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Design and Evolution of Functional Nucleic Acids

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Design and Evolution of Functional Nucleic Acids

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May, 2003

For Harry, my mother and my father.

Dad, now I, too, have advanced graduate degrees.

Acknowledgements

I would like to thank Andy and all of the members of the Ellington lab, past and present. In particular, I'd like to thank Michael Robertson, who not only first helped show me how to do science in Stanley Miller's lab, but helped me learn molecular biology in Andy's lab. I would also like to thank Amos Yan, Jay "Buddy" Hesselberth, Scott Knudsen, Colin "Bunny" Cox, Jeff Tabor and Kristin Thompson for all of their help and fruitful scientific conversations. In addition, I'd like to thank Dr. Kenneth A. Johnson for his assistance with kinetics. Also, a special thanks to Stanley L. Miller for first teaching me how to do research in his lab and sparking my interest in origins research. Finally, I'd like to thank all of my friends in Texas, San Diego and New York, as well as my family, especially my mother Judy, father Walter, brother Alex, stepmother Gene, stepsisters Kate and Kristie (Matt and Emma, too) and stepfather Harvey, for all of their love and support over the years.

Design and Evolution of Functional Nucleic Acids

Publication No._____

Matthew Levy, Ph. D.

The University of Texas at Austin, 2003

Supervisor: Andrew D. Ellington

Functional nucleic acids provide insight into the 'RNA world,' the proposed period in Earth's history where RNA served both as catalyst and as the genetic material. Additionally, they are potentially valuable tools for biotechnical applications. Like catalytic RNA, DNA has proven capable of catalyzing chemical reactions. Using a novel ligation chemistry, we have employed *in vitro* selection to isolate a catalytic DNA molecule (deoxyribozyme) that is capable of forming an unnatural nucleotide linkage. In addition, we have used a combination of rational design and *in vitro* selection to construct effector-dependent deoxyribozymes that are allosterically activated by the small molecule ATP. These results further strengthen the idea that life arose from a nucleic acid based metabolism and also bode well for the design of more stable nucleic acid based analyte detection systems.

In addition to the design of novel DNA catalysts, we have been interested in the origin of the first simple self replicating systems. Simple replicators based on short oligonucleotides as well as peptides have been demonstrated. We have designed a cross-catalytic system based on a well characterized peptide-RNA aptamer interaction, in which the peptide serves as a template for the ligation of RNA aptamer half-molecules. Our results demonstrate that the peptide could specifically enhance the rate of RNA ligation, and suggest the possibility for increased diversity of early replicators.

We have also designed an autocatalytic system based on the fast and efficient RNA cleaving 10-23 deoxyribozyme. In this system, two complementary deoxyribozymes have been inactivated by circularization. The circular deoxyribozymes are capable of serving as substrates for the linear enzymes such that linearization results in a cascade of cleaving reactions that exhibits exponential growth. A selection scheme based on this reaction resulted in optimal sequence selection and demonstrates the first in vitro selection experiment conducted in the absence of proteins.

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Chapter 1: A Framework

THE MACHINERY OF THE RNA WORLD

While the foundations of the RNA world were first laid down by Orgel (1968), Crick (1968), Woese (1967) and White (1976), it wasn't until the discovery of catalytic RNA (Kruger et al., 1982; Guerrier-Takada et al., 1983) and a correspondence in Nature by Gilbert (1986) that the enzymatic capability of RNA was realized and the name "RNA world," the period where RNA served both as catalyst and genetic material, was coined. Since then, the discovery of other naturally occurring catalytic RNA molecules (reviewed in Doudna and Cech, 2002) and the development of *in vitro* selection techniques (reviewed in Pan, 1997; Wilson and Szostak, 1999) have truly demonstrated that RNA is not only a competent catalyst, but capable of a variety of reactions.

By far, it has been *in vitro* selections that have provided the complex machinery necessary for reconstructing an RNA world (**Figure 1-1**). For example ribozymes capable of acting as kinases (Lorsch and Szostak, 1994), capases (Huang and Yarus, 1997) and ligases (Bartel and Szostak, 1993; Hager and Szostak, 1997; Robertson and Ellington, 1999) provide a minimal set of tools that would be required for replication. While ribozymes capable of carbon-carbon bond formation (Tarasow et al., 1997; Seelig and Jaschke, 1999), Michael addition (Sengle et al., 2001), amide bond formation (Wiegand et al., 1997; Sun et al., 2002), sulfur and nitrogen alkylation (Wilson and Szostak, 1995; Wecker et al., 1996), nucleotide synthetase activity (Unrau and Bartel, 1998), and even

redox chemistry (personal communication, H.Suga) begin to provide the chemical infrastructure necessary for the development of an RNA based metabolism. Perhaps one of the most exciting findings has been that in addition to acting as a catalyst, RNA is also capable of serving as molecular switch (Tang and Breaker, 1997; Robertson and Ellington, 1999; Soukup and Breaker, 1999). This discovery provides the RNA world with a level of regulation and control that would be necessary for any complex RNA based metabolism. In addition, the more complex and essential replication function of nucleotide polymerization has also been demonstrated. Two different ribozymes have proven capable of nucleotide polymerization on an external template (Johnston et al., 2001; McGinness and Joyce, 2002). The most exciting of these is the RNA polymerase from the Bartel lab that is capable of general RNA-templated primer extension of up to 14 nucleotides (Johnston et al., 2001). Perhaps the pinnacle of the RNA world is the ribosome itself which now, more than ever, appears to be a ribozyme (reviewed in Moore and Steitz, 2003). But of course this magnificent invention was the beginning of the end for the RNA world.

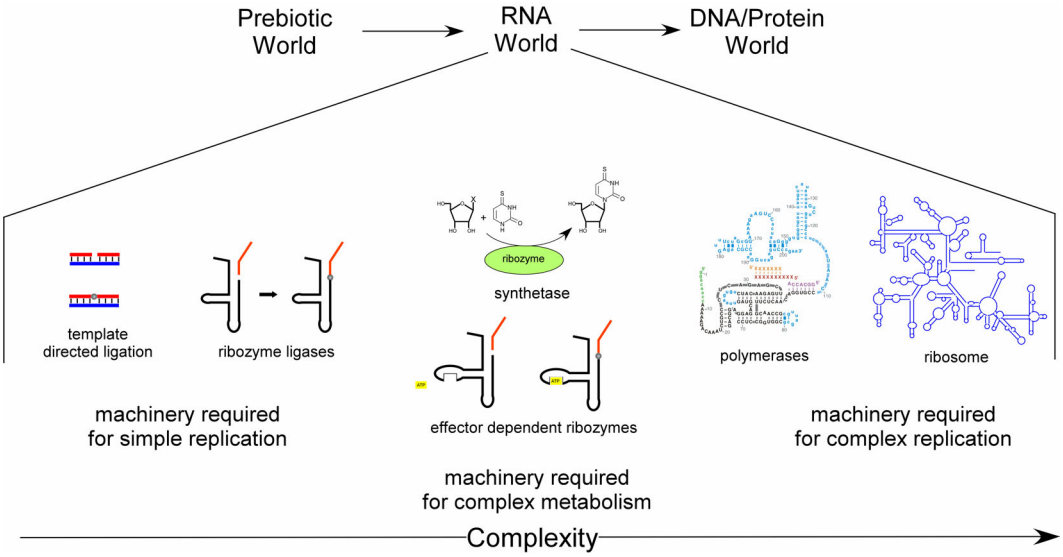

Figure 1-1. The machinery of the RNA world.

While the examples listed above are catalytic RNA molecules (ribozymes) a substantial amount of work has also been done with the discovery of catalytic DNA molecules (deoxyribozymes). Unlike ribozymes, deoxyribozymes have not been found in nature. This is perhaps not all that surprising considering that the role of DNA in biology confines it to a double stranded form. And while the lack of a 2'OH may make it less capable of forming complex tertiary structures (Cate et al., 1996) and limits its chemical repertoire when compared to RNA, single stranded DNA has proven more than capable of catalysis (reviewed in Emilsson and Breaker, 2002). A variety of catalytic functions paralleling those of catalytic RNA have been demonstrated. These include: RNA cleavage (for some examples see Breaker and Joyce, 1994; Geyer and Sen, 1997; Santoro and Joyce, 1997), DNA cleavage (Carmi et al., 1996), N-glycosylase activity (Sheppard et al.,

2000), porphyrin metalation (Li and Sen, 1996), kinase activity (Li and Breaker, 1999), capase activity (Li et al., 2000) and even ligase activity (Cuenoud and Szostak, 1995; Levy and Ellington, 2001b; Emilsson and Breaker, 2002).

This last activity was particularly intriguing to us both as origins researchers and as biotechnologists. New synthetic DNA reagents allowed for the incorporation of stable activating groups which facilitated the selection of a novel DNA ligase (Levy and Ellington, 2001b; Levy and Ellington, 2002b, Chapter 2). In addition, using strategies that have been effective for the design of allosteric ribozymes (Tang and Breaker, 1997; Robertson and Ellington, 1999; Soukup and Breaker, 1999), we were able to design an ATP-effector dependent allosteric DNA ligase (Levy and Ellington, 2002a, Chapter 3). These results coupled with recent results from the Sen lab illustrating an alternative mechanism by which DNA "molecular switches" can be designed (Wang and Sen, 2001; Wang et al., 2002) show that like RNA, DNA is capable of catalyzing chemical reactions, and acting as a regulatory unit, giving it the same added level of control that would be required for complex metabolisms. This is not to say that the notion of the "RNA world" should be replaced with one of a "DNA world", but simply to suggest that we should perhaps think more "loosely" when we consider the nature of the first genetic material.

EVOLUTION OF THE RNA WORLD

While **Figure 1-1** serves to present some of the catalytic functions of RNA in an attempt to illustrate the increasing level of complexity required for

development of the RNA world, a crucial problem still remains. How did things get started?

To begin to address this issue, we must first make two non-trivial assumptions: 1) all of the required components (e.g. nucleosides, amino acids) were prebiotically available, and 2) prebiotic polymerization mechanism existed to produce a variety of short oligonucleotides. Indeed a several of methods for prebiotic synthesis do exist (Miller and Orgel, 1972), and although there is no really good prebiotic condensing agent (Keefe and Miller, 1995), prebiotically plausible polymerizations have been demonstrated (Ferris et al., 1996; Ferris, 2002). In addition, we have not taken into account compartmentalization and the evolution of membranes. While this was undoubtedly an important event in the evolution of life and is currently being investigated by some of the leaders in the field (Szostak et al., 2001), including it in our evolutionary scheme only makes this discussion more complicated.

Once these details are out of the way, we can begin to speculate about how things got going. This is illustrated in **Figure 1-2**. While this view of molecular evolution may be as flawed as that of the original by Zallinger (**Figure 1-2**, top portion) it serves to help us focus on some of the evolutionary steps that might have been required (Levy and Ellington, 2001a).

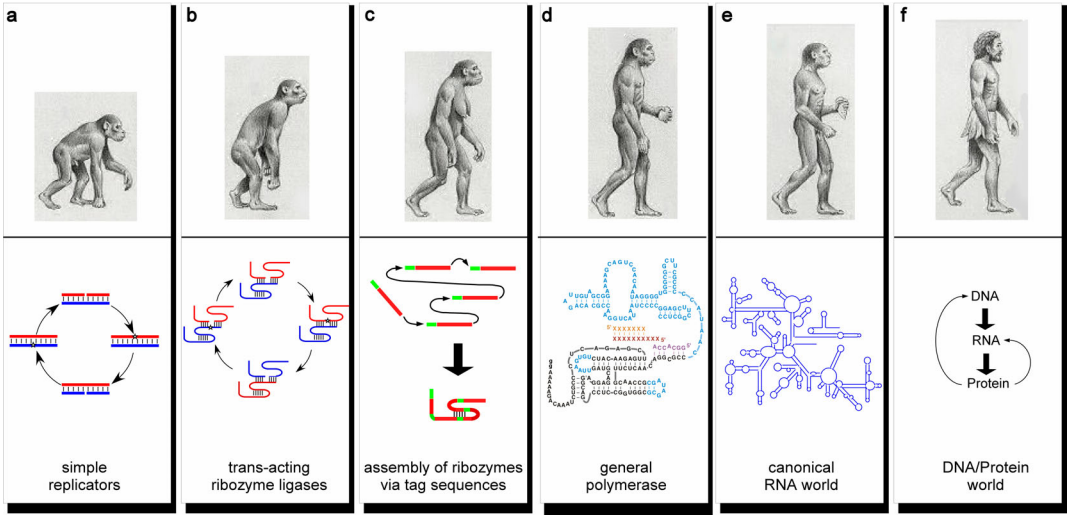

Figure 1-2. Evolution of the RNA World.

An attractive possibility for a starting point is that early simple replicators first evolved from systems not unlike those originally designed by von Kiedrowski (von Kiedrowski, 1986) and Orgel (Zielinski and Orgel, 1987), in which short oligonucleotides served as catalysts for the template-directed ligation of oligonucleotides (**Figure 1-2a**). However, alternate non-nucleic acids based replicators have also been demonstrated. For example, the Ghadiri lab has shown that short peptide sequences are capable of self replication (Lee et al., 1997; Severin et al., 1997). Indeed, these two systems are not necessarily exclusive. We have recently shown that peptides are capable of serving as templates in the directed ligation of oligonucleotides (Levy and Ellington, 2003b, Chapter 4) suggesting the possibility for interactions between these two types of replication systems. In addition, molecular replication is not just limited to biogenic polymers and has been extended to synthetic systems (Nowick et al., 1991; Bag

and Von Kiedrowski, 1999), although a connection from these replicators to the RNA world is not as obvious.

If indeed the original self-replicators utilized simple, template-directed oligonucleotide ligation systems like those of von Kiedrowski or Orgel, then it seems reasonable that the next function to be acquired by a nascent self-replicator may have been the ability to better catalyze self-assembly via ligation. This idea is rather attractive considering the discovery of numerous, short ribozyme ligases (for examples see Ekland et al., 1995; Robertson and Ellington, 1999; Rogers and Joyce, 2001). While *in vitro* selections are limited to the selection of cis-acting ribozymes (e.g. ribozymes that act on themselves) it has generally proven possible to re-engineer these molecules to function in trans (e.g. on other molecules). Therefore, it seems likely that the evolution of trans-acting ribozymes capable of assembling ribozymes and templates encoding ribozymes might be the next step in our progression (**Figure 1-2b**). In fact, Paul and Joyce (2002) have recently designed a self-replication system based on a small RNA ligase as a template for the synthesis of other functional ribozymes. Similarly, we (Levy and Ellington, 2003a, Chapter 5) have recently designed a replication system based on a catalytic nucleic acid. Our system differs from that of Paul in Joyce, and is based on a cleavage reaction rather than ligation

In the absence of larger starting materials, smaller oligonucleotide fragments might be assembled to produce a functional catalyst (**Figure 1-2c**). This idea has given rise to the notion that the first polymerase may have been an oligonucleotide polymerase as opposed to a canonical nucleotide polymerase

(James and Ellington, 1997; James and Ellington, 1999). The idea of an oligonucleotide polymerase is attractive for many reasons. Such a polymerase would have had to carry out fewer sequential reactions. While the best ribozyme polymerase can only make ~14 successive nucleotide additions (Johnston et al., 2001), the addition 14 oligonucleotides even as short as trimers would be sufficient to produce a viable ribozyme. For example, the minimal size for the hammerhead ribozyme is ~28 nucleotides long (Tuschl and Eckstein, 1993) and that of the 10-23 deoxyribozyme is ~32nt (Santoro and Joyce, 1998). Moreover, it is likely that any prebiotic nucleotides or their derivatives would have been present in a variety of chemical and stereochemical forms (Shapiro, 1984). This poses a problem for early replicators, because the addition of 'unnatural' nucleotides during strand elongation leads to 'enantiomeric poisoning' (Joyce et al., 1984). However, replicases that relied upon oligonucleotide ligation, would have been relatively impervious to enantiomeric poisoning as long as those nucleotides were not located at the ligation junctions and did not constrain templating function during additional rounds of replication (Ellington, 1993).

The assembly of functional catalysts may have been aided by the use of "tag" sequences which would have helped a given replicase identify itself and out-compete the evolution of parasites. Perhaps a simple example of such as tag can be seen in the ligation reaction catalyzed by the Group I intron, which has been shown capable of performing sequential template-directed ligation reactions (Doudna and Szostak, 1989; Green and Szostak, 1992). The 5' guanosine leaving group in this reaction can also be thought of as a "tag", as sequences without it are

not substrates (Been and Cech, 1988). We have recently selected for a DNA ligase that discriminates between substrates with common ligation junction and have suggested that such interaction may have also contributed to replicase self-recognition (Levy and Ellington, 2002b).

The evolution of ribozyme oligonucleotide polymerases would have allowed for the generation of larger products which would in tern allow for the evolution of more complex ribozymes (Sabeti et al., 1997). However, template-directed ligations would be limited in comparison to monomer polymerization (James and Ellington, 1999), and polymerases perhaps not too unlike the Bartel polymerase (Johnston et al., 2001) or others that have been selected (McGinness and Joyce, 2002; McGinness et al., 2002) may have evolved (**Figure 1-2d**). The smaller substrates used by these polymerases would not only allow access to untapped resources but also provided protection against the incorporation of errors during polymerization (James and Ellington, 1999) leading to the production of even larger more complex genomes.

Our "march to progress" ends with the emergence of the ribosome (**Figure 1-2-e**) leading to the rise of protein catalysis and the DNA/Protein world (**Figure 1-2f**).

RECONCILING ORIGINS RESEARCH WITH BIOTECHNOLOGY

In some respects, origins of life research is perhaps the ultimate project in molecular engineering. After all, we're trying to build life. But there is another side to all of this. That, of course, is the application of origins research to

biotechnology. This is perhaps most apparent in the case of allosteric ribozymes and deoxyribozymes which have shown the potential for being valuable diagnostic tools (Hesselberth et al., 2000; Breaker, 2002). For example, arrays of allosteric ribozymes based on both the hammerhead ribozyme (Seetharaman et al., 2001) and the L1 ribozyme ligase (Hesselberth et al., 2003), have demonstrated the potential for the detection of diverse metabolites ranging from ions to small molecules to proteins. The construction of such arrays might be facilitated by the recent demonstration of allosteric deoxyribozymes (Wang and Sen, 2001; Levy and Ellington, 2002a; Wang et al., 2002) which are more amenable to chemical synthesis and offer increased hydrolytic stability than RNA. Clearly, the principles of molecular recognition, regulation and catalysis lead to a large overlap between biotechnology and the requisite machinery for the construction of an RNA world. Even the work we have done with peptide templated RNA ligation (Levy and Ellington, 2003b, Chapter 4) is an aptazyme of sorts, albeit a poor one.

The detection of analytes with allosteric ribozymes has also been coupled with amplification systems such as RT-PCR (Robertson and Ellington, 1999), offering the potential for great increases in sensitivity. It's easy to see how the deoxyaptazyme that we have designed could be used in a similar fashion. However, what is perhaps more exciting is the potential of combining aptazyme technology with an all nucleic acid based (protein free) signal amplification system like the one we have designed with the 10-23 deoxyribozyme (Levy and Ellington, 2003a, Chapter 5).

Aptazymes have also found a role in a variety of other biotechnical applications, such as drug discovery (Hartig et al., 2002), and gene regulation (Atsumi et al., 2001; Thompson et al., 2002). It will be interesting to see how these applications develop over the coming years.

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Chapter 2: Selection of a DNA Ligase

INTRODUCTION

The selection of RNA ligase ribozymes has been facilitated by the fact that RNA transcripts naturally possess a 5'triphosphate which provides a "built-in" pyrophosphate leaving group (**Figure 2-1a**). Indeed, this chemistry is the same as that utilized by protein polymerases in biology. For example, the selection of the first ribozyme ligases by Bartel and Szostak isolated ribozymes that catalyzed the attack of the 3'-hydroxyl of an RNA substrate on the naturally occurring 5'triphosphate at the end of an RNA pool (Bartel and Szostak, 1993). In fact, the majority of ribozyme selections that catalyze an RNA ligation reaction have taken advantage of this built in pyrophosphate leaving group (Bartel and Szostak, 1993; Jaeger et al., 1999; Landweber and Pokrovskaya, 1999; Robertson and Ellington, 1999; Teramoto et al., 2000; Rogers and Joyce, 2001; Reader and Joyce, 2002). The only alternate chemistry employed for the selection of ribozyme ligases that catalyze the ligation of RNA was employed by Hager and Szostak who used an AMP activated RNA (Hager and Szostak, 1997) (**Figure 2-1b**). This type of activation mimics the natural substrate utilized by protein ligases.

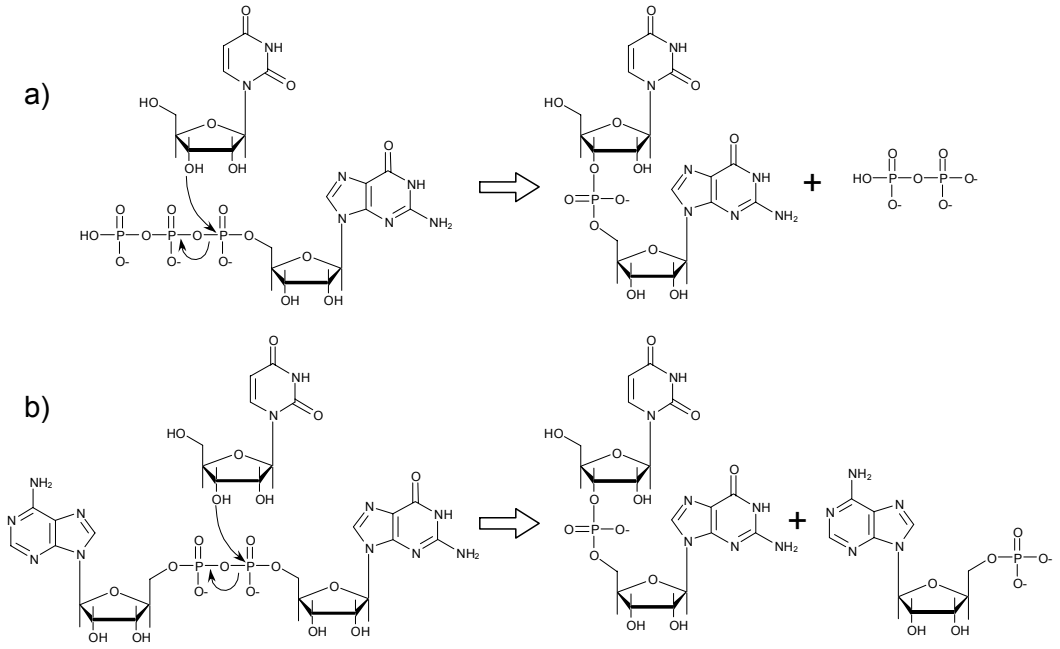


Figure 2-1. Chemistry employed for the selection ribozyme of ligases. (a) with pyrophosphate as the leaving group. (b) with AMP as the leaving group

Because DNA does not naturally bear a triphosphate, selections for DNA ligase deoxyribozymes require an additional activation step. Nonetheless, deoxyribozyme ligases have been selected. For example, the Breaker lab has reported the selection of a DNA ligase that utilizes an AMP capped substrate similar to that employed by Hager and Szostak (**Figure 2-1b**) (Emilsson and Breaker, 2002). A similar albeit unnatural, strategy was also used by Cuenoud and Szostak who selected for a DNA enzyme that catalyzed the ligation of its 5' hydroxyl to a target oligonucleotide bearing an activated 3'phosphorimidazolide (Cuenoud and Szostak, 1995) (**Figure 2-2**). More recently, a deoxyribozyme capable of RNA ligation has been selected by Flynn-Charlebois et al. This deoxyribozyme catalyzes the attack of a 5'-hydroxyl on an oligonucleotide

bearing a 2'3'-cyclic phosphate to produce a 2'-5' internucleotide linkage (Flynn-Charlebois et al., 2003).

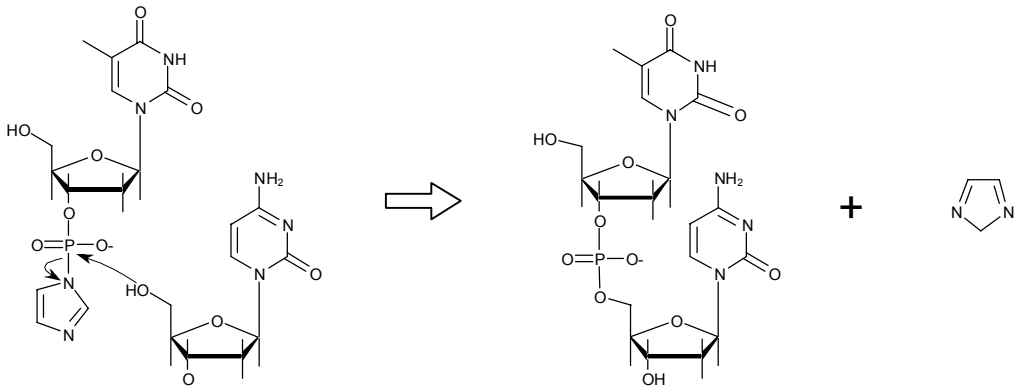


Figure 2-2. Chemistry employed for the selection of a deoxyribozyme ligase by Cuenoud and Szostak. Formation of the phosphorimazolide requires an additional step after the synthesis of the DNA.

The development of a novel ligation chemistry by Eric Kool and his co-workers (Xu and Kool, 1997) in which a 3' phosphorothioate displaces a 5' iodine forming an internucleotide phosphorothioester bond (**Figure 2-3**) prompted us to pursue the selection of a DNA ligase capable of catalyzing this reaction. This chemistry is attractive because it can be incorporated directly into oligonucleotides during chemical synthesis and therefore avoids the additional activation steps required for the generation of phopohorimazolides, AMP caps or 2'3'cyclic phosphates. While this sulfur-iodine chemistry has previously been shown to be efficient for the ligation and circularization of oligonucleotides (Xu and Kool, 1999), the reaction is relatively slow, typically requiring many hours to

accumulate appreciable ligation products. Using in vitro selection we hoped to enhance the overall speed of the reaction via the selection of a catalyst.

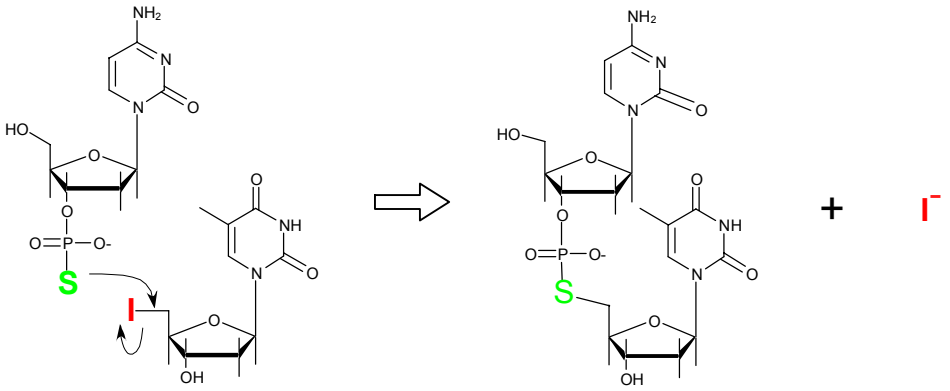


Figure 2-3. Phosphorothioate - Iodine chemistry employed in our selection of a deoxyribozyme ligase. The iodine leaving group is put on DNA during PCR simplifying the selection process.

As biotechnologists we, were interested in selecting a novel DNA ligase with prospects of using it for the design of biosensors (Hesselberth et al., 2000). To this end, we hoped that the selection would facilitate the isolation of a deoxyribozyme ligase that would be amenable to further engineering efforts such as the design of effector dependent ligases much like the L1 ligase ribozyme that was previously selected in our laboratory (Robertson and Ellington, 1999).

As origins researchers, we were compelled by the desire to try to bridge the gap between simple ligators and polymerases. To address this goal, we hoped to construct (via selection) a DNA ligase that we could subsequently deconstruct into component oligonucleotides such that the pieces could reassemble into the catalytic whole. In this way we hoped that the reassembled enzyme could further

facilitate oligonucleotide ligation potentially leading to the design of an autocatalytic replicase.

RESULTS AND DISCUSSION

Selection of deoxyribozyme ligases.

In order to select deoxyribozyme ligases capable of catalyzing the formation of a bridging 5' phosphorothioester internucleotide linkage, we employed a selection strategy similar to that originally employed by Bartel and Szostak (1993) and successfully adapted by our laboratory (Robertson and Ellington, 1999). As shown in **Figure 2-4**, the starting pool contained 90 random sequence positions flanked by two constant regions.

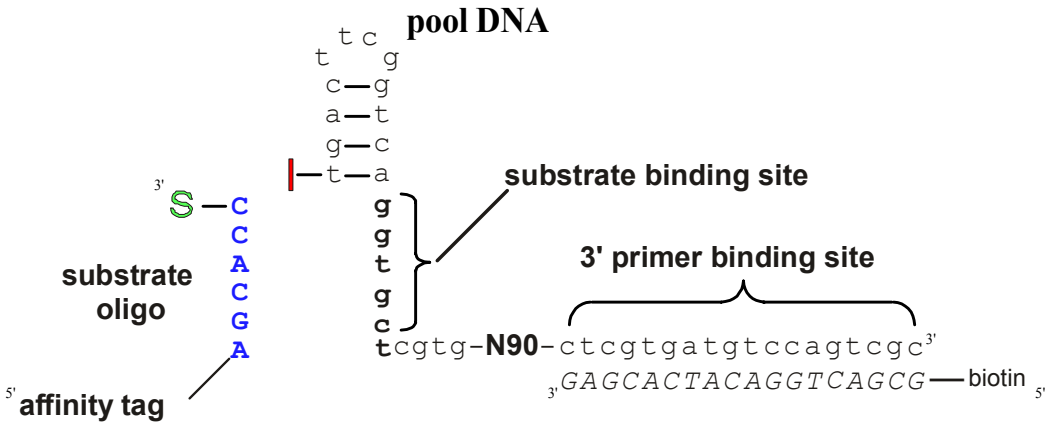


Figure 2-4. Design of the 5IN90-DNA pool for the selection of deoxyribozyme ligases. Constant regions are shown in lowercase with the designed substrate binding site in bold. The 5'-iodine is shown in red and the 3'-phosphorothioate shown in green. The hexanucleotide sequence common to all substrates used in the selection is shown in blue. The 3' primer bearing a 5'biotin used for pool immobilization and the generation of single stranded DNA is shown in upper case italics.

The 5' constant region was designed to form a hairpin and contained a region of 6 nucleotides that were complementary to the 3'end of the substrate.

The 5'I-dT was incorporated into the 5'primer and subsequently incorporated into the pool via PCR.

Deoxyribozyme ligases were iteratively selected by amplifying those variants that successfully joined a substrate oligonucleotide to themselves (Figure 2-5). The ligation products that accumulated upon incubation of substrates with the iodinated pool could be readily amplified via PCR.

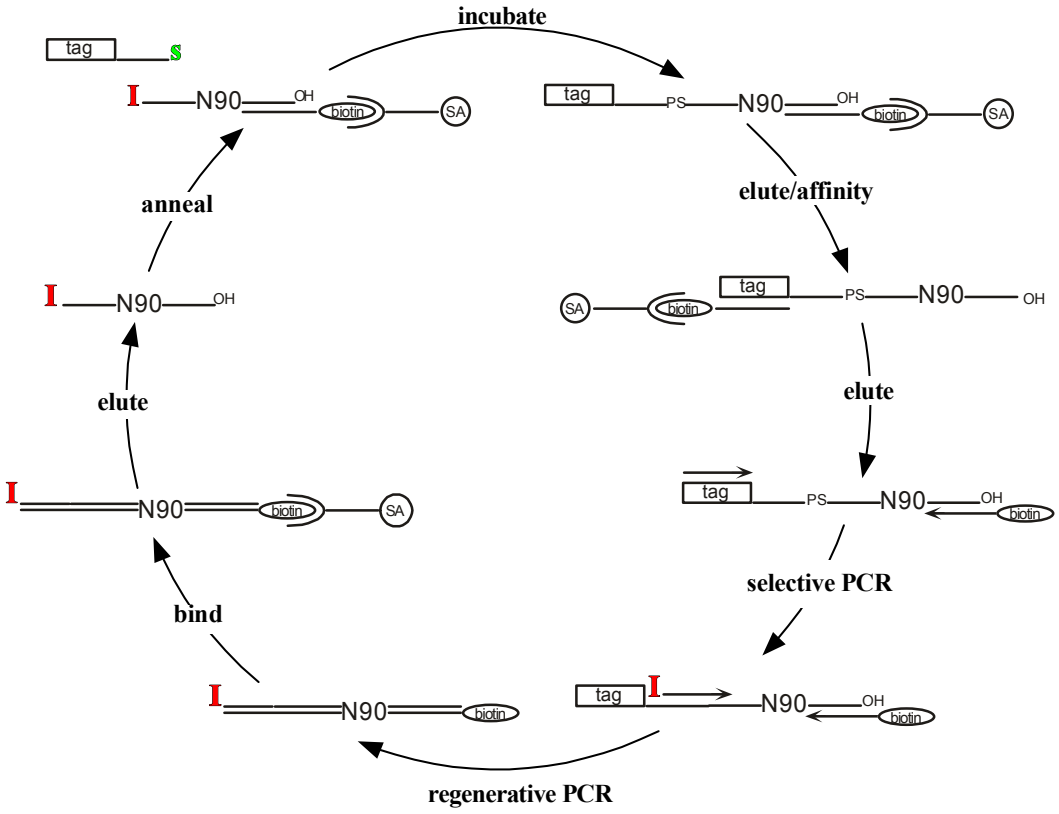


Figure 2-5. In vitro selection scheme used for the isolation of deoxyribozyme ligases. Details of each step are given in the Experimental Protocols.

