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Selection of Deoxyribozyme Ligases That Catalyze the Formation of an Unnatural Internucleotide Linkage

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Abstract—The chemical ligation of DNA molecules can be mediated by terminal phosphorothioate displacement of a 5′ iodine. We have selected deoxyribozymes that can catalyze the formation of such phosphorothioester internucleotide linkages. The selected deoxyribozymes enhance the rate of ligation in part through the provision of a template that aligns the ligation junction and do not appear to require metal ions for catalysis. © 2001 Published by Elsevier Science Ltd.

Introduction

DNA has been shown to be as competent a catalyst as RNA.[9] However, there has to date been only a single selection of a deoxyribozyme ligase.[2] This DNA enzyme catalyzed the ligation of its 5′ hydroxyl to a target oligonucleotide bearing an activated 3′phosphorimidazolide, forming a new phosphodiester bond. The selected catalyst required divalent cations (Zn^{2+} or Cu^{2+}) for its activity, suggesting that ligases selected from DNA pools are similar to those that have previously been selected from RNA pools, which in general have also used metals to catalyze the attack of hydroxyl moieties on activated phosphates (typically phosphoanhydrides) to form 2′-5′ or 3′-5′ phosphodiester linkages.[1,7,11]

We were interested in determining whether deoxyribozymes might also be capable of facilitating ligase reactions via chemistries not typically found in nucleic acids. To this end, we adapted a selection procedure to a novel ligation chemistry that had previously been developed by Kool and co workers.[14] In this chemistry, a 5′ iodine is displaced by a 3′ phosphorothioate to form a bridging 5′ phosphorothioester linkage (Fig. 1a). While this chemistry has been shown to be very efficient for the ligation and circularization of oligonucleotides, it is relatively slow; ligation reactions typically require many hours to accumulate appreciable ligation products.[16] Using in vitro selection we hoped to enhance the overall speed of the reaction via the selection of a catalyst.

After 11 rounds of selection and amplification we have identified deoxyribozymes that can catalyze the formation of this unnatural internucleotide linkage. Interestingly, the deoxyribozyme reaction has proven to be metal-independent.

Materials and Methods

5′IN90 pool construction

The pool was designed and synthesized according to previously reported methods.[11] In brief, synthesis was carried out 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). Primers containing 5′ I-dT were synthesized with ultramild amidites. The 3′ phosphorothioates were synthesized on 3′ phosphate-CPG by replacing the normal oxidizer with the sulfurizing reagent thiosulfonate. The pool contained a central core region of 90 random nucleotides flanked on both sides by constant sequence regions (5′ TGACTTCGGTCAGGTGCTCGTG-N90-CTCGTGATGTCCAGTCGC) (Fig. 1b). The randomized mixture contained a 1.2:1.3:1.5:1 molar ratio of

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dA/dC/dG/dT (David Bartel, Whitehead Institute, personal communication).

The 5′ iodine was added to the pool via PCR. A large scale PCR reaction (180 mL) was carried out using a 5′ primer containing iodine and a 3′ primer containing biotin (5′ iodine-TGACTTCGGTCAGGTGCTCGTG