

### Stepwise Evolution

All RNA-catalyzed reactions for stepwise evolution were carried out in the presence of 30 mM *N*-[2-hydroxyethyl]piperazine-*N'*-[3-propane-sulfonic acid] (EPPS, pH 8.5), 50 mM KCl, 5 mM DTT, and 2 mM spermidine. The initial round of selection was performed in a 1.6 ml volume containing 10  $\mu$ M pool RNA, 10  $\mu$ M substrate (S) having the sequence 5'-CTTGACGTCAGCCTGGACTAATACGACT CACUUAU-3' (the T7 RNAP promoter sequence is underlined; RNA residues are in bold), and 60 mM MgCl<sub>2</sub>, which was incubated at 22°C for 5 min, then at 37°C for 30 min. The reaction was quenched with disodium ethylenediaminetetraacetate (Na<sub>2</sub>EDTA [pH 8.0]). The second and third rounds (E102, E103) were carried out with 0.5  $\mu$ M ribozyme, 5  $\mu$ M substrate (S - 1) having the sequence 5'-CTT GACGTCAGCCTGGACTAATACGACTCACUUAU-3', 2.5  $\mu$ M reverse transcription primer having the sequence 5'-GCTGAGCCTGC GATTGG-3', 10 mM ATP, and 25 mM MgCl<sub>2</sub>, which were incubated at 22°C for either 1 hr (E102) or 30 min (E103), followed by quenching with Na<sub>2</sub>EDTA (pH 8.0). Rounds E156-158 and E164-168 were carried out in a similar manner with 1  $\mu$ M ribozyme, 5  $\mu$ M substrate (S - 2) having the sequence 5'-CTTGACGTCAGCCTGGACTAATAC GACTCACUA-3', 2  $\mu$ M reverse transcription primer, 2 mM each of ATP, CTP, GTP, and UTP, 0.2 mM each of dATP, dCTP, dGTP, and TTP, and 25 mM MgCl<sub>2</sub>, which was incubated at 37°C for 30 min, followed by quenching with Na<sub>2</sub>EDTA (pH 8.0).

The reacted molecules were purified in a denaturing 6% polyacrylamide gel, reverse transcribed with the primer 5'-GCTGAGCCTGC GATTGG-3', and subsequently PCR amplified with upstream primer 5'-CTTGACGTCAGCCTGGA-3' and a downstream primer having the same sequence as the reverse transcription primer. The same primers were employed for all mutagenic PCR procedures [17, 18] and for PCR amplification of plasmid DNA. For rounds E102 and E103, a second PCR amplification was carried out with the upstream primer 5'-GGACTAATACGACTCACTATA-3' (the T7 RNAP promoter sequence is underlined) and the same downstream primer that was used for the first PCR amplification. The PCR products were first either extracted with phenol and chloroform:isoamyl alcohol (24:1) or purified with a Qiagen PCR purification kit and subsequently transcribed as previously described [30].

The RNA-catalyzed reactions for rounds E104 and E105 were carried out in the presence of 10  $\mu$ M S - 1 substrate, 10 mM ATP, and 35 mM MgCl<sub>2</sub>, which were incubated at 22°C for 30 min. After the reaction, the mixture was adjusted to contain 4 mM ATP, 2 mM each of CTP, GTP, and UTP, 0.2 mM each of dATP, dCTP, dGTP, and TTP, 2  $\mu$ M reverse transcription primer, and 8.6 U/ $\mu$ l reverse transcriptase, then incubated at 37°C for 10 min. This was followed by the addition of 20 U/ $\mu$ l T7 RNAP and  $6.4 \times 10^{-5}$  U/ $\mu$ l inorganic pyrophosphatase, then further incubation at 37°C for 2 hr. Amplifica-

tion was quenched with Na<sub>2</sub>EDTA (pH 8.0), followed by heat denaturation of the protein enzymes at 65°C for 5 min and removal of 5  $\mu$ l of material for use as input into the next round of evolution.

#### Continuous Evolution

Continuous-evolution experiments usually were carried out in the presence of 25 mM MgCl<sub>2</sub>, 50 mM KCl, 30 mM EPPS (pH 8.5), 5 mM DTT, 2 mM spermidine, 5  $\mu$ M substrate oligonucleotide, 2  $\mu$ M reverse transcription primer, 0.2 mM each of dATP, dCTP, dGTP, and TTP, 2 mM each of ATP, CTP, GTP, and UTP, 0.006  $\mu$ Ci/ $\mu$ l [ $\alpha$ -<sup>32</sup>P]ATP, 4 U/ $\mu$ l T7 RNAP, and 8 U/ $\mu$ l reverse transcriptase in a 25  $\mu$ l reaction volume. During rounds E106–E155, the concentration of ATP was increased to 10 mM, MgCl<sub>2</sub> was increased to 35 mM, and the substrate was increased to 10  $\mu$ M. During rounds E156–E170, the concentration of ATP was 5 mM, that of MgCl<sub>2</sub> was 30 mM, and that of the substrate was 5  $\mu$ M. During rounds E106–E108, the substrate concentration was 2.5  $\mu$ M. During rounds E211–E223, the concentration of UTP was 10 mM, and that of MgCl<sub>2</sub> was 50 mM, then the concentration of MgCl<sub>2</sub> gradually was reduced from 42 mM at round E224 to 25 mM at round E230.

