A molecular description of the evolution of resistance

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Background: *In vitro* evolution has been used to obtain nucleic acid molecules with interesting functional properties. The evolution process usually is carried out in a stepwise manner, involving successive rounds of selection, amplification and mutation. Recently, a continuous *in vitro* evolution system was devised for RNAs that catalyze the ligation of oligonucleotide substrates, allowing the evolution of catalytic function to be studied in real time.

Results: Continuous *in vitro* evolution of an RNA ligase ribozyme was carried out in the presence of a DNA enzyme that was capable of cleaving, and thereby inactivating, the ribozyme. The DNA concentration was increased steadily over 33.5 hours of evolution, reaching a final concentration that would have been sufficient to inactivate the starting population in one second. The evolved population of ribozymes developed resistance to the DNA enzyme, reducing their vulnerability to cleavage by 2000-fold but retaining their own catalytic function. Based on sequencing and kinetic analysis of the ribozymes, two mechanisms are proposed for this resistance. One involves three nucleotide substitutions, together with two compensatory mutations, that alter the site at which the DNA enzyme binds the ribozyme. The other involves enhancement of the ribozyme's ability to bind its own substrate in a way that protects it from cleavage by the DNA enzyme.

Conclusions: The ability to direct the evolution of an enzyme's biochemical properties in response to the behavior of another macromolecule provides insight into the evolution of resistance and may be useful in developing enzymes with novel or enhanced function.

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Introduction

An important objective of evolutionary biology is to understand the molecular processes that underlie genotypic and phenotypic changes that occur in response to a toxicant or predator. Spiegelman and coworkers [1] first demonstrated that these changes could be studied *in vitro* at the molecular level. Their evolution system utilized Qβ replicase, a template-specific RNA-dependent RNA polymerase, and variants of Qβ genomic RNA that can be copied by the replicase in the test tube. Serial transfer experiments were carried out to apply selective pressure to the population of replicating RNAs, resulting in the evolution of variants that could be copied more efficiently. In these experiments, the population was allowed to amplify continuously in a single reaction mixture and then a small aliquot was transferred to a fresh reaction mixture. The process was repeated for many successive transfers. Not surprisingly, the final surviving molecules were those that were as short as possible yet could still be copied by the replicase. Such individuals were the fittest because they could give rise to the largest number of copies prior to each transfer [1].

In subsequent studies with the Qβ system, a variety of impediments to RNA amplification were introduced as added selection constraints. For example, progressively increasing concentrations of ethidium bromide were added to the reaction mixture; the ethidium bromide acted as a toxicant that altered the structure of the RNA and inhibited its amplification. Serial transfer experiments were carried out to evolve variant RNAs that had become resistant to ethidium bromide [2]. One such molecule contained three nucleotide substitutions that were shown to be responsible for the resistant phenotype [3]. In another study, variant forms of Qβ RNA were obtained that had evolved resistance to ribonuclease A, which cleaves the phosphodiester linkage following pyrimidine residues of RNA. The pyrimidine residues within the evolved nuclease-resistant RNAs tended to be either buried within secondary and tertiary structure or restricted to one of the two replicating strands [4].

In recent years, studies involving the *in vitro* molecular evolution of nucleic acids have expanded considerably [5–7]. This was made possible by the development of techniques for the amplification of nucleic acids of almost any sequence, including those with catalytic function. One such technique is isothermal RNA amplification, also known as self-sustained sequence replication (3SR) [8], nucleic-acid sequence-based amplification

(NASBA) [9] or transcript-mediated amplification (TMA) ([10]; available at http://www.gen-probe.com/pdfs/tma_whiteppr.pdf). Isothermal RNA amplification employs both a reverse transcriptase and a DNA-dependent RNA polymerase in a reaction mixture that is maintained at a constant temperature of about 37°C. The mixture also contains the four NTPs, four dNTPs, primers for first- and second-strand DNA synthesis, $MgCl_2$ and a buffer. When a small amount of RNA is added to the mixture, it is copied by reverse transcriptase to cDNA and then to double-stranded DNA. The primer for second-strand DNA synthesis contains the sequence of a promoter element for the RNA polymerase, which allows the double-stranded DNA to be transcribed by the RNA polymerase to produce multiple copies of RNA. These in turn are reverse transcribed, leading to still more RNA, and so on until one or more of the reaction components is exhausted.

Predator–prey dynamics have been studied at the molecular level in the context of isothermal RNA amplification [11]. Two different RNA species were amplified in the same reaction mixture, with the cDNA strand of the 'prey' serving as the primer for second-strand DNA synthesis of the 'predator'. Coupling was observed between the two species, although parasitic side products soon emerged and grew to dominate the system. In another study, cooperative behavior was observed among two different RNAs undergoing isothermal RNA amplification [12]. In that case, the respective cDNAs were made to prime each other's second-strand DNA synthesis. The cooperative system was maintained over several serial transfers, and led to the emergence of variants that could amplify both selfishly and cooperatively, depending on the concentration regime.