Following the PCR amplification of successful variants, deoxyribozymes were regenerated via amplification with the 5'-iodinated 5'-primer and a 5'-biotinylated 3'-primer. Single stranded DNA was generated by immobilized streptavidin capture followed by alkali denaturation.

The overall stringency of the selection was progressively increased by decreasing the substrate concentration as well as the time allowed for ligation (**Figure 2-6a**). The efficiency of ligation was assayed at each round (**Figure 2-6b**).

a

Round	Pool Concentration (μM)	Substrate Concentration (μM)	Substrate	Time
1	1	1.5	1	1 hr
2	0.1	0.15	2	1 hr
3	0.1	0.15	1	15 min
4	0.1	0.15	2	5 min
5	0.1	0.15	1	1 min
6	0.1	0.15	1	1 min
7	0.1	0.15	2	15 s
8	0.1	0.15	3	1 min
9	0.1	0.15	6	1min
10	0.1	0.15	7	15 s
11	0.1	0.15	6	5s
12	0.1	0.01 w/ 10X competitor	2	5 s
13	0.1	0.01 w/ 10X competitor	6	5s

b

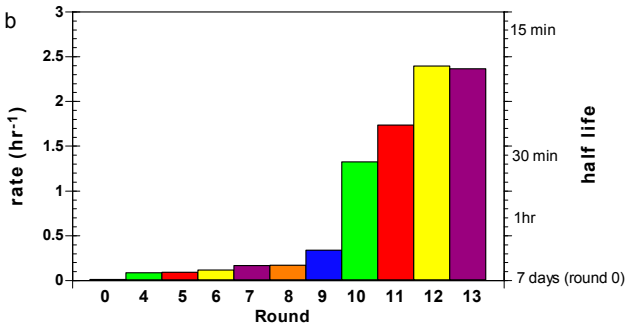


Figure 2-6. Progression of the deoxyribozyme selection. **(a)** Conditions used during the course of the selection. **(b)** Rate of ligation for each rounds of the selection.

To guard against the accumulation of ligases that developed extensive templating abilities and to encourage the selection of catalysts that could act on a variety of substrates, a total of 5 different substrates were employed during the course of the selection. All contained a 3' phosphorothioate and the terminal hexanucleotide sequence 5' TGCACC-S, but otherwise differed extensively in their sequences (**Figure 2-7**).

```
KSS1        AAAAAAAAAAAAAAAAAAAAAAGCACC-S
KSS2        TACATGTCTATCGATCTGACTAAGCACC-S
KSS3             GATCTAGTCGATCGTAGAGCACC-S
KSS6    GCATACGACAAACTGGACCTACTGAAGCACC-S
KSS7    TCTACGCTGATCTGTACTGAATGGCAGCACC-S
```

Figure 2-7. Substrates used for the selection of deoxyribozyme ligases. All substrates share the same 3'-prime hexanucleotide sequence (blue) but have different affinity tags (black). The phosphorothioate is indicated by the green "S".

After 13 rounds of selection, the selected pool showed no further improvement in catalysis (**Figure 2-6b**). The final rate of ligation for the pool was 2.4 hr-1 which is a 600-fold increase over the rate observed for the starting pool (0.004 hr-1). These rates are comparable to or slightly slower than those seen with other selected nucleic acid ligases, however, the overall rate enhancement for our ligase is much smaller because the nascent phosphorothioate nucleophile is much more reactive than a canonical hydroxyl moiety (Bartel and Szostak, 1993; Cuenoud and Szostak, 1995; Hager and Szostak, 1997; Robertson and Ellington, 1999). It is unlikely that the selection prohibited further catalytic improvement as the selection steps in the final rounds were carried out in approximately five seconds and would have strongly favored any faster ligases that were present.

Characterization of selected deoxyribozymes.

Deoxyribozymes from Rounds 10, 11, and 13 were cloned and sequenced. Most of the ligases could be grouped into a single major class (**Figure 2-8**) based on shared sequence motifs. A conserved octamer sequence (5' TGCTTTTT, blue) was found near the 3' end of the clones. This octamer was frequently accompanied by an adjacent pentamer sequence (5' GACGG, purple). The major

class of deoxyribozymes also possessed a second octamer motif nearer the 5' end (5' G[A/T]CAGGTT, red). This latter motif was partially complementary to the common hexanucleotide found in all of the substrates (5' TGCACC-S; complementary regions underlined). As in several previous selections for ligases (Ekland and Bartel, 1995; Ellington and Robertson, 1999; Robertson and Ellington, 1999), it appears as though the selected catalysts may not have utilized the substrate-binding site provided and instead created their own unique substrate binding site from random sequence.

Overall, the selection was dominated by two clones, C14 and C15, which first appeared in Round 10. While both clones appeared 3 times (including mutant variants) out of 13 clones in Round 10, C14 predominated in Round 11 (7 copies out of 12 clones), and C15 predominated in Round 13 (9 copies out of 15 clones, including mutational variants). Interestingly, C15 disappeared as a dominant clone in Round 11, and then re-emerged in Round 13, while C14 was present throughout (2 copies remain in Round 13). The rates of these clones were representative of the rate of the pool as a whole. The rates of ligation for clones C14 and C15 with substrate KSS2 were 1.9 hr^{-1} and 2.7 hr^{-1}, respectively.

ROUND 10

```
clone
15(3) ACGCGTACGAACGGAGCCGTGTCAGGTTTGCTCGCGAGTAATGTGATTCGCGTACGAACGAGATGCTTTTTTGACGGATCTTTGGCTGGTG
14(2) CATGAATCGAAGACAGGTTGCCGAGTCTCTCGTAGCGAGCAAGGATCGCTGCGATTGGGGCGAGGCTGCTTTTTGACGGCCAGATCGGTG
1     TATGAATCGAAGACAGGTCGCCGAGTCTCTCGTAGCGAGCAAGGATCGCTGCGATTGGGGCGAGGCTGCTTTTTGACGGCCAGATCGGTG
13    CAACGCAGAAAAGAAGTNCTGCAACCACAGTCAGGTTCCCCCATCCCGACCTACGGGTGAATTGGGGTGCTTTTTTTAGATCGTTGAC
19    AATCGTAGAGACATGTTTATTTTTGCTTTTGTGTGGGAGCTAGGTCAACTCTTGAGTTTTTCGCGCCCAGCGTGGATTGACGTGTTCC
2     GTCTACATTAGACGACAACCCTGGAGGTCGTTTACGCAAGCCGAATTAGACTTCATTATGTTTTTTTCTCAATGTATAGGATCGATATG
3     GTCGCATCGACTGTGTCCGAGTTCCGCACCAGAATGGTCGCATACATTAGCGGTGCTCGGATTTTTCTTATTTTTTGTCTGGGCAGGCCCGCC
12    GATTGGTCGACGCTTGTTTTGTCTTTTTACCCTATTGTTTCGAGTGCTATCGGACAGGCTCCCGTGTAGTTCCGATACGGCATCTATTGC
8     GAACCGGCTAGGTACCTTGGCTACCGTAGTTGGGCAGCCGGAGAACGTTTTTCTTTTTCGCCTTGGCGGATAGTTGTCAGATTGTAGC
7     TTCCGGTAGGGAGGATGACCTAACTGACGGCTCCGTCGTGTAGGCAACGTCACTCTGGTCTAACACTTTTTTTCGATAGATTCGTGGTCC
```

ROUND 11

```
                                                                                          rate(hr-1)
CLONE
[r10 clone C14] 15(7) CATGAATCGAAGACAGGTTGCCGAGTCTCTCGTAGCGAGCAAGGATCGCTGCGATTGGGGCGAGGCTGCTTTTTGACGGCCAGATCGGTG    1.9
[r10 clone C13] 7 (2) TAACGCAGAAAACGAAGTGCCTGCAACCACAGTCAGGTTCCCCCATCCCGACCTACGGGCGAATTGGGGTGCTTTTTTTAGATCGTTGAC    1.0
                1     GTCCCAGGAAGCGAAGTCAGGTTTGCCGGGCTTGGCAAGCGCTTAGCTTGCAGCACCCGGATGCTTTTTTTAGACGATTAGTACAACAGG    2.0
                3     TTCAGGTCGAAAACTCACGCAGTTTCGTATTTTATTTTTTGCATTTGTAATTTTTCTGCTGGCGATTGTCTGAGCGGGCTCTTGTTGTGCC    0.1
                9     GATCAGATCATGAATGACCAATTTTTTTTTTGCGTTACGGCGGCCTCGTAATGCGAGATCGGGACGCCCACAGGGACAGGCTTGTTCAGC    0.1
```

ROUND 13

```
                clone
r10 clone C15   1     ACGCGCACGAACGGAGCCGTGTCAGGTTTGCTCGTGAGTAGTATGATTCGCGTACGAACGAGATGCTTTTTTGACGGATCTTTGGCTGGTG
variants        2     ACGCGCACGAACGGAGCCGTGTCAGGTTTGCTCGCGAGTAGTATGATTCGCGTACGAGCGAGATGCTTTTTTGACGGATCTTTGGCTGGTG
                9     ACGCGCACGAACGGGGCCGTGTCAGGTTTGCTCGTGAGTAGTATGATTCGCGTACGAACGAGATGCTTTTTTGACGGATCTTTGGCTGGTG
                11(2) ACGCGCAGCAACGGAGCCGTGTCAGGTTTGCTCGCGAGTAGTGTGATTCGCGTACGAACGAGATGCTTTTTTGACGGATCTTTGGCTGGTG
                13    ACGCGCACGAACGGAGCCGTGTCAGGTTTGCTCGCGAGTAGTGTGATTCGCGTACGAACGAGATGCTTTTTTGACGGATCTTTGGCTGGTG
                16(2) TCGCGCACGAACGGAGCCGTGTCAGGTTTGCTCGCGAGTAGTATGATTCACGTACGAACGAGATGCTTTTTTGACGGATCTTTGGCTGGTG
                20    ACGCGCAGCAACGGAGCCGTGTCAGGTTTGCTCGCGAGTAGTATGATTCGCGTACGAGCGAGATGCTTTTTTGACGGATCTTTGGCTGGTG
                3     TGCAACCCAGCACTGCAGTCCAAGTCAGGTTTGCTCGCGAGTAGTATGATTTCGCGTACGAGCGAGATGCTTTTTTTTGTACAGATGTTGACGT
                7     TGCAACCCAGCACTGCAGTCCAAGTCAGGTTTACTTGCCGTGAGCCTGGCTCCCGCAGTTGCTTAG----TTTTTTTGGTACAGATGTTGACGT
                4 (2) TTTCGTACGCGTTAATCGCCGATTAGTCAGGTTTTCCGCAACGTGCGTGCGGATGCTTTTTTCAGGTCCGGTAGTTGGCGCTCCACTTTC
                8 (2) CATGAATCGAAGACAGGTTGCCGAGTCTCTCGTAGCGAGCAAGGATCGCTGCGATTGGGGCGAGGCTGCTTTTTGACGGCCAGATCGGTG
```

Figure 2-8. Selected random region sequences from Rounds 10, 11, and 13. The number of times a clone appeared is shown in parentheses. The 3' conserved motif is shown in blue, while an accompanying, conserved pentamer is shown in purple. The 5' conserved motif is shown in red. The rates for individual clones from Round 11 are shown at right.

Doped reselection of dominant clones

The partial randomization and re-selection of selected ribozymes has previously been used to identify and verify secondary structures (Ekland and Bartel, 1995; Robertson and Ellington, 1999). In addition, this type of re-selection often potentiates the discovery of more catalytically active variants (Ekland and Bartel, 1995; Robertson and Ellington, 1999). We therefore carried out a doped sequence selection on the two dominant clones from the selection. Both C14 and C15 (from Round 10) were re-synthesized so that each position within the original 90 nucleotide random region contained 70% wild-type residues and 30% non-wild-type residues. For example, the first position of the 5' octamer motif in C14 contained 70% G, 10% A, 10% T, and 10% C. Each population was kept separate during the re-selection. The entire process was carried out in manner similar to the original selection and is described in detail in the Experimental Protocols.

The initial population of synthesized variants showed minimal catalytic activity (<0.01 hr^{-1}). However, after 5 rounds of selection and amplification with substrate KSS6 (the last substrate used during the original selection) both populations had returned to near wild-type rates (~1 hr^{-1}). An additional round of selection showed no further catalytic improvement and individual variants were cloned and sequenced from Round 6 (**Figure 2-9**). Sequence analysis indicated that a relatively small number of residues were absolutely conserved relative to the wild-type enzyme during the course of the re-selection. The most conserved residues were localized in the 5'octamer motif (boxed) previously identified by

sequence analysis. For the C14 variants, six of the eight residues in this motif appeared to be essential for deoxyribozyme function (G[A/T]CAGGTT; conserved residues bolded), while in C15, three of the eight residues were essential (G[A/T]CAGGTT).

Figure 2-9. Sequences of reselected variants of C14 and C15. Residues conserved in more than 75% of re-selected clones are shown in red; those conserved in more than 90% of reselected clones are shown in boxes.

Identification of the substrate binding site

To determine whether this 5' conserved motif played a role in substrate binding, we performed a site-directed co-variation analysis on both the designed and the putative evolved binding sites for both C14 and C15 (**Figure 2-10**). For C14 the results were unequivocal. The G:C pairing in the evolved substrate binding site could be exchanged for a C:G pairing , although the rate of ligation dropped to ~30% (**Figure 2-10a**, evolved). When the same exchange was attempted with the original designed substrate-binding site, there was no recovery of activity (**Figure 2-10a**, designed). For C15, the mutation of the substrate led to a significant loss of activity regardless of whether compensatory changes were introduced into the evolved or designed substrate binding sites (**Figure 2-10b**), suggesting that the 3' cytidine of the substrate is important for activity or that this deoxyriboyzme assumes a different active structure.

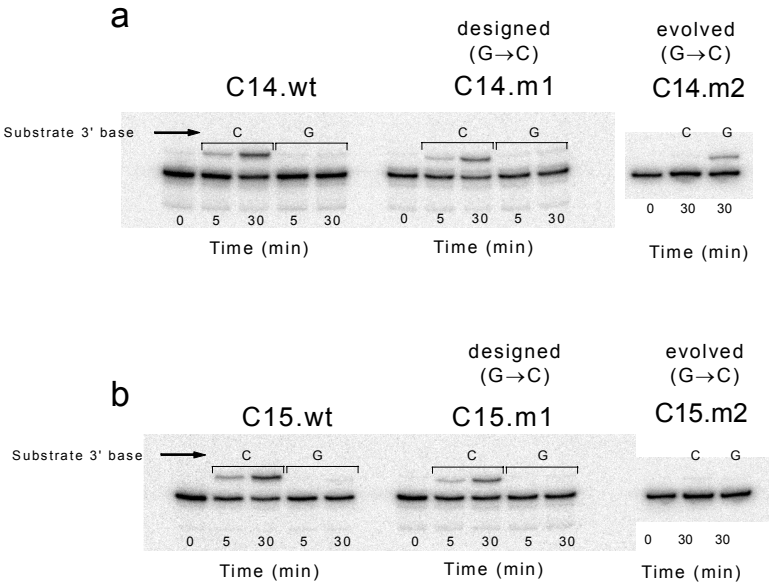


Figure 2-10. Identification of the substrate binding site. **(a)** Analysis of C14. The wild type construct C14.wt is sensitive to the C to G mutation at the 3' end of the substrate. Mutant C14.m1 which contains a G to C mutation in the 5' portion of the designed primer binding site is also sensitive to the substrate mutation. C14.m2 contains a G to C mutation in the hypothesized internal binding site and reacts only with the mutant substrate indicating that these two positions pair with one-another. **(b)** Analysis of clone C15. Mutation of the substrate led to a significant loss of activity regardless of whether compensatory changes were introduced into the evolved or designed substrate binding sites.

Deoxyribozymes C14, C15, and their re-selected variants from the doped re-selection were folded into secondary structural models using the program RNAStructure 3.5 (SantaLucia, 1998; Mathews et al., 1999). While multiple secondary structures were possible when the propsed internal substrate-binding site was fixed, clones C14, C15, and their re-selected variants predominantly adopted a single, similar secondary structure (**Figure 2-11**). In this structure, the 5' constant region folded against the random region to form a stem structure

(Stem A) which apparently aligned the 3' ends of oligonucleotide substrates for ligation. A second stem structure (Stem B) contained a number of residues that were conserved upon re-selection and were apparently important for function, but did not participate in substrate templating. Finally, the 3' portion of the random sequence region could participate in base-pairing with the 5' end of substrate KSS6, forming a third stem (Stem C).

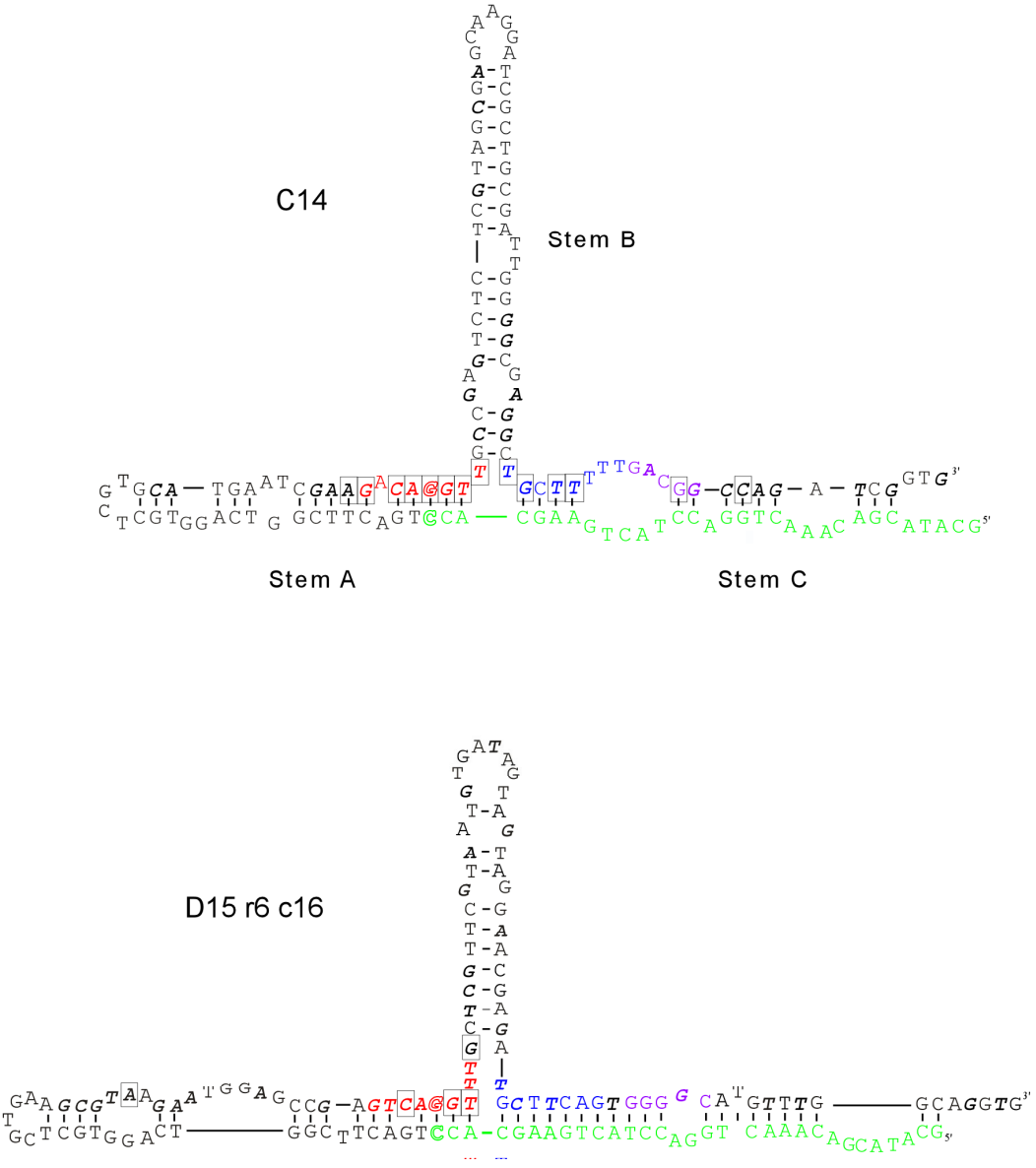
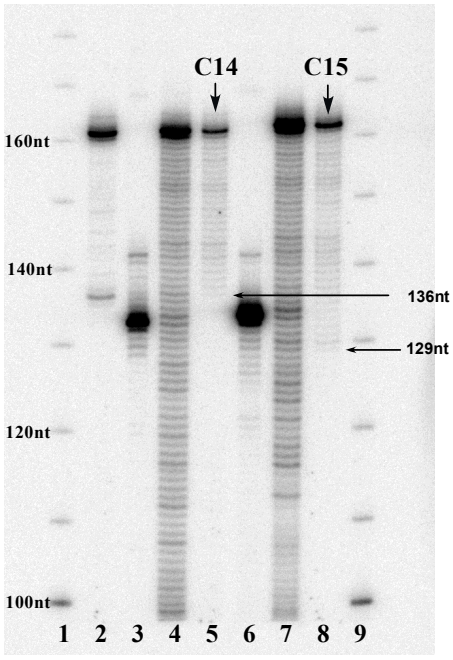


Figure 2-11. Putative secondary structures for C14 and a variant of C15. Substrate 6 is shown in green. Residues conserved in more than 75% of reselected clones are shown in bold; those conserved in more than 90% of re-selected clones are shown in boxes. All other colors are as in Figure 2-8. D15r6c16 is a variant of C15 from the doped selection. It shows extensive base-pairing with the oligonucleotide substrate as observed for many variants during the reselection. Conserved residues in D15r6c16 are the same as those found in the parental clone (C15). The outlined G and C residues were involved in site-directed mutagenesis experiments to identify the evolved substrate binding site.

3' End mapping analysis

In order to determine the minimal length for selected deoxyribozymes and further assess the role of the 3' end of the random region, a 3' deletion analysis was performed on the dominant clones C14 and C15. A 3' deletion ladder was generated by reverse transcription. To do this, clones C14 and C15 were first amplified with PCR primers that attached a T7 promoter to the 3' end of the clone such that transcription of the double stranded PCR product produced the RNA complement of each clone. After purification, the RNA complements were partially alkaline hydrolyzed producing a hydrolysis ladder of RNA. This was subsequently converted to DNA by reverse transcription using the 5'-iodinated primer. Following extension of the primer, the RNA was removed by treatment with RNAse and the series of deletion mutants allowed to react with 5'^{32}P-labeled substrate KSS2. Samples were then analyzed by denaturing gel electrophoresis (**Figure 2-12**).

In the case of C14, the smallest ligated product appears to be ~136nt long, which is 27 nucleotides shorter than the full length ligated product (158nt). For C15, the smallest ligated products appeas to be ~129nt long, which is 33nt shorter than the full length ligated product (160nt) (**Figure 12**; arrows). It therefore appears that for both clones, the 3' conserved region (**Figure 2-8**; blue and purple) appears to be important for efficient substrate ligation.



lane 1: 10bp ladder
lane 2: full length r10c14 ligation product
lane 3: clone r10c14
lane 4: digestion ladder
lane 5: r10c14 digestion ligation reaction
lane 6: clone r10c15
lane 7: digestion ladder
lane 8: r10c15 digestion ligation reaction
lane 9: 10bp ladder

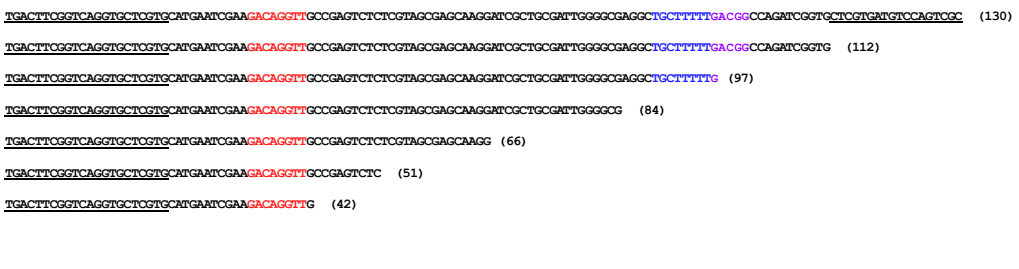
c14
TGACTTCGGTCAGGTGCTCGTGCATGAATCGAAGACAGGTTGCCGAGTCTCTCGTAGCGAGCAAG
GATCGCTGCGATTGGGGCGAGGCTTGCTTTTTTGACGGCCAGATCGGTGCTCGTGATGTCCAGTCGC

c15
TGACTTCGGTCAGGTGCTCGTGACGCGTACGAACGGAGCCGTGTCAGGTTTGCTCGCGAGTAATG
TGATTCGCGTACGAACGAGATGCTTTTTTTGACGGATCTTTGGCTGGTGCTCGTGATGTCCAGTCGC

Figure 2-12. 3' Deletion analysis of clones C14 and C15. Deletion mutants were reacted with radiolabeled substrate KSS2. (top) Contents of each lane are as indicated. (bottom) Arrows indicate the length of the minimal construct for each clone. Colors are as indicated in Figure 2-8.

In order to test the role of the 3' end further, six 3'deletion mutants of clone C14 were generated via PCR and biotin capture, and the rate of substrate ligation was measured. As can be seen in **Figure 2-13**, the full length (130nt) and

112nt constructs reacted at approximately the same rates. However truncation to 97nt (and into the 3' conserved region) resulted in a substantial loss of activity supporting the importance the 3' end.



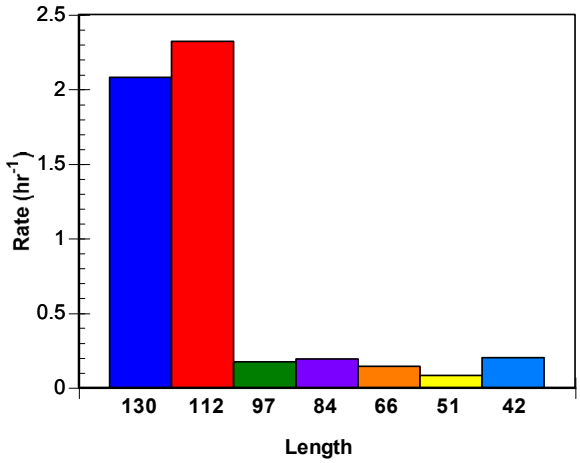
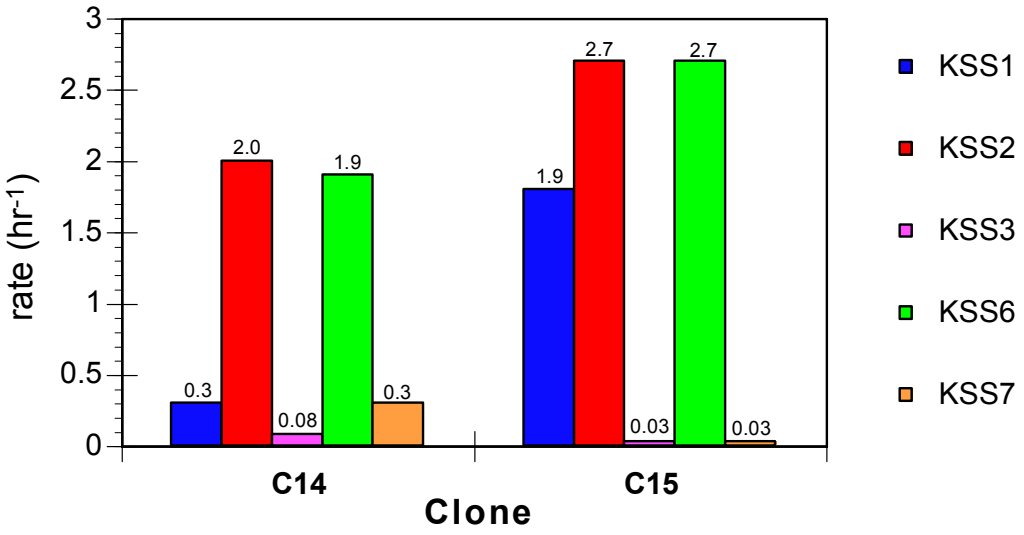


Figure 2-13. Rate analysis of C14 3'-deletion mutants. The sequences of the mutants are as shown, Colors are as in **Figure 2-8**. Consistent with the results shown in Figure 2-12, the 3' conserved region (purple) appears to be required for full activity.

Substrate specificity

Sequence analysis from rounds 10, 11, and 13 showed variation in the presence of dominant clones C14 and C15. The most likely explanation for this change in representation was that the different deoxyribozymes favored different oligonucleotide substrates and, thus, were disproportionately represented in the population as the substrates changed between rounds of selection. To assess this,

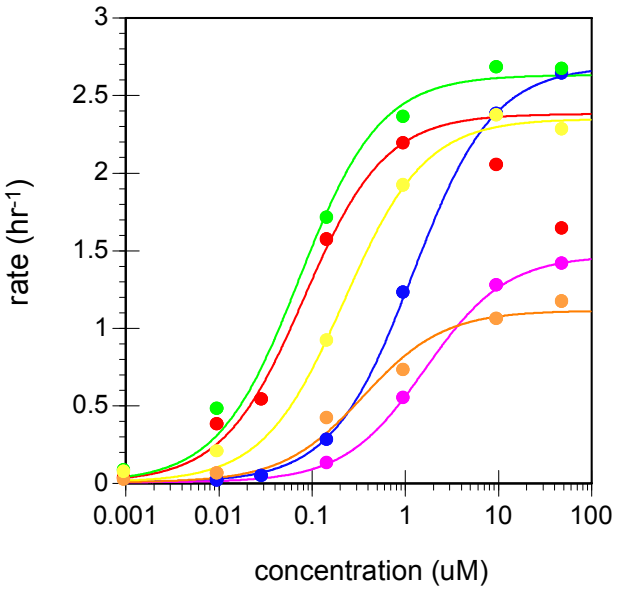
we assayed C14 and C15 with each of the substrates used during the course of the selection. Catalysis over background rates was observed with each substrate, albeit to different extents (**Figure 2-14**). Both C14 and C15 preferred substrates KSS2 and KSS6 and reacted poorest with KSS3 and KSS7 (although these latter substrates were still utilized at rates > 10-fold above background). C14 appeared to be more of a 'generalist,' in that it could utilize KSS3 and KSS7 better than C15 could while C15 was somewhat faster than C14 with the preferred substrates KSS2 and KSS6 as well as KSS1.




```
KSS1        AAAAAAAAAAAAAAAAAAAAAAAGCACC-S
KSS2        TACATGTCTATCGATCTGACTAAGCACC-S
KSS3            GATCTAGTCGATCGTAGAGCACC-S
KSS6    GCATACGACAAACTGGACCTACTGAAGCACC-S
KSS7    TCTACGCTGATCTGTACTGAATGGCAGCACC-S
```

Figure 2-14. Substrate specificities of C14 and C15. The rates of the reaction were calculated as described in Experimental Protocols and are given above each bar on the graph. The subtrates used for each assay are as indicated in the figure. The sequences of each substrate are given below the figure. The common hexanucleotide portion of each substrate is shown in blue, and the phosphorothioate indicated by a green "S".

In order to better characterize the underlying kinetic differences between the substrates, the rate of reaction of C14 was determined as a function of substrate concentration (**Figure 2-15**). Assuming that the rate of reaction (k_{chem}) is slow relative to the equilibration of the oligonucleotide substrates with the deoxyribozyme then it should be possible to determine the relative affinities of the deoxyribozymes for the different substrates. As anticipated, the 'good' substrates KSS2 and KSS6 had a higher apparent affinity for C14 than did the other substrates. Interestingly, the catalytic rates also appeared to be sensitive (within a factor of two) to the sequence and / or structure of the substrate:deoxyribozyme complexes.