All reaction components, except the protein enzymes, were prewarmed to 37°C prior to initiation of the reaction by the addition of purified RNA, PCR DNA, or a portion of the mixture from the previous round of evolution. For analysis, a small portion of the reaction mixture was removed and diluted 10-fold in a solution containing 10 mM Tris-HCl (pH 7.5) and 1 mM Na<sub>2</sub>EDTA, then mixed with an equal volume of gel loading buffer containing 9 M urea, 20% sucrose, 90 mM Tris-borate (pH 8.3), 0.05% bromophenol blue, 0.05% xylene cyanol, and 0.1% SDS. Unreacted and reacted RNAs were separated by electrophoresis in a denaturing 6% polyacrylamide gel and quantitated with a Molecular Dynamics phosphorimager. Each round of continuous evolution was carried out over 0.5–2 hr, with a 100- to 1000-fold dilution occurring between successive rounds (Figure 3).

#### RNase T1 Digestion Assay

RNA-catalyzed reactions were carried out in the presence of 1  $\mu$ M ribozyme, 5  $\mu$ M 5'-<sup>32</sup>P-labeled S – 2 substrate, 50 mM KCl, 30 mM EPPS (pH 8.5), 5 mM DTT, 2 mM spermidine, and a MgCl<sub>2</sub> concentration that was in 21 mM excess over the total concentration of NTPs, which were at a concentration of 2 mM each. Some of the reaction mixtures also contained 4 U/ $\mu$ l T7 RNAP. The reacted RNAs were purified by electrophoresis in a denaturing 6% polyacrylamide gel, ethanol precipitated, and resuspended in a mixture containing 25 mM sodium citrate (pH 5.0), 5.9 M urea, 1 mM Na<sub>2</sub>EDTA, 0.03% bromophenol blue, 0.03% xylene cyanol, and 4 U/ $\mu$ l RNase T1, which was incubated at 50°C for 30 min. The digested RNAs then were separated by electrophoresis in a denaturing 20% polyacrylamide gel.

Variant ribozymes that contained different templating nucleotides for NTP addition were generated by PCR amplification of E278-19 plasmid DNA with upstream primers 5'-TAATACGACTCACTATCA GAAGAATACATXXTAGTGACAGG-3' (X = A, C, G, or T) and downstream primer 5'-GCTGAGCCTGCGATTGG-3'; transcription followed. RNA-catalyzed reactions were carried out as described above in the presence of a 2 mM concentration of the appropriate NTP. The reaction products were analyzed via the T1 digest assay, except for ribozymes that had directed the addition of GTP. In that case the products were digested with RNase CL3 in a reaction mixture that contained 35 mM Tris-HCl (pH 8.0), 5.9 M urea, 1 mM Na<sub>2</sub>EDTA, 0.03% bromophenol blue, 0.03% xylene cyanol, and 1.25  $\times 10^{-5}$  U/ $\mu$ l RNase CL3, which were incubated at 50°C for 15 min.

#### [ $\alpha$ -<sup>32</sup>P]-NTP Incorporation Followed by Partial Alkaline Hydrolysis

RNA-catalyzed reactions were carried out in the presence of 1  $\mu$ M ribozyme, 5  $\mu$ M S – 2 substrate, 25 mM MgCl<sub>2</sub>, 50 mM KCl, 30 mM EPPS (pH 8.5), 5 mM DTT, 2 mM spermidine, 2 mM each of CTP and UTP, and 2.5  $\mu$ Ci/ $\mu$ l of either [ $\alpha$ -<sup>32</sup>P]CTP or [ $\alpha$ -<sup>32</sup>P]UTP; this mixture was incubated at 37°C for 2 hr. A control reaction contained unlabeled NTPs and 5'-<sup>32</sup>P-labeled S – 2 substrate. The reacted RNAs were digested with RNase T1 as described above, and the resulting products were separated by electrophoresis in a denaturing 20% polyacrylamide gel. The bands corresponding to the addi-

tion of either one or two NTPs were isolated from the gel, and the eluted material was subjected to partial alkaline hydrolysis by incubation with 0.1 M NaOH at 37°C for 20 min, followed by neutralization with 0.1 M HCl and the addition of an equal volume of gel loading buffer. The partial hydrolysis products were separated by electrophoresis in a denaturing 20% polyacrylamide gel.