The Qβ system and the isothermal amplification system described above allow one to carry out studies in 'molecular ecology', in which evolutionary phenomena such as resistance, predation and cooperativity are captured in the behavior of informational molecules. In these systems, however, the evolving molecules have no function of their own other than to serve as a substrate for the relevant polymerase proteins. Recently, a system was developed for the continuous *in vitro* evolution of catalytic RNAs (ribozymes) that perform an RNA ligation reaction [13]. An oligonucleotide substrate is provided that contains the sequence of an RNA polymerase promoter element. The catalytic RNAs are required to bind to the substrate through Watson–Crick pairing and catalyze attack of the 3′ hydroxyl group of the substrate on the 5′ triphosphate of the ribozyme, resulting in formation of a 3′,5′-phosphodiester linkage with release of inorganic pyrophosphate. This RNA-catalyzed reaction replaces second-strand synthesis as an essential component of the isothermal amplification cycle. Only those cDNAs generated from reacted ribozymes will contain a functional promoter and become amplified. The 5′ triphosphate of the ribozyme is restored during synthesis of the progeny RNAs because it is provided by the NTP that initiates transcription.

Continuous *in vitro* evolution was used previously to obtain variants of the class I ligase ribozyme [14,15] that operate with a catalytic efficiency, k_{cat}/K_m, of about 10^7 M^{-1} min^{-1} under the conditions of continuous *in vitro* evolution [13]. In a separate study, traditional stepwise evolution was used to develop an RNA-cleaving DNA enzyme — the 10–23 motif — that can be made to cleave almost any target RNA substrate with a catalytic efficiency of $\geq 10^7$ M^{-1} min^{-1} [16]. Here, as an exercise in molecular ecology, the DNA enzyme has been directed to cleave a population of continuously evolving ligase ribozymes (Figure 1). In order to avoid a trivial mechanism of escape, the DNA enzyme was designed to recognize and cleave the same site that the ribozyme uses to bind its oligonucleotide substrate. The population of evolving ribozymes was required to develop resistance to the DNA enzyme while maintaining ligase activity. The sequence of the DNA enzyme was kept constant while the ribozymes were free to evolve. The DNA enzyme should therefore be viewed as a toxicant rather than a predator of the ribozyme. The concentration of DNA enzyme was increased steadily over the course of evolution to maintain stringent selection pressure on the population of ribozymes.

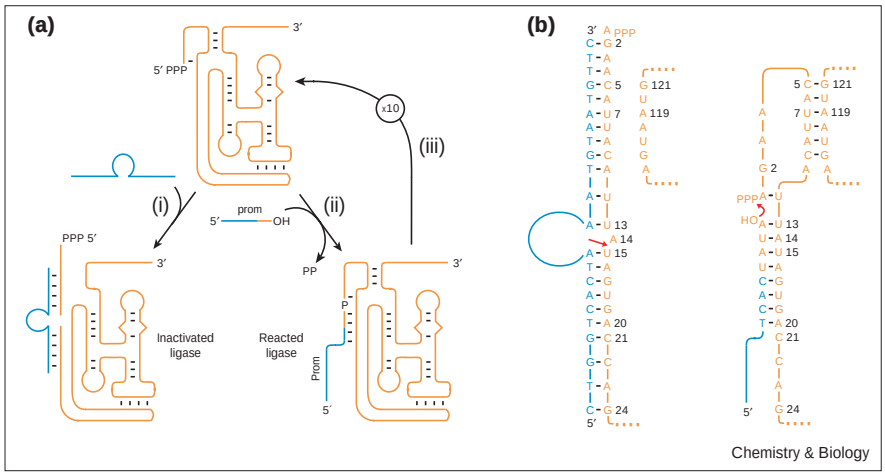
There are several possible mechanisms by which organisms evolve resistance to a toxicant or predator [17]. Two broad categories of resistance are those that are either pharmacodynamically or pharmacokinetically derived. The first involves a decrease in sensitivity to the toxicant. The second involves processes that reduce the effective concentration of the toxicant, such as decreased uptake, increased export, sequestration or detoxification. Both categories of evolved resistance can ultimately be traced to genetic changes that alter either the expression level or the functional properties of an enzyme, receptor or other functional macromolecule. Factors that influence the ability of a population to develop resistance include its genetic diversity, survival rate, replication rate, frequency of mutation and probability of generating favorable mutants. It is usually the case that initial exposure to a sub-lethal dose of toxicant provides greater opportunities for generating resistance than exposure to a larger dose that would greatly reduce the genetic diversity of the population.

Results

Evolution of resistant ribozymes

Continuous *in vitro* evolution in the presence of the DNA enzyme was initiated with randomized variants of a ligase ribozyme that had been obtained previously following 52 hours of continuous evolution [13]. The parent ribozyme, designated as 'ligase A' (Figure 2a), contained