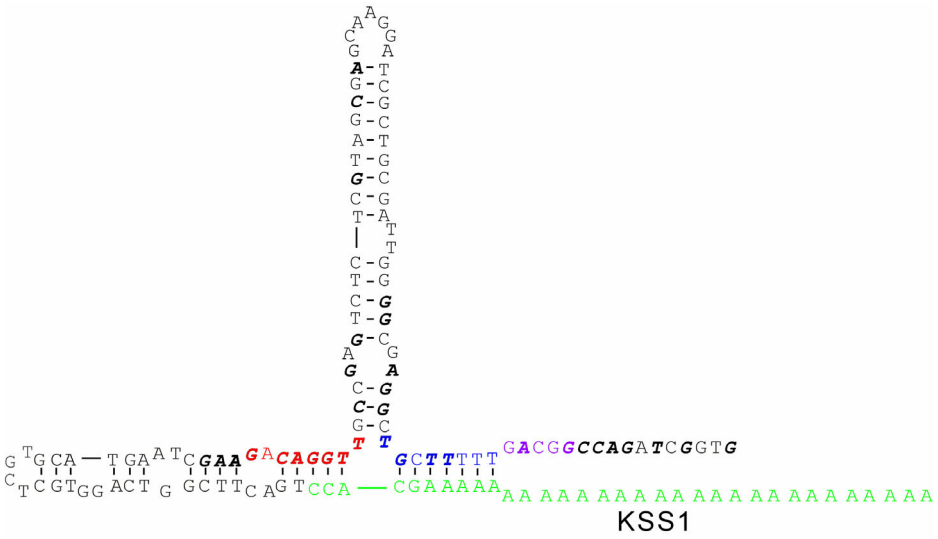
substrate	K_d^{app} (μM)	k_{chem} (hr^{-1})
KSS1	1.2	2.7
KSS2	0.08	2.4
KSS3	1.7	1.5
KSS6	0.07	2.6
KSS7	0.36	1.1
KSS7.m1	0.23	2.3

Figure 2-15. Apparent Kd of C14 for oligonucleotide substrates. Substrate sequences are as shown in Figure 2-14 and in Experimental Protocols. K_d^{app} and k_{chem} were calculated from the equation; $k_{obs}=k_{chem}[S]/(K_d^{app} + [S])$, where [S] is the substrate concentration. Substrate KSS2 inhibits the deoxyribozyme at high concentrations and values were therefore derived from lower concentration points.

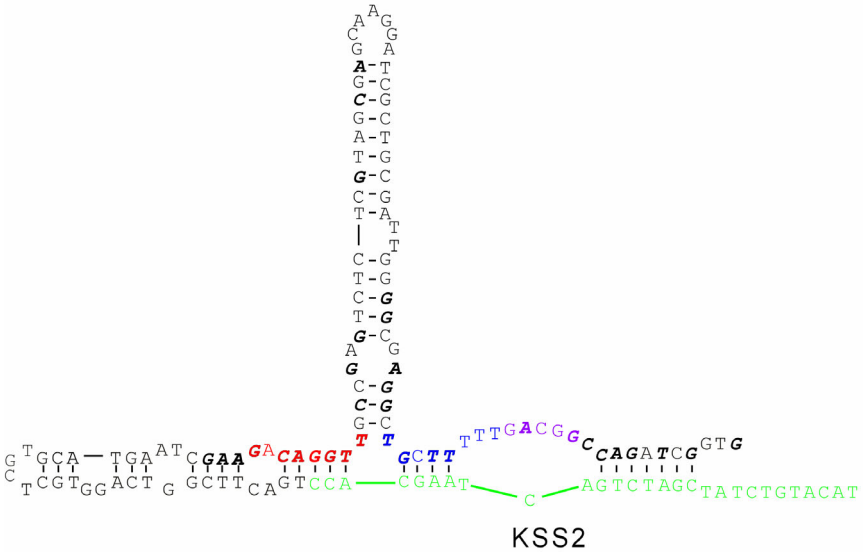
The substrate preference can be explained to some extent by differential binding. However, the preference of both clones for substrates KSS1, KSS2, and KSS6 may also be related to the fact that all three of these substrates have an adenosine residue immediately 5' of the common hexanucleotide sequence (position '-7') shared by all of the substrates. This adenosine can potentially pair with a thymidine residue that was found in both C14 and C15 and was shown to be functional following re-selection with substrate KSS6. The importance of position –7 was further supported by mutational analysis. For example, the introduction of a C to A mutation at position -7 of substrate KSS7 (substrate KSS7.m1) that created an additional A:T pair between the deoxyribozyme and the substrate, resulted in a rate enhancement (**Figure 2-15,** compare orange and yellow). Consistent with this result, the mutation of an A to G in the substrate KSS2 that disrupted an A:T pairing, resulted in a loss of activity (data not shown). The inability of the dominant clones C14 and C15 to utilize KSS3 and KSS7 may therefore simply reflect the fact that the substrates utilized 12 of the 13 total rounds of the selection possessed an A at position -7 (**Figure 2-6**, Rounds 8 and 10).

In order to try to further determine how the different oligonucleotide substrates might interact with the catalysts, we folded clone C14 with each of the different substrates (**Figure 2-16**). While each could potentially form multiple base-pairs with the deoxyribozyme, each substrate utilized the conserved motifs (**Figure 2-8**, red, blue and purple) to different extents. The common 3' end of each oligonucleotide substrate was hypothesized to pair with the substrate-binding

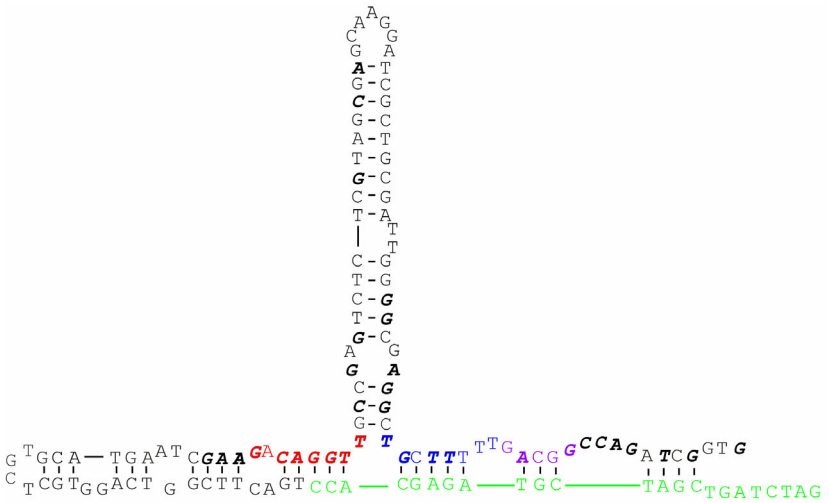
site as defined in **Figure 2-10a**, while the 5' end of each substrate differentially paired with the 3' end of the deoxyribozyme. The role of the 3' end of the deoxyribozyme was supported by the 3' deletion experiments (**Figure2-12** and **2-13**). In addition, several residues in Stem C that could pair with substrate KSS6 were conserved upon reselection (**Figure 2-11**) and that most of the reselected species were predicted to base-pair with this substrate more extensively than the parental clone (for example see deoxyribozyme D15r6c16 in **Figure 2-11**). It appeared that when given the chance to use one substrate rather than many substrates, the deoxyribozyme quickly optimized its templating region for that substrate. The evolution of extended substrate binding sites has previously been observed during the selection of other deoxyribozyme ligases (Cuenoud and Szostak, 1995). However, it should be noted that in the case of our deoxyribozyme, the extent of pairing observed does not in, and of itself, predict the substrate preferences observed.



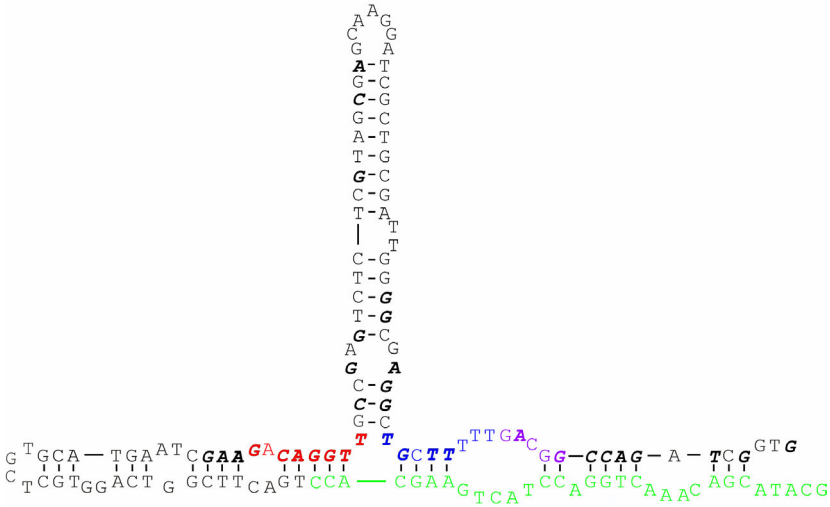
KSS1



KSS2



KSS3



KSS6

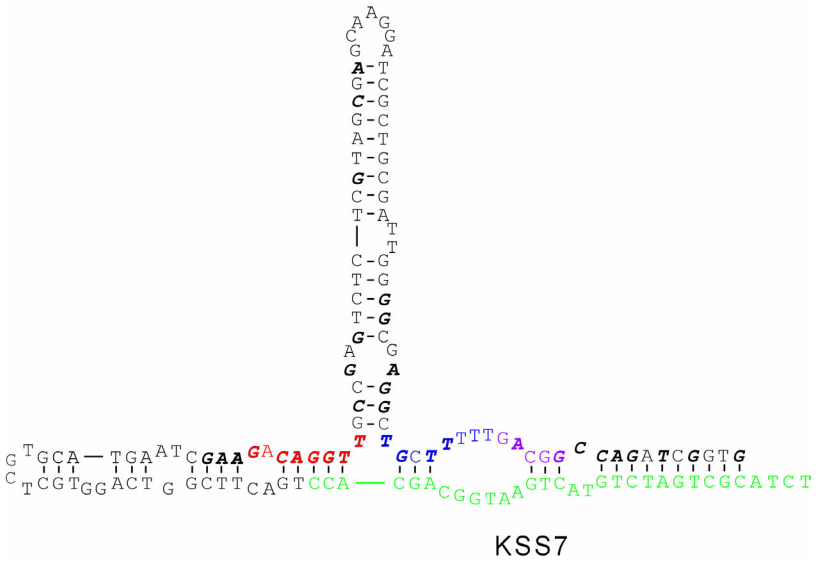


Figure 2-16. Potential secondary structures of C14 paired with different substrates. The oligonucleotide substrates are as indicated and are shown in green, other colors are as in **Figure 2-8**.

Metal independence of selected ligases

Almost all natural and selected nucleic acid enzymes have been shown to utilize metal ions for structure and/or function (Faulhammer and Famulok, 1997; Li and Breaker, 1999; Scott, 1999). Since our selection was conducted in the presence of 10mM $MgCl_2$, we investigated the dependence of our selected deoxyribozymes on divalent metals. Previous experiments with this ligation chemistry by Kool's lab have typically included magnesium. It was therefore possible in the current instance that a metal ion would contribute to the active structure of the deoxyribozyme. We examined the metal dependence of clone C14, with oligonucleotide substrate KSS2. Reactions carried out in both the presence and absence of the divalent metal Mg^{2+}, as well as in the presence of 10mM EDTA showed <2 fold difference in rates (**Figure 2-17**). Replacing Mg^{2+}

with the much more thiophilic Mn^{2+} had little effect on the reaction rate (data not shown). Thus it appeared as though much of the ligation chemistry was dictated by the inherent nucleophilicity of the phosphorothioate and strength of the iodine leaving group. This apparently made it possible for the selected deoxyribozyme ligases to avoid or ignore the chemical advantages that metal ions provide for most catalysts, although it was somewhat remarkable that no magnesium stabilized the active structure of the deoxyribozyme.

	relative rate
No Mg^{2+}	0.65
10mM Mg^{2+}	1.0
10mM EDTA	0.61

Figure 2-17. Metal independent rate of ligation. The rates of ligation were determined as descried in the Experimental Protocols, and are reported relative to the rate in selection buffer which contains 10mM $MgCl_2$

Template contribution to catalysis

In order to determine the relative contribution of templating versus other catalytic mechanisms, derivatives of clone C14 were generated that lacked the stem structure (**Figure 2-11**, Stem B), but contained the extended substrate-binding site (**Figure 2-11**, blue and purple). As can be seen in **Figure 2-18**, the deletion constructs can still catalyze oligonucleotide ligation better than the original pool, but not as well as full-length C14, suggesting that catalysis arises from mechanisms other than just simple templating.

This is supported further by the fact that nucleotides in Stem B (and away from the predicted substrate templating region) were conserved during the doped reselection. In addition, the fact that different substrates yielded different maximal velocities (Figure 14) can be most readily explained by assuming that the sequence and structure of the substrate:deoxyribozyme complex influenced the catalytic ability of the deoxyriboyzme. Nonetheless, much of the rate improvement observed in clone C14 can obviously be attributed to templating.

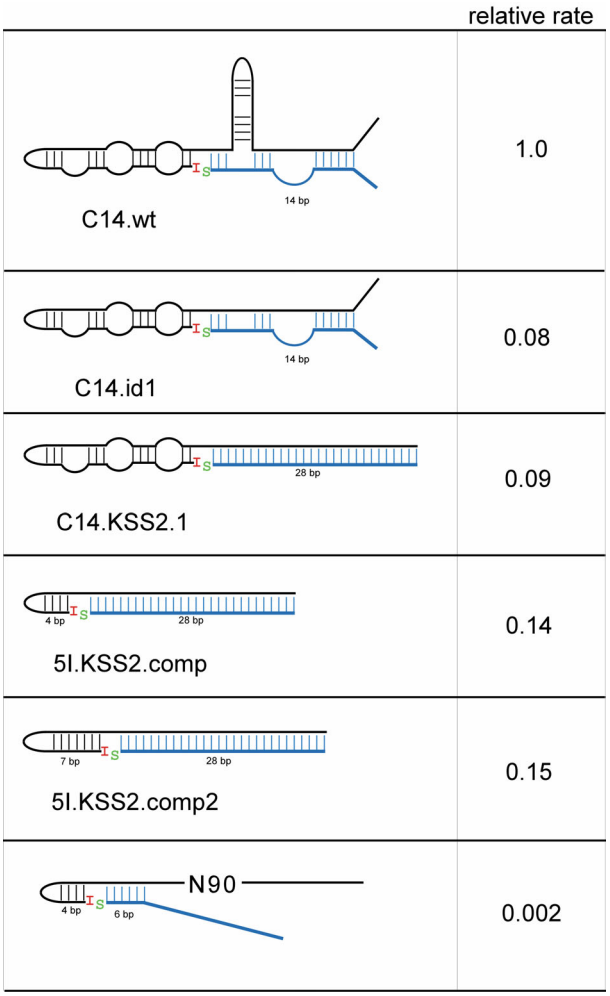


Figure 2-18. Analysis of C14 internal deletion mutants. Deoxyribozymes and the internal deletion constructs are shown in black with the substrate in blue. The number of base pairs is as indicated. The deletion constructs were synthesized as described in the Experimental Protocols.. All rates are reported relative to clone C14 which has a rate of $1.9hr^{-1}$. All assays were conducted using standard assay conditions as described in the Experimental Protocols.

CONCLUSIONS

The characterization of the selected deoxyribozymes suggests that in order to survive selection with multiple substrates, the deoxyribozymes relied heavily on templating as a catalytic mechanism. The selected variants appear to have developed an extended substrate-binding site that could differentially pair with each of the substrates. Similar results were obtained by Hager and Szostak (1997) who found that selected ribozyme ligases challenged with only two different oligonucleotide substrates could utilize both substrates (differences in k_{obs} were on the order of 10-fold; compare with the differences in apparent K_d values shown in **Figure 2-15**). Although the rate overall enhancement over background observed by Hager and Szostak was much greater than that observed here.

The strategy adapted by successful deoxyribozymes in our selection differs from that seen in other ligase selections (both RNA and DNA) in which the successful variants were extremely efficient at just recognizing and ligating the hexanucleotide sequence common to each substrate. For example, Bartel and Szostak (1993) selected ribozyme ligases that used either the 2' or 3' hydroxyl of a RNA substrate to displace a 5' pyrophosphate and form a phosphodiester bond. In the course of these selections, the RNA pool was challenged with 3 different oligonucleotide substrates. These selected ribozymes showed little or no substrate preferences, but showed much greater rate enhancements than did our deoxyribozymes. They also showed a requirement for divalent metals for function. This difference may simply be a property of the chemistry involved at the ligation junction, as hydroxyl nucleophiles may provide a greater opportunity

for optimization than phosphorothioate nucleophiles. Thus it appears that the successful deoxyribozymes may have more readily improved their catalytic potential by focusing on templating rather than by attempting to enhance an already facile catalytic mechanism.

Implications for Origins

If oligonucleotide ligases were some of the earliest self-replicases, then the earliest oligonucleotide ligases may have been those that relied upon extremely facile ligation chemistry, similar to the phosphorothioate displacement of iodine examined here. Thus, even though 5' iodo-nucleotides are unlikely prebiotic compounds, the in vitro evolution of oligonucleotide ligases should nonetheless mimic some of the earliest steps of chemical evolution.

To this extent, the apparent selection strategy adopted by our deoxyribozymes might be similar to that employed by early replicators. That is, in order to use the widest variety of prebiotically available oligonucleotide substrates, early replicators might have developed a multi-faceted substrate binding site in proximity to a common ligation junction. The common but defined sequence at the ligation junction would have potentially provided a way for these early replicators to avoid copying of templates other than themselves.

It has been suggested that during the RNA world, RNA catalysts may have bound cofactors (like NAD FAD, amino acids or peptides) by oligonucleotide linkers or "handles" (White, 1982; Orgel, 1989; Szathmary, 1993). What remains of these oligonucleotide linkers has in the case of cofactors like NAD and FAD

subsequently been reduced to a single nucleotide (White, 1976) or these linkers may be distant relations to what is now tRNA (Orgel, 1989; Szathmary, 1993) and even the genetic code (Knight and Landweber, 1998; Knight and Landweber, 2000), although this last hypothesis is in some dispute (Ellington et al., 2000). Nonetheless, the strategy adopted by our deoxyribozyme ligases suggests that recognition by oligonucleotide codes would have been an effective strategy and may have therefore aided in the evolution of more elaborate metabolisms.

EXPERIMENTAL PROTOCOLS

Pool construction

The DNA pool was designed and synthesized according to previously reported methods (Robertson and Ellington, 1999). The synthesis was carried out in our laboratory on an Expedite 8909 DNA synthesizer (PE Biosystems, Foster City, CA) using standard phosphoramidite chemistry. Synthesis reagents were purchased from Glen Research (Sterling, VA). Oligonucleotide deprotection was conducted in NH_4OH for 16 hr at 55° C for all oligonucleotides except for those bearing a 5'iodine. To minimize the loss of the 5' iodine upon deprotection, DNA molecules containing 5' I-T were synthesized with the UltraMILD phosphoramidite reagents Pac-dA and iPr-Pac-dG and deprotected for 12 hours at room temperature in 0.05 M potassium carbonate in methanol. The 3' phosphorothioates were synthesized on 3' phosphate-CPG by replacing the normal oxidizing reagent with the sulfurizing reagent thiosulfonate (0.05 M in acetonitrile). The pool contained a core region of 90 random nucleotides flanked on both sides by constant sequence regions (5' TGACTTCGGTCAGGTGCTCGTG-N90-CTCGTGATGTCCAGTCGC) (**Figure 2-4**). The randomized phosphoramidite mixture contained a 1.2:1.3:1.5:1 molar ratio of dA:dC:dG:dT .

The 5' iodine (5 'I-T) was added to the pool via PCR. A 180ml PCR reaction was carried out with a 5' primer containing iodine and a 3' primer containing biotin (5' I-TGACTTCGGTCAGGTGCTCGTG and 5' biotin-GCGACTGGACATCACGAG). The double-stranded DNA was purified by

native acrylamide electrophoresis to remove excess biotinylated primer. After extraction from the gel, the double stranded PCR product was immobilized on streptavidin agarose (Fluka, Switzerland) and the single-stranded, iodinated strand was eluted with 0.2 N NaOH (Hultman et al., 1988). The eluate was immediately neutralized by the addition of 3 M NaOAc pH 5.2, and precipitated with ethanol.

Selection for catalysts

Thirteen rounds of in vitro selection were performed according to a procedure originally described by Bartel and Szostak (1993) and adapted by our laboratory with some modifications (Robertson and Ellington, 1999) (Figure 4b). In short, the single stranded DNA pool was annealed to a biotinylated DNA primer (5' biotin-GCGACTGGACATCACGAG) and immobilized on streptavidin agarose (Gibco BRL, Gaithersburg, MD). The beads were washed and equilibrated in selection buffer (500 mM NaCl, 50 mM Tris pH 7.4, 10 mM $MgCl_2$). The ligation reaction was initiated by the addition of the substrate oligonucleotide bearing a 3' phosphorothioate. Incubation times and reactant concentrations were as shown in **Figure 2-6**. Reactions (0.2-1 ml) were stopped by dilution into 20 ml of wash buffer (500 mM NaCl, 30 mM Tris pH 7.4, 1 mM EDTA) and washed thoroughly (~100 ml) to remove unligated substrate. DNA was eluted from the streptavidin agarose column with base and neutralized. Ligated species were isolated by affinity purification on a column that contained an oligonucleotide complementary to the substrate sequence. Columns were washed to remove any unligated DNA, and the deoxyribozymes were subsequently eluted with alkali. After neutralization and precipitation the eluted

population was amplified using a selective 5' primer that contained the same sequence as the substrate used in the reaction and a common 3' primer. Double-stranded DNA was purified on a 3% agarose gel, and extracted using the Qiagen gel extraction kit (Qiagen, Valencia, CA). The selective PCR product served as a template for regenerative PCR with a nested 5' primer containing iodine and a 3' primer containing biotin, as outlined above. Single-stranded DNA was isolated for additional rounds of selection and amplification.

The first round of selection employed 120 micrograms of single-stranded DNA pool (~$1.7x10^{15}$ sequences). Five different substrate oligonucleotides were employed during the selection: KSS1 5' AAAAAAAAAAAAAAAAA-AAAAAGCACC; KSS2 5' TACATGTCTATCGATCTGACTAAGCACC-S; KSS3 5' GATCTAGTCGATCGTAGAGCACC-S; KSS6 5' GCATACGACAA-ACTGGACCTACTGAAGCACC-S; and KSS7 5' TCTACGCTGATCTG-TACTGAATGGCAGCACC-S, where S denotes the presence of the3' phosphorothioate. All substrates were treated with DTT prior to reaction to reduce any disulfides that may have formed during storage. The final concentration of DTT in the selection mixture was 50 uM.

Doped selection

Doped pools were synthesized as previously described (Robertson and Ellington, 1999). The random sequence regions from C14 and C15 were re-synthesized with 30% non-wild-type residues (D14 5' I-TGACTTCGGTC-AGGTGCTCGTGcatgaatcgaagacaggttgccgagtctctcgtagcgagcaaggatcgctgcgattggg gcgaggctgctttttgacggccagatcggtgCTCGTGATGTCCAGTCGC; D15 5'I-TGA-

CTTCGGTCAGGTGCTCGTGacgcgtacgaacggagccgtgtcaggtttgctcgcgagtaatgtgatt
cgcgtacgaacgagatgctttttgacggatctttggctggtgCTCGTGATGTCCAGTCGC

degenerate bases in lowercase). The 5'I-T was added to the initial pool during synthesis. Following deprotection, gel purification, and quantitation, the degenerate pools were used for selection with out any amplification.

The selection was carried out as described above, except that only a single substrate, KSS6, was used. The starting pool contained 120 micrograms of single-stranded DNA. The extension efficiency of the pool was estimated at ~15%. This corresponds to ~10^{14} sequences. In rounds 2 through 6 the pool was not immobilized but instead hybridized to a non-biotinylated 3' primer. In addition these rounds of selection were conducted using a biotinylated oligonucleotide substrate. Ligation reactions were terminated by the addition of 7 M urea and a 100-fold excess of KSS6 lacking a 3' phosphorothioate, and ligated species were captured on streptavidin agarose in the presence of the stop mixture. After thorough washing, the agarose beads (with captured, ligated variants) were used to seed a selective PCR. Regenerative PCR and ssDNA isolation were as described above. The stringency of the selection was progressively increased by decreasing the incubation time and by adding a competitor substrate containing a 3' phosphate rather than a phosphorothioate in Rounds 4-6. Incubation times were 1 minute for Rounds 1 and 2, and 5 seconds for Rounds 3 through 6.

Cloning deoxyribozymes

PCR products from Rounds 10, 11, and 13 were cloned using the Topo TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced using the dideoxy method (Sequitherm EXCEL II, Epicenter Technologies, Madison, WI).

Deoxyribozyme kinetics

The standard ligation assay for pools and individual clones were conducted in selection buffer at 25° C with 0.1 μM single-stranded DNA, 0.15 μM substrate, and 0.15 μM 3' primer. Individual deoxyribozymes were amplified from clonal plasmid DNA. The ssDNA catalysts were 3' end-labeled using terminal deoxynucleotide transferase (Gibco BRL, Gaithersburg, MD) and dideoxyadenosine 5'-[α-^{32}P]-triphosphate (Amersham Parmacia Biotech, Piscataway, NJ). The ssDNA catalysts and the 3' primer were heat denatured at 70° C and cooled to 25° C in selection buffer prior to the addition of DTT-treated substrates. Reactions were terminated by the addition of 95% formamide gel-loading buffer. Ligated and unligated species were separated on denaturing (7M urea) 8% acrylamide gel and quantitated using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Rates of reaction and apparent dissociation constants were determined via ligation assays with clone C14. Reactions were carried out in selection buffer with $\leq$ 1nM DNA and 10 nM 3' primer, as described above. Reactions were initiated by the addition of DTT-treated substrate. The final DTT concentration was 500 μM. All assays were conducted in silanized tubes to avoid non-specific DNA binding. Assays were conducted with all 5 substrates as well as a mutant

substrate KSS7.m1 (5' TCTACGCTGATCTGTACTGAATGGAAGCACC-S; underline indicates the mutation).

Ligation reactions monitored to completion typically proceeded to >70% reaction. Pseudo-first order rates were determined by the best fit through two points in the linear range of the reaction (<10% reaction). Apparent K_d's were calculated by fitting the equation $k_{obs} = k_{chem}[S] / (K_d^{app} + [S])$, where S is the substrate concentration, kchem is the rate at saturating substrate concentrations, and K_d^{app} is the apparent binding constant. The use of this equation assumes that k_{chem} is the rate-limiting step. This is a reasonable assumption: oligonucleotides hybridize either to their complements or to nucleic acid enzymes such as the Tetrahymena self-splicing ribozyme with kon values on the order of 10^8 $M^{-1}min^{-1}$ (Herschlag and Cech, 1990). In contrast, the kchem values that we observed at saturating oligonucleotide concentrations were on the order of 1 hr^{-1}. Thus, at the apparent K_d values, the rates of both association and dissociation of oligonucleotide substrates were likely much faster than the rate of reaction. For example, for substrate KSS2 the apparent K_d is 70 nM, and at this concentration the values for $k_{on}[S]$ and k_{off} would have been on the order of 7 min^{-1} (420 hr^{-1}).

Deletion constructs

Deoxyribozyme 3'deletion ladders bearing 5'-iodine were generated by reverse transcription of an alkaline hydrolyzed reverse compliment RNA of the selected clone. In brief, clone c14 and c15 were amplified by PCR using 5I.22.90, and 20.T7.18.90a (TTCTAATACGACTCACTATAGGAGCGACTGG-ACATCACGAG, the T7 promoter is underlined) which added a T7 promoter to

the 3'end of the clone. Following transcription with T7 RNA polymerase the transcripts (now the RNA complimet of the original DNA clone) were gel purified. Alkaline hydrolysis of the RNA transcript wwas conducted in pH 10, 0.1M sodium carbonate for 20minutes at 90° C. After neutralization, the resulting RNA digest ladder was reverse transcribed with Super Script II reverse transcriptase (Invitrogen, Carlsbad, CA)and the 5' primer 5I.22.90. The RNA was then remopved by treatment with RNase A.

Ligation reactions with deoxyribozyme 3'deletion ladders were conducted with 15 μM 5'^{32}P labeled KSS2 under standard assay conditions except for the concentration of 18.90a which was 5 μM. Reactions were conducted for 15 min and stopped by the addition of an equal volume of 95% formamide loading dye. Reactions were analyzed on 8% sequencing size gel and analyzed using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Clone C14 3'deletion mutants used in rate assays (**Figure 2-13**). Were generated by PCR using a the common 5' primer 5I.22.90 and one of the following 3' primers, C14.3d1 (5B-CACCGATCTGGCCGTCAAAAAGC), C14.3d2 (5B-CAAAAAGCAGCCTCGCCCCAA), C14.3d3 (5B-CGCCCCAA-TCGCAGCGATCCTTG), C14.3d4 (5B-CCTTGCTCGCTACGAGAGACTC), C14.3d5 (5B-GAGACTCGGCAACCTGTCTT), C14.3d6(5B-CAACCTGTC-TTCGATTCATG), where 5B is a 5' biotin. Single stranded DNA was generated as described previously. The ligation reactions were conducted with 15 μM KSS2 under standard assay conditions. Rates were determined as described previously.

Internal deletion constructs (**Figure 2-18**) were synthesized with 5'I-T and gel-purified prior to use. The sequences of the deletion constructs were: 5I KSS2.comp I-TGACTTCGGTCAGGTGCTTAGTCAGATCGATAGACA-TGTA; 5I KSS2.comp2 I-TGACGACTTCGGTCGTCAGGTGCTTAGTCAG-ATCGATAGACATGTA; C14.KSS2.1 I-TGACTTCGGTCAGGTGCTCGT-GCATGAATCGAAGACAGGTGCTTAGTCAGATCGATAGACATGTA; and C14.wt.id1 I-TGACTTCGGTCAGGTGCTCGTGCATGAATCGAAGACA-GGTGCTTTTTGACGGCCAGATCGGTG.

Assays were carried out with substrate KSS2 as described above, except that ligated and unligated species were separated on denaturing 8% acrylamide gels containing both 7 M urea and 40% formamide. To ensure that the measured rates of reaction were similar for deoxyribozymes that were synthesized directly and those that were generated by PCR amplification, both C14.wt.id1 and C14.KSS2.1 were also generated via PCR using the appropriate 5' and 3' primers (5' iodine-TGACTTCGGTCAGGTGCTCGTG; 5'-biotin-CACCGATCT-GGCCGTCAAAAAGC; 5'-biotin TACATGTCTATCGATCTGACTAAGC-ACC). Rates for chemically synthesized deoxyribozymes and those generated by PCR were in good agreement.

Site-directed mutagenesis

Deoxyribozymes containing mutations in the designed substrate-binding site were generated by PCR with a mismatched, iodinated 5' primer (I-TGACTTCGGTCACGTGCTCGTG; the underline indicates the mismatch). Deoxyribozymes containing mutations in the evolved substrate-binding site were

generated by PCR with mismatched, non-iodinated 5' primers (C14 5' TGACTTCGGTCAGGTGCTCGTGCATGAATCGAAGACACGTTGCCGAGT, and C15 5' TGACTTCGGTCAGGTGCTCGTGACGCGTACGAACGGA-GCCGTGTCACGTTTGCTCGC; the underline indicates mismatches). The 5' iodine was then introduced by PCR with a 5' iodinated primer. Mutant deoxyribozymes were assayed with both KSS2 and a mutant substrate, KSS2.m2 (TACATGTCTATCGATCTGACTAAGCACG-S; the underline indicates the mutation).

Secondary structural models

Secondary structural models were generated using the program RNAStructure 3.5 (Mathews et al., 1999) with parameters appropriate for DNA (SantaLucia, 1998). The secondary structures that were generated were derived from the sequences of ligated products. For most structures, the base-pair between the 3' C of the substrate and the first G residue of the conserved 5' octamer motif (white boxed) was assumed to be a known parameter, based on the mutational analyses described in the text.

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Chapter 3: Design and Selection of Allosteric Deoxyribozymes

INTRODUCTION

The first allosteric ribozymes were designed by Tang and Breaker, who found that they could modulate the activity of the hammerhead ribozyme simply by appending a known anti-adenosine aptamer to the ribozyme. Depending on the design construct, binding of the effector (ATP) to the aptamer domain resulted in either activation or inactivation of the ribozyme (Tang and Breaker, 1997). Since then, a combination of rational design and *in vitro* selection techniques have been employed to generate allosteric ribozymes that are modulated by a variety of effector molecules ranging from small molecules like ATP, FMN and quinolone derivatives (Tang and Breaker, 1997; Soukup and Breaker, 1999b; Robertson and Ellington, 2000; Piganeau et al., 2001) to oligonucleotides (Porta and Lizardi, 1995; Robertson and Ellington, 1999; Komatsu et al., 2000) to peptides and proteins (Faulhammer and Famulok, 1997; Robertson and Ellington, 2001).