#### Kinetic Analysis of Direct Ligation

RNA-catalyzed reactions were carried out with ribozymes that had been transcribed in the presence of [ $\alpha$ -<sup>32</sup>P]ATP. Ligation reactions with the full-length substrate employed 2 nM ribozyme, 20–150 nM substrate (S') having the sequence 5'-CTTGACGTCAGCCTGGAC TAATACGACTCACAUC-3' (the T7 RNAP promoter sequence is underlined; RNA residues are in bold), 21 mM MgCl<sub>2</sub>, 50 mM KCl, 30 mM EPPS (pH 8.5), 5 mM DTT, and 2 mM spermidine. Reactions involving the S – 1 substrate employed 5 nM ribozyme and 50–600 nM substrate; reactions involving the S – 2 substrate employed 50–200 nM ribozyme and 0.25–17  $\mu$ M substrate. The data were fit to a linear regression plot of  $\ln[1 - F_{\text{reacted}}]$  versus time to determine  $k_{\text{obs}}$  for each substrate concentration. The  $k_{\text{obs}}$  values were fit to a standard Michaelis-Menten equation to provide estimates of  $k_{\text{cat}}$  and  $K_M$ .

#### Kinetic Analysis of NTP Addition Followed by Ligation

RNA-catalyzed reactions were carried out under conditions similar to those above, except that they employed 50–200 nM ribozyme, 0.75–17  $\mu$ M 5'-<sup>32</sup>P-labeled S – 2 substrate, 2 mM CTP, 2 mM UTP, and 25 mM MgCl<sub>2</sub>. The reaction mixtures were incubated at 37°C for 4–8 hr, and aliquots were taken at various times and quenched by the addition of an equal volume of 25 mM Na<sub>2</sub>EDTA, 9 M urea, 20% sucrose, 90 mM Tris-borate (pH 8.3), 0.05% bromophenol blue, 0.05% xylene cyanol, and 0.1% SDS. The products were separated by denaturing polyacrylamide gel electrophoresis. The overall rate of the reaction was determined by plotting  $\ln[1 - F_{\text{reacted}}]$  versus time to obtain a value for  $k_{\text{obs}}$  for each substrate concentration and then fitting these values to a standard Michaelis-Menten equation to obtain estimates of  $k_{\text{cat}}$  and  $K_M$ . In addition, the reacted products were isolated from the gels and subsequently digested with RNase T1 as described above. The products of RNase T1 digestion were separated in a denaturing polyacrylamide gel, and the materials corresponding to direct ligation, one NTP addition followed by ligation, and two NTP additions followed by ligation were quantitated with a Molecular Dynamics Phosphorimager. These data were processed in a manner similar to that described above to obtain estimates of  $k_{\text{cat}}$  and  $K_M$  for each of the component reactions.

#### Direction of NTP Addition

For reactions that employed a nucleotide analog at the 5' end of the ribozyme, E278-19 PCR DNA was transcribed as previously described [30], except that ATP was replaced by 2 mM  $\alpha$ - $\beta$ -methylene ATP (AMP-CPP) and 0.2 mM ATP- $\gamma$ -S. Passing the transcripts over a 10  $\mu$ l thiopropyl agarose column that had been prewashed three times with 25  $\mu$ l of 1 M Tris-HCl (pH 8.0) enhanced the proportion of molecules that contained AMP-CPP at their 5' end. After transcription, the RNAs were purified by extraction with phenol and chloroform:isoamyl alcohol (24:1), ethanol precipitated, then incubated with the washed agarose at 22°C for 20 min with agitation. RNAs that were not retained on the column were purified in a denaturing 6% polyacrylamide gel, then used to carry out the RNA-catalyzed reaction in the presence of 5'-<sup>32</sup>P-labeled S – 2 substrate, as described above.

Reactions with radiolabeled NTPs were carried out under similar conditions, except that they employed 0.2  $\mu$ M ribozyme, 10  $\mu$ M S – 2 substrate, 2 mM UTP, and 2  $\mu$ Ci/ $\mu$ l [ $\alpha$ -<sup>32</sup>P]CTP and were incubated at 22°C for 4 hr. Reactions in which NTP addition could not occur onto the 3' end of the substrate employed an all-DNA substrate having the sequence 5'-CTTGACGTCAGCCTGGACTAA TACGACTCACTA-3'. Reactions in which NTP addition could not occur onto the 5' end of the ribozyme employed ribozymes that had been dephosphorylated in a mixture containing 2  $\mu$ M ribozyme, 50 mM Tris-HCl (pH 8.5), 0.1 mM Na<sub>2</sub>EDTA, and 0.2 U/ $\mu$ l calf intestinal phosphatase, which were incubated at 37°C for 1 hr. The reaction products were ethanol precipitated twice, with 2  $\mu$ g of carrier tRNA, then separated by denaturing polyacrylamide gel electrophoresis.

# Supplemental Material

To receive a pdf of the Supplemental Material, including information about unusual T7 RNAP promoters, a table showing the transcription efficiency of these mutant promoters, and an autoradiogram depicting catalytic activity of the E278-19 ribozyme in the three-step reaction, please contact the *Chemistry & Biology* production department at chembiol@cell.com.

# Acknowledgments

This work was supported by Research Grant NAG5-9386 from the National Aeronautics and Space Administration.

Received: November 29, 2001

Revised: February 21, 2002

Accepted: March 18, 2002

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