Figure 1

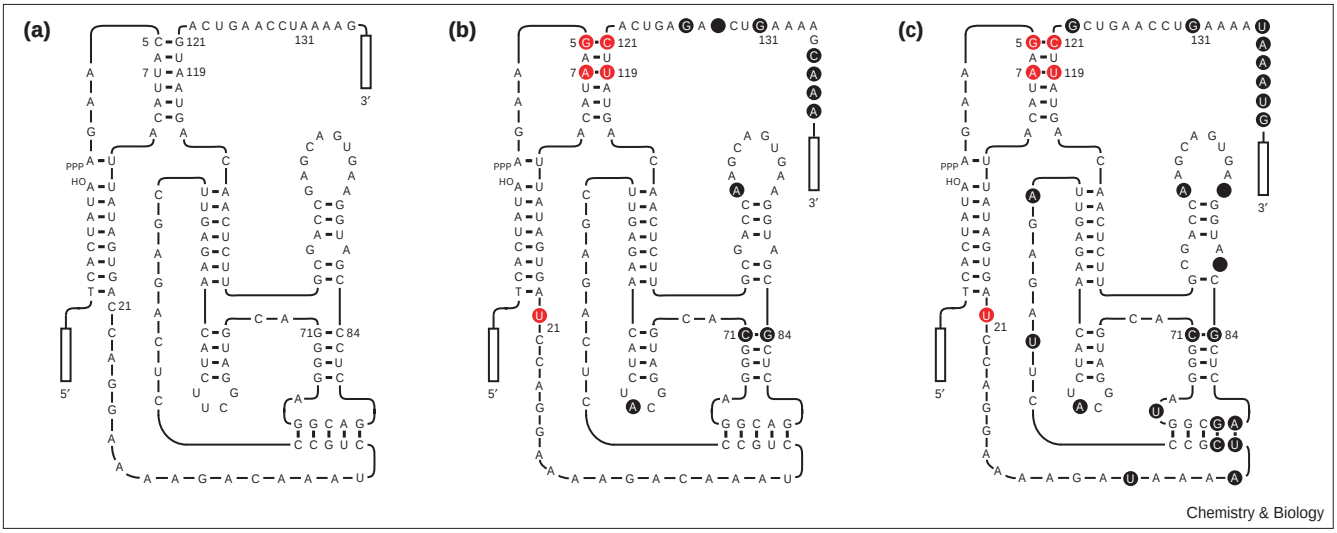


In vitro evolution of ligase ribozymes that are resistant to cleavage by the DNA enzyme. **(a)** Selective amplification of the ribozyme (orange line) requires that it escape the DNA enzyme (blue line) and react with the chimeric DNA–RNA substrate (blue–orange line). (i) The DNA enzyme cleaves and thereby inactivates the ribozyme; (ii) the ribozyme ligates the promoter-containing substrate to its own 5′ end; (iii) reacted ribozymes are reverse transcribed, then forward transcribed to produce multiple copies of RNA. **(b)** Base-pairing interactions involved in the binding of DNA enzyme and ribozyme (left) and the binding of ribozyme and oligonucleotide substrate (right). Straight red arrow indicates the site of cleavage by the DNA enzyme. Curved red arrow indicates the site of ligation. Numbered nucleotide positions are discussed in the text.

29 mutations relative to the class I ligase first described by Bartel and Szostak [14,15]. Ligase A was mutagenized at a frequency of 10% per nucleotide position using a hypermutagenic polymerase chain reaction (PCR) procedure [18]. This resulted in an average of 13 mutations per molecule, with the starting population of 10^{14} RNAs containing all possible one-, two-, three- and four-error mutants and a sampling of the higher-error mutants.

A DNA enzyme was constructed on the basis of the 10–23 catalytic motif [16] and directed to cleave ligase A (Figure 1b). The DNA enzyme contains a catalytic core of 15 nucleotides, flanked by substrate-recognition domains that bind the RNA substrate through Watson–Crick pairing. The 3′ and 5′ substrate-recognition domains were designed to be complementary to ribozyme nucleotides 2–13 and 15–24, respectively. Nucleotide 14 is an unpaired adenylate that immediately precedes the cleavage site. Nucleotides 12–20, which surround the cleavage site, are used by the ribozyme to bind its own oligonucleotide substrate. DNA-catalyzed cleavage of the ribozyme and RNA-catalyzed ligation leading to selective

Figure 2



Mutations that arose over the course of the evolution. **(a)** Ligase A, which was used to generate the starting population of ribozymes; **(b)** ligase B, which was resistant to the DNA enzyme; **(c)** ligase C, which was similar to ligase B but contained several additional mutations. Red circles indicate the five highly conserved mutations; black circles indicate other mutations relative to ligase A. Open rectangles correspond to the 5′ portion of the substrate and the fixed primer binding site at the 3′ end of the ribozyme (see the Materials and methods section).

Table 1

DNA-catalyzed cleavage of the ligase ribozymes.

	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\ min^{-1}$)
Starting DNA enzyme			
Ligase A	0.4	0.04	1.0×10^7
Ligase B	0.04	8.0	5.0×10^3
Mutated DNA enzyme			
Ligase B	0.2	0.02	1.0×10^7

See the Supplementary material for an autoradiogram depicting the cleavage reaction and for Michaelis–Menten saturation plots used to determine the kinetic parameters.

amplification were therefore designed to be mutually exclusive events (Figure 1a).