Further study has shown a variety of different allosteric mechanisms leading to ribozyme activation (or inactivation) including effector-induced steric interference (Tang and Breaker, 1998), secondary structure stabilization (Tang and Breaker, 1998; Robertson and Ellington, 1999; Zhou et al., 2000), antisense interactions (Porta and Lizardi, 1995; Robertson and Ellington, 1999; Komatsu et al., 2000), or a combination of these (Kuwabara et al., 1998; Zhou et al., 2000). In general, it appears that the ligand-dependent stabilization of secondary and

tertiary structure results in a concominant stabilization of the catalytic domain and thus activation of catalysis (Soukup and Breaker, 1999c).

Unlike RNA, no DNA enzymes have been found in nature, which has given rise to the prejudice that RNA must be more catalytically 'fit' than DNA (Sigurdsson and Eckstein, 1995; Cate et al., 1996; Li and Breaker, 1999a). Yet, using the techniques developed for the in vitro selection of RNA, researchers have shown that DNA is capable of a variety catalytic activity including cleaving RNA and DNA (Breaker and Joyce, 1994; Carmi et al., 1996; Faulhammer and Famulok, 1997; Geyer and Sen, 1997), DNA ligation (Cuenoud and Szostak, 1995), phosphorylation (Li and Breaker, 1999b) and capping with AMP (Li et al., 2000).

While the design and selection of effector-dependent ribozymes is now well established, there has not been a great deal of complementary work with deoxyribozymes. Breaker and Roth were able to isolate RNA-cleaving deoxyribozymes that required the cofactor histidine (Roth and Breaker, 1998). However, the cofactor appears to function as a general base in the cleavage reaction rather than as an allosteric effector. More recently, Wang and Sen have used rational design to engineer a RNA-cleaving 10-23 deoxyribozyme (Santoro and Joyce, 1997) that requires an oligonucleotide effector for activity (Wang and Sen, 2001). The so-called 'expansively regulated' deoxyribozyme catalyst forms a three-way helical junction with the oligonucleotide substrate and a novel effector oligonucleotide. This method of activation has been applied to deoxyribozymes and ribozymes alike. In their system, the binding of the effector

oligonucleotide enhances the binding of the substrate by effectively extending the substrate-binding site. The effector oligonucleotide can thus be considered a co-substrate of the reaction. More recently, this method has been expanded to regulation by small molecules. By incorporating an anti-adenosine DNA aptamer (Huizenga and Szostak, 1995) in place of the oligo-effector binding site, Wang et al. (2002) were able to generate 'expansively regulated' deoxyribozymes capable of being activated by up to ~30-fold in the presence of ATP.

We have attempted to determine if the methods for generating allosteric ribozymes which respond to small organic effector molecules such as ATP and FMN, could be successfully extended to deoxyribozymes. We have previously selected a deoxyribozyme ligase that catalyzes the formation of an unnatural internucleotide linkage (Levy and Ellington, 2001; Levy and Ellington, 2002), starting from a random DNA pool that contained a 5' iodine and a substrate that contained 3' phosphorothioate. A previously selected anti-adenosine, DNA aptamer that was known to undergo a significant conformational change on ligand-binding (Lin and Patel, 1997) was appended to the deoxyribozyme. As in previously designed allosteric ribozymes, the ligand-dependent conformational change is relayed to the catalytic core of the deoxyribozyme, and, in optimized constructs, ATP was found to modulate the rate of catalysis by up to 460-fold. The allosteric deoxyribozyme also appears to employ a novel mechanism, in that the conformation assumed in the absence of the effector actually depresses the rate of reaction below background, templated levels.

RESULTS AND DISCUSSION

Further Characterization and Minimization of a Deoxyribozyme Ligase

Deoxyribozyme ligases that could catalyze the formation of an unnatural nucleotide linkage via the reaction of a 3' phosphorothioate and a 5' iodine residue were originally selected from a DNA pool that spanned 90 random sequence positions (Levy and Ellington, 2001; Levy and Ellington, 2002). After 13 rounds of selection two dominant clones (clone 14 and 15) were isolated and characterized. An additional series of experiments was designed to further characterize clone 14, which had a rate of 1.9 hr-1 and showed over a 400-fold rate enhancement over the unselected pool.

Co-variation analysis and the doped reselection of clone 14 suggested that the active ligases adopted the structure shown in **Figure 3-1**. In order to better

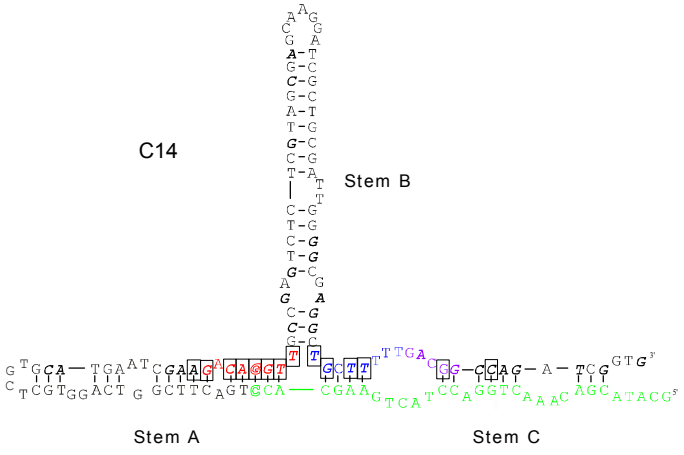


Figure 3-1. Putative secondary structure for deoxyribozyme C14 with substrate KSS6. Residues conserved in more than 75% of re-selected clones are shown in bold; those conserved in more than 90% of re-selected clones are shown in outlines. Substrate KSS6 is shown in green, all other colors are as in Figure 2-8.

map the core of the deoxyribozyme, we designed a series of constructs in which we deleted different portions of the deoxyribozyme (**Figure 3-2**). As suggested from the doped selection, the predicted internal loops and nucleotide bulges proved to be relatively unimportant for catalysis, and the removal of the 3' conserved region could be compensated for by extending the substrate binding adjacent to 'Stem B'. However, consistent with our previous observations, the conserved residues in 'Stem B' appeared to be important for catalysis contributing an additional ~10-fold to the rate of ligation in a standard assay (Levy and Ellington, 2001). In addition, these deletion analyses were

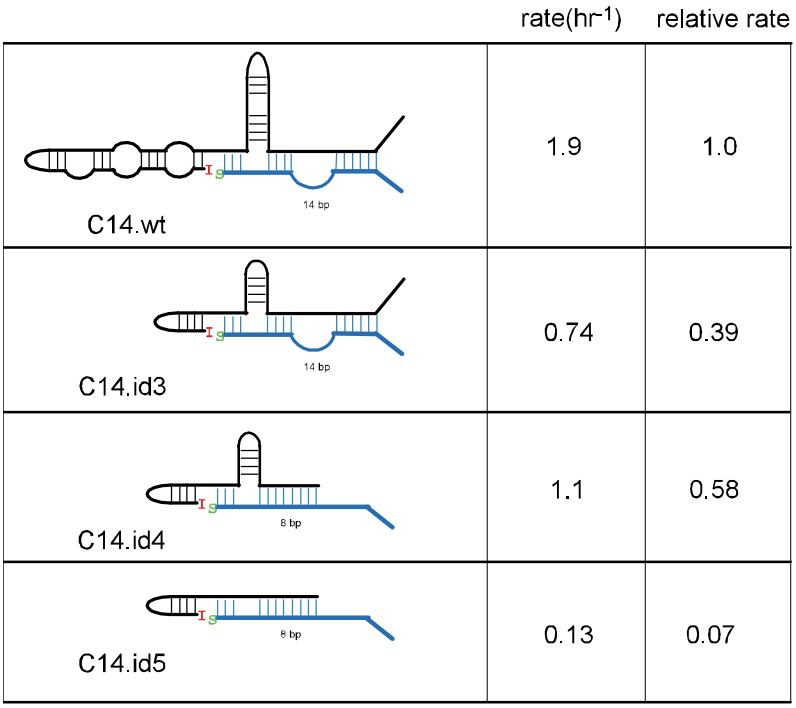


Figure 3-2. Ligation rates of 'cis'deoxyribozyme deletion constructs. These deoxyribozymes ligate a single oligonucleotide substrate to themselves. Substrate (KSS2) is shown in blue. The number of base-pairs between the each construct and its substrate are schematically indicated. Initial rates are reported per hour, and relative rates are normalized with respect to the parental deoxyribozyme C14.

consistent with the secondary structural hypothesis but more importantly, yielded a 36 nucleotide construct that could be readily synthesized. In addition, by opening the tetra loop at end of the minimal 36 nucleotide construct, it proved possible to generate a deoxyribozyme that could act on oligonucleotide substrates in 'trans' (**Figure 3-3**). Here, again, some aspect of 'Stem B' proved to be important for catalysis beyond simple templating function.

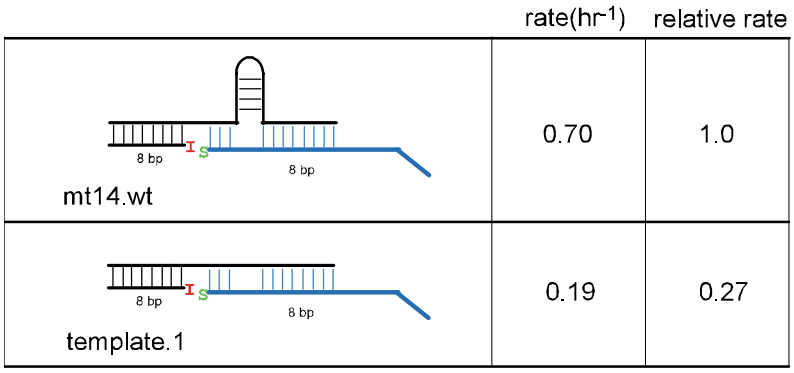
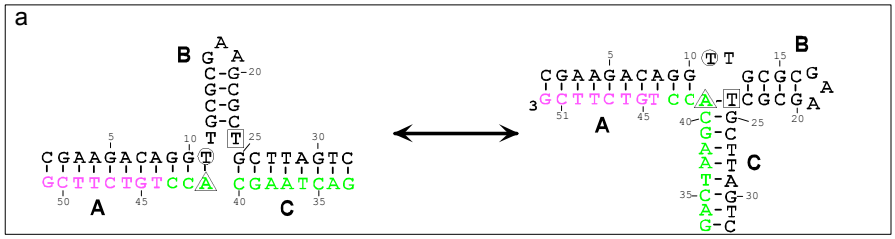


Figure 3-3. Ligation rate of a 'trans'deoxyribozyme construct. These deoxyribozymes catalyze the ligation of two oligonucleotide substrates. Initial rates are reported per hour, and relative rates are normalized with respect to mt14.wt.

The secondary structure analysis of the minimized deoxyribozyme constructs suggested two possible conformations for the deoxyribozyme. One had two unpaired thymidine residues on either side of 'Stem B', and the other had two unpaired thymidine redisues adjacent to the stem (**Figure 3-4a**). To more fully determine the role of 'Stem B' in catalysis, a series of mutations was designed to determine which residue, T11 (circle) or T24 (square), was nominally unpaired in the deoxyribozyme secondary structure. When the originally predicted T11:A41 base-pair was changed to a C:G base-pair, the deoxyribozyme

lost over 2,000-fold activity (**Figure 3-4b**; mt14.7). However, when the alternative T:A base-pair between T24 and A41 was changed to a C:G base-pair, the deoxyribozyme showed wild-type or better activity (**Figure 3-4b**; mt14.8). In general, mutations that included the predicted T24:A41 pairing were roughly as active as the predicted strength of the pairing, with Watson-Crick pairings showing greater activity than wobble pairings (**Figure 3-4c**). Thus, while we had previously drawn the three-way junction for the deoxyribozyme as shown in **Figure 3-1** and in **Figure 3-4a** (left), the more likely secondary structure appeared to be the one depicted in **Figure3-4a** (right).



b

construct	pos 11	pos 24	pos 41	rate (hr^{-1})	relative rate
mt14.wt	T	T	A	0.70	1
mt14.1	T	C	A	0.016	0.022
mt14.2	C	T	A	$9.2x10^{-4}$	$1.3x10^{-3}$
mt14.3	G	T	A	0.056	0.08
mt14.4	A	T	A	0.021	0.029
mt14.5	5MeC	T	A	$1.5x10^{-3}$	$2.2x10^{-3}$
mt14.6	dU	T	A	0.83	1.2
mt14.7	C	T	G	$3.4x10^{-4}$	$4.9x10^{-4}$
mt14.8	T	C	G	0.95	1.4
mt14.9	T	T	G	0.044	0.063

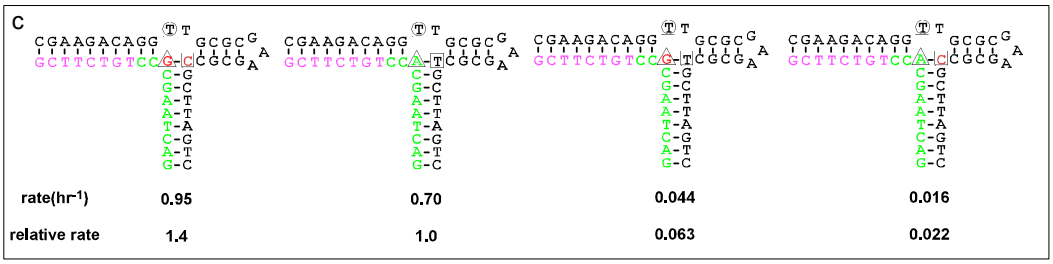


Figure 3-4. Mutational analysis of the 'trans' deoxyribozyme mt.14.wt. **(a)** Two potential deoxyribozyme conformations. Iodinated substrate 5I.8.C14.m1 is shown in pink and the eleven 3'nucleotides of substrate KSS2 are shown in green. **(b)** Ligation rates of mutant deoxyribozymes. All rates were determined with substrate KSS2, under standard assay conditions. The identities of position 11, 24 and 41 are explicitly shown. Initial rates are reported per hour, and relative rates are normalized with respect to mt.14.wt. **(c)** Covariation analysis of positions 11 (circle), 24 (square), and 41 (triangle). Rate of ligation corresponds to the strength of the base pair between position 24 and 41.

While the identity of position 41 appeared to correlate strongly with the identity of the nucleotide at position 24, the dramatic loss in ligation activity with the mutation of position T11 prompted us to further explore the role of this residue. When T11 was mutated to a cytidine, the activity of the deoxyribozyme

dropped by over 750-fold. (**Figure 3-4b**; mt14.2). This residue appeared especially sensitive to mutation, as changes to either A or G or 5-methyl C all resulted in a substantial loss of activity (**Figure 3-4b**; mt14.3-5). Only the substitution to deoxyuridine retained activity (**Figure 3-4b**; mt14.6).

Taken together, these results strengthened our hypothesis that the templating function and other catalytic mechanisms in the deoxyribozyme were intimately linked. Previously experiments using phosphorothioate-iodide ligation chemistry have shown that mismatches between the iodinated oligonucleotide and its template resulted in a loss of >100-fold in the rate of ligation (Xu and Kool, 1999). In these experiments, the changes in ligation efficiency were expected due to the interruption of base-pairing between the substrates and the target sequence. However, in the case of our deoxyribozyme, while constructs mt14.wt and m14.7 should have been able to form the same number of base-pairs with the template, their activities differed by over 2,000-fold. This was especially surprising given that the evolved catalyst was only 400-fold more active than the nascent pool. Thus, the mutation of a single base that was uninvolved in base-pairing with the substrate was capable of misaligning the template to the point where catalysis was lower even than the background, templated reaction.

These results could to some extent be rationalized based on what is known about the three-dimensional structures and relative stabilities of three-way junctions. It has been shown that the introduction of unpaired bases at the branch site of three-way junctions (e.g., residues T11 and T12) could have a stabilizing effect on these structures (Leontis et al., 1991). The introduction of such bulged

residues has also been shown to reduce the flexibility of these three-way junctions (Yang and Millar, 1996) and to allow for the coaxial stacking of two of the three helices (Rosen and Patel, 1993b; Rosen and Patel, 1993a; van Buuren et al., 2000). In the case of the oligonucleotide effector-dependent 10-23 deoxyribozyme designed by Wang and Sen, which included a three-way helical junction, both the identity and position of two bulged adenosines at the junction were found to be important for activation (Wang and Sen, 2001). While we do not know the three-dimensional structure of our deoxyribozyme ligase, it is possible that the unpaired thymidine residues aid in the formation of an extended helix and, hence, in the alignment of the substrate oligonucleotide.

Designing allosteric deoxyribozymes

The finding that the selected deoxyribozyme was sensitive to mutations at position 11 in the TT bulge and that the apparent misalignment of the template could drastically reduce ligase activity immediately suggested a method for designing allosteric deoxyribozymes. Breaker and his co-workers had previously found that by appending an anti-adenosine aptamer to the hammerhead ribozyme, they could modulate the activity of the ribozyme (Tang and Breaker, 1997). Binding of the ligand produced a conformational change in the aptamer that was transmitted to the catalytic core resulting in either activation or inactivation, depending upon the 'communication module' that connected the aptamer and the catalytic core, (Tang and Breaker, 1997).

Using this same methodology, we hoped to control the rate of the selected deoxyribozyme ligase by appending an anti-adenosine aptamer. The DNA ATP

aptamer is a bulged DNA hairpin that binds ATP with micro-molar affinity (6 ± 3 μM, Huizenga and Szostak, 1995). NMR structural analysis of the aptamer has revealed that in the absence of ligand the aptamer adopted an open structure (**Figure 3-5**, upper). However, in the presence of ATP, the aptamer formed a compact stem-bulge structure that could simultaneously bind two ATP ligands (Lin and Patel, 1997) (**Figure 3-5**, lower). By appending this aptamer to one of the stems of the deoxyribozyme ligase we hoped to make use of this conformational change within the aptamer to align or misalign the template and thus control catalytic activity.

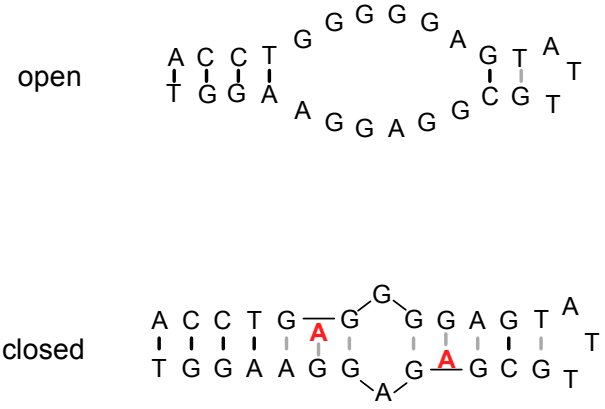


Figure 3-5. Conformational change in the ATP-binding DNA aptamer. In the absence of ATP, the aptamer adopts an open configuration (upper). In the presence of ATP the aptamer adopts a closed conformation (lower). Each aptamer binds two ATP molecules, shown in red. Figure adapted from (Lin and Patel, 1997).

We initially designed several constructs in which the DNA aptamer was appended to 'Stem B' of the minimized deoxyribozyme trans-ligase (**Figure 3-6a**) using a connecting region that was based on the stem region used by Lin and Patel (1997). In order to optimize the potential effect of the conformation change, the joining region (**Figure 3-6a**; blue) between the aptamer and the deoxyribozyme

was varied from 0 to 3 base-pairs in length. Deoxyribozyme chimeras were then assayed for their ability to ligate two oligonucleotide substrates in either the presence or absence of the cognate effector ATP and a non-cognate effector, GTP (**Figure 3-6b, c**). While the presence of the effector had no effect on deoxyribozymes that had no appended aptamer (data not shown), three of the four designed constructs showed a strong ATP-dependence with no activation above background in the presence of GTP. Surprisingly, a single base-pair in the stem appeared to be sufficient to mediate ATP-dependent activation, although the greatest activation was seen with a stem of three nucleotides. Deoxyribozymes that contained stems greater than 3 base pairs in length appeared constitutively active (data not shown). The dependence of stem-length in aptamer-mediated activation of catalysis has previously been observed with hammerhead ribozyme chimeras. For example, Tang and Breaker observed an optimal length of 4-5 base pairs for the joining region between the hammerhead ribozyme and the anti-adenosine RNA aptamer (Tang and Breaker, 1998). Similarly, Araki et al. found a stem length of 3 base pairs to be optimal for the linker between an anti-FMN aptamer and the hammerhead (Araki et al., 1998).

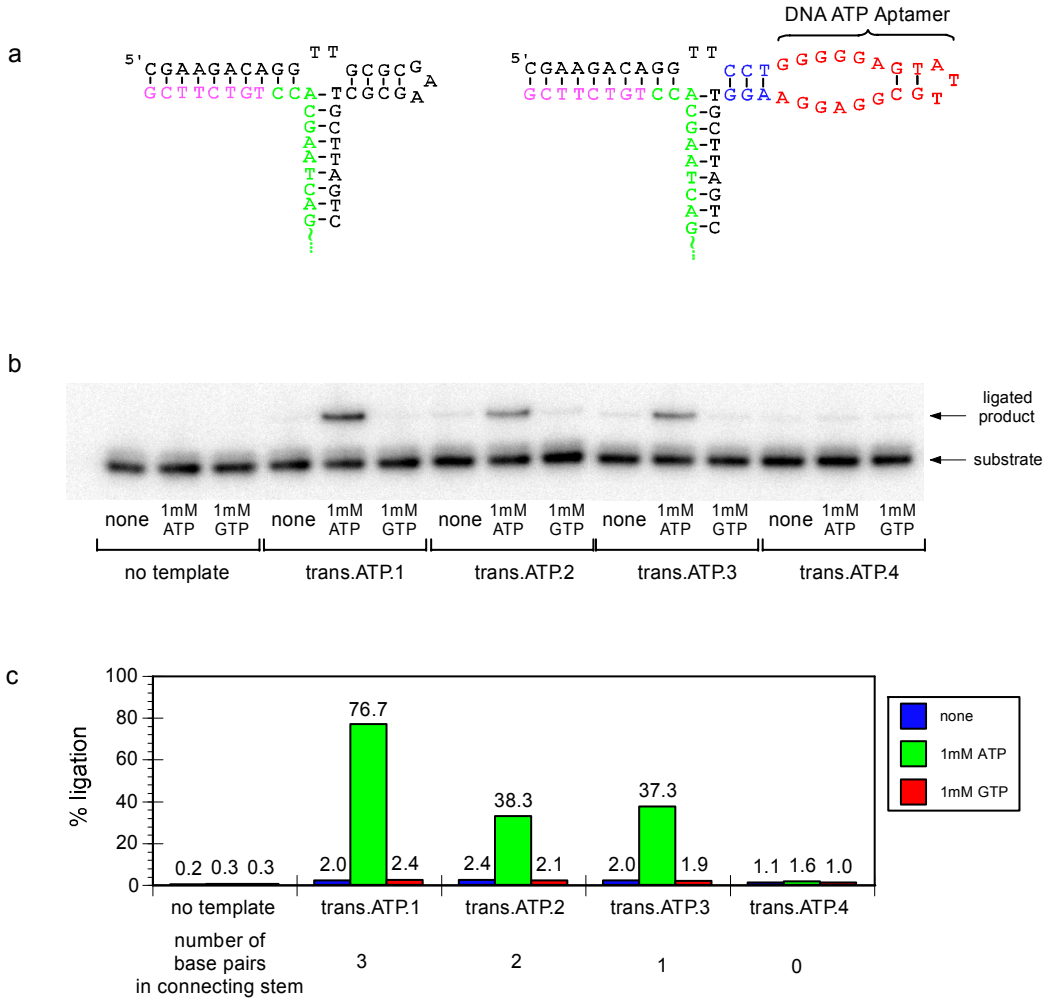


Figure 3-6. Rational design of ATP-dependent deoxyribozyme ligases. **(a)** Putative secondary structures of parental (mt.14.wt, left) and effector-activated deoxyribozyme ligase (trans.ATP.1, right). The iodinated substrate 5I.8.C14.m1 is shown in pink and the eleven 3'nucleotides of substrate KSS2 are shown in green. The anti-adenosine aptamer is shown in red and the joining region in blue. **(b)** Ligation assay for ATP-dependent deoxyribozyme ligases. The constructs trans-ATP.1, trans-ATP.2, trans-ATP.3 and trans-ATP.4 contained different numbers of base-pairs in the joining region are indicated in (c). Reactions were carried out with no effector, 1 mM ATP, or 1 mM GTP. **(c)** The % ligation for the reactions shown in (b). Values have been adjusted to reflect the fact that there was a 2-fold excess of labeled substrate in the reaction mixture.

Additional analysis of the best construct, trans.ATP.1 indicated that the initial rate of reaction increased 250-fold upon the addition of ATP (**Figure 3-7**).

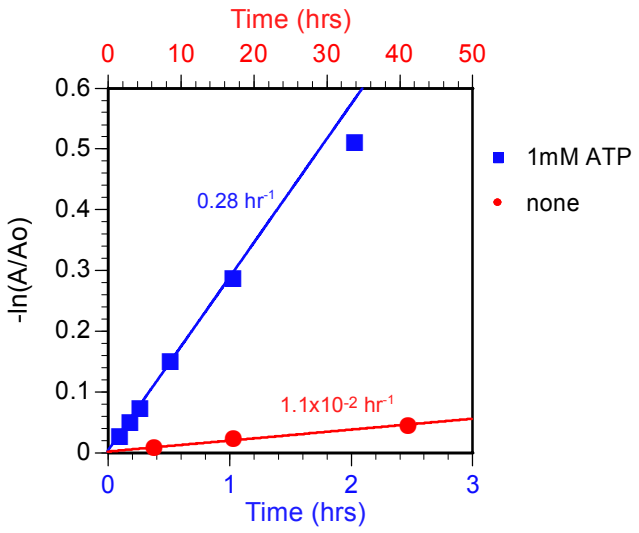


Figure 3-7. Kinetics of ATP-activation of trans-ATP.1. Initial rate determination for the reaction of trans-ATP.1 in the presence (blue) and absence (red) of 1 mM ATP. A is the amount of unreacted substrate, while Ao is the amount of substrate at time 0. The rates of ligation are as indicated.

Activation was also found to occur irrespective of the pre-incubation time, ruling out the possibility that the activation was coincident with the addition of ATP rather than dependent upon it (**Figure 3-8**). The addition of ATP did not change the solution pH ruling this out as a possible trigger. In addition, the measured rates did not change when Mg^{2+} concentration was increased from a 5-fold molar excess relative to ATP to a 50-fold excess making it unlikely that ATP-dependent changes in metal ion concentration affected catalysis.

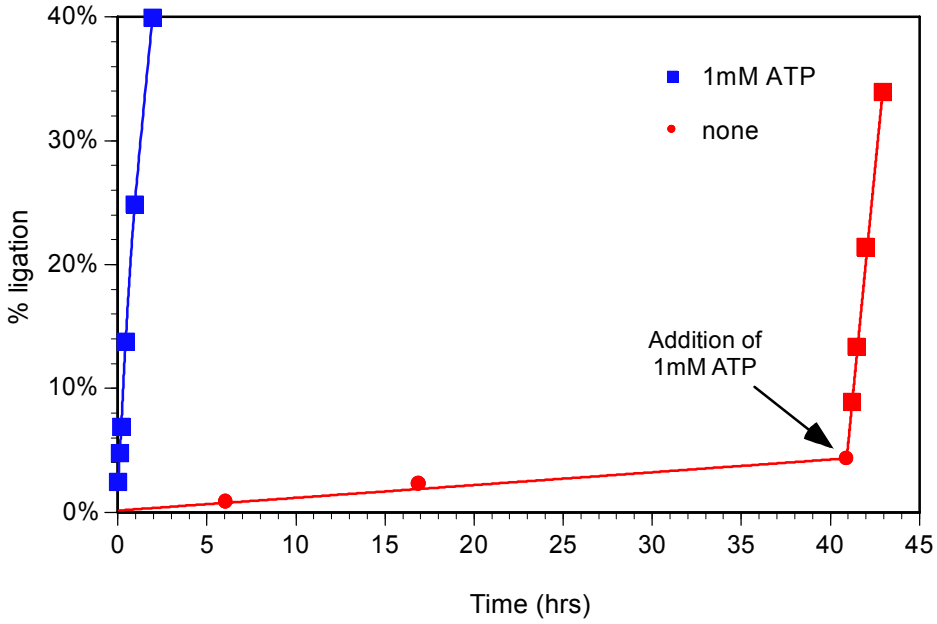


Figure 3-8. Extent of reaction as a function of time for trans-ATP.1. The blue line indicates the extent of reaction when ATP is included in the reaction mixture. The red line indicates the initial extent of reaction when ATP is excluded from the reaction mixture; ATP is then added at 41 hours.

As a final proof of allosteric activation, we examined the effect of the ATP concentration on the rate of ligation. The NMR structure had revealed that two ATP molecules were bound to the aptamer, and the symmetry of binding site suggested that they might bind cooperatively. Indeed, cooperative binding of ATP to the aptamer had previously been observed by our (Jhaveri et al., 2000) and other labs (Battersby et al., 1999). Consistent with these observations, the allosteric deoxyribozyme showed almost perfect cooperativity as a function of ATP concentration (**Figure 3-9**, red line). This was indicated by a Hill coefficient of 1.98. A value of 2.0 would be perfect. The data could not be fit if no cooperativity was assumed (**Figure 3-9**, blue line). The equilibrium constant for

ternary complex formation was 1.6×10^{-7} M^2, and further analysis of the data with the Adair equation suggested that the first binding constant was on the order of 10 mM, while the second was on the order of 10 μM. The initial K_d of the deoxyribozyme chimera is considerably larger than that of the aptamer alone (6 uM, Huizenga and Szostak, 1995). An increase in the apparent K_d has previously been observed with other effector-dependent ribozymes (Soukup and Breaker, 1999a; Robertson and Ellington, 2000; Soukup et al., 2000), and may reflect the increase in free energy required to drive a conformational transition in the catalytic core of the deoxyribozyme. Interestingly, Jose et al. (Jose et al., 2001) have previously shown that different effectors can act cooperatively on distinct allosteric sites; our results reveal a mechanism by which the same allosteric site can mediate cooperative induction.

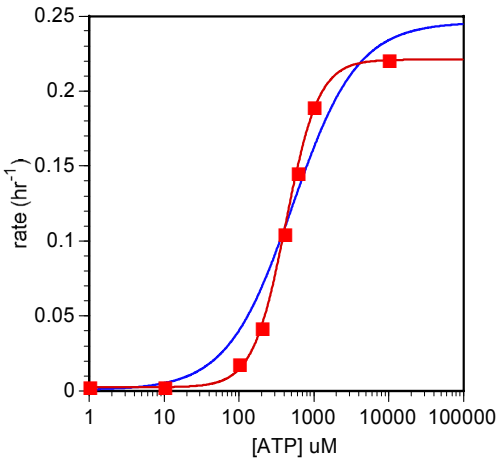


Figure 3-9. Activation of trans-ATP.1 as a function of ATP concentration. Individual rates were determined by the best fit of three initial time points, as described in the Methods and Materials. The red line was generated by non-linear regression analysis with the Hill equation: $v = v_o + (V_{max} x^n / K^n_{0.5} + x^n)$, where v is the rate, v_o is the initial rate, V_{max} is the maximum rate, $K_{0.5}$ is the equilibrium concentration at half-saturation, and n is the Hill coefficient. The blue line represents the best fit to the data assuming no cooperativity (n=1).

In the presence of saturating amounts of ATP, the ligation rate of trans-ATP.1 is 0.22 hr-1, which is only about three fold lower than that of the parental trans-construct mt14.wt (**Figure 3-3**; top, 0.70 hr-1), and similar to the basal rate of non-catalyzed template-directed ligation (**Figure 3-3**; lower, 0.19 hr-1) under the same conditions. However, in the absence of ATP, the initial rate of ligation of trans-ATP.1 is 0.0011 hr-1(**Figure 3-7**; red), which is over 100-fold slower than the non-catalyzed templated rate. The most consistent interpretation of these results is that in the absence of ATP, the ligation substrates are mis-aligned on their template while in the presence of ATP the conformational change in the aptamer aligns the substrate oligonucleotides. This hypothesis is also consistent with our previous finding that a single mutation to position 11 in the deoxyribozyme (**Figure 3-4**; mt14.2) could depress the rate of catalysis well below that of the background templated reaction.

The depression of background reaction rates is a novel mechanism for allosteric activation that has previously not been observed for nucleic acid catalysts. For example, Soukup and Breaker found that an FMN-activated hammerhead ribozyme still catalyzed phosphodiester bond cleavage ~100,000 fold faster than a corresponding background reaction (Soukup and Breaker, 1999a). Similarly, in the absence of effector, the rates of ligation of the FMN dependent L1 ligase variants selected by Ellington and Roberston have ligation rates that are at least 100-fold faster than that of the templated background reaction (Robertson and Ellington, 2000). Some protein catalysts depress the rate

of background reactions in order to direct reactivity. For example, carbamoyl phosphate synthetase and glutamine phosphoribosyl amidotransferase channel reactive intermediates to avoid hydrolysis (Huang et al., 2001). However, in general, protein catalysts activate substrates for reaction and promote the formation of high energy transition states rather than depressing the ground state of reactions.

