be preceded by a condensation of the chromosomes. After association with the emerging central spindle, they separate into two clusters that move to opposite spindle poles. They remain at the spindle poles during spindle elongation until they are in close proximity to the nuclear envelope. Their asymmetrical distribution within the nucleus is maintained after spindle disassembly until late S phase when they are distributed almost randomly near the nuclear envelope. As the ploidy is uncertain and individual minichromosomes cannot be visualized owing to the lack of large enough specific target DNA sequences, it is not clear whether there is faithful segregation of each minichromosome. However, detailed microscopic analysis of many cells (>100) showed that the minichromosomal clusters segregated on the spindle and inherited by each daughter cell were of equivalent size, indicating a precise segregation mechanism.

The existence of a highly coordinated segregation mechanism for minichromosomes suggests that they play an important role in the biology of this parasite. In addition, owing to their small size, minichromosomes may serve as an excellent model for the study of mitotic segregation, particularly with respect to the evolution of DNA partition mechanisms. The diploid large chromosomes, as exemplified by the chromosome harboring the 5S ribosomal gene, are likely segregated by peripheral pole-to-kinetochore microtubules. There is, however, an intriguing discrepancy between the number of large chromosomes, estimated to be at least 20 for the diploid set (*4*, *21*), and the number of kinetochore-like structures, estimated to be approximately 10 (*11*, *24*).

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16. For combined immunofluorescence and fluorescence in situ hybridization, procyclic trypanosomes (strain 427) were washed once in phosphate-buffered saline (PBS), transferred to slides coated with aminopropyltriethoxysilane (Sigma), and fixed with 4% formaldehyde–5% acetic acid in PBS for 15 min at 20°C. After washing twice with PBS, cells were permeabilized with 0.1% NP-40 in PBS for 5 min, washed once in PBS, and incubated with the first antibody in PBS for 1 hour, then washed three times for 5 min each in PBS and incubated with a secondary, fluorescein isothiocyanate (FITC)–conjugated antibody (Dako) for 45 min. After washing, cells were postfixed in 3% formaldehyde for 20 min. Cells were then prehybridized for 60 min in 50% formamide, 2× saline sodium phosphate–EDTA (SSPE), 10% dextran sulfate [hybridization buffer (HB)] at 37°C, and subsequently hybridized as described in (*17*).
17. For in situ hybridization, digoxigenin- or biotin-labeled DNA probes were coprecipitated with herring sperm DNA (10 mg/ml) and yeast tRNA (10 mg/ml), respectively, and resuspended in HB. After prehybridization, probes in HB were transferred to the slide, sealed with a plastic frame (Geneframe, Hybaid, Teddington, UK), and denatured simultaneously with the cellular DNA on an in situ block (Hybaid) at 95°C for 5 min and hybridized at 37°C for 16 hours. Washing was done in 50% formamide, 2× standard saline citrate (SSC) for 30 min at 37°C, 10 min in 2× SSC at 50°C, 60 min in 0.2× SSC at 50°C, and 10 min in 4× SSC at 20°C. For detection of digoxigenin-labeled probes, cells were then incubated with a sheep anti-digoxigenin Fab fragment (Boehringer Mannheim) in 100 mM maleic acid, 150 mM NaCl, 1% blocking reagent (Boehringer) (MEB) for 1 hour at 20°C, washed in tris-buffered saline plus 0.1% Tween-20, and incubated with an FITC-conjugated antibody to sheep immunoglobulin G (Jackson Laboratory) for 45 min in MEB. For detection of biotinylated DNA, cells were incubated with a streptavidin-CY3 conjugate (Sigma) for 1 hour at 20°C in MEB. After washing as above, cells were mounted in Vectashield (Vector Laboratories) containing 4′,6′-diamidino-2-phenylindole (DAPI; 0.1 μg/ml). Cells were analyzed on a Leitz DMRB microscope, and images were captured by means of a slow-scan cooled charge-coupled device camera (Photometrics) and IPLab software (Sigma Analytics). Images were deconvoluted with HazeBuster software (VayTek) and pseudocolored and merged in Adobe Photoshop on a Macintosh 9500/132 computer.
18. The minichromosomal 177-bp repeat element was amplified by polymerase chain reaction (PCR) with the primers (5′-GCGAATTCTAAATGGTTCTTATACGA-ATG) and (5′-TACGAAGCTTAACACTAAAGAACAG-CGTTG) (*6*, *7*). The ribosomal 5S DNA was amplified with the primers (5′-GCGAATTCAATCTATGC-CAATC) and (5′-TACGAAGCTTCTGATCGCCGTA-CTAACGAG) (*20*). Purified PCR products were labeled by PCR with either digoxigenin (ribosomal probe) or biotin (minichromosomal probe) with the same sets of primers and a 1:2 ratio of digoxigenin or biotin-dUTP and dTTP. Labeled PCR products were purified on Qiagen spin columns.
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26. We thank members of our lab for discussion. DNA probes of the tubulin locus were obtained from S. Melville, University of Cambridge. The work in our lab was supported by a Wellcome Trust Programme and Equipment Grant. K.E. is supported by a Wellcome Trust Postdoctoral Fellowship.