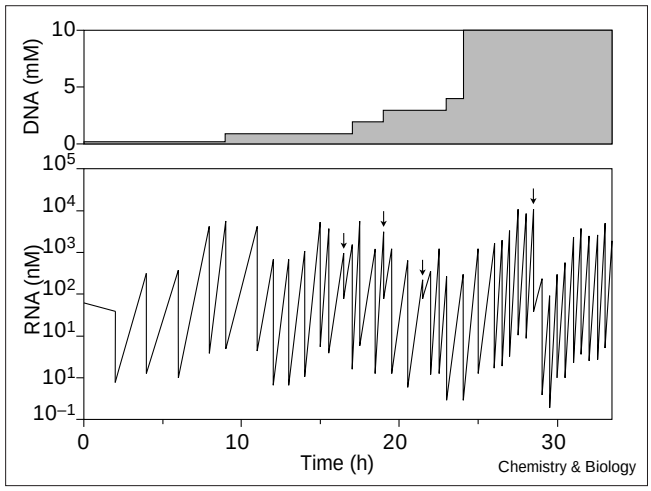
DNA-catalyzed cleavage of ligase A occurred with a k_{cat} of 0.4 min^{-1} and K_m of 40 nM, measured under multiple-turnover conditions similar to those employed during continuous *in vitro* evolution (Table 1). Preliminary experiments with the starting population of randomized variants of ligase A demonstrated that the population quickly fell to extinction in the presence of > 100 nM DNA enzyme. A starting population of 4 nM ribozymes was amplified by 150-fold over 30 min in the presence of 100 nM DNA enzyme, but by only sevenfold in the presence of 500 nM DNA enzyme and by only twofold in the presence of 1000 nM DNA enzyme. A starting concentration of 100 nM DNA enzyme was therefore chosen to initiate the continuous *in vitro* evolution process.

The course of evolution was monitored by measuring the concentration of RNA before and after each transfer event (Figure 3). The time between transfers was adjusted so that the ribozyme population increased from ~1 nM starting concentration to ~1 μM final concentration in each reaction mixture. Mutagenic PCR was performed several times during the evolution process to maintain genetic diversity in the population [19]. The concentration of DNA enzyme was increased steadily over the course of evolution, reaching 1 μM after 9 hours and 10 μM after 24 hours. Following 33.5 hours of continuous *in vitro* evolution, corresponding to 40 transfers and an overall dilution of 10^{107}, the population of ribozymes was able to amplify rapidly in the presence of 10 μM DNA enzyme.

Biochemical properties of the resistant ribozymes

After the 40th transfer, individuals were isolated from the population by a shotgun cloning procedure and their nucleotide sequence was determined. Among the 17 ligases that were sequenced, all contained eight specific point mutations compared with ligase A (Figure 2; Table 2). One individual, termed 'ligase B', contained these eight mutations and nine others. Another individual, termed 'ligase C', contained the eight conserved mutations,

Figure 3



Time course of continuous *in vitro* evolution. The saw-toothed line (bottom) depicts successive cycles of RNA amplification and dilution over the course of the serial-transfer procedure. Arrows indicate the times when mutagenic PCR was performed. The stepped graph (top) depicts the increasing concentration of DNA enzyme in the reaction mixture.

five of the other nine mutations found in ligase B, and 15 additional mutations. Three of the five conserved mutations (5:C→G, 7:U→A and 21:C→UC) occurred within the portion of the ribozyme that is recognized by the DNA enzyme, but just outside the region that binds the oligonucleotide substrate. The other two conserved mutations (121:G→C and 119:A→U) were compensatory changes that maintained Watson–Crick complementarity with the mutated residues at positions 5 and 7, respectively.

The catalytic efficiency, k_{cat}/K_m, for cleavage of ligase A by the DNA enzyme was $1 \times 10^7\ M^{-1}\ min^{-1}$, measured under multiple-turnover conditions. In contrast, DNA-catalyzed cleavage of ligase B occurred with an efficiency of only $5 \times 10^3\ M^{-1}\ min^{-1}$. This 2000-fold reduction was due to a tenfold decrease in k_{cat} and 200-fold increase in K_m (Table 1). The increase in K_m for DNA-catalyzed cleavage of ligase B compared with ligase A was thought to be due to the conserved mutations that occurred within the portion of ligase B recognized by the DNA enzyme. Two of these mutations, 5:C→G and 7:U→A, disrupt the 3′ substrate-recognition domain of the DNA enzyme by introducing unfavorable G–G and A–A mismatches, respectively. The other mutation, 21:C→UC, disrupts the 5′ substrate-recognition domain by inserting an unpaired uridine residue. In order to test the effect of these mutations, a new DNA enzyme was constructed that restored Watson–Crick complementarity between the DNA enzyme and ligase B. The new enzyme cleaved ligase B with a catalytic efficiency of $1 \times 10^7\ M^{-1}\ min^{-1}$, with a fivefold improvement in k_{cat} and 400-fold improvement in K_m compared with the original DNA enzyme (Table 1).

Table 2

Mutations occurring in the 17 resistant ribozymes that were sequenced.