It is also interesting to compare the design scheme of our ATP effector deoxyribozyme with the 'expansively regulated' ATP-effector dependent deoxyribozymes designed by Wang and Sen (Wang et al., 2002). Unlike our construct design in which the absence of effector appears to inactivate the deoxyribozyme by disrupting the core of the deoxyribozyme and misaligning the template, 'expansive regulation' relies solely on the disruption and subsequent restoration of the substrate binding site in the presence of the effector molecule. Thus, the low levels of activation seen with the expansively regulated ATP-effector dependent deoxyribozymes (2 to 30-fold) may result from the fact that the core of the deoxyribozyme is left in an active conformation and, therefore, still capable of reaction in the absence of effector. Nonetheless, while these two designs enable effector-dependent control of catalysis via two different mechanisms (effector-dependent template alignment versus effector-dependent substrate-binding), both designs center on the formation and stabilization of a three-way helical junction, suggesting that this may be a useful structure for the generation of other allosteric nucleic acid catalysts.

Optimization of allosteric deoxyribozyme ligases

In order to try to optimize the activation of the deoxyribozyme ligase, we designed a pool in which the stem region joining the deoxyribozyme and aptamer domains was randomized. This strategy was previously employed by Soukup and Breaker with the hammerhead ribozyme (Soukup and Breaker, 1999b) and by Robertson and Ellington with the L1 ligase ribozyme (Robertson and Ellington, 2000).

A DNA pool was synthesized based on the minimized cis-acting DNA ligase (**Figure 3-2**; C14.id4) with the ATP aptamer attached to the ligase through a randomized connecting stem (**Figure 3-10a**). The stem length was either 3 or 4 nucleotides in length and contained all possible combinations of sequences and lengths (102,400 variants). The in vitro selection was conducted using a two-stage selection procedure that has previously been employed in our laboratory and others (Soukup and Breaker, 1999b; Robertson and Ellington, 2000) (**Figure 3-10b**). In the first round of selection, 10 pmoles (~$6x10^7$ copies of each sequence) of the single stranded DNA pool was incubated with the 5'biotinylated substrate oligonucleotide in the absence of the effector molecule. This negative selection was allowed to proceed for 21 hr at which point ligated species were removed from the population by binding to a streptavadin agarose beads followed by centrifugation. The effector (1 mM ATP) and additional substrate were added directly to the eluant which contained the remaining unligated species. The positive selection was carried out for 1 hr after which the ligated species were again captured on steptavidin agarose. After stringent washing, the bound DNA

was selectively amplified and the ssDNA pool regenerated by biotin capture and alkaline denaturation.

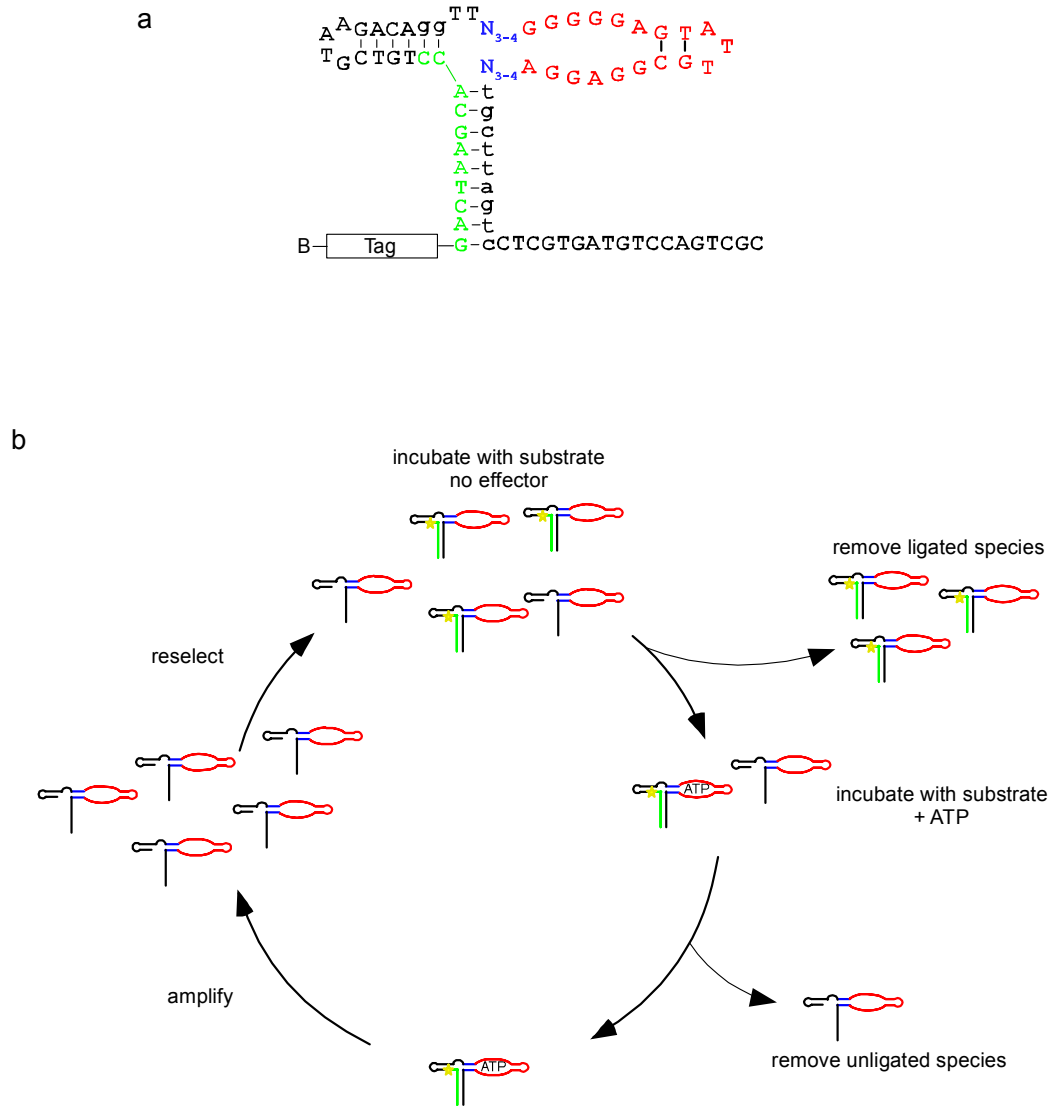


Figure 3-10. Optimization of effector-activated deoxyribozymes. **(a)** Pool construction and design. The aptamer portion is shown in red and the randomized stem region is shown in blue. The pool was designed to make a total of 11 base-pairs (lowercase) with the 3'end of substrate KSS2. **(b)** Selection scheme for isolation of ATP-dependent deoxyribozyme ligses. Selection protocol is described in detail in the Experiment Protcols.

The *in vitro* selection was carried out for four rounds under conditions of increasing stringency (**Figure 3-11a**). The pool was assayed after each round for the ability to ligate the substrate (KSS2) in the presence and absence of ATP (**Figure 3-11b**). An increase in the ligation activity in the presence of ATP was observed for the first two rounds of selection. However, the assay of the Round 3 pool indicated a loss in the overall level of activation. This loss was the result of a 3-fold increase in the rate in the absence of ATP compared to only a 2-fold increase in its presence. In order to minimize ATP-independent catalysis the stringency of Round 4 was increased further. The negative selection was carried out for 19 days and was accompanied by a 10-fold increase in the concentration of substrate KSS2 (1.5 μM vs. 0.15 μM). In addition, an alternative substrate (KSS8), which shares the same eleven 3' terminal nucleotides as substrate KSS2 but contains an alternative 5' tag sequence, was used for the positive selection. The use of the alternative substrate should have prohibited the amplification of any ligated species that survived the negative selection. Analysis of the Round 4 pool showed an increase in effector-dependent activation to over 30-fold. Interestingly, the rate of the ligation reaction in the presence of effector was almost 2-fold slower than observed in the previous round and similar to that observed in Round 2, suggesting that those ligases that had the greatest effector-dependence were not necessarily the fastest catalysts. Trade-offs between kinetic parameters during evolutionary optimization have previously been a hallmark of protein evolution (Cherry et al., 1999; Gonzalez-Blasco et al., 2000; Nielsen et al., 2001).

a

Round	negitive	positive	substrate (-/+)
1	21hrs	1hr	2/2
2	44hrs	30min	2/2
3	95hrs	30sec	2/2
4	454hrs	30sec	(10X) 2/8

b

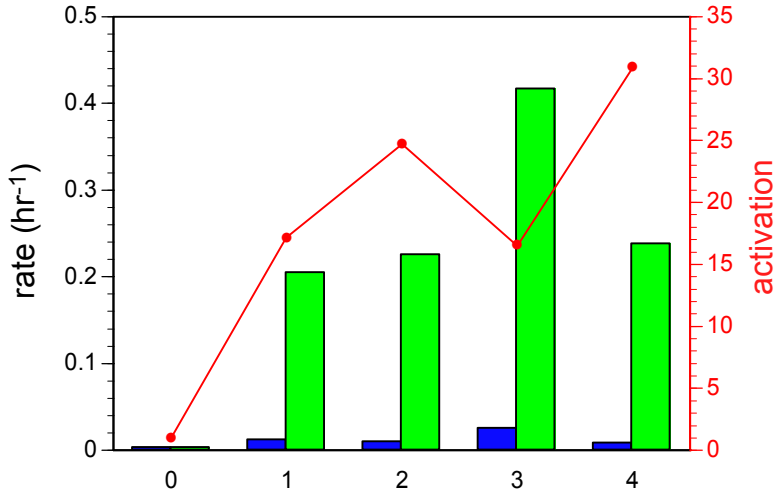
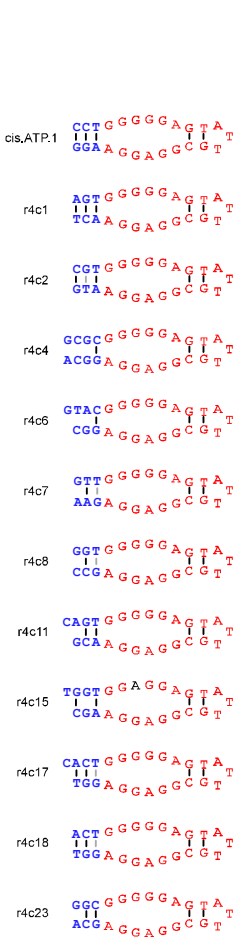


Figure 3-11. Selection of optimized ATP-effector dependent deoxyribozyme ligases. **(a)** Conditions used during the course of the selection. The lengths of both negative and positive selection steps are indicated. Substrate KSS2 (denoted here as '2') was used for both the (- ATP) and (+ ATP) selection steps except for the final round of selection in which the alternative substrate KSS8 (denoted here as '8') was used in the (+) selection. The concentration of substrate was 0.15 μM for all rounds of the selection except for the negative selection step in the final round where it was increased to 1.5 μM (denoted here as 10X) **(b)** Progress of the selection for ATP-dependent ligases. The initial rates of ligation of pools in the absence (blue) and presence (green) of 1 mM ATP are shown (scale on the left). The progression of the ratios of these rates is shown as a red line (scale on the right).

After four rounds of selection, the DNA pool was cloned and sequenced. Analysis of the Round 4 deoxyribozymes revealed the presence of multiple different clones in the population (**Figure 3-12**). Despite the fact the initial population was small, no consensus was observed. However, this diversity was

perhaps not that surprising considering that our rationally-designed, effector-dependent construct showed ~50-fold activation in cis (cis-ATP.1, **Figure 3-12**).



	cis			trans		
	activation	rate (hr^{-1}) (+) ligand	(-) ligand	activation	rate (hr^{-1}) (+) ligand	(-) ligand
cis.ATP.1	55	0.46	0.0085	250	0.28	0.0011
r4c1	13*	0.15	0.011	-	-	-
r4c2	340	0.26	0.0007	33	0.038	0.00012
r4c4	1*	0.003	0.0046	-	-	-
r4c6	51*	0.079	0.0015	26	0.051	0.002
r4c7	460	0.32	0.0007	41	0.16	0.004
r4c8	10*	0.41	0.04	-	-	-
r4c11	110	0.13	0.0011	12	0.044	0.0038
r4c15	60*	0.11	0.0018	-	-	-
r4c17	340	0.67	0.0020	400	0.30	0.00075
r4c18	270	0.28	0.0011	190	0.15	0.00078
r4c23	160	0.31	0.0019	-	-	-

Figure 3-12. Activation parameters for selected optimized ATP-dependent deoxyribozymes. Putative secondary structures of the allosteric aptamer domains of optimized deoxyribozymes are shown on the left. The anti-adenosine aptamer is shown in red, while the selected residues in the joining region are shown in blue. Additional mutations are shown in black. Initial rates were determined by the best first through three initial data points, except where indicated (*; rates based on a single data point).

Analysis of individual clones revealed a wide range of activation parameters ranging from almost no activation (r4c4) to over 450-fold activation (r4c7). In general, the rates of ligation in the absence of effector were significantly less than that of the background templated reaction (Figure 2; C14.id5, 0.13 hr-1). Clones that showed the most activation (r4c2, r4c7, r4c17, and r4c18) showed the largest decreases in rate in the absence of effector relative to the background reaction. The activated rates varied less and were not greater than those of the parental deoxyribozyme (**Figure 3-2**; C14.id4). Overall, these results were consistent with the hypothesis that the mechanism of activation relied primarily upon decreasing the rate of the background (effector-independent) reaction rather than increasing the rate of the catalyzed reaction.

While our best rationally designed construct had shown 250-fold activation for the trans-ligation reaction, it only showed 55-fold activation when the reaction was done in cis (**Figure 3-12**, cis.ATP.1, compare left and right column). Therefore, we assayed the selected variants for their ability to catalyze ligation reactions in trans, hoping to see a similar increase in activation (**Figure 3-12**, right). Unfortunately, almost all of the clones tested with the exception of r4c17 showed a decrease in their effector-dependence in trans. In most cases, the decrease appeared to be the result of a decreased rate in the presence of the effector, but in some cases (e.g., r4c7), it was due to an increase in the ligation rate in the absence of effector. These results suggest that there was no direct correlation between cis- and trans-activation rates. More importantly, it may illustrate an inherent limitation on the direct selection methods currently used in

most nucleic acid catalyst selections (Pan, 1997; Ellington and Robertson, 1999; Wilson and Szostak, 1999; Jaschke and Seelig, 2000). That is, by selecting for single-turnover (cis) reactions, it may prove difficult or impossible to identify catalysts that are exceptional at multiple-turnover (trans) reactions.

CONCLUSIONS

Application to biotechnology

Effector-dependent nucleic acid enzymes or aptazymes can be adapted to function as biosensors (reviewed in Hesselberth et al., 2000; Breaker, 2002). Breaker and his co-workers, for example have generated an array of effector-activated hammerhead ribozymes and showed the specific and quantitative detection of a variety of ligands, from cobalt to cyclic mononucleotides (Seetharaman et al., 2001).

Nucleic acid ligases should prove to be even more versatile biosensors, since they can potentially co-immobilize a wide variety of reporter molecules conjugated to oligonucleotide substrates (Marshall and Ellington, 1999). In addition, the ligation products of nucleic acid ligases are amplifiable and can be detected with high sensitivity by conventional amplification technologies such as PCR. For example the combination of effector dependent ligation and RT-PCR allowed for a 1000 fold increase in sensitivity (from 1 μM to 1 nM) in the level of ATP detected by the ATP dependent L1 ligase ribozyme (Robertson and Ellington, 1999). In addition, the greater stability of DNA and its ease of chemical synthesis when compared to RNA make DNA a better choice for most

diagnostic applications. Therefore, the demonstration that deoxyribozymes are amenable to many of the same methods used for the generation of allosteric ribozymes is an important step towards the continued generation of useful biosensors and biosensor arrays.

The small molecule effector dependent DNA ligases that we have selected performs as well as some of the best designed small molecule hammerhead and ligase ribozymes (Tang and Breaker, 1997; Robertson and Ellington, 1999; Soukup and Breaker, 1999b; Robertson and Ellington, 2000). Additionally, the ease with which a small (32 nucleotide) core catalytic domain could be detached from its substrate may allow for greater design flexibility allowing for the development of new assay formats such as the trans-ligation of short oligonucleotides bearing reporters to short oligonucleotides immobilized on solid supports or chips.

EXPERIMENTAL PROTOCOLS

Sequences and Primers

The sequence of the C14 deoxyribozyme ligase is I-TGACTTCGGTCAGGTGCTCGTGCATGAATCGAAGACAGGTTGCCGAGTCTCTCGTAGCGAGCAAGGATCGCTGCGATTGGGGCGAGGCTGCTTTTTGACGGCCAGATCGGTGCTCGTGATGTCCAGTCGC, where I-T denotes the 5' iodinated thymine. Single stranded DNA was generated from a double-stranded PCR product using the 5' primer 5I.22.90 (I-TGACTTCGGTCAGGTGCTCGTG) and the 3'primer 5B.18.90a (B-GCGACTGGACATCACGAG; where B is a 5' biotin), as previously described (Levy and Ellington, 2001; Levy and Ellington, 2002).

Deletion mutants C14.id3 I-TGTCGTAAGACAGGTTGCGC-GAAGCGCTGCTTTTTGACGGCCAGATCGGTG; C14.id4 I-TGTCGTA-AGACAGGTTGCGCGAAGCGCTGCTTAGTC; C14.id5 I-TGTCGTAAG-ACAGGTGCTTAGTC; trans-constructs mt14.wt CGAAGACAGGTT-GCGCGAAGCGCTGCTTAGTC; template.1 CGAAGACAGGTGCTTAGTC; mt14.1 CGAAGACAGGTTGCGCGAAGCGCCGCTTAGTC; mt14.2 CGAA-GACAGGCTGCGCGAAGCGCTGCTTAGTC; substrates 5I.8.C14m1 I-TGTCTTCG; KSS2 TACATGTCTATCGATCTGACTAAGCACC-PS, where PS denotes a 3' phosphorothioate; 5B-KSS2 TACATGTCTATCGA-TCTGACTAAGCACC-PS; KSS2.G41 TACATGTCTATCGATCTGACTAA-GCGCC-PS; 5B-KSS8 B-TGCTACTCATCTAGTCAGTCATCAAGAC-

TAAGCACC; pool primers 5I.16.N3-4 I-TGTCGTAAGACAGGTT; 5B.18.90.a B-GCGACTGGACATCACGAG; and ATP constructs trans-ATP.1 CGAAGACAGGTTCCTGGGGGAGTATTGCGGAGGAAGGTGCTTAGTC; trans-ATP.2 CGAAGACAGGTTCTGGGGGAGTATTGCGGAGGAAGTGCT-TAGTC; trans-ATP.3 CGAAGACAGGTTTGGGGGAGTATTGCGGAGGAAT-GCTTAGTC; trans-ATP.4 CGAAGACAGGTTGGGGGAGTATTGCGGAGG-ATGCTTAGTC were all synthesized in our laboratory on an Expedite 8909 DNA synthesizer (PE Biosystems, Foster City, CA) using standard phosphoramidite chemistry. All synthesis reagents were purchased from Glen Research (Sterling, VA). The 3' phosphorothioates were synthesized on 3' phosphate-CPG by replacing the normal oxidizing reagent with the sulfurizing reagent thiosulfonate. Oligonucleotides containing 5' I-dT were deprotected in NH_4OH at room temperature for 24 hrs; all other oligonucleotides were deprotected at 55°C for 16hrs. Trans-constructs mt14.3 CGAAGACAGGGTGCGCGAAGCGCT-GCTTAGTC; mt14.4 CGAAGACAGGATGCGCGAAGCGCTGCTTAGTC; mt14.5 CGAAGACAGG5MedCTGCGCGAAGCGCTGCTTAGTC; and mt14.6 CGAAGACAGGdUTGCGCGAAGCGCTGCTTAGTC were purchased from IDT (Coralville, Iowa). All oligonucleotides were purified by denaturing PAGE in the presence of 7 M urea prior to use.

Pool design and in vitro selection of allosteric deoxyribozyme ligases

The ssDNA pool TGTCGTAAGACAGGTTN_{3-4}GGGGGAGTATTGCG-GAGGAN_{3-4}TGCTTAGTCCTCGTGATGTCCAGTCGC; where the aptamer

domain is underlined, and N= A,G,C or T, was synthesized using an ABI 394 DNA synthesizer as previously described (Robertson and Ellington, 2000). Positions denoted N were generated by equi-volume mixing of phosphoramidites by the synthesizer. The pool was synthesized with a 5'I-dT moiety. After deprotection and gel purification the ssDNA was used directly for selection.

The selection protocol for effector-dependent deoxyribozyme ligases was derived from the original selection protocol for deoxyribozyme ligases (Levy and Ellington, 2001; Levy and Ellington, 2002) and selection protocols for effector-dependent ribozyme ligases (Robertson and Ellington, 2000; Robertson and Ellington, 2001) and is shown in Figure 10b. The selection protocol relies on coupled negative and positive selection steps. In the first round, 10 pmoles of the pool (final concentration 0.1 μM) was incubated with 15 pmoles of substrate 5B-KSS2 in selection buffer (500 mM NaCl, 50 mM Tris pH 7.4, 10 mM $MgCl_2$, 500μM DTT) in the absence of effector for 21 hrs. Ligated species were then removed from the population by passing the reaction through a column of streptavidin agarose (Sigma, St Louis Missouri). The eluant was replenished with 15 pmoles of substrate 5B.KSS2 and 100 pmoles of ATP (final concentration 1mM) and incubated at 25°C for 1 hr. This positive selection step was stopped by the addition of 1 ml of stop buffer (7 M urea, 300 mM sodium acetate pH 5.2). Ligated species were captured on streptavidin agarose in the presence of the stop buffer. Ligated species were selectively amplified directly from the resin using a selective 5' primer that contained the same sequence as the substrate used in the reaction and a common 3' primer. The selective PCR product served as a

template for regenerative PCR with a nested 5' primer containing iodine and a 3' primer containing biotin. Single-stranded DNA was isolated and used for additional rounds of selection and amplification as previously described (Levy and Ellington, 2001a; Levy and Ellington, 2002). Selection stringency was increased by increasing incubation times in the absence of effector and decreasing incubation times in the presence of effector (Figure 11a). For Round 4, the concentration of the substrate KSS2 in the negative selection was increased from 0.15μM to 1.5μM. In addition, to avoid contamination by ligated species from previous rounds of selection an alternative substrate, 5B-KSS8, was employed in the positive selection step in Round 4. This substrate shared the same eleven 3' terminal nucleotides as 5B-KSS2, but contained a different 5' tag sequence.

Cloning deoxyribozymes

PCR products from Round 4 were cloned using the Topo TA cloning kit (Invitrogen, Carlsbad, CA). The dideoxy method was used for sequencing and sequences were acquired on a CEQ 2000XL automated sequencer (Beckman Coulter, Fullerton, CA)

Ligation Assays

Ligation assays for all cis-acting deoxyribozymes (including deletion constructs, selection Rounds 0 though 4, and isolated effector-dependent clones) were carried out at 25°C with trace (~nM) amounts of 3' end radiolabeled ssDNA and 0.15 uM substrate, as previously described (Levy and Ellington, 2001). The ssDNA was 3' end-labeled using terminal deoxynucleotide transferase (Gibco

BRL, Gaithersburg, MD) and dideoxyadenosine 5'-[α-32P]-triphosphate (Amersham Parmacia Biotech, Piscataway, NJ). The ssDNA was heat-denatured at 70°C and cooled to 25°C in selection buffer prior to the addition of DTT-treated substrates and effector. The final concentration of DTT in reactions was 500 uM. Reactions were initiated by the addition of effector (10-100 mM ATP, pH7, or water in the case of mock reactions) and substrates.

Trans-ligation reactions were carried out in selection buffer at 25°C with 0.5 uM ssDNA constructs and 1.0 uM 5I.8.C14m1 with substrate KSS2. Constructs mt14.7, 14.8, and 14.9 are the same as constructs mt14.2, 14.1, and 14.wt, but these reactions were carried out with substrate KSS2.G41. The iodinated 5I.8.C14m1 was 3' end-labeled as described above. All reactions were carried out in selection buffer except for those in which the activity of KL.ATP.5 was measured as a function of ATP concentration; these reactions contained 50 mM $MgCl_2$ rather than 10 mM $MgCl_2$.

All reactions were terminated by the addition of 95% formamide gel-loading buffer. Ligated and unligated species were separated on denaturing 8% acrylamide gels containing 7 M urea and quantitated using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Ligation reactions were initially linear as a function of time, and pseudo-first order rates were determined by the best fit through three points (< 10% reaction) except where indicated. Ligation reactions typically proceeded to > 70% reaction. Unreacted species may have lost the iodine leaving group or folded into an unreactive conformation.

None of the trans-constructs were capable of performing multiple-turnover reactions. Reaction rates were therefore calculated assuming single-turnover, pseudo-first order kinetics. Rate analyses on c14.Trxn.4 were carried out 4 times, and the calculated rates differed by ± ~20%.

The cooperativity of effector-dependence was determined by non-linear regression using the program SigmaPlot (SPSS Science, Chicago, IL). Data was fit with the Hill equation:

$$v = v_o + (V_{max}\, x^n / K^n_{0.5} + x^n)$$

where v is the rate, v_o is the initial rate, V_{max} is the maximum rate, $K_{0.5}$ is the equilibrium concentration at half maximal saturation, and n is the Hill coefficient. Calculated values were as follows; v_o = 0.002 ±0.002 hr^{-1}, V_{max} = 0.22 ±0.003 hr^{-1}, $K_{0.5}$ = 430±10.0 M^2, n = 1.98±0.07. Individual binding constants were estimated by non-linear regression using the Adair equation for two binding sites:

$$= \frac{\left(\frac{[s]}{k_1} + \frac{2*[s]^2}{k_1 k_2}\right)}{2*\left(1 + \frac{[s]}{k_1} + \frac{[s]^2}{k_1 k_2}\right)} \times V_{max} + v_0$$

where k_1 and k_2 are the binding constants, s is the concentration of effector, Vmax is the maximum rate and vo is the initial rate. The calculated values were as follows: k_1 = 13±24 mM, k_2 = 14±29 μM, V_{max} = 0.22±0.003 hr^{-1} and v_o = 0.002±0.002 hr^{-1}.

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Chapter 4: Peptide-Templated Nucleic Acid Ligation

INTRODUCTION

A critical problem that remains in understanding life's origins is to figure out how the self-replication of the various prebiotic materials arose. While the self-replication of short oligonucleotides has been demonstrated by von Kiedrowski (Sievers and von Kiedrowski, 1994) and Orgel (Zielinski and Orgel, 1987), it is still unclear how these short replicators may have eventually expanded to become a RNA world (Levy and Ellington, 2001a).

In addition to self-replicating nucleic acids, the design of self-replicating leucine zipper peptides has also been demonstrated (Lee et al., 1997; Severin et al., 1997). Without a doubt, the precursors necessary for the synthesis of simple peptides were available on the early earth (Miller, 1953), and a variety of potentially prebiotic synthetic methods for peptides have been described (Fox and Harada, 1960; Ferris et al., 1996; Liu and Orgel, 1997; Liu and Orgel, 1998). This has led to the suggestion that peptides might have played a role early in origins by potentially aiding in RNA catalysis and replication (Orgel, 1989; Wong, 1991; Di Giulio, 1997; Bergstrom et al., 2001). Certainly, peptides played a role in the rise of protein catalysis and the invention of translation (Woese, 1967; Crick, 1968; Orgel, 1968; Orgel, 1989). However, the design of peptide replicators suggests the potential for an even earlier role in evolution, perhaps even preceding that of nucleic acid replicators.

To the extent that peptides and small nucleic acids interacted early on in the evolution of life, we have attempted to design a nucleic acid – peptide cross-catalytic system. As a first step in this process, we have used peptides to template the ligation of nucleic acids (Levy and Ellington, 2003).

RESULTS AND DISCUSSION

A model system for peptide-templated nucleic acid ligation

In order to determine if peptides are capable of serving as templates for the ligation of nucleic acids, we chose a modern, well-characterized peptide:nucleic acid interaction, of the HIV-1 Rev protein and the Rev response element (RRE), as our model system. The HIV-1 Rev protein is known to bind tightly (K_d ~1 nM) to a short stem-internal loop-stem RNA structure within the RRE known as the RBE (Zapp and Green, 1989; Bartel et al., 1991; Iwai et al., 1992; Tan et al., 1993). Direct interaction of the RNA stem-loop with the protein is mediated through an arginine rich motif (ARM) comprised of 17 amino acids (**Figure 4-1a**). When synthesized in isolation, this short peptide adopts an alpha-helical conformation and is still capable of specifically binding the RBE (Tan et al., 1993).

In vitro selection experiments have been used to identify RNA aptamers that bind to the Rev protein with even higher binding affinities (K_d ~0.1 nM) than the native RBE (Bartel et al., 1991; Giver et al., 1993b). The NMR structure of a 35 nucleotide RNA aptamer variant from this selection complexed with the isolated 17 amino acid Rev peptide indicats that, like the RBE, the aptamer

interacts with the alpha-helical conformation of the peptide (**Figure 4-1c,d**) (Ye et al., 1996). The aptamer:peptide complex is similar to that formed between the peptide and the wild type RBE, with the peptide lying in a widened major groove formed from non-canonical G:A and A:A base pairings. As with the RBE, the arginine residues of the peptide make contacts with phosphates on both sides RNA duplex (**Figure 4-1c**). In addition to sharing some sequence and structural similarity with the wild type RBE, the selected aptamer also contains an arginine-binding pocket composed of and U:A;U base triple(**Figure 4-1b**, circles; **Figure4-1c,d**, red) The arginine-binding pocket consists of a small bulge loop adjacent to G:C and A:U base-pairs. Arginine forms a specific interaction with the Hoogsteen face of the guanosine residue in the G:C base-pair, and a uridine in the bulge-loop then forms a base triple with the A:U base-pair pulling negatively charged phosphates in the bulge-loop into apposition with the positively charged guanidino group of arginine. This interaction is similar to the tertiary structure found between another HIV-1 ARM peptide, the TAR element, and its cognate RNA (Ye et al., 1996; Patel, 1999)

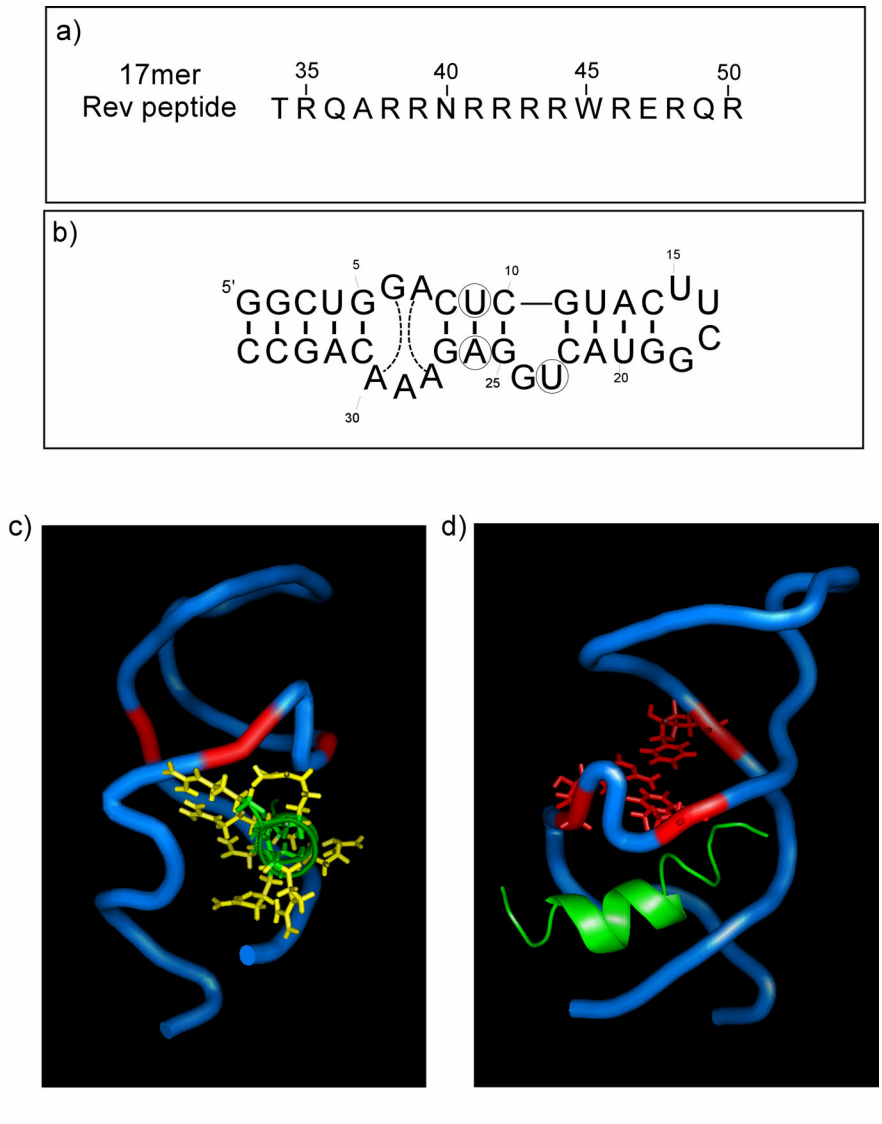

Figure 4-1. Specific peptide:RNA interaction of the anti-Rev aptamer and the 17amino acid Rev peptide. **(a)** The sequence of the 17-mer HIV-1 Rev arginine-rich motif (residues 34-50 of the HIV-1 Rev protein). **(b)** The sequence and secondary structure of an anti-Rev aptamer (Giver et al., 1993a; Ye et al., 1996). Dotted lines indicate non-Watson-Crick base pairs observed in the NMR structure (Ye et al., 1996). Circled residues participate in a base-triple interaction. **(c)** Ribbon drawing of the structure between the arginine side chains (yellow) of the Rev ARM peptide (green) and the anti-Rev aptamer (blue). Residues involved in the U:A:U triple are shown in red. **(d)** Alternate view of the Rev ARM peptide and the widened major groove of the anti-Rev aptamer. Stuctures adapted from (Ye et al., 1996).