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Continuous in Vitro Evolution of Catalytic Function

Martin C. Wright and Gerald F. Joyce*

A population of RNA molecules that catalyze the template-directed ligation of RNA substrates was made to evolve in a continuous manner in the test tube. A simple serial transfer procedure was used to achieve approximately 300 successive rounds of catalysis and selective amplification in 52 hours. During this time, the population size was maintained against an overall dilution of 3×10^{298}. Both the catalytic rate and amplification rate of the RNAs improved substantially as a consequence of mutations that accumulated during the evolution process. Continuous in vitro evolution makes it possible to maintain laboratory "cultures" of catalytic molecules that can be perpetuated indefinitely.

The principle of Darwinian evolution is applicable in vitro when a population of informational macromolecules is subjected to repeated rounds of selective amplification and mutation. An earlier extracellular Darwinian evolution experiment was done with variants of Qβ bacteriophage genomic RNA that were amplified on the basis of their ability to serve as a substrate for the Qβ replicase protein (*1*). Evolution was made to occur in a continuous manner by serial transfer of the RNAs to successive reaction vessels. In recent years, in vitro evolution procedures have been generalized to encompass almost any nucleic acid molecule, including those that have catalytic function (*2*). Unlike the Qβ evolution experiments, however, the evolution of catalytic function has been carried out in a stepwise rather than continuous fashion. Stepwise evolution requires intervention by

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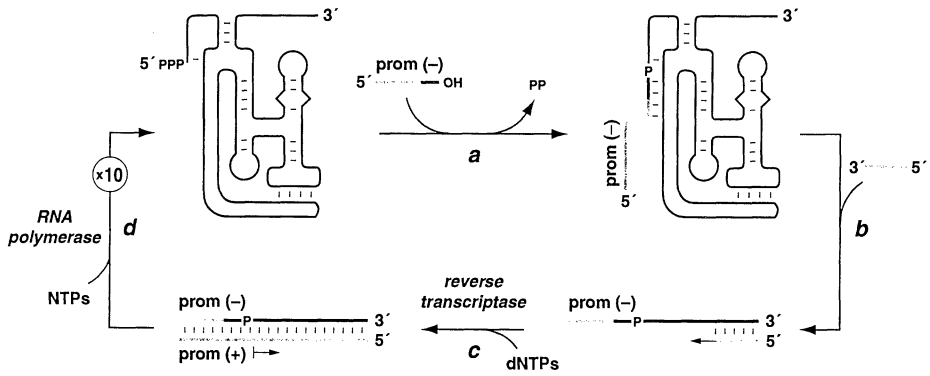

Fig. 1. Scheme for coupled catalysis and amplification. RNA and DNA are depicted as black and gray lines, respectively. (*a*) RNA molecules catalyze their own ligation to a chimeric RNA-DNA substrate that contains one strand of a bacteriophage RNA polymerase promoter (prom). (*b*) A DNA primer hybridizes to the 3′ end of the RNA and initiates reverse transcription. (*c*) Reverse transcriptase produces a complementary DNA copy from the ligated RNA, reading through the ligation junction to copy the attached promoter sequence. (*d*) RNA polymerase recognizes the newly formed double-stranded promoter element and produces multiple copies of progeny RNA. The 5′-triphosphate moiety is restored by the NTP that initiates transcription.

the experimenter at successive steps of catalysis, selective amplification, and mutation, and proceeds hundreds of times more slowly than would be possible with continuous evolution.