Clone	62:U	78:C	100:G	104:G	122:A	126:A	128:C	134:A	135:G
1 (ligase C)	A		A		G				UAAAUG
4	A	U	U	–	G				UAAAG
5	A		A	U	G				AUAAA
6		U	A		G				UAAAG
8	A	U	A						UAAAG
9			A	A		–			GCAAA
12	A		A			AG	–		UAA
13	A		A		G				UAAAG
14			A			AG	–	–	GCAAAA
15			A			AG	–	–	GCAAAA
25			A		GA	AG	–	–	GCAAA
26	A	U	A		G			–	UAAAAG
31 (ligase B)	A		A			AG	–		GCAAA
32			A		G			–	CCUUAAAA
34			A	A		–		–	CCUGAAAAGCAAA
36	A		A	A					GCAAA
38		U	C		G		–		UAAAG

Columns refer to nucleotide positions at which a mutation occurred in at least five of the 17 clones. In addition, all clones contained: 5:C→G, 7:U→A, 21:C→UC, 71:G→C, 84:C→G, 119:A→U, 121:G→C and 131:A→GA. Additional mutations were: clone 1 32:C→U, 36:U→A, 37:C→U, 38:U→C, 44:C→U, 49:C→A, 75:A→AU, 79:A→G, 80:G→A, 86:G→–, 91:A→–; clone 5 86:G→A; clone 12 32:C→U, 36:U→A, 37:C→U, 38:U→C, 44:C→U, 49:C→A, 75:A→AU, 79:A→G, 80:G→A; clone 13 25:G→–,32:C→U, 36:U→–, 38:U→G, 44:C→U, 49:C→A, 75:A→AU, 79:A→G, 80:G→A; clone 14 133:A→–; clone 15 25:G→A, 133:A→–; clone 32 25:G→A, 94:U→C, 133:A→–; and clone 34 133:A→–. – indicates a nucleotide deletion.

Regarding the catalytic properties of the various ligase ribozymes, the starting ligase A and the two evolved ligases B and C all had a catalytic efficiency of about 1×10^7 M^{-1} min^{-1} (Table 3). Ligase C was somewhat more efficient because of a slightly lower k_{cat} but threefold improvement in K_m. This difference is likely to be due to one or more of the 15 mutations that were present in ligase C but not ligase B.

Continuous evolution of the resistant ribozymes

Although the kinetic analyses described above give insight into the molecular mechanism of evolved resistance to the DNA enzyme, the true measure of fitness is the amplification rate of the ribozymes in the context of continuous *in vitro* evolution. The amplification profile was determined for ligases A and B in either the presence or absence of 10 μM DNA enzyme (Figure 4). In each case, amplification was initiated by adding 1 nM ligase to the continuous *in vitro* evolution mixture. In the absence of the DNA enzyme both ligases amplified efficiently, with an exponential growth rate of 0.13 min^{-1}, corresponding to a doubling time of 5.3 min. The amplification profiles fit well to a logistic curve, reflecting exponential growth constrained by the finite resources of the reaction mixture. As the supply of oligonucleotide substrate (initially present at 2.5 μM) became depleted, the growth rate fell to sub-exponential levels and eventually leveled off, with the ribozyme attaining a final concentration of 5–10 μM after 60 min.

Amplification of ligase A was greatly reduced in the presence of the DNA enzyme, attaining a final concentration of only about 60 nM after 60 min. In contrast, ligase B was amplified almost as efficiently in the presence of the DNA enzyme as it was in the absence, reaching a concentration of about 6 μM. In both cases, the ribozyme approached a maximum concentration that was roughly equal to the K_m value for cleavage by the DNA enzyme, which was 40 nM for ligase A and 8 μM for ligase B (Table 1).

Discussion

Two mechanisms of resistance

The evolved population of ribozymes contained five highly conserved mutations that conferred resistance to the DNA enzyme by disrupting binding of the DNA enzyme to the ribozyme. Previous studies have shown that even a single base mismatch in one of the two substrate-recognition

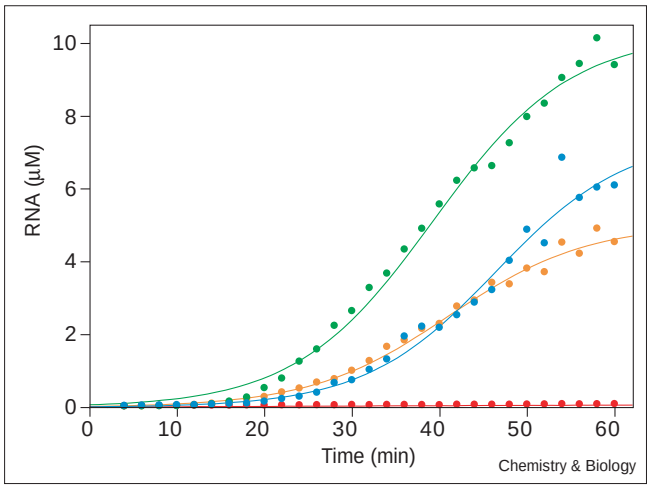
Table 3

RNA-catalyzed RNA ligation.

	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1})
Ligase A	21	1.7	1.2×10^7
Ligase B	14	1.3	1.1×10^7
Ligase C	8	0.5	1.6×10^7

See the Supplementary material for modified Eadie–Hofstee plots used to determine the kinetic parameters.