The close association of the alpha-helical Rev ARM with the major groove of the anti-Rev aptamer, in particular the fact that specific arginine contacts were located on both sides of the RNA duplex and that binding of the ARM peptide stabilized the formation of the arginine-binding pocket, suggested that it might be possible for the peptide to serve as a template and appropriately align two aptamer fragments for ligation. An ideal site for dividing the aptamer in two appeared to be between two canonical base pairs (Figure 4-2, yellow) in a region adjacent to the arginine pocket. We hypothesized that in the presence of the Rev ARM peptide, the arginine-binding pocket would form, the overall conformation of the aptamer would be stabilized, and the half-aptamers would be aligned for ligation.

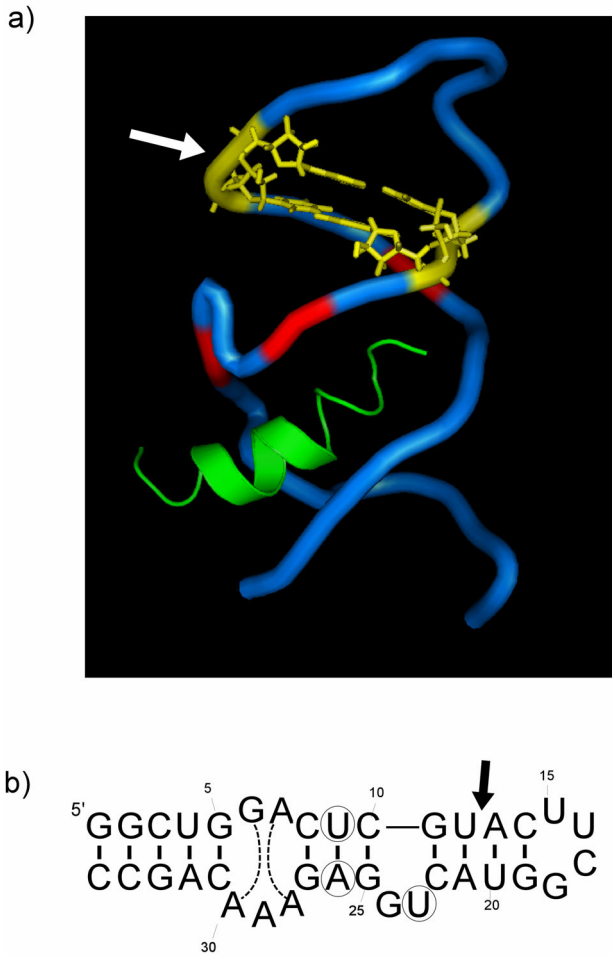

Figure 4-2. Break site for testing peptide templates nucleic acid ligation. **(a)** Initial break site (white arrow) in the aptamer (blue) was introduced between two canonical base pairs (yellow) with in a short stem adjacent to the U:A:U triple (red). The peptide is shown in green. **(b)** The position of the break site (black arrow) on a map of the anti-Rev aptamer secondary structure. Labels are as in Figure 4-1b. Structures adapted from (Ye et al., 1996)

In order to test this hypothesis we examined the ability of the Rev ARM to assist two different types of ligation events: one in which RNA oligonucleotide termini were transiently activated for ligation, and one in which RNA oligonucleotide termini were more stably activated. Although it cannot be known

what activating or leaving groups may have been present prebiotically or during early evolution, by looking at these two reaction classes hoped to determine whether peptide-assisted ligation might have been available to the earliest replicators.

Peptide-templated ligation with transient activation

Cyanogen bromide activation and ligation is known to occur on a timescale of seconds to minutes (Dolinnaya et al., 1991; James and Ellington, 1997), and thus may be a reasonable mimic of transient activations that might have occurred during the evolution of self-replicating oligonucleotides.

The Rev ARM and half-aptamers were pre-incubated to allow complex formation to occur. The complexes were then treated with cyanogen bromide, which quickly promotes the ligation of appropriately aligned 5' phosphates and 3' hydroxyls (Dolinnaya et al., 1991; Shabarova et al., 1991). In line with this expectation, a time-course of template-directed oligonucleotide ligation showed maximal ligation at the first time-point monitored (30 seconds). No further increase in ligation was observed as a function of time.

In the presence of the Rev ARM, the ligation of the anti-Rev half-aptamers (rev.35A12 and rev.35B23, (**Figure 4-3a**) was substantially enhanced (**Figure 4-3b**). Ligation increased as a function of peptide concentration. In control experiments in which half-aptamers had no relationship to the aptamer sequence (**Figure 4-3c**) little or no peptide-mediated ligation was observed. At high peptide concentrations, however, a band appeared that was slightly different in size than the correctly ligated oligonucleotide pair. This product formed even

when only one aptamer fragment was present (**Figure 4-3d**) suggesting it represented a covalent linkage between the peptide and the activated oligonucleotide. In order to test this hypothesis, the ligation reactions were carried out at a high peptide concentration in the presence and absence of a non-specific competitor, tRNA (**Figure 4-3b, c, d**, right hand lanes). In the presence of tRNA, the higher molecular weight species disappeared, and only peptide-templated ligation of the anti-Rev aptamer was observed. This observation is consistent with the tRNA acting as a buffer to disrupt the non-specific interactions between the Rev ARM and the half-aptamer. However the presence of competitor tRNA also decreased the efficiency of templated ligation. This is not completely unexpected given that the highly basic Rev ARM is known to associate non-sepcifically with a RNA molecules (Tan et al., 1993). Therefore, it is not surprising that a 20-fold excess of tRNA acted as a buffer that drew the peptide away from its specific target. Nonetheless, as expected, the decrease in non-specific binding in the presence of tRNA was greater than the decrease in specific binding.

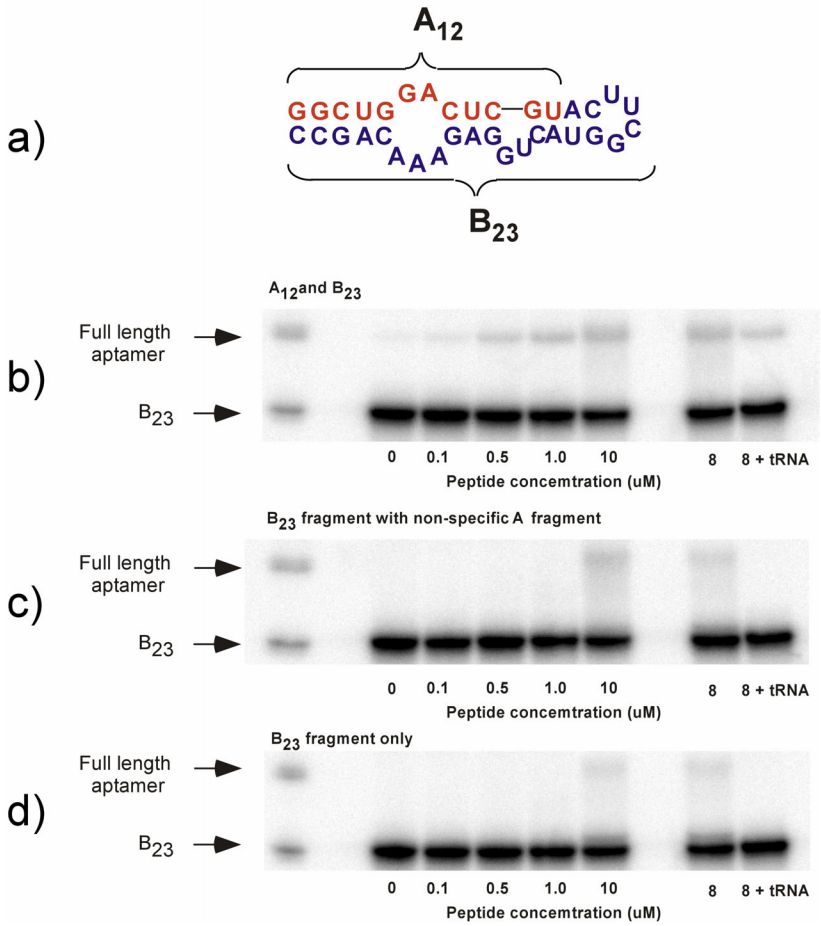

Figure 4-3. Peptide-templated ligation with cyanogen bromide. **(a)** Sequences of the half-aptamers A12 and B23. **(b)** Ligation of A12 and B23 as a function of peptide concentration. Transfer RNA was added to some samples ('+ tRNA') as described in Materials and Methods. The positions of the radiolabeled substrate B23 and the radiolabeled, ligated product are indicated. The peptide-RNA conjugate product migrates near the full length aptamer. **(c)** Ligation of B23 with a non-specific A fragment Ansp as a function of peptide concentration. **(d)** Ligation of B23 as a function of peptide concentration.

In addition to the Rev peptide we also examine the ability of other peptides to serve as a template for the ligation reaction. As shown in **Figure 4-4**, these other peptides had little or no effect on the amount of full length aptamer formed. This was true not only for the relatively unrelated peptide bradykinin,

but also for another arginine-rich motif, the HIV-1 Tat ARM. While all arginine-rich motifs can non-specifically bind to RNA molecules due to electrostatic interactions (Daly et al., 1993; Tan et al., 1993; Tao and Frankel, 1993), it appears that it is the specific tertiary interactions between the anti-Rev aptamer and the Rev ARM that lead to peptide-templating of cyanogen bromide-catalyzed ligation.

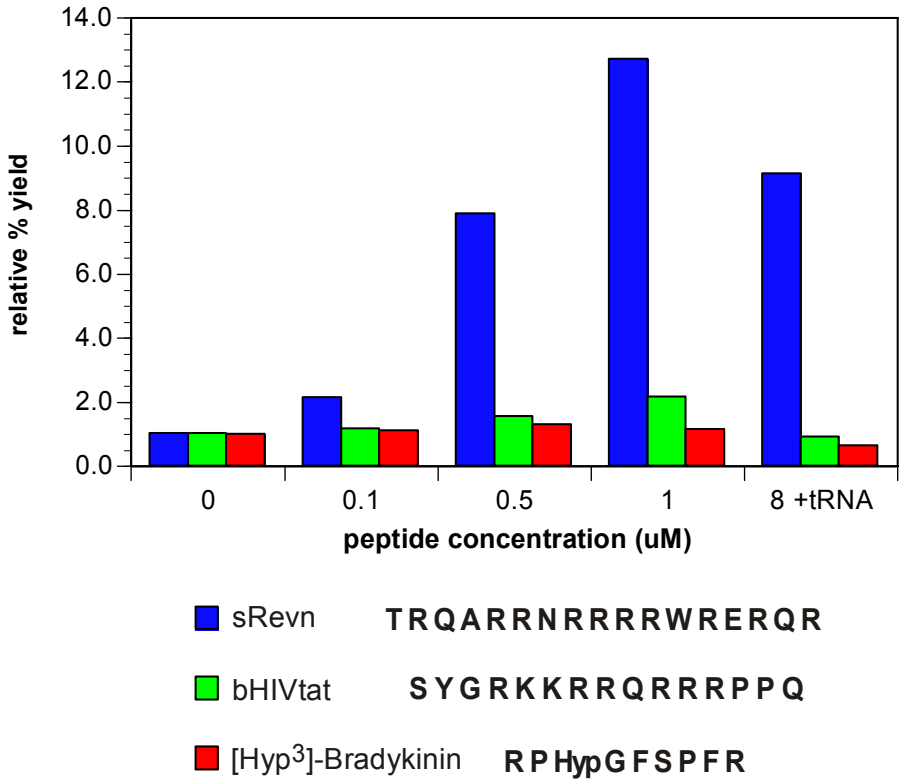

Figure 4-4. Peptide specificity of ligation with cyanogen bromide. Increases in yield relative to standard reactions carried out in the absence of peptide are shown for the HIV-1 Rev ARM (blue bars), the HIV-1 Tat ARM (green bars), and [Hyp3]-bradykinin (red bars).

Peptide-templated ligation with stable activation

In addition to looking at transient activation and ligation events, we also wanted to examine whether the Rev ARM could also assist in the ligation of more stably activated ligation junctions. To do this, we chose to use the phosphorothioate-iodine ligation chemistry developed by Eric Kool (Xu and Kool, 1997) and used previously by our lab for the selection of a DNA ligase (Levy and Ellington, 2001b; Levy and Ellington, 2002).

Reagents for the synthesis of oligonucleotides containing 3' phosphorothioate and 5' iodo-thymidine moieties are commercially available making this type of activation simple, as it requires no additional chemical modifications after synthesis. However, the incorporation of these chemical modifications into the half-aptamer molecules required the insertion of deoxyribose residues to prevent hydrolysis of the ligation product, a bridged internucleotide 5'-phosphorothioate (Kuimelis and McLaughlin, 1995). In addition, the iodinated residue is only available as a deoxythymidine (5'I-dT) which also required a modification of the original aptamer sequence. In order to determine if the introduced mutations affected binding, modified RNA containing uridine residues at the same positions that would contain thymidine residues were synthesized by run off transcription. The mutant aptamers were assayed for their ability to bind to the Rev protein (**Figure 4-5**). Consistent with results from previous in vitro selection experiments (Giver et al., 1993a), the region adjacent to the hairpin loop (**Figure 4-2a,** yellow) could be altered as long as base-pairing was maintained.

construct	relative binding affinity
rev.35	1.0
rev.rmut.3	0.7
rev.rmut.5	0.8
rev.mut.CC	0.03

Figure 4-5. Relative binding affinity of mutant anti-Rev aptamers. Binding affinities are reported relative to the wild type aptamer. Binding assays were conducted as described in the Experimental Protocols. Sequences of the aptamers are given in the Experimental Protocols.

Based on these results, mutant anti-Rev half-aptamers were synthesized with the requisite sequence modifications. The 5' half-aptamer contained a uridine to deoxythymidine change at position 12 and terminated in a 3' phosphorothioate. The 3' half-aptamer began with a 5' iodine, and contained an adenosine to deoxythymidine change at position 13 and a uridine to adenosine change at position 20 (**Figure 4-6a**; mut.3). Three negative controls that contained the same breakpoint were also synthesized (**Figure 4-6a**; mut3B, C, and D). Each of the negative control constructs contained mutations in residues that had previously been identified to be critical for binding (Bartel et al., 1991; Iwai et al., 1992).

Wild-type and mutant half-aptamers were assayed for their ability to ligate in the absence and presence of the Rev ARM (**Figure 4-6b**). The Rev ARM enhanced the ligation of the wild-type half-aptamers by 7.6-fold in the standard assay, but did not enhance the ligation of the negative controls. The effect appeared to be specific to the Rev ARM, as non-cognate peptides including other arginine-rich sequences such as the Tat ARM did not activate ligation (**Figure 4-6b**). As with cyanogen bromide-catalyzed ligation, it appeared as though the

ligation of stably-activated oligonucleotides could only be templated by specific peptide:RNA interactions.

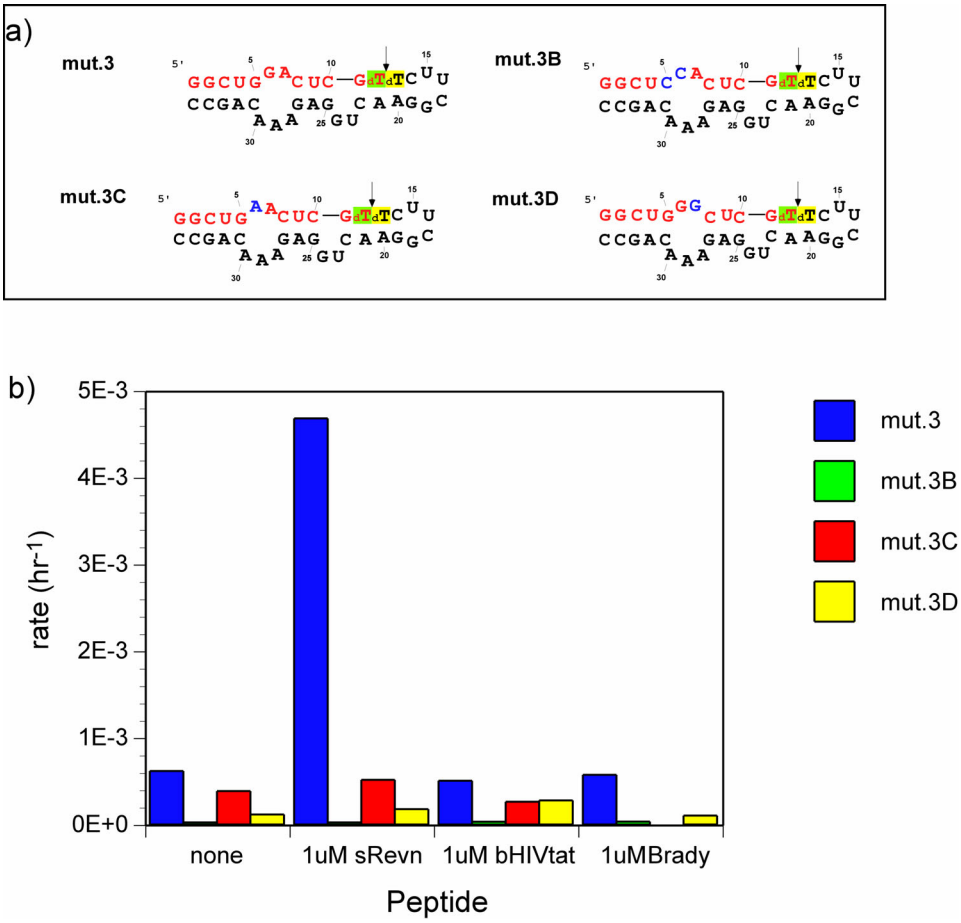

Figure 4-6. Peptide-templated ligation forming a bridged internucleotide 5'-phosphorothioate linkage. **(a)** A series of RNA variants were synthesized based on the same half-aptamers described in Figure 4-2 and 4-3a. The A12 equivalent is in red, and the B23 equivalent is in black; the ligation junction is indicated by an arrow. Mut.3 was wild-type except for the presence of deoxythymidine residues (in green and yellow highlights) at the ligation junction. Mut.3B was similar to Mut.3, but contained G5C and G6C substitutions (blue). Mut.3C contained a G6A substitution (blue). Mut.3D contained a A7G substitution (blue). **(b)** The influence of peptide sequence on the rates of variant ligation. Rates were measured in a standard assay for the RNA variants in the presence of either the Rev ARM (sRevn), the HIV-1 Tat ARM (bHIVtat), or the [Hyp3]-bradykinin peptide (Brady). The color-coding of the variants is shown to the right of the figure.

While the ligation reaction appears to be specific for both the RNA and peptide portions of the complex, it is unclear to what extent peptide-templating is generalizable. Therefore, we also broke the anti-Rev aptamer on the other side of the helix, creating a junction between a 3' deoxyadenosine residue (phsophorothioated) a 5' deoxythymidine (iodinated; **Figure 4-7a**). Again, the ligation of the half-aptamers appears to be specific for both the peptide and the RNA partners with an enhancement in the rate of ligation of 5.9 fold over ligation in the absence of peptide (**Figure 4-7b**).

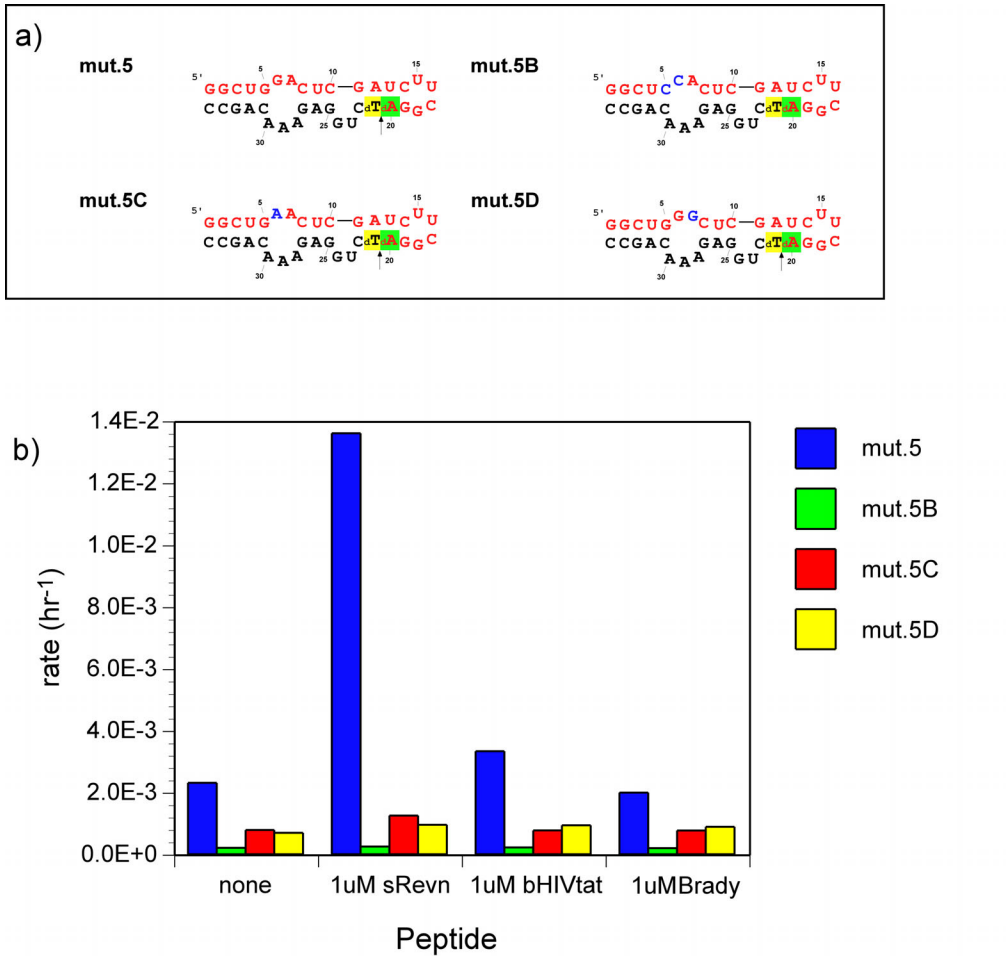

Figure 4-7. Peptide-templated ligation of alternative constructs that form a bridged internucleotide 5'-phosphorothioate linkage. **(a)** A series of RNA variants were synthesized based on half-aptamers broken between residues 20 and 21. Mutations and color-coding are as in Figure 4-6. The ligation junction is indicated by an arrow. **(b)** The influence of peptide sequence on the rates of variant ligation. Peptides and color-coding are as indicated..

CONCLUSIONS

Implications for origins

The catalytic capabilities of RNA and the importance of RNA-based cofactors in biology (like NAD and FAD) (White, 1976) strongly point to the existence of an RNA world (Gesteland and Atkins, 1993). However, problems with the prebiotic synthesis of nucleosides (Fuller et al., 1972a; Fuller et al., 1972b), the instability of ribose (Larralde et al., 1995), and the difficulty with prebiotic polymerizations suggest the potential for a "pre-RNA world"(Lazcano and Miller, 1996). If we believe that an RNA world did indeed exist, then any precursor genetic material would have eventually succumbed to RNA. To this end, information transfer between RNA and DNA, and a variety of nucleic acid analogues (prebiotic or not) such as PNA (peptide nucleic acids) (Bohler et al., 1995; Schmidt et al., 1997) HNA (hexose nucleic acid) (Kozlov et al., 1999) and TNA (threose nucleic acid) (Chaput et al., 2003) and other nucleic acid like polymers (Chaput and Switzer, 2000) have been demonstrated indicating that such genetic takeovers are possible.

Recently Ghadiri and co-workers have demonstrated that peptides can template their own ligation (Lee et al., 1997; Severin et al., 1997) leading to the possibility that peptide replicators may have played a role in the origin of life. Even if this were the case, it seems that nucleic acid replicators would have taken over from peptide replicators at some point. One way in which this transition could have occurred would be if peptides templated the production of particular

nucleic acids. In this model, pre-existing replicative peptides would have built the first nucleic acid templates, which could then have replicated further either with or without assistance from peptides. The demonstration that peptides can specifically template the ligation of longer nucleic acids provide experimental support for this model.

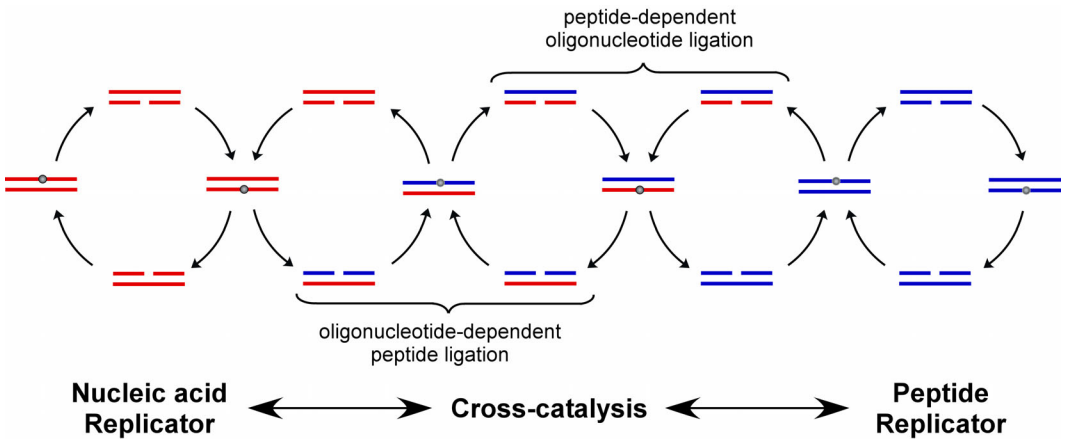

Figure 4-8. Model for the co-replication of peptides and nucleic acids. A nucleic acid replicator (red) and a peptide replicator (blue) may be capable of catalyzing the ligation of one another via peptide-directed oligonucleotide ligation (as demonstrated in this chapter) and oligonucleotide-directed peptide ligation reactions. Ligation junctions are indicated by gray circles.

By extension from our results, it should be possible to design, or even select for oligonucleotides that can assist in the template-directed replication of peptides via activated amino acids. Ribozymes capable of peptidyl transfer have already been demonstrated (Illangasekare and Yarus, 1999; Sun et al., 2002), and there is substantial evidence that the ribosome is indeed a ribozyme (Moore and Steitz, 2003). To the extent that this is possible, then these results provide a potential mechanism by which peptides and oligonucleotides may have interacted

and potentially reciprocated both sequence and function. In this regard, it is interesting to think about our results with respect to the scenario set out by Eigen in his description of hypercycles and the origin of translation (Eigen and Schuster, 1977) in which peptides assist in the replication of nucleic acids, which in turn catalyze the synthesis of peptides. To the extent that different peptides can in fact template and catalyze the replication of different nucleic acids (and vice versa), it may be possible to eventually build a hypercycle similar to the one generically described in **Figure 4-8**. Our results lay the foundation for the design and construction of more complex peptide:RNA molecular ecosystems.

EXPERIMENTAL PROTOCOLS

RNA Synthesis

Oligoribonucleotide synthesis was carried out on an Expedite 8909 synthesizer (PE Biosystems, Foster City, CA) using standard phosphoramidite chemistry. All synthesis reagents were purchased from Glen Research (Sterling, VA). RNA molecules bearing a 3' phosphorothioates were synthesized on 3' phosphate-CPG and oxidized during the first cycle of synthesis with the sulfurizing agent thiosulfonate (0.5 M in acetonitrile). All oligoribonucleotides were deprotected in 3:1 NH_4OH:EtOH for 17 hr at 55° C except for the constructs bearing a 5'-iodothymidine, which were deprotected for 24 hr at room temperature. Samples were lyophilized, and the 2'-hydroxyl was deprotected according to previously published methods by the addition of 250 μL of N-methylproyrolidone:Triethylamine:Triethylamine trihydrofluoride at a ratio of 2:1:1.3 at 65° C for 1.5 hrs (Wincott et al., 1995). Oligoribonucleotides bearing a 5'-iodothymidine were deprotected at room temperature for 24 hrs. After the addition of 25 μL 3 M NaOAc (pH 5.2), samples were butanol-precipitated and purified on 15% denaturing (7 M urea) polyacrylamide gels.

Ligations with CNBr

The half-aptamer constructs Rev.35A12 (5'GGCUGGACUCGU), Rev.35B23 (5'ACUUCGGUACUGGAGAAACAGCC) and the non-specific aptamer fragment A_{nsp} (GAUUCGUUGAGGG) were synthesized as described above. The Rev peptide (suc-TRQARRNRRRRWRERQR-n, where suc is an

amino-terminal succinate, and n a carboxy-terminal amide) and the HIV-Tat peptide (B-SYGRKKRRQRRRPPQ, where B is an amino-terminal biotin) were purchased from Genmed Synthesis, Inc. (San Francisco, CA) and purified by HPLC on a 250 mm x 4.6 mm Vydac C18 protein and peptide column using a liner gradient of 10-40% Solvent B (A: 0.1% TFA in H2O; B: 0.1% TFA in acetonitrile). The [Hyp3]-bradykinin peptide (RPHypGFSPFR; where Hyp is hydroxyproline) was purchased from Novabiochem (Laufelfingen, Switzerland) and used without further purification. Peptide concentrations were determined via absorption measurements at 280 nm.

Ligation reactions were conducted at 0° C in 0.25 M MES:$(C_2H_4)_3N$ pH 7.5, 33 mM NaCl, and contained final concentrations of 10 uM Rev.35A12 and 1 uM Rev.35B23. Rev.35B23 was phosphorylated using T4 polynucleotide kinase (Invitrogen, Carlsbad, CA) and adenosine 5'-[γ-^{32}P] triphosphate (>7000Ci/mmol ICN, Irvine, CA). The aptamer fragments were combined in reaction buffer, denatured for 3 min at 70° C and slowly cooled to 23° C. After the addition of peptide and / or E. coli tRNA (Boehringer Mannheim, Indianapolis, IN) the reactions were placed at 0° C and equilibrated for 2 hr. Ligation was initiated by the addition of 1/10 volume 5 M cyanogen bromide (CNBr) in dry acetonitrile and allowed to proceed for 2 min. Reactions were stopped by diluting them in 100 uL of 7M urea, 300 mM NaOAc (pH 5.2), and 3 mM EDTA. Extraction with phenol:chloroform (1:1) removed the peptide and prevented it from interfering with the electorphoretic mobility of RNA molecules. Following ethanol precipitation, the ligated and unligated species were separated on a denaturing (7

M urea) 20% acrylamide gel and quantitated using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Filter Binding Assays

The 35-nucleotide Rev aptamer and the mutant aptamers Rev.rmut.3, Rev.35.CC and Rev.rmut.5, were synthesized by run-off transcription of double-stranded DNA templates using the Ampliscribe T7 Transcription Kit (Epicentre Technologies, Madison, Wisconsin). 1 μL uridine 5'-[α-^{32}P] triphosphate (3000 Ci/mmol, NEN LifeScience Products) was added to each 10 μL transcription reaction. Transcription products were gel-purified and precipitated. The sequences of the transcribed aptamer and mutants were as follows: Rev.35, 5' GGCUGGACUCGUACUUCGGUACUGGAGAAACAGCC; Rev.rmut.3, 5' GGCUGGACUCGUUCUUCGGAACUGGAGAAACAGCC; Rev.35.CC, 5' GGCUCCACUCGUACUUCGGUACUGGAGAAACAGCC; Rev.rmut.5, 5' GGCUGGACUCGAUCUUCGGAUCUGGAGAAACAGCC. HIV-1 Rev protein was prepared as described by Orsini et al.(Orsini et al., 1995). The Rev protein was purified on SP Sepharose (BioRad, Hercules, CA) and its identity was confirmed by SDS-PAGE and mass spectrometry.