We devised a scheme for continuous in vitro evolution of catalytic RNAs that directs the condensation of an oligonucleotide 3′-hydroxyl substrate and their own 5′-triphosphate terminus, generating a 3′,5′-phosphodiester linkage and releasing pyrophosphate (3) (Fig. 1). The sequence of the oligonucleotide substrate was designed to correspond to the promoter element of a bacteriophage RNA polymerase. Thus, reverse transcription of reacted, but not unreacted RNA molecules would yield complementary DNAs that included one strand of the promoter element at their 3′ end. The other strand of the promoter would be provided by the substrate oligonucleotide, allowing selective transcription of DNAs that had been copied from reacted RNAs. Transcription would produce progeny RNA molecules that no longer carried the substrate at their 5′ end, but instead had a 5′-triphosphate, allowing them to participate in a subsequent RNA-catalyzed reaction. This process of coupled catalysis and selective amplification would continue indefinitely, so long as an ongoing supply of substrate, polymerases, and nucleic acid monomers was made available.

Several years ago we attempted to carry out continuous evolution of catalytic RNA, beginning with a population of variants of the BI1 group II ribozyme of yeast mitochondria (3). This ribozyme has a weak ability to catalyze the target reaction (4). Our attempt was thwarted, however, by the appearance of "selfish" RNAs that had circumvented the selection constraint by generating a heritable promoter sequence within themselves rather than acquiring the promoter through catalysis (3).

Bartel and Szostak used in vitro evolution to develop ribozymes that catalyze the highly efficient template-directed condensation of an oligonucleotide 3′-hydroxyl and an oligonucleotide 5′-triphosphate (5). If one of their "ligase" ribozymes could be made to operate in the coupled catalysis and amplification system described above, the ribozyme might outcompete the selfish RNAs and allow the evolution of catalytic function to proceed continuously. We constructed a modified form of the b1-207 ligase ribozyme (6) in which the template sequence, originally 5′-GACUGG-3′, was changed to 5′-UAUAGU-3′, making it complementary to a substrate having the sequence of the T7 RNA polymerase promoter (Fig. 2A). Unfortunately, this change reduced the catalytic rate of the ribozyme from 14 min^{-1} to 1.4×10^{-3} min^{-1} when an all-RNA substrate was used, and to $<10^{-4}$ min^{-1} when a largely DNA substrate that contained a single 3′-terminal ribonucleotide was used.

The T7 RNA polymerase promoter consists of a double-stranded DNA, but we determined that a maximum of four ribonucleotides at the 3′ terminus of the substrate were compatible with efficient promoter function. Proceeding initially with a substrate containing a single ribonucleotide, and employing a population of 10^{14} randomized variants of the prototype ligase ribozyme, we performed 15 rounds of stepwise evolution (7). This resulted in individual ribozymes with a catalytic rate of 0.05 to 0.15 min^{-1}. These molecules were not capable of undergoing continuous evolution. Their catalytic rate was still too slow under optimal conditions and even slower in the presence of active reverse transcriptase and RNA polymerase.

Using the ribozymes that resulted from the stepwise evolution, we next turned to a rapid evolution procedure in which the ribozymes first were allowed to react for 5 minutes with a substrate containing four

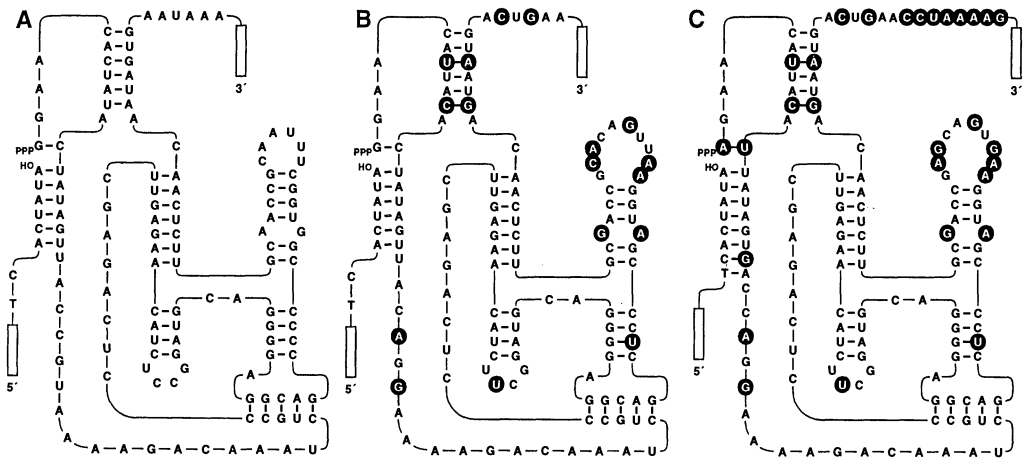

Fig. 2. Mutations that arose during ribozyme evolution. Open rectangles indicate the 5′ portion of the substrate and the primer binding site at the 3′ end of the ribozyme (5′-CCAAUCGCAG-GCUCAGC-3′), both of which are immutable during evolution. Highlighted residues are those that were mutated relative to the starting ribozyme. The secondary structural representation is based on Ekland *et al.* (6). (**A**) Prototype ribozyme used to construct the initial pool. (**B**) An individual ribozyme isolated before beginning continuous evolution. (**C**) An individual ribozyme isolated after 52 hours of continuous evolution.

3′-terminal ribonucleotides, and then were transferred to a selective amplification mixture that allowed reverse transcription and subsequent forward transcription. Catalysis and selective amplification were performed in an alternating manner and a total of 100 cycles were completed over a 3-week period (8). This procedure yielded ribozymes that were highly active in the target reaction. Individuals were isolated from the population and characterized. A typical example (Fig. 2B) contained 17 mutations relative to the starting RNA and had a catalytic rate of >1 min^{-1} under conditions that are compatible with polymerase function.

Using the population of ribozymes that resulted from the rapid evolution procedure, we initiated continuous evolution. Approximately 10^{11} RNA molecules were added to a reaction mixture containing substrate, Moloney murine leukemia virus reverse transcriptase, DNA primer (used to initiate reverse transcription), deoxyribonucleotide triphosphates (dNTPs), T7 RNA polymerase, NTPs, and buffer. After 60 min, a small portion of this mixture was transferred to a fresh reaction vessel. The process was continued for 100 serial transfers and resulted in an overall amplification of 3×10^{298} (Fig. 3A). At no time during the process were the RNA molecules isolated or purified. They continued to breed true, "purified" only by selective amplification based on their catalytic function.

As the evolution proceeded, the pace of selective amplification quickened. At the outset, the doubling time was about 12 min. During transfers 51 to 58, exponential growth occurred with a doubling time of less than 2 min (Fig. 3B). We chose to stop after the 100th transfer, but see no reason why the process could not be continued indefinitely. On the basis of the efficiency of transcription under our reaction conditions, we estimate that, on average, each catalytic event led to the production of 10 progeny molecules. Thus, approximately 300 successive catalytic events occurred during the 52 hours of continuous culture.

We isolated individuals from the population after the 100th transfer. A typical example (Fig. 2C) had 29 mutations relative to the starting ribozyme and an average of 15 mutations relative to the ribozymes that were isolated before beginning continuous evolution. Of the mutations that arose during continuous evolution, three are particularly noteworthy. One resulted in extension of the template region of the ribozyme so that it was complementary to eight rather than six nucleotides of the substrate. Two other mutations involved replacement of the 5′-terminal guanosine triphosphate by adenosine triphosphate (ATP) and a compensatory change of the templating nucleotide, thereby converting a pppG·C pair to a pppA·U pair.

The evolved ligase ribozyme (Fig. 2C) was a very efficient catalyst, with $k_{cat} > 20$ min^{-1} and $k_{cat}/K_m = 1 \times 10^7$ M^{-1} min^{-1} in the target reaction (9). This compares with a catalytic efficiency of 800 M^{-1} min^{-1} for the starting ribozyme (Fig. 2A). The evolved ribozyme was remarkably efficient in the selective amplification process, with an exponential "growth rate" of 1×10^{12} $hour^{-1}$ (Fig. 4). In contrast, the starting ribozyme did not exhibit exponential growth, and a ribozyme isolated prior to beginning continuous evolution had a growth rate of only 400 $hour^{-1}$. A disabled form of the evolved ribozyme that lacked catalytic activity was not selectively amplified (*10*).

The three mutations described above are largely, but not entirely, responsible for the enhanced growth rate of the evolved ribozyme. Reverting the mutation that resulted in extension of the template region reduced catalytic efficiency by a

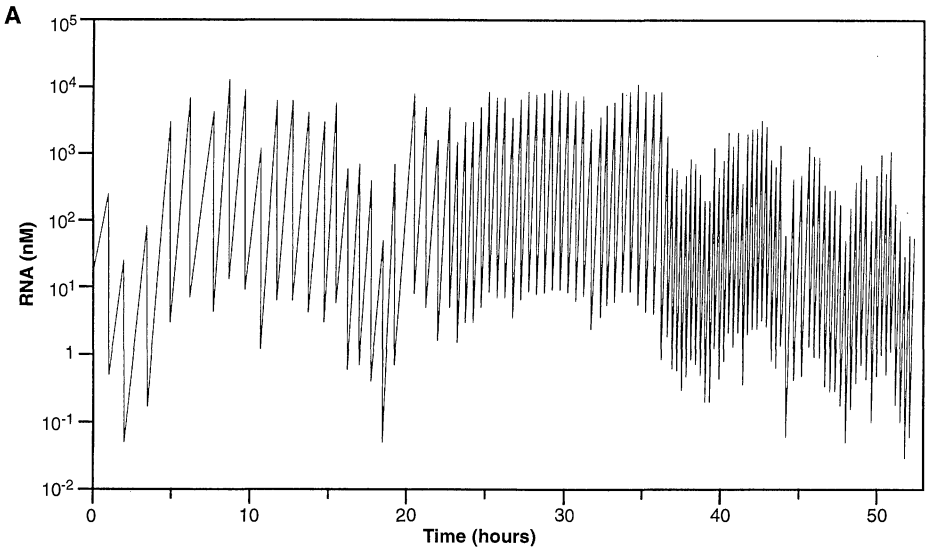

Fig. 3. Time course of continuous evolution. (**A**) One hundred serial transfers over 52 hours. (**B**) Detail of RNA amplification during transfers 51 to 58. RNA was amplified at 37°C in a 20-μl volume containing 5 μM substrate (5′-CTTGACGTCAGCCTGGACTAAT-ACGACTCAC**UAUA**-3′; promoter sequence underlined, RNA residues shown in bold), 2.5 μM DNA primer (5′-GCTGAGCCTGCGATTGG-3′), each dNTP at 0.2 mM, each NTP at 2 mM, [α-^{32}P]ATP at 0.5 μCi μl^{-1}, Moloney murine leukemia virus reverse transcriptase at 10 U μl^{-1}, T7 RNA polymerase at 2 U μl^{-1}, 25 mM $MgCl_2$, 50 mM KCl, 4 mM dithiothreitol, 2 mM spermidine, and 50 mM EPPS (pH 8.5). RNA was quantitated by separation in a denaturing polyacrylamide gel and Phosphorimager analysis of [α-^{32}P]ATP incorporation into full-length transcription product, on the assumption of an average of 40 adenosine residues per molecule. The overall amplification is the product of the amplification that occurred prior to each transfer. Adjustments to the time between successive transfers were made in an ad hoc manner, aiming for 1000-fold amplification per transfer. A diluted sample of the mixture could be stored at −20°C before being added to a fresh reaction vessel. During transfers 1 to 20, the $MgCl_2$ concentration was increased to 35 mM to enhance the ligation rate; during transfers 51 to 74 and 91 to 100, the substrate concentration was reduced to 2.5 μM.

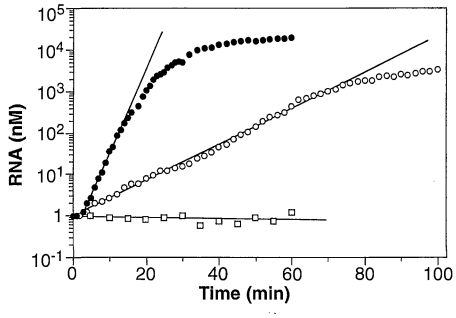

Fig. 4. Amplification profile of individual ribozymes shown in Fig. 2. Amplification conditions and quantitation of RNA were as described in the legend to Fig. 3, with 1 nM RNA input, 2.5 μM substrate, and 25 mM $MgCl_2$. The rate constant was calculated from the exponential phase, indicated by the line. □, Ribozyme shown in Fig. 2A (no amplification); ○, ribozyme shown in Fig. 2B (doubling time, 6.9 min; growth rate, 400 $hour^{-1}$); ●, ribozyme shown in Fig. 2C (doubling time, 1.5 min, growth rate, 1×10^{12} $hour^{-1}$).

half and reduced the exponential growth rate to 10^5 hour^{-1}. A compensatory mutation in the substrate, re-establishing an eight–base pair interaction between ribozyme and substrate, restored catalytic efficiency. Reverting the pppA·U pair to pppG·C did not significantly reduce catalytic efficiency, but reduced the growth rate to 10^9 hour^{-1}. Surprisingly, ribozymes that have either pppA or pppG at their 5′ terminus were transcribed with comparable efficiency, despite the usual preference of T7 RNA polymerase to initiate transcription with guanosine triphosphate (*11*).

The continuous in vitro evolution system described in this study cannot be generalized to all RNA-catalyzed reactions. In principle, however, it can be applied to other reactions that involve attachment of a promoter-containing substrate to a ribozyme that has a reactive group at its 5′ end, so long as the reactive group can be incorporated during transcription and the product of the RNA-catalyzed reaction can be "reverse-transcribed" to yield complementary DNA that contains the second strand of the promoter. The template-directed condensation of an oligonucleotide 3′-hydroxyl and ribozyme 5′-triphosphate is perhaps the most interesting example of this class of reactions. It is similar to the reaction catalyzed by an RNA replicase protein. If multiple NTP additions could be performed by a ribozyme, then the ribozyme itself, rather than polymerase proteins, might be able to sustain continuous in vitro evolution.

The b1-207 ligase ribozyme described by Ekland *et al.* (*6*) catalyzes the template-directed polymerization of NTPs that are bound at one to three template positions located immediately downstream from the usual ligation junction (*12*). The ligase ribozyme that resulted from our continuous evolution procedure (Fig. 2C) catalyzes a similar reaction in which ATP, bound at a template position located immediately upstream from the usual ligation junction, adds to the 3′ end of a truncated promoter-containing substrate that lacks the 3′-terminal adenylate (*13*). This suggests that continuous in vitro evolution might be used to develop ribozymes with RNA polymerase activity.

Prior to this study, only 63 successive rounds of catalysis and selective amplification in vitro had ever been accomplished, a task that required several years (*14*). With continuous in vitro evolution, this number of rounds could be completed in less than 6 hours. The rapid pace of continuous in vitro evolution makes it practical to address questions concerning evolutionary mechanism that require longitudinal analysis of a population subject to defined selection constraints. Compared to stepwise evolution, continuous in vitro evolution provides a more realistic model of biological evolution because of the reduced intervention by the experimenter and the direct link between phenotype and fecundity of individuals in the population. The fittest ribozymes are not only more likely to produce progeny, but also likely to produce more progeny in a given period of time.

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7. An RNA population was constructed based on the prototype ribozyme (Fig. 2A), with 112 randomized nucleotide positions and 8% degeneracy [J. Tsang and G. F. Joyce, *Methods Enzymol.* **267**, 411 (1996)]. An average of 12 copies each of 10^{14} different RNA sequences were incubated for 2 hours at 24°C in a 2-ml volume containing 10 μM substrate (5′-biotin-AAACTTGACGTCAGCCTGGACTAATACGACTCACTATA-3′; the promoter sequence is underlined and riboadenylate is shown in bold), 60 mM $MgCl_2$, 200 mM KCl, and 50 mM 4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid (EPPS; pH 8.5). Reacted molecules were captured on streptavidin-agarose beads, reverse-transcribed, and selectively amplified by the polymerase chain reaction (PCR) with primers 5′-GCTGAGCCTGCGATTGG-3′ (primer 1) and 5′-CTTGACGTCAGCCTGGA-3′ (primer 2). The PCR products were transcribed with T7 RNA polymerase to produce progeny RNAs. Rounds 2 to 15 were performed with a nonbiotinylated substrate in a 10-μl volume, under conditions as described above, decreasing the reaction time progressively from 2 hours to 5 min. The reaction mixture was diluted into a 40-μl volume containing primer 1 at 1 μM, dNTPs at 0.2 mM each, NTPs at 2 mM each, 5 mM dithiothreitol, 2 mM spermidine, reverse transcriptase (Superscript, Gibco-BRL) at 5 U μl^{-1}, and T7 RNA polymerase at 10 U μl^{-1}, and was incubated at 37°C for 2 hours. The protein enzymes were inactivated by heating to 65°C for 5 min in the presence of 15 mM disodium EDTA; 8 μl of this mixture was used to initiate the next round. After rounds 5, 10, and 15, the population was treated with reverse transcriptase, selectively amplified by PCR, and transcribed in order to reduce amplification artifacts.
8. Evolution was made to occur as described (*7*), except that the reaction was at 37°C in the presence of 2.5 μM substrate (5′-CTTGACGTCAGCCTGGACTAATACGACTCAC**UAUA**-3′; the promoter sequence is underlined and ribonucleotides are shown in bold), 15 mM $MgCl_2$, 50 mM KCl, 2 mM spermidine, and 50 mM EPPS (pH 8.5). Ribozyme was present in excess of substrate to ensure that a constant amount reacted during each round. Reverse transcription was carried out at 37°C for 5 min, then T7 RNA polymerase was added and transcription was continued for 30 to 45 min. After heat inactivation of the polymerase proteins, 5 μl of the amplification mixture was diluted into 20 μl of a fresh reaction mixture. The population was purified every 10 rounds by PCR amplification of the complementary DNA and polyacrylamide gel electrophoresis of the RNA.
9. Ribozymes were labeled by transcription in the presence of [α-^{32}P]ATP. Catalytic rates were determined by measuring the fraction ligated as a function of time in the presence of various concentrations of substrate [15 mM $MgCl_2$, 50 mM KCl, 2 mM spermidine, and 50 mM EPPS (pH 8.5)] at 37°C. Reaction rates greater than 20 min^{-1} could not be determined by manual pipetting methods. The proportion of correctly folded ribozymes depended on the method of purification. The values reported were obtained with in vitro transcribed RNA that was purified by phenol extraction and Sephadex chromatography, reacting to 60% completion.
10. A disabled form of the ribozyme was constructed by reverse transcription of reacted RNA with a mutagenic DNA primer (5′-GCTGAGCCTGCGATTGGCTTTTAGGTTCAGTGTAATGTGTTGAGAACGCTGGC-3′, mutations underlined), followed by PCR amplification. The double-stranded DNA product was cloned and sequenced to confirm the presence of the desired mutations. The mutant ribozyme could be amplified nonselectively in the presence of reverse transcriptase and T7 RNA polymerase with the use of primer 1 (*7*) and an alternative version of primer 2 that did not require attachment of the substrate to the 5′ end of the RNA.
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13. RNA-catalyzed addition of [α-^{32}P]ATP to a substrate lacking the 3′-terminal adenylate was performed at 24°C in the presence of 60 mM $MgCl_2$, 200 mM KCl, 0.6 mM disodium EDTA, and 30 mM tris-HCl (pH 8.0). The RNA was dephosphorylated prior to reaction so as to prevent its direct ligation to the substrate.
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