Figure 4



Amplification profiles for the starting and evolved ribozymes in either the absence or presence of 10 μM DNA enzyme. Orange line, ligase A without DNA enzyme; red line, ligase A with DNA enzyme; green line, ligase B without DNA enzyme; blue line, ligase B with DNA enzyme. The starting concentration of ribozyme was 1 nM. Aliquots were withdrawn at frequent intervals and the amount of ribozyme was determined by measuring the incorporation of [α-^{32}P]ATP (see the Materials and methods section). Data were fit, using the Levenberg–Marquardt algorithm, to the logistic function: [RNA] = $a/(1 + be^{-ct})$, where a is the maximum extent of amplification and c is the exponential growth rate. Curvilinear regression coefficients were in the range 0.971–0.997.

domains of the DNA enzyme can greatly reduce its catalytic efficiency [20]. This is especially true when the domains are short or when the mismatch is located close to the cleavage site. In the present study, a DNA enzyme was employed that contained 3′ and 5′ substrate-recognition domains of 12 and 10 nucleotides, respectively. These were sufficiently long that a single mismatch would not be expected to prevent DNA-catalyzed cleavage. Furthermore, the ligase ribozyme was not free to change the three nucleotides immediately upstream and six nucleotides immediately downstream from the cleavage site because these nucleotides were involved in recognition of its own oligonucleotide substrate. A selectively advantageous outcome was, therefore, the occurrence of multiple mutations at ribozyme positions just outside the region required for substrate recognition, but still within the region that is recognized by the DNA enzyme.

Two of the five highly conserved mutations were nucleotide substitutions in the portion of the ribozyme that is bound by the 3′ substrate-recognition domain of the DNA enzyme. These residues are involved in Watson–Crick base pairing with other nucleotides of the ribozyme, requiring compensatory mutation of their pairing partners in order to maintain ribozyme activity (Figure 2). The fifth highly conserved mutation was a nucleotide insertion immediately downstream from the portion of the ribozyme that binds the oligonucleotide substrate, but still within the region that is bound by the 5′ substrate-recognition domain of the DNA enzyme. As a result of the three disruptive and two compensatory mutations, the K_m for cleavage of the ribozyme by the DNA enzyme increased by 200-fold, whereas the catalytic efficiency of the ribozyme was unchanged. The ability of the DNA enzyme to cleave the resistant ligase could be fully restored by changing three nucleotides of the DNA enzyme to re-establish its complementarity to the ribozyme (Table 1).

The second strategy for evolved resistance, as exemplified by ligase C, involved a threefold improvement in the K_m value of the ribozyme for its oligonucleotide substrate (Table 2). The decrease in K_m was subtle, from 1.7 to 0.5 μM, but it is likely to have had a significant effect on the fitness of the ribozyme under the conditions of continuous *in vitro* evolution. The substrate was provided at an initial concentration of 2.5 μM, which would have been less than 60% saturating for the starting ribozyme but more than 80% saturating for ligase C. Any ribozyme molecule that bound an oligonucleotide substrate, whether or not it had reacted, would have been protected from cleavage by the DNA enzyme. The ribozyme must still ligate the bound substrate if it is to be selectively amplified, but it could have done so without the risk of first being inactivated by the DNA enzyme.

An extreme version of the substrate protection strategy would be an evolved ribozyme that had a K_m value of less than 1 nM. In that case, it would be fully saturated with the oligonucleotide substrate and not at all susceptible to cleavage by the DNA enzyme. The second-order rate constant for RNA ligation would, however, still be limited by the rate of helical nucleation between the ribozyme and complementary substrate, which is about 10^9 M^{-1} min^{-1} [21]. A K_m of < 1 nM would therefore be accompanied by a k_{cat} of < 1 min^{-1}. This would put the ribozyme at a selective disadvantage compared with molecules such as ligase A, which has a k_{cat} of > 10 min^{-1}. In order to have a high fitness value, a ribozyme must not only avoid inactivation by the DNA enzyme, but also compete favorably against other ribozymes in the population.

One can imagine other mechanisms of resistance that were not observed among the evolved ribozymes. For example, the ribozymes might have developed a decoy site for the DNA enzyme that sequestered the DNA within a nonproductive complex. However, this would not have been effective immediately following transfer of the population to a fresh reaction mixture when the concentration of DNA enzyme greatly exceeded the concentration of ribozyme. Another mechanism of resistance might have been the evolution of ribozymes that catalyzed inactivation of the DNA enzyme upon its encounter with the

ribozyme. RNA is capable of cleaving DNA in a sequence-specific manner [22,23] and other means of nucleic-acid-catalyzed inactivation of DNA are possible [24]. It would seem far more difficult, however, for the population to have developed a second catalytic activity than to discover the escape mutants that were observed.

Conflicting demands of function and resistance

When a substrate and a toxicant compete for the same active site of an enzyme, mutations that confer resistance must alter the binding site for the toxicant without disrupting formation of a productive enzyme–substrate complex. This requires either alteration of features of the active site that are not important for catalysis or remodeling of the active site so that it is more discriminating between the substrate and toxicant. The highly conserved mutations that arose in the present study are not likely to have substantially remodeled the active site of the ribozyme. Instead they appear to tease at the fringes of the active site in a way that prevents attack by the DNA enzyme but causes only a minor alteration of the structure of the enzyme. This alteration was offset by the two compensatory mutations and perhaps other less highly conserved mutations.