Filter-binding assays were conducted as previously described (Jhaveri and Ellington, 2000). In brief, radiolabeled RNA (0.1 uM final concentration) was incubated with Rev protein (0.5 uM final concentration) and tRNA (2.0 uM final concentration) in 50 uL of 50 mM Tris pH 8.0, 50mM KCl for 2 hrs at room temperature. The solution was filtered through stacked nitrocellulose and nylon membranes and washed with 2 x 200 uL buffer. The fraction of RNA bound by

the protein was determined by using a Phosphorimager to compare the counts retained on the nitrocellulose and nylon membranes. Binding affinity is expressed relative to the wild type 35-mer RNA.

Phosphorothioate-Iodine Ligation Reactions

Half-aptamer constructs were synthesized as described above. The sequence of the constructs were: mut.3.1, 5' GGCUGGACUCGdT-PS; mut.3.2, 5' I-dTCUUCGGAACUGGAGAAACAGCC; mut.3B.1, 5' GGCUCCACUC-GdT-PS; mut.3C.1, 5' GGCUGAACUCGdT-PS; mut.3D.1, 5' GGCUGGGCU-CGdT-PS; mut.5A.1, 5' GGCUGGACUCGAUCUUCGGdA-PS; mut.5.2, 5' I-dTCUGGAGAAACAGCC; mut.5B.1, 5' GGCUCCACUCGAUCUUCGGdA-PS; mut.5C.1, 5' GGCUGAACUCGAUCUUCGGdA-PS; and mut.5D.1, 5' GGCUGGGCUCGAUCUUCGGdA-PS, where I-dT denotes a 5' iodinated deoxythymidine, dA denotes deoxyadenosine, dT denotes deoxythymidine, and PS denotes 3' phosphorothioate.

Ligation reactions were carried out at 25° C in 50 mM Tris pH 8.0, and 50 mM KCl. The 5'-iodinated aptamer fragments were radiolabeled at their 3' termini using poly-A polymerase (USB, Cleveland, OH) and dideoxyadenosine 5'-[α-32P] triphosphate (>5000 Ci/mmol; Amersham Pharmacia Biotech, Piscataway, NJ). Radiolabeled, 5'iododinated fragments (0.5 uM final concentration), tRNA (250 ng/uL final concentration) and peptide (1uM final concentration) were combined in reaction buffer. Reactions were initiated by the addition of the 3' phosphorothioated aptamer fragments (1.0 uM final concentration) and stopped by the addition of 95% formamide gel loading buffer.

Ligated and unligated species were separated from one another on denaturing 20% acrylamide gels containing 7 M urea and 35% formamide. The extent of ligation was quantitated using a Phosphorimager. Ligation reactions were initially linear as a function of time, and pseudo-first-order rates were determined by the best fit line through three points that occurred prior to 10% of the substrate reacting.

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Chapter 5: Design, Evolution and Exponential Growth in a Cross-Catalytic Deoxyribozyme Cycle

INTRODUCTION

Molecular replication is not only an indispensable life process but also a valuable tool for the biotechnologist in search of new ways of generating and amplifying signals. In biology, molecular replication and signal generation is dominated by protein enzymes which partake in a wealth of signal cascades providing both positive and negative feedback loops. The discovery, evolution and design of nucleic acid catalysts raises the possibility that similar nucleic acid based auto-catalytic systems and signaling cascades might also be designed.

Nucleic acid based autocatalytic cycles were first demonstrated by von Kiedrowski (von Kiedrowski, 1986) and Orgel (Zielinski and Orgel, 1987) who showed that short palindromic oligonucleotides can serve as templates for the ligation of shorter oligonucleotides. Further experiments from these labs have shown that short oligonucleotide templates are also capable of semi-conservative (cross-catalytic) replication (Sievers and von Kiedrowski, 1994), and that substrates as short as single nucleotides can be utilized (Inoue and Orgel, 1983; Inoue et al., 1984; Achilles and Von Kiedrowski, 1993). However, because of the strong interactions between nucleic acids, the products formed in these ligation based systems bind strongly to one another, and the reaction kinetics of these systems are thus limited to parabolic, rather than exponential growth (von Kiedrowski, 1993).

Various strategies have been implemented to avoid the problem of product inhibition. For example, Li and Nicolaou utilized pH changes as well as the addition of excess complimentary oligonucleotide to release ligated product from its template (Li and Nicolaou, 1994). Work from the von Kiedrowski lab facilitated product release by template immobilizion followed by denaturation washing (Luther et al., 1998).

Recently, Paul and Joyce have designed an autocatalytic cycle based on a small RNA ligase ribozyme (the 3RC ligase) that is capable of limited exponential growth (Paul and Joyce, 2002). This replicator avoids the problem of product inhibition through the formation of intramolecular contacts within the newly formed product / template. However, the Paul replicator does not show extensive exponential growth in part due to a different binding problem: the association of the substrates with one another.

In order to avoid the problems that appear to be inherent in the design of autocatalytic ligation cycles and to design nucleic acid based system capable of exponential rather than parabolic growth, we have designed an autocatalytic cycle based on cleavage, rather than ligation. In this respect our system closely resembles a protein based proteolytic cascade such as blood clotting (Jones and Mann, 1994; Butenas and Mann, 2002), and apoptosis (Zimmermann et al., 2001), in which inactive zymogens, proenzymes and procofactors are activated via cleavage.

As a starting point for our autocatalytic cleavage cycle, we have chosen to use the fast (k_{cat} = ~0.1min^{-1}), highly efficient ($k_{cat}/K_m > 10^9$ M^{-1} min^{-1}), and

programmable 10-23 deoxyribozyme (Santoro and Joyce, 1998). Circularization of one 10-23 deoxyribozyme results in its inactivation and yields a novel substrate that can be acted upon by a linear, complementary 10-23 deoxyribozyme. Circularization of both complementary deoxyribozymes effectively eliminates catalytic activity, but positions the system so that the formation of even a single active linear species could potentially result in a cascade of cleavage reactions (**Figure 5-1c**). In addition to its potential use in biotechnology, we also hoped that we could use this system as a means of examining the evolution of a sequence in autocatalytic networks. As an initial test of this hypothesis, we performed a selection experiment, in the absence of proteins, to select for optimal deoxyribozymes from a pool of randomized variants.

RESULTS AND DISCUSSION

Cascade design

The design process for the cascade was governed by the requirements of the 10-23 deoxyribozyme (**Figure 5-1a**) (Santoro and Joyce, 1997; Santoro and Joyce, 1998). The core of the DNA enzyme consists of 15 invariant nucleotides (**Figure 5-1**, black) flanked by two binding arms (**Figure 5-1**, blue) which can be designed to base pair with almost any substrate sequence (**Figure 5-1**, green). The deoxyribozyme shows a preference for the cleavage of a phosphodiester bond between an unpaired purine and a pyrimidine nucleotide with AU and GU being cleaved most rapidly. The cleavage site must be composed of two ribose residues for efficient cleavage (see Appendix A), however, the remainder of the substrate could be either RNA or DNA (Ota et al., 1998). For the purpose of our cascade, the sequence AU was arbitrarily chosen as the cleavage site for both of the design constructs.

A previous study with the 10-23 deoxyribozyme indicated that the optimal length of the binding arms was between 7 and 10 base pairs (Santoro and Joyce, 1998), however, these values were highly dependent on sequence (Cairns et al., 1999) as well as application. For example 10-23 deoxribozyme with arm lengths of up to 24 nucleotides have been used for real-time PCR applications (Impey et al., 2000). For the design of our cascade system, an arm length of 7 nucleotides was chosen (**Figure 5-1b**).

The constructs were designed such that a linear (and active) species could base pair with the circular form of its complement (**Figure 5-1c**). Once designed, the initial linear constructs where folded using the computer program RNAStructure 3.7 (SantaLucia, 1998; Mathews et al., 1999), and the amount of self-structure as well as the formation of stable interactions between the two constructs (other than those required for binding arms) was minimized by mutation. Finally, the constructs were synthesized as described in the Experimental Protocols.

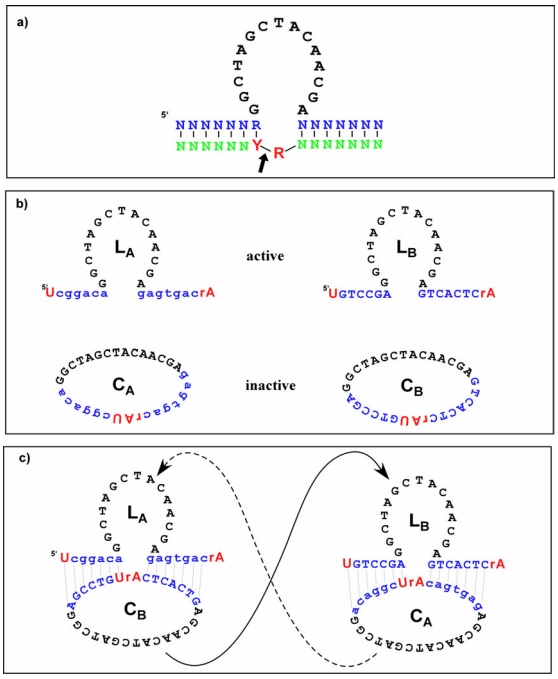

Figure 5-1. Design of linear and circular 10-23 deoxyribozymes. **(a)** The 10-23 RNA-cleaving deoxyribozyme. The deoxyribozyme-substrate complex is formed by the canonical base pairing between the binding arms (blue) and the substrate (green). The cleavage site (arrow) requires the presence of two RNA resiudes (red). The 15 nucleotide core of the ribozyme is shown in black. R = A or G, Y= U or C, N = A,G,C , or T. (**b**) Active linear deoxyribozymes L_A and L_B and inactive circular deoxyribozymes C_A and C_B. Colors are as in (a), with the complementary arms denoted by upper and lowercase letters. (**c**) Schematic for the autocatalytic 10-23 deoxyribozyme cleavase cycle. Cleavage of inactive C_B by active L_A produces active L_B which can in turn cleave inactive C_A to reproduce active L_A.

Circularization and inactivation of deoxyribozymes

In order to circularize the 10-23 deoxyribozyme, linear permutation of constructs L_A and L_B were synthesized containing two ribose residues and a 5'-phosphate (**Figure 5-2a**). The constructs were then radiolabeled by an exchange reaction with T4 polynucleotide kinase and γ-^{32}P-ATP. Circularization was accomplished using T4 DNA Ligase and a splint oligonucleotide which was complimentary to 8 nucleotides on either end of the construct (**Figure 5-2b)**. The reactions were allowed to proceed for 16hr at room temperature and the circular products (50-70%) were then isolated by denaturing (7M urea) gel electorophoresis on 20% polyacrylamide gels. Circular species ran slighter slower than the linear species under these conditions (**Figure 5-2c**). After extraction from the gel, the circular constructs were treated with Exonuclease VII to remove any remaining liner construct.

The first step in the determining whether or not it would be possible to design a deoxyribozyme cascade based on the 10-23 deoxyribozyme was to determine if the deoxyribozyme could be inactivated by circularization. To test this, we initially designed non-cleavable versions of both circular variants of C_A and circle C_B that contained no RNA residues. Because the substrates for these circular enzymes would also be circular, we assayed these non-cleavable constructs for their ability to cleave their circular counterparts that did contain RNA linkages.

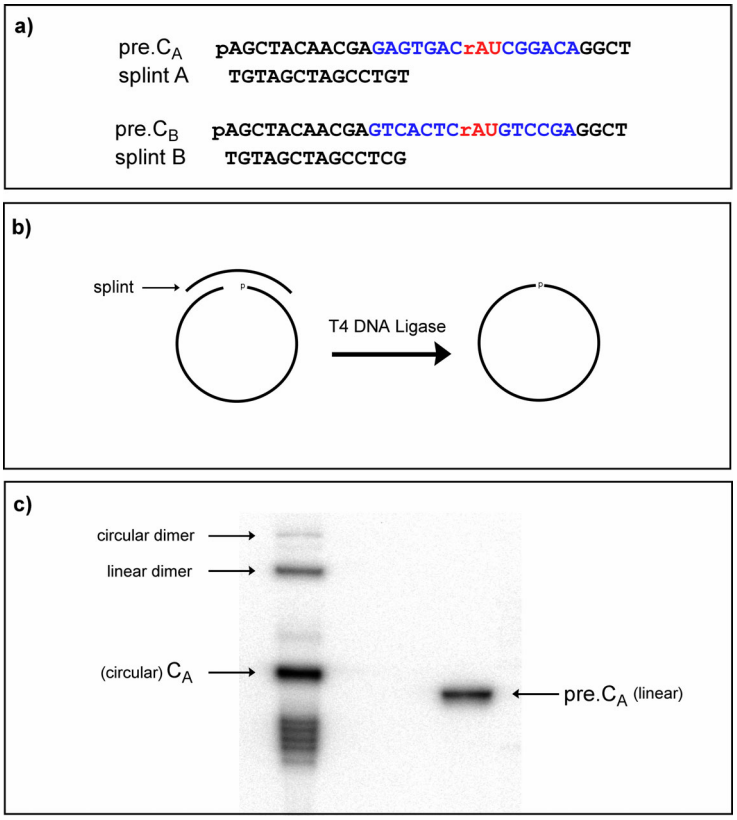

Figure 5-2. Circularization of deoxyribozymes. **(a)** Linear permutations of L_A and L_B (pre.C_A and pre.C_B) were synthesized containg two ribose residues (red). **(b)** Ligation was accomplished with T4 RNA ligase and a splint oligonucleotide. **(c)** Typical purification of circular deoxyribozymes by denaturing 20% polyacrylamide gel electrophoresis. Other products from the reaction are as indicated.

When 200 nM non-cleavable, circular enzyme (C_A) was incubated with 200 nM cleavable, circular substrate, the rate of ring-opening was found to be 1nM hr^{-1}, more than 250-fold slower than if the catalyst was linear (L_A). Similarly, non-cleavable C_B was found to cleave C_A at a rate of 0.2 nM hr^{-1}, >1000-fold slower than L_B and roughly equivalent to the rate of background hydrolysis of the RNA linkage due to spontaneous cleavage.

Because the sequence of the substrate-binding arms of the 10-23 deoxyribozyme has been shown to influence catalysis (Santoro and Joyce, 1998; Cairns et al., 1999), the activities of the linear deoxyribozymes were also assayed (**Figure 5-3a**). When L_A and L_B were assayed with linear substrates that had the same sequences as the circular deoxyribozymes, higher k_{cat}'s and lower K_M's were observed (**Figure 5-3b)**. Overall, while the catalytic efficiencies of the linear deoxyribozymes were similar to those previously reported (Santoro and Joyce, 1997; Ota et al., 1998; Santoro and Joyce, 1998), circularization decreased substrate utilization.

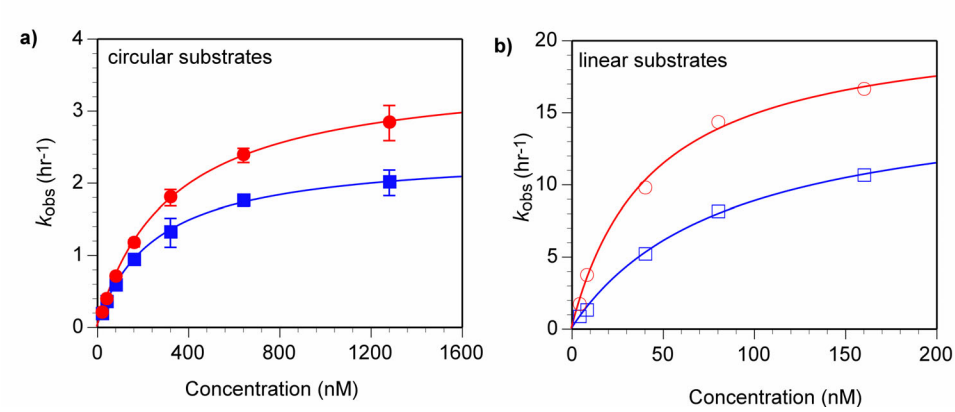

Figure 5-3. Kinetic analysis of linear deoxyribozymes with circular and linear substrates. **(a)** Cleavage kinetics of linear deoxyribozymes with circular substrates. (■) indicates reaction of L_A with C_B and (●) indicates reaction of L_B with C_A. Lines are the best fit with the Michaelis-Menten equation which gave a K_M of 260 ± 9nM and a k_{cat} of 2.4 ± 0.03 hr^{-1} for L_A with C_B and a K_M of 320 ± 7nM and a k_{cat} of 3.6 ± 0.03 hr^{-1} for L_B with C_A. **(b)** Cleavage kinetics of linear deoxyribozymes with linear substrates. (□) indicates the reaction of L_A with pre.C_B and (○) indicates the reaction of L_B with pre.C_A. Linear substrates (pre.C_A and pre.C_B) are the inactive linear permutations of the linear deoxyribozymes. Lines are best fit with the Michaelis-Menten equation which gave a K_M of 43 ± 7nM and a k_{cat} of 21 ± 1.0 hr^{-1} for L_A with pre.C_B and a K_M of 85 ± 9nM and a k_{cat} of 16 ± 0.6 hr^{-1} for L_B with pre.C_A.

A Deoxyribozyme Cascade

Having established that the circularized deoxyribozymes were catalytically impaired yet could still act as substrates, a mixture of the circles C_A and C_B were incubated together and the reaction monitored for the production of linear species (**Figure 5-4**). A time course of the reaction revealed an initial lag phase of approximately 3 hours followed by an exponential growth phase. This was observed irrespective of whether the reaction was monitored for the cleavage of C_A or C_B (**Figure 5-4a** red squares, **Figure 5-4b** open red squares) indicating that both linear species were being formed in tandem. When reactions were triggered by the addition of linear L_A or linear L_B at 1 part in 40, the reaction kinetics were still exponential, but the lag phase was reduced to less than 1 hour (**Figure 5-4a**,**b**, triangles and circles). All reactions routinely proceeded to roughly 80% completion, possibly due to the misfolding of some substrates.

In contrast, when each circle was incubated separately, very little linear product was formed, consistent with the measured rates of background hydrolysis in buffer ($k_{hyd}^{C_A}$ = 2.6x10^{-4} hr^{-1}, $k_{hyd}^{C_B}$= 3.9x10^{-4} hr^{-1}). When only one circle was present, the addition of complementary trigger resulted in a linear rather than an exponential reaction (**Figure 5-4a** yellow diamonds). Additionally, when antisense oligonucleotides that bound to and inhibited the function of both L_A and L_B were added to the mixture at 1 part in 4, the reaction was inhibited for the duration of the experiment (**Figure 5-4a**; purple squares), and the remaining unreacted even after 24hrs (data not shown). In each instance, the observed

reaction kinetics were fully consistent with the engineered cross-catalytic cycle originally shown in **Figure 5-1c**.

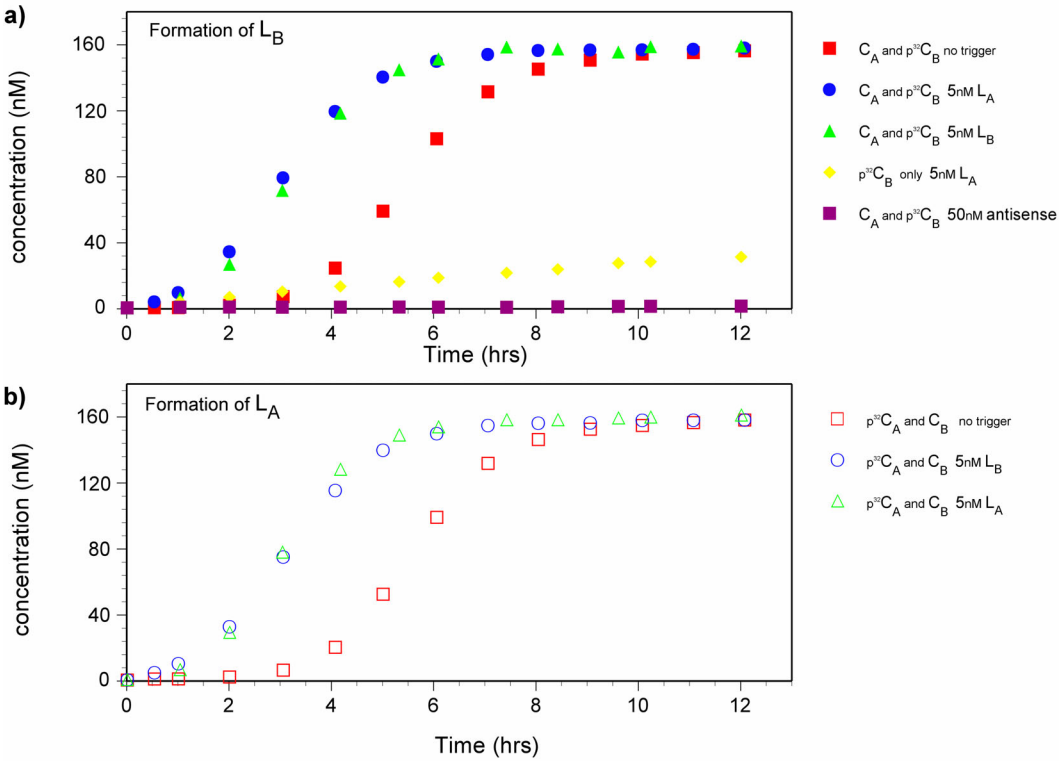

Figure 5-4. Time-course of autocatalytic cleavage. **(a)** Formation of L_B from C_B. Reactions contained 200nM C_A and 200nM ^{32}P-labeled C_B. The concentrations of added trigger or antisense oligonucleotide were as indicated. **(b)** Formation of L_A from C_A. Reaction conditions were the same as in (a) except that C_A was radiolabeled.

To further demonstrate these results, cleavage assays were conducted in the presence of increasing amounts of linear trigger L_B (**Figure 5-5**). This resulted in the shortening of the lag phase of the reaction until little or no sigmoidicity was observed. A simulation of the cascade reaction using the previously determined Michaelis-Menten parameters and background rates of

hydrolysis was in good agreement with the observed data, as shown by the overlaid curves (**Figure 5-5**).

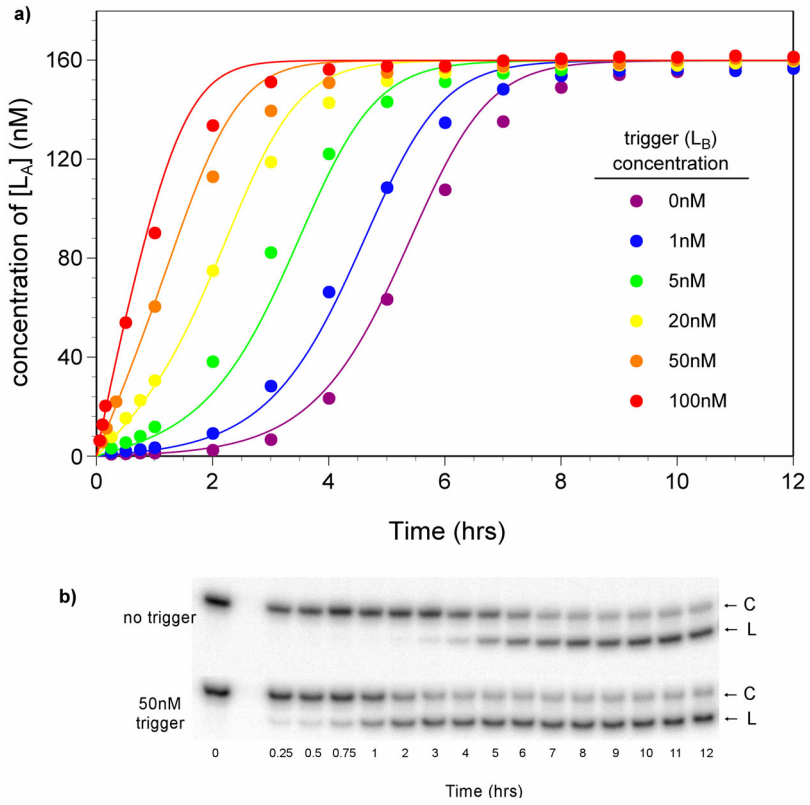

Figure 5-5. Autocatalytic cleavage of circle C_A with increasing concentrations of trigger L_B. **(a)** Reactions contained 200nM ^{32}P-labeled C_A 200nM C_B and were monitored for the formation of L_A. The concentration of added linear trigger L_B is indicated. Overlaid, theoretical curves were derived based on the kinetic schema described in Experimental Protocols and rates given in the text. **(b)** Denaturing (7M urea) 20% polyacrylamide gel analysis of an autocatalytic cleavage reaction time course. These particular data contributed to Figure 5-5a, no trigger or 50nM trigger. Circular (C) and linear deoxyribozymes (L) are indicated.

The kinetic behavior of self-replicating systems can be described by the replicator equation: $d[L]/dt]_{initial} = k_a[L_0]^p + k_b$ (von Kiedrowski, 1993; Paul and Joyce, 2002). While this equation is typically applied to ligase systems, it is also

applicable to our cleavase system where the initial rate of cleavage is proportional to the initial trigger concentration, L_0. In order to show that the reaction was truly exponential and that the deoxyribozymes were turning over, the initial rate of reaction was determined as a function of trigger (L_A) concentration (**Figure 5-6**). The exponential nature of the system is clearly demonstrated by the linear relation ship between $[L_0]^p$ and [d[L]/dt], where p =1 (von Kiedrowski, 1993). The slope of the line yields a autocatalytic rate constant (k_a) of 1.5 +/- 0.04 hr^{-1} which is in relatively good agreement with the rate of reaction of L_A with C_B under the sub-saturating conditions of the assay (k_{obs} at [160nM] = 1.1 +/- 0.03 hr^{-1}). The y-intercept, k_b, gives the rate for the background reaction of 1.3 +/- 0.4nM hr^{-1} which is consistent with the value obtained by direct measurement using the non-cleavable circle C_A (1nM hr^{-1} at 160nM C_A and C_B).

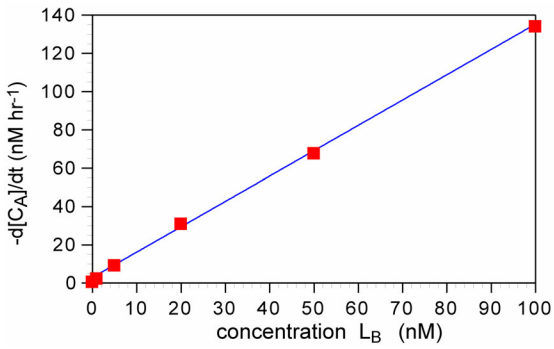

Figure 5-6. Initial rate of reaction as a function of trigger concentration. Rates were determined by the best linear fit through the first 3 or 4 time points from each curve shown in Figure 5-5a. The line on the graph represents the best fit of all of the rate data. However, only the first 4 points were used for the determination of the slope and y-intercept as reported in the text.

Recently, Paul and Joyce have engineered a ligase ribozyme that was capable of limited self-replication (Paul and Joyce, 2002). Unfortunately, strong intermolecular interactions between the ligase substrates permitted exponential growth to occur only when external template was added, and even then, the exponential portion of the reaction generated products that corresponded to only about one third of the input template concentration. However, it is interesting to note that although these two systems function in completely different ways, the autocatalytic efficiency of the exponential portion of the of the ligase system ($\varepsilon = k_a / k_b = 3.3 \times 10^8\ M^{-1}$) is similar that of our10-23 cleavase cycle ($\varepsilon = 1.2 \times 10^9\ M^{-1}$).

Evolution of functional sequences

The successful design of a cross-catalytic cycle provided us with a means to examine how the exponential growth of catalytic activity might aid in the evolution of functional sequences. In order to do this, two pools, C.E1 and C.E2, based on circles C_A and C_B, were constructed in which three nucleotides on opposing arms of the deoxyribozymes were randomized (**Figure 5-7a,b**). The pools were incubated together and allowed to generate linear active species, after which a portion of the reaction (10%) was transferred to a new tube containing unreacted circles, effectively 'seeding' the new reaction with linear triggers. In theory, individual sequences capable of finding their appropriate partner and becoming linear (and active) would be expected to amplify exponentially. Even though the 'seed' contained both circular and linear species, the active linear species are expected to find their complements, initiate a new cascade and therefore predominate over several serial dilutions.

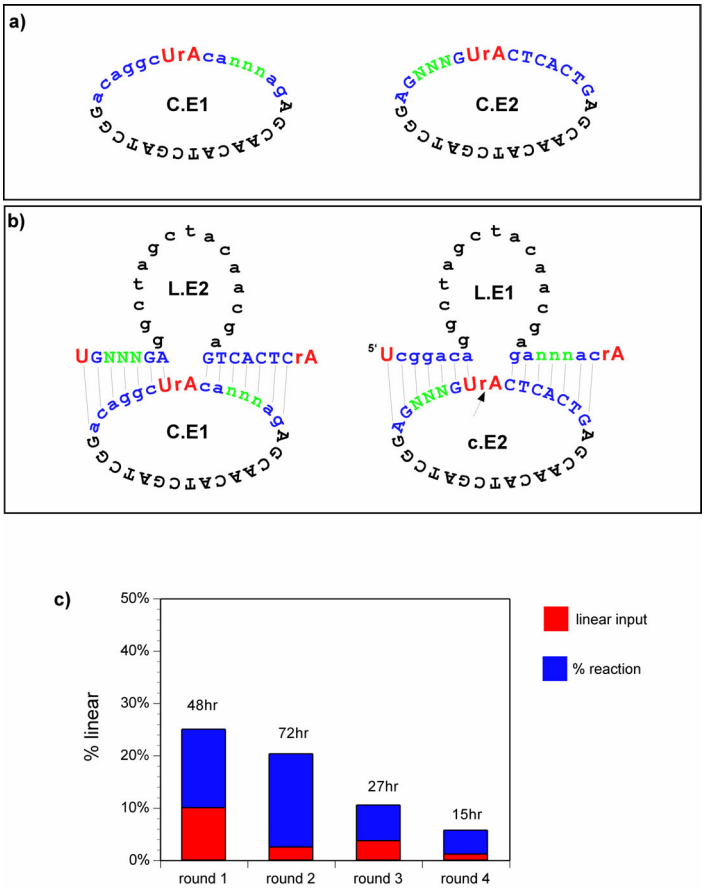

Figure 5-7. Construction and evolution of 10-23 deoxyribozyme N3 pools. **(a)** Design of N3 pools. **(b)** Hypothesized interaction of linear and circular species during selection. **(c)** Progress of the selection. The amount of linear species of was determined by gel electrophoresis as described in in the Experimental Protocols.

The initial round of the selection contained 4 μM of each circular pool, and 0.4 μM of each linear, active pool to serve as initial catalysts. After 48 hrs, 10% of the Round 1 reaction was transferred to another tube containing an additional 4 μM of each circular pool. Serial transfers were conducted for 3 more rounds of selection with increasingly stringent incubation times of 72, 27, and 15hr. In addition to shortening the incubation time, Rounds 3 and 4 were also

conducted with lower circle concentrations (2 μM each). The progress of the selection was monitored by gel electrophoresis, and the results are shown in **Figure 5-7c**. During the course of the selection, the fraction of the population that was linear increased following the first round of selection, was slightly decreased when the stringency was increased between Rounds 2 and 3, and then increased again following Round 3.

In order to determine if sequence selection had indeed occurred, linear species from Round 0 and Round 4 were cloned and sequenced. While members of both Round 0 populations appeared random with no apparent sequence bias (**Figure 5-8a**), both Round 4 pools have become appreciably fixed on a given sequence (**Figure 5-8a**). For the C.E1 pool two sequences predominated, the expected complement GTG (13 occurrences) and an alternate sequence GGG (9 occurrences). The GGG might be expected to form a stable duplex with the complement CAC in the C.E2 pool through the formation of a non-canonical A:G pair. Similarly, for the Round 4 C.E2 pool, the expected complement TCC (20 occurrences) was found, along with the alternate sequences GCC (3 occurrences), TCA (3 occurances), and several additional variants. Again, the minor variants that were recovered might still be expected to base-pair in the cross-catalytic cycle by the formation of non-canonical (G:A and A:G) base-pairs. If evolution of functionality as well as sequence had occurred, it would be predicted that the 'fitness' of the recovered species should be roughly proportional to their representation in the population. To determine this, we assayed the major and minor variants (GTG and GGG) from C.E1 pool with the parental C.E2 sequence,

C_B, in an exponential growth assay (**Figure 5-8b**). As predicted, GTG was more functional than GGG.