This study demonstrates how a functional RNA can develop resistance to the inhibitory effect of a second nucleic acid molecule. It has implications for attempts to inhibit the expression of RNA using antisense agents, aptamers or catalytic nucleic acids. If there is a strong selective advantage in escaping the inhibitory effect, for example, in the outgrowth of a cancer cell or pathogen, then it should be possible to acquire mutations that disrupt the interaction between the target RNA and inhibitory nucleic acid. Structured RNAs typically have a high degree of Watson–Crick complementarity, making it straightforward to discover compensatory mutations that preserve their overall structure. Targeting a region of RNA that has essential features of primary structure would make it more difficult for the RNA to evolve resistance.

Predator–prey dynamics

Predator–prey systems occur frequently in nature and generally result in one of three stable outcomes: extinction of the predator and continued survival of the prey; attainment of an equilibrium state in which the predator and prey have coevolved to express a constant phenotype; or continued evolutionary change of both the predator and prey resulting in a coupled and sustained oscillation [25]. The continuous *in vitro* evolution system employed in this study allowed evolution of the ribozyme, but not the DNA enzyme, and therefore did not exemplify predator–prey ecology. If the fixed DNA enzyme had been dependent on cleavage of the ribozyme for its survival, then it would have fallen to extinction. A mutant DNA enzyme could be constructed, however, that contained three nucleotide substitutions that fully restored the ability of the DNA to cleave the ribozyme (Table 1). This suggests that an evolving population of DNA enzymes would have been able to respond to the evolutionary changes of the ribozyme, extending a predator–prey dynamic to a new round of competition. In accordance with the Red Queen hypothesis [26], the evolving population of predators and prey might exhibit sustained oscillations of phenotype as a result of repeated cycles of mutation and counter-mutation.

Unlike natural evolving systems, most *in vitro* evolution systems do not allow spatial separation of individuals. In the present study, all of the ribozymes shared a common reaction environment, making it impossible for them to escape the DNA enzyme through dispersal [27]. There are other ways for the ribozymes to escape that take advantage of specific concentration regimes, however. The rate of association of ribozyme and DNA enzyme is about 100-fold slower than the rate of their diffusional encounter [20]. At very low concentrations of both molecules (< 1 nM each), the rate of duplex association would be very slow (< 1 min^{-1}). If the oligonucleotide substrate was present at much higher concentration (> 1 μM), then each ribozyme molecule would be far more likely to associate with a substrate molecule than with a DNA enzyme. Immediately after each transfer, the starting concentrations in the reaction mixture were: ~1 nM ribozyme, 0.1–10 μM DNA enzyme and 2.5 μM oligonucleotide substrate. Under these conditions, all association rates were fast and saturation behavior would have determined whether a given ribozyme molecule formed a complex with the DNA enzyme or the oligonucleotide substrate (see above).

During the first 13 transfers of continuous evolution, the concentration of DNA enzyme was ≤ 1 μM and the concentration of ribozyme rose from ~1 nM to ~1 μM in each reaction mixture. Once the concentration of ribozyme reached saturation for the DNA enzyme (K_m = 40 nM), any further accumulation of ribozyme would not result in additional DNA-catalyzed RNA cleavage events. The constant number of DNA enzymes would be operating at V_{max} and the additional ribozymes would not be cleaved prior to their reaction with the oligonucleotide substrate. Thus, even in a homogeneous reaction mixture, there can be refuge for the prey at both very low and very high concentrations of the predator.

Significance

An *in vitro* system for the continuous evolution of ribozymes has made it possible to follow the emergence of resistance to a toxicant in real time. The toxicant was a DNA enzyme that cleaved the ribozyme at its active site, thereby preventing the ribozyme from carrying out its function and undergoing subsequent selective

amplification. Over the course of 33.5 hours of evolution, the population of ribozymes developed resistance to the DNA enzyme while retaining their own catalytic activity. This was accomplished by mutations that disrupted binding of the DNA enzyme to the ribozyme and enhanced binding of the ribozyme to its substrate, as well as compensatory mutations that maintained the active structure of the ribozyme. As an example of *in vitro* molecular ecology, this study has relevance to predator–prey systems in nature. It also provides insight into the evolution of resistance at the level of a target RNA, as has been observed in the emergence of antibiotic-resistant bacteria.

Materials and methods

Materials

Synthetic oligodeoxynucleotides were obtained from Operon Technologies. The DNA enzyme employed during *in vitro* evolution had the sequence 5′-*CTGGTCACTA*GGCTAGCTACAACGA*AATGTAATGT-TC*-3′ (substrate-recognition domains italicized). The mutated DNA enzyme that cleaved the evolved ribozyme had the sequence 5′-*CTG-G**A**TCACTA*GGCTAGCTACAACGA*AATGTA**TT**C**TTC*-3′ (compensatory mutations shown in bold). The primer for cDNA synthesis was 5′-GCTGAGCCTGCGATTGG-3′ and the second primer used in PCR amplification was 5′-CTTGACGTCAGCCTGGA-3′. The substrate for the ribozyme was a chimeric DNA–RNA oligonucleotide having the sequence 5′-CTTGACGTCAGCCTGGAC*TAATACGACTCAC***UAU-A**-3′ (promoter sequence italicized; RNA residues shown in bold). It was synthesized on a Pharmacia LKB Gene Assembler Special using standard phosphoramidite chemistry. All oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis and desalted on a Sephadex NAP-25 column (Pharmacia).