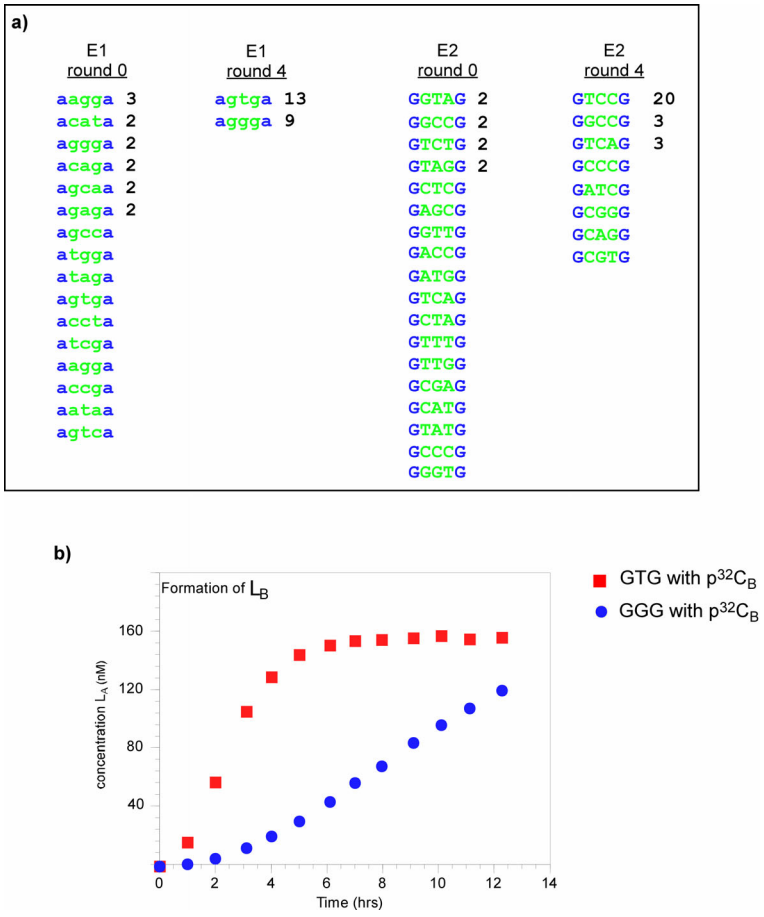

Figure 5-8. Sequence and functional evolution. **(a)** Round 0 and Round 4 sequences. Sequences of the randomized region of the linearized variants (green) with one flanking nucleotide (blue). **(b)** Time-course of autocatalytic cleavage of selected sequences. Circularized deoxyribozymes containing either GTG (C_A) (red squares) or GGG (blue circles) were incubated with radiolabeled circle C_B. Reactions were set up as described in Experimental Protocols, and contained each circular deoxyribozyme at 200 nM and 20 nM L_B as an initial trigger.

Implications for origins

Previous studies with both autocatalytic and cross-catalytic nucleic acid based replicators have shown the possibility for amplification as well as information transfer in the absence of proteins. For example, von Kiedrowski and co-workers have demonstrated that short oligonucleotides can be replicated from smaller complementary fragments via chemical ligation (von Kiedrowski, 1986; Von Kiedrowski et al., 1991; Sievers and von Kiedrowski, 1994). Unfortunately, because the products of these reactions bind the starting template better than the reactants, the reactions are prohibited from achieving exponential growth (Von Kiedrowski et al., 1991; von Kiedrowski, 1993). This prevents the use of these systems for signal amplification. In addition, even in systems with competition between replicators, parabolic replication prevents the complete extinction of less efficient competitors. In the long run, the net result is coexistence rather than selection (Szathmary and Gladkih, 1989). However, this limitation may be overcome by stepwise non-autonomous replication procedures (Li and Nicolaou, 1994; Luther et al., 1998) and has been mathematically analyzed to be less limiting in autonomous replication schemes that involve surfaces (von Kiedrowski and Szathmary, 2000).

Amplification and information transfer are also possible in the cleavage based system that we have developed. In our system, if a sequence is initially a successful catalyst, proportionately more of it will be converted into a linear molecule at later times. Unlike the ligase based systems this allows for uninhibited sequence amplification. However, with respect to evolution, this

system too leads to coexistence. While this might not be obvious, especially in light of the successful selection, since all sequences are present in equal concentrations at every round of the selection and since there is a background hydrolysis reaction, in the long term, any sequence can potentially become activated and re-emerge in the population. Thus, no sequence can ever be driven to extinction.

The problem of a background reaction (like hydrolysis) is not just limited to our system, but to any replicating system that lacks competition. In fact, even if ligase systems, such as those of von Kiedrowski, were not inhibited by parabolic replication, they would still ultimately result in coexistence because these systems also possess a background ligation reaction and lack the competition for resources which would be required for the evolution of a dominant species and the extinction of competitors.

However, if we assume for a moment that in an ideal system there would be no background reaction, then an interesting result emerges. That is, while individual species can predominate because they drive other species to extinction through competition for resources, it is also possible for species to predominate in the absence of direct competition simply because they replicate faster. Nonetheless, even with an appreciable background reaction (as in our system), dominant species do emerge, at least in the short term.

While the results of the current selection were essentially predetermined, the random regions opposed constant regions, the incorporation of larger over-lapping random regions should allow for the direct selection and optimization of

autocatalytic RNA-cleaving deoxyribozyme pairs. To the extent that we can design and/or select multiple deoxyribozymes capable of participating in autocatalytic systems, this will also provide us with a means of designing, modeling and observing simple molecular ecosystems.

Applications to biotechnology

As mentioned in Chapter 3, nucleic acid catalysts have proven to be useful for the design of biosensors (Marshall and Ellington, 1999; Hesselberth et al., 2000). In particular, it has proven possible to design and select "aptazymes", allosteric nucleic acids that can be activated or inhibited by molecular effectors ranging from small organic molecules to oligonucleotides to proteins (Tang and Breaker, 1997; Soukup and Breaker, 1999; Robertson and Ellington, 2000; Robertson and Ellington, 2001). Of particular interest is the recent design of effector dependent 10-23 deoxyribozymes by Sen and coworkers that respond to small molecules such as ATP as well as oligonucleotides (Wang and Sen, 2001; Wang et al., 2002). The combination of an aptazyme trigger with the cleavase cascade would potentially allow the design of novel highly sensitive detection and amplification systems. It's easy to envision the introduction of a fluorophore and a quencher on opposite arms of the circular 10-23 deoxribozyme that would effectively "light-up" due to the exponential accumulation of fluorescence. Currently the some of the most sensitive nucleic acid detection systems rely on amplification with protein enzymes (such as PCR) to amplify a very small signal (Robertson and Ellington, 1999; Fredriksson et al., 2002). In contrast, a

diagnostic cascade would preclude the use of proteins or any other reagents other than sample addition. In addition, nucleic acid cascades might also find use in the growing field of DNA machines where they could potentially assist in the rapid assembly or disassembly of DNA structures (Yurke et al., 2000; Eckardt et al., 2002; Niemeyer and Adler, 2002; Yan et al., 2002; Seeman, 2003) or find use in DNA computation (Benenson et al., 2001) where they could provide a role in rapid protein independent sequence-specific readouts.

EXPERIMENTAL PROTOCOLS

Oligonucleotide Synthesis and Circularization

All oligonucleotides were synthesized on a Expedite 8909 synthesizer (PE Biosystems, Foster City, California) using standard DNA and RNA phosphoramidite chemistry. All synthesis reagents were purchased from Glen Research (Sterling, Virginia).

The linear deoxyribozymes L_A (UCGGACAGGCTAGCTACAACGAG-AGTGACA-p) and L_B (UGTCCGAGGCTAGCTACAACGAGTCACTCA-p) were chemically synthesized with terminal RNA residues (underlined) and a 3' phosphate (denoted 'p') to more closely mimic the product of the cleavage reaction, which produces a 2',3' cyclic phosphate.

Circular deoxyribozymes were synthesized from linear permutations of L_A and L_B bearing a 5' phosphate (denoted 'p') (pre.C_A, p-AGCTACAACGA-GAGTGACAUCGGACAGGCT; pre.C_B p-AGCTACAACGAGTCACTCAUG-TCCGAGGCT, where underlined residues are RNA). Circularization reactions were carried out with T4 DNA ligase (NEB, Beverly, MA) and a splint oligonucleotide (splint.a TGTAGCTAGCCTGT; splint.b GCTCCGATC-GATGT). The concentration of the linear substrate was 100 nM, and the splint oligonucleotide was present in 1.2-fold molar excess. The reactions were allowed to proceed for 16 hr at room temperature and the circular products (50-70%) were isolated on denaturing (7M urea) 20% polyacrylamide gels. Following gel purification, C_A and C_B were treated with exonuclease VII to remove any

remaining linear nucleic acids. Where appropriate, the constructs were radiolabeled prior to circularization via an exchange reaction with T4 polynucleotide kinase and γ-^{32}P-ATP (>7000Ci/mmol; ICN, Irvine, CA).

Circular pools C.E1 and C.E2 were synthesized from linear precursor pools bearing three randomized nucleotides, a 5'phosphorylated ribouridine and a 3'riboadenosine (pre.CE1, p-UCGGACAGGCTAGCTACAACGAGANNNACA; pre.ce2, p-UGNNNGAGGCTAGCTACAACGAGTCACTCA, where 'N' denotes randomized positions). Circularization reactions in this instance performed were with T4 RNA ligase (Promega, Madison, WI) (Pan and Uhlenbeck, 1992). The circular constructs were purified as described above.

Reaction Conditions and Deoxyribozyme Kinetics

All reactions were equilibrated at 70°C in 50 mM Tris, pH 8.0, 0.1% SDS, and slow cooled to 23°C prior to initiation by the addition of 30 mM $MgCl_2$. Reactions were stopped by the addition of 95% formamide loading dye containing a 2-fold excess of EDTA. Circular and linear species were separated on denaturing (7 M urea) 20% acrylamide gels and quantitated using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Multiple turnover reactions with circular substrates C_A and C_B (**Figure 5-3a)** were conducted at 20, 40, 80, 160, 320, 640, and 1280 nM. The concentrations of the linear deoxyribozyme catalysts (L_A and L_B) varied from 2-10 nM, but was always at least 10-fold less than that of their circular substrates. Initial velocities were determined by the best linear fit through 3 time points within the first ~10% of the reaction. Each point on the graph represents the

average of 3 rate measurements. The values k_{cat} and K_m were determined by fitting a plot of k_{obs} versus substrate concentration. This gave $k_{cat}(L_A)$ = 2.4 hr^{-1}, $k_{cat}(L_B)$ = 3.6 hr^{-1}, $K_M(L_A)$ =260 nM, $K_M(L_B)$ =320 nM,

Multiple turnover reactions with linear substrates pre.C_A and pre.C_B, (**Figure 5-3b)** were conducted at 4, 8, 40, 80, and 160 nM. The initial velocities and the values k_{cat} and K_m were determined as before. Each point represents a single rate measurement.

The rate of background hydrolysis (k_{hyd}) of circles C_A and C_B were determined by the best fit line though three time points of a graph of ln(1-% reaction) vs. time. This gave a values of $k_{hyd}^{C_A}$ = 2.6x10^{-4} hr^{-1}, $k_{hyd}^{C_B}$ = 3.9x10^{-4} hr^{-1}.

The rates of cleavage of the circular from of the deoxyribozymes were determined using non cleavable versions of circles C_A and C_B. These circles were identical to the cleavable C_A and C_B, but contained not ribose residues. The concentration of both non-cleavable and cleavable circles in these reactions was 200nM. Second order rates were estimated from a pseudo first order fit of two time points at less than 10% reaction. When adjusted for the rate of background hydrolysis, the pseudo first order fit gave values of 5.0x10^{-4} hr^{-1} for the cleavage of C_A by non-cleavable C_B, and 4.5 x10^{-3} hr^{-1} for the cleavage of C_B by non-cleavable C_A. The estimated second order rate constants were 3100 M^{-1} hr^{-1} (4.99x10^{-4} hr^{-1}/160nM) for non-cleavable C_B and 28,100 M^{-1} hr^{-1} (4.53x10^{-3} hr^{-1}/160nM) for non-cleavable C_A.

***In vitro* selection**

The selection was carried out using the circular pools C.E1 and C.E2. All rounds were conducted in reaction buffer (Tris, pH 8.0, 0.1% SDS, 10ul final volume), and initiated by the addition of 30 mM $MgCl_2$.

The initial round of selection contained 40 pmol of each circular pool and 4pmol of each linear pool. The linear pool was catalytically active and could initiate the autocatalytic cycle. After initiation with $MgCl_2$, the reaction was allowed to proceed for 48hrs. A fraction of this reaction (1 μL) was then transferred to another tube containing an additional 40 pmol of each circular pool. This reaction (Round 2) was again initiated by the addition of $MgCl_2$. The process was repeated for an additional two rounds. This should have resulted in a vast dilution of the original, linear pools. Rounds 3 and 4 contained only 20pml of each pool. The incubation times for each round are as shown in **Figure 5-7b**.

After each serial transfer, the remaining 9 uL from each round was quenched by the addition of an equal volume of 95% formamide loading dye containing a 2-fold excess of EDTA over the concentration of $MgCl_2$. Circular and linear species were separated on denaturing (7 M urea) 20% acrylamide gels and the amounts of nucleic acid present in individual bands were quantitated via fluorescent staining with SyberGold (Molecular Probes, Portland, Oregon) and a Fluorimager (Molecular Dynamics, Sunnyvale, CA).

The linear species from Round 4 were extracted from the gel for cloning and sequencing. After gel extraction, linear DNA molecules were treated with T4 polynucleotide kinase to remove the 2',3' cyclic phosphate (Schurer et al., 2002)

and then radiolabeled with an excess of γ-^{32}P-ATP (>7000Ci/mmol; ICN, Irvine, CA) in standard buffer. Primer binding sites were ligated to the 3' or 5' end of a portion of the purified, radiolabeled pool using T4 RNA ligase (L3, p-UUUCTGAGACGTAGACAGCACGAT-c3; L5, TCGTACTACTAGCATCG-TTATGGAAA; the underlined residues are RNA a, 'p' a 5' phosphate, and 'c3' denotes a three carbon alkyl blocking group). The ligated products were purified on denaturing (7M urea) 8% acrylamide gels, and amplified using primers specific for either C.E1 or C.E2 (for C.E1; P5.E1 CGTTATGGAAATCGGA-CAGGC and P3.E1 ATCGTGCTGTCTACGTCT-CAG; for C.E2, P5.E2 CGTACTACTAGCATCGTTATGG and P3.e2 TGAGTGACTCGTTGTAGC). After amplification, PCR products were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and sequences acquired via standard dideoxy, cycle sequencing methods on a CEQ 2000XL automated sequencer (Beckman Coulter, Fullerton, CA). To obtain sequences from Round 0, the circularized pools were treated with RNase U2 prior to the ligation of primer binding sites, PCR amplification, and sequencing.

Reaction simulations

The following reactions were used to model the autocatalytic cycle:

(1) $C_A + L_B \overset{K_m^{L_B}}{\rightleftharpoons} C_A \bullet L_B \xrightarrow{k_{cat}^{L_B}} L_A + L_B$

(2) $C_B + L_A \overset{K_m^{L_A}}{\rightleftharpoons} C_B \bullet L_A \xrightarrow{k_{cat}^{L_A}} L_A + L_B$

(3) $C_A \xrightarrow{k_3} L_A$

(4) $C_B \xrightarrow{k_4} L_B$

(5) $C_A + C_B \xrightarrow{k_5} C_A + L_B$

(6) $C_A + C_B \xrightarrow{k_6} L_A + C_B$

Where reactions (1) and (2) represent the catalyzed reactions; (3) and (4) represent the background rates of hydrolysis; and reactions (5) and (6) represent cleavage reactions catalyzed by the circular (as opposed to linear) deoxyribozymes. For these latter two reactions, we have used 2nd order rate constants based upon the initial rates of substrate cleavage by the non-cleaveable enzymes circle C_A and C_B.

Simulations were conducted using the program SigmaPlot 7.0 (SPSS Science, Chicago, IL) and the following set of differential equations:

$$\frac{dL_A}{dt} = k_2[L_B][C_A] + k_3[C_A] + k_6[C_B][C_A]$$

$$\frac{dL_B}{dt} = k_1[L_A][C_B] + k_4[C_B] + k_5[C_A][C_B]$$

$$\frac{dC_A}{dt} = -k_2[L_B][C_A] - k_3[C_A] - k_6[C_B][C_A]$$

$$\frac{dC_B}{dt} = -k_1[L_A][C_B] - k_4[C_B] - k_5[C_A][C_B]$$

where:

$$k_1 = \frac{k_{cat}^{L_A}}{K_M^{L_A} + [C_B]}$$

$$k_2 = \frac{k_{cat}^{L_B}}{K_M^{L_B} + [C_A]}$$

and k_3, k_4, k_5, k_6 are defined by equations (3), (4), (5) and (6). The values used for the simulation are: $k_{cat}(L_A) = 2.4\ hr^{-1}$, $k_{cat}(L_B) = 3.6\ hr^{-1}$, $K_M(L_A)$ =260 nM, $K_M(L_B)$ =320 nM, $k_3 = 2.6 \times 10^{-4}\ hr^{-1}$, $k_4 = 3.9 \times 10^{-4}\ hr^{-1}$ $k_5 = 28{,}100\ M^{-1}\ hr^{-1}$ $k_6 = 3{,}100\ M^{-1}\ hr^{-1}$. These values were determined as described previously. The initial concentrations of C_A and C_B have been set at 160 nM. The concentrations of trigger L_B are shown in **Figure 5-5a.**

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Appendix A: Initial deoxyribozyme cascade designs

The initial incarnation of the 10-23 cleaving cascade was based on the three circle system shown in **Figure A-1**. The linear permutations of the circles, Ls.1, Ls.2 and Ls.3, were synthesized bearing a 5'I-dT and a 3' phosphorothioate which allowed for circularization using the non-enzymatic ligation strategy designed by the Kool Lab (Xu and Kool, 1997). These are shown in **Figure A-2a** along with their splint oligonucleotides **Figure A-2b**. In order to test whether the designed 10-23 deoxyribozymes were active, short oligonucleotide substrates (**Figure A-3a)** designed to mimic the cleavage site in each of the three circle constructs were synthesized. The substrates were then assayed for cleavage by with the corresponding 10-23 deoxyribozymes. As shown in **Figure A-3b**, while cleavage appeared specific, all of the substrates were cleaved poorly by their corresponding 10-23 deoxyribozymes.

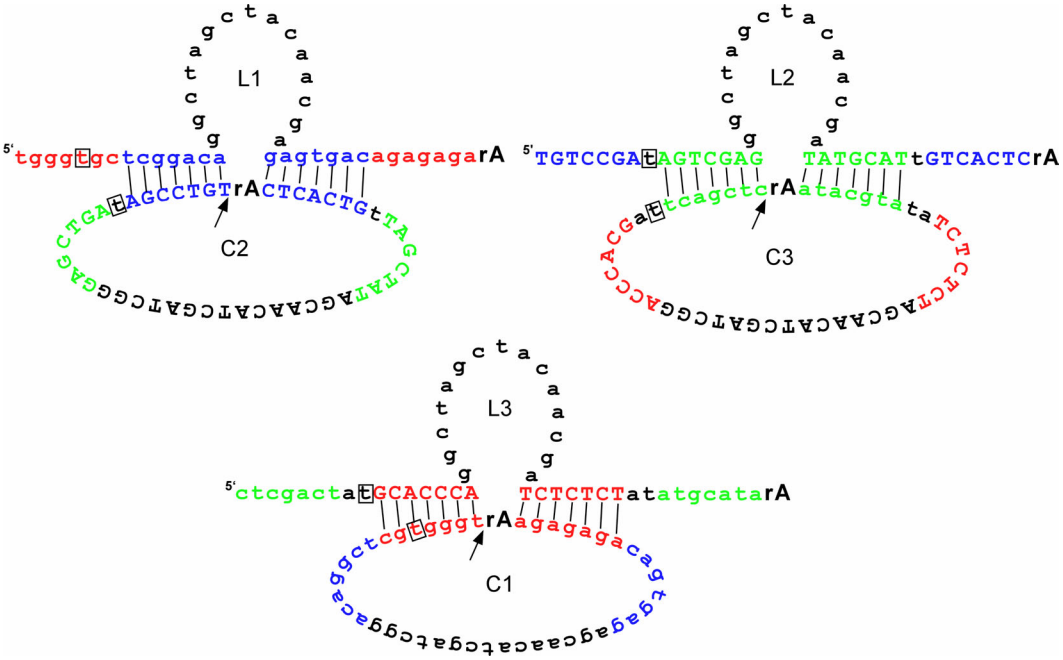

Figure A-1. Three circle cascade scheme with a single ribose residue. The core of the deoxyribozyme is indicated in black lower case. The complementary binding arms are indicated by upper and lower case and share the same color. The single ribose residue is indicated by **rA**. Residues not involved in binding are also indicated in black lowercase. The boxed t represents the position of the 5'-I dT used to ligate the circles. The cleavage site is indicated by the arrow.

a)
Ls.1 tcggacaggctagctacaacgagagtgacagaaagarAtgggtgc-ps
Ls.2 tagtcgaggctagctacaacgatatcgattgtcactcrAtgtccga-ps
Ls.3 taGCACCCAGGCTAGCTACAACGATCTTTCTatatgcatarActcgact-ps

b)
dc.splint.1 TGTCCGAGCACCCA
dc.splint.2 TCGACTATCGGACA
dc.splint.3 GGTGCTAAGTCGAG

Figure A-2. Oligonucleotides for the synthesis of a three piece deoxyribozyme cascade. (**a**) Linear permutations of circles C1, C2, and C3. The underlined residues bear a 5'-iodine and ps denotes a phosphorothioate. Other colors are as in Figure A-1. (**b**) Splint oligonucleotides designed for circularization of linear speicies shown in (a).

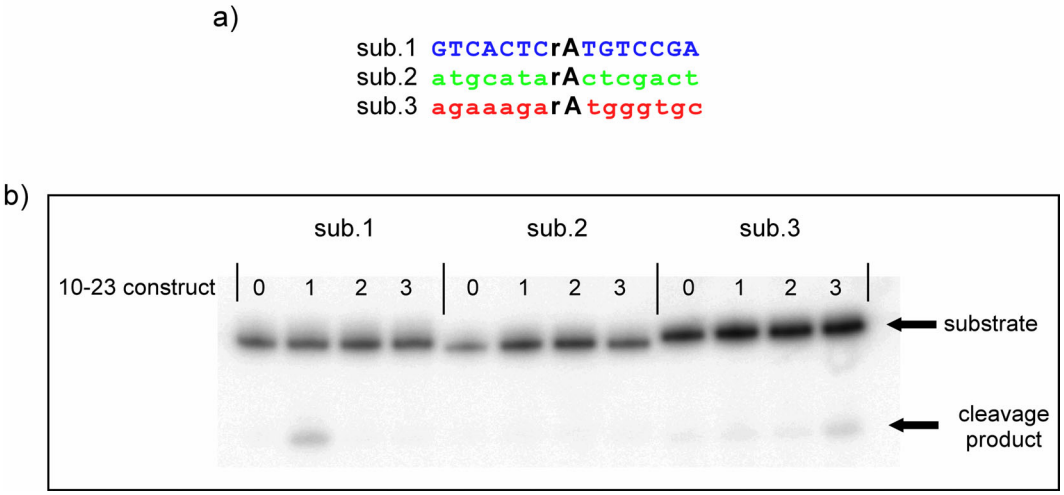

Figure A-3. Cleavage of substrates with a single ribose residue. (**a**) The sequence of the three substrates used. **rA** is the single ribose residue. (**b**) Substrate cleavage reactions. 10-23 construct 1 is designed to cleave sub.1, 10-23 construct 2 is designed to cleave sub.2, 10-23 construct 3 is designed to cleave sub.3. A 0 denotes the absence of any deoxyribozyme. Substrates were radiolabeled with γ-^{32}P ATP prior to analysis. The cleavage assays were conducted in 50 mM Tris pH 8.0, 30 mM MgCl2 and contained ~10 μM enzyme and ~1 μM substrate. Assays were run for 1hr at 37° C.

A survey of the literature suggested that the poor rate of cleavage might be due to the need for two ribose residues at the cleavage site (Ota et al., 1998; Santoro and Joyce, 1998; Impey et al., 2000). Circles C1, C2, and C3 were subsequently redesigned containing two ribose residues. The corresponding circles C4, C5, and C6 are shown in **Figure A-4**. The linear permutations of the new circles, Ls.4, Ls.5 and Ls.6, and splint oligonucleotides are shown in **Figure A-5a,b**.

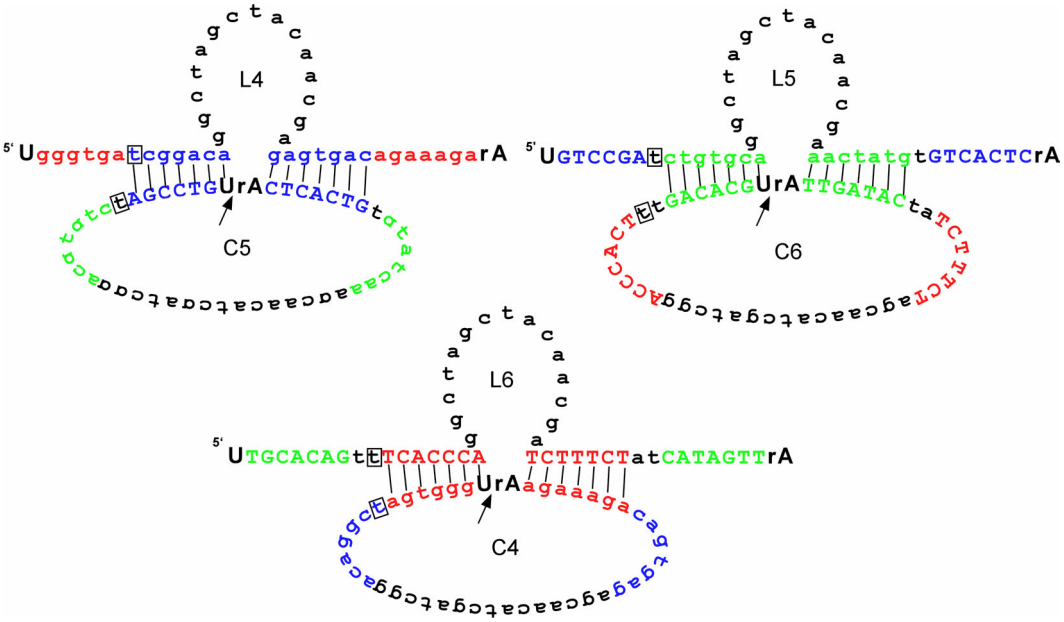

Figure A-4. Three circle cascade scheme with two ribose residues. All colors and symbols are as indicated in Figure A-1. **rA** and **U** denote the ribose residues.

a)

Ls.4 tcggacaGGCTAGCTACAACGAgagtgacagaaagarAUgggtga
Ls.5 tCTGTGCAGGCTAGCTACAACGAaactatgtGTCACTCrAUGTCCGA
Ls.6 tTCACCCAGGCTAGCTACAACGATCTTTCTatCATAGTTrAUGCACAGt

b)

dc.splint.4	TGTCCGATCACCCA
dc.splint.5	GCACAGATCGGACA
dc.splint.6	GGGTGAAACTGTGC

Figure A-5. Oligonucleotides for the synthesis of a three piece deoxyribozyme cascade with two ribose residues. (a) Linear permutations of circles C4, C5, and C6. (b) Splint oligonucleotides designed for circularization of linear speicies shown in (a). Colors and symbols are as in Figure A-5.

New substrates were also designed that contained two ribose residues mimicking the cleavage site of C4, C5, and C6 (**Figure A-6a)**. These new substrates were then tested for cleavage by their corresponding 10-23 deoxribozymes. As shown in **Figure A-6**b, the new substrates containing two ribose residues were cleaved efficiently and specifically.

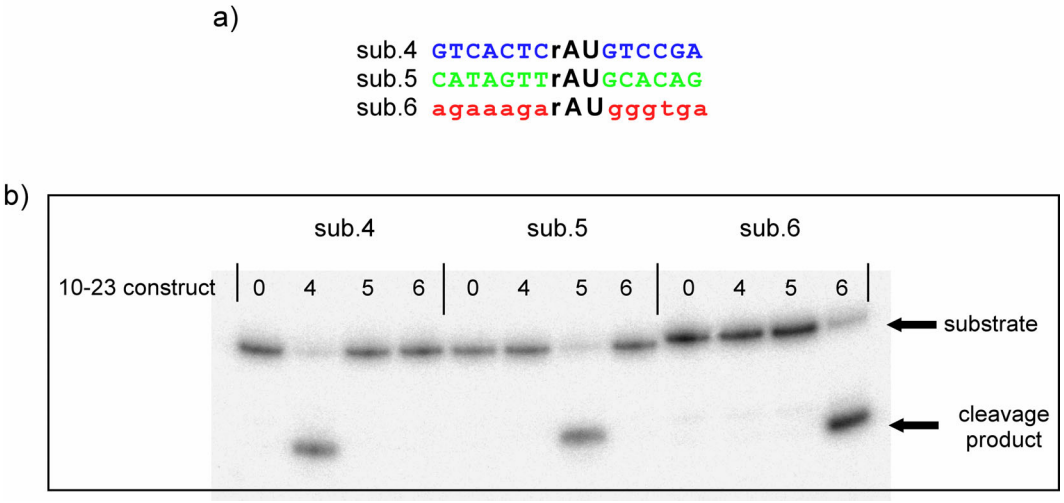

Figure A-6. Cleavage of 10-23 substrates with two ribose residues. (**a**) Sequences of the three substrates. **rA** and **U** denote the ribose residues. (**b**) Substrate cleavage reactions. 10-23 construct 4 is designed to cleave sub.4, 10-23 construct 5 is designed to cleave sub.5, 10-23 construct 6 is designed to cleave sub.6. A 0 denotes the absence of any deoxyribozyme. Assays were conducted as described in Figure A-3.

Following the successful cleavage assay, circular deoxyribozymes C4, C5, and C6 were generated from the linear permutations. Non-enzymatic circularization reactions were carried out in 50 mM Tris pH 7.4 containing 500 mM NaCl and 10 mM $MgCl_2$ and contained 2.5 nmoles of linear circle and 5 nmoles of splint oligonucleotide. Each reaction was 50 µL. Reactions were digested with exonuclease VII for 5 hr to remove any linear species prior to gel

purification on a 15% denaturing (7M urea) gel. The circles were subsequently eluted from the gel and recovered by ethanol precipitation.

In order to determine if the circular deoxyribozymes were capable of cleaving one another as circles, cleavage reactions containing purified circles C4 and C5, C5 and C6, and C6 and C4 were conducted (**Figure A-7**).

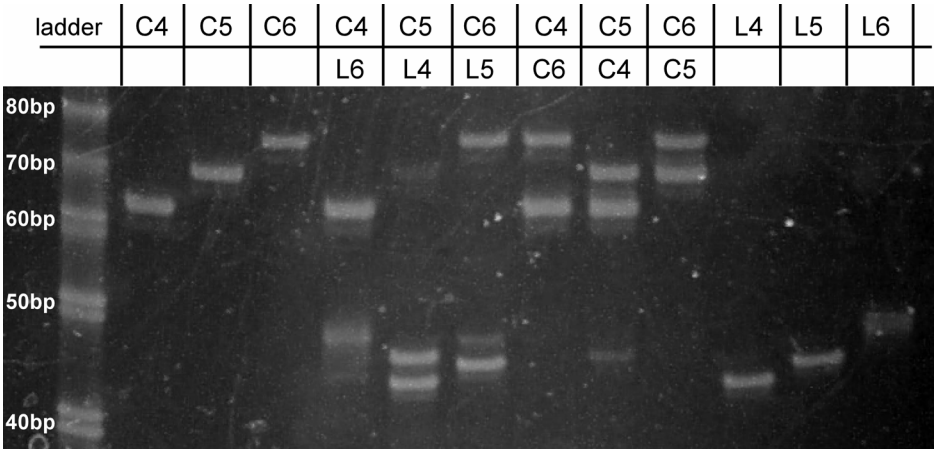

Figure A-7. Cleavage reactions with circles C4, C5 and C6. Linear tiggers L4, L5, and L6 mimic the cleavage product of each circle and cleave circles C5, C6 and C4 respectively. Reactions were conducted in 50 mM Tris pH 8.0, 30 mM MgCl2 and contained 0.5 μM of each circle or linear trigger. Reactions were incubated for 5.5hr at 37C and then analyzed by a 15% denaturing (7M urea) gel electrophoresis. The gel was stained with SybrGold and photographed on a UV transilluminator.

As shown in **Figure A-7**, while the reaction of C4 and C6 and C5 (lane 7) and C5 and C6 (lane 9) showed no cleavage products, the reaction of C4 and C5 (lane 8) produced a smaller product approximately the same size as L5 indicating that C4 was capable of cleaving C5. It is possible that the cleavage product observed was the result of contaminating L4 produced from the hydrolysis of C4. However, analysis of the C4 stock solution (lane 1) does not show any

contaminating linear species, although this does not rule out the presence of trace amounts of L4 contaminants.

Because of the leaky interaction of C4 and C5 the three circle system was abandoned in favor of the simpler two circle system (C_A and C_B) described in Chapter 5. Additionally, while the non enzymatic ligation scheme used for the construction of circle C1, C2, C3, C4, C5, and C6 worked well, it too was abandoned for enzymatic ligations, which removed the requirement of a 5' IdT, making the design process simpler.

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Vita

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Levy M, Ellington AD (2003a) Exponential growth in a cross-catalytic deoxyribozyme cycle. Proc Natl Acad Sci U S A in press

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This dissertation was typed by the author.