T7 RNA polymerase was prepared from the cloned gene [28] and purified as described previously [29]. Superscript II RNase H- reverse transcriptase was obtained from Gibco-BRL, *Taq* DNA polymerase was from Stratagene, Sequenase 2.0 modified T7 DNA polymerase was from US Biochemical, T4 polynucleotide kinase was from New England Biolabs and calf intestine phosphatase was from Boehringer. Nucleoside 5′-triphosphates (NTPs) and deoxynucleotide 5′-triphosphates (dNTPs) were from Pharmacia, dideoxynucleoside 5′-triphosphates (ddNTPs) were from U.S. Biochemical and [γ-^{32}P]ATP and [α-^{32}P]ATP were from ICN Radiochemicals.

Construction of the initial pool

The initial pool of ribozymes was prepared by hypermutagenic PCR [18] starting with the cloned gene of ligase A, which was isolated in a previous study [13]. This mutagenesis procedure results in ~10% error rate per nucleotide position, with a substantial bias towards GC→AT transitions [18]. The mutagenized DNA was transcribed *in vitro* and the resulting RNA was purified using denaturing polyacrylamide gel electrophoresis and Sephadex chromatography. The extinction coefficient of the ribozyme was determined by hydrolyzing a precise aliquot under strongly alkaline conditions, neutralizing and comparing the absorbance of the resulting monomers to that of the polymer [30]. For ligase A the value of A_{260} was 2034 mM^{-1} cm^{-1}.

Continuous in vitro *evolution*

RNAs were selectively amplified in a 25 µl volume containing 2.5 µM substrate, 2 µM cDNA synthesis primer, 0.2 mM each dNTP, 2 mM each NTP, 0.2 µCi μl^{-1} [α-^{32}P]ATP, 8 U μl^{-1} Superscript II reverse transcriptase, 2.8 U μl^{-1} T7 RNA polymerase, 25 mM $MgCl_2$, 50 mM KCl, 5 mM dithiothreitol, 2 mM spermidine, 50 mM EPPS (pH 8.5) and 0.1–10 µM DNA enzyme, which was incubated at 37°C for 30–120 min. After each incubation, a small aliquot of the completed reaction mixture (typically 0.1%) was transferred to a fresh reaction mixture. Mutagenic PCR was performed after transfers 12, 16 and 19, resulting in a mutation frequency of 0.7% per nucleotide position [19]. Hypermutagenic PCR was performed after transfer 30.

Analysis of the amplification profile of individual ribozymes was performed in a 100 µl volume under the same conditions as above, employing 1 nM input RNA and either none or 10 µM DNA enzyme. The amount of ribozyme was determined by withdrawing 2 µl aliquots at various times, quenching the reaction with Na_2EDTA, separating the reaction products in a denaturing polyacrylamide gel and comparing the amount of unincorporated [α-^{32}P]ATP to the amount that had been incorporated into ribozyme.

Kinetic analysis of DNA-catalyzed RNA cleavage

Cleavage of the ribozyme by the starting DNA enzyme was performed under multiple-turnover conditions, employing nine different concentrations of ribozyme ranging from 1–500 nM and a concentration of DNA enzyme that always was at least tenfold lower than the concentration of ribozyme and at least fivefold lower than the K_m. The ribozyme was labeled by incorporation of [α-^{32}P]ATP during *in vitro* transcription and the DNA enzyme was unlabeled. The reaction mixture also contained 25 mM $MgCl_2$, 50 mM KCl, 5 mM dithiothreitol, 2 mM spermidine and 50 mM EPPS (pH 8.5), and was incubated at 37°C. Two independent measurements of k_{obs} were obtained for each concentration of ribozyme, on the basis of seven data points obtained over the first 10–15% of the reaction. Values for k_{cat} and K_m were determined from a standard Michaelis–Menten saturation plot.

Cleavage of ligase B by the starting DNA enzyme could not be measured under multiple-turnover conditions because the high value for K_m made it impossible to attain saturating concentrations of ribozyme. Instead, cleavage was measured under single-turnover, enzyme excess conditions, employing 4 nM [5′-^{32}P]-labeled ribozyme and 12 different concentrations of DNA enzyme ranging from 0.2–40 µM. The reaction conditions and analytic procedure were as described above.

Kinetic analysis of RNA-catalyzed RNA ligation

Ligation reactions were performed under single-turnover, enzyme excess conditions employing 5 nM [5′-^{32}P]-labeled oligonucleotide substrate and 12 different concentrations of unlabeled ribozyme ranging from 0.05–6 µM. The reaction mixture also contained 25 mM $MgCl_2$, 50 mM KCl, 5 mM dithiothreitol, 2 mM spermidine and 50 mM EPPS (pH 8.5), and was incubated at 37°C. Values for k_{obs} were determined for each concentration of ribozyme on the basis of seven data points obtained over the first three half-lives of the reaction. Values for k_{cat} and K_m were determined from the *y*-intercept and negative slope, respectively, of a modified Eadie–Hofstee plot of k_{obs} versus k_{obs}/[ribozyme].

Supplementary material

Supplementary material including an autoradiogram of the DNA-catalyzed cleavage reaction, and Michaelis–Menton saturation and Eadie–Hofstee plots used to calculate kinetic parameters for the DNA enzyme and ribozyme, respectively, is available at http://current-biology.com/supmat/supmatin.htm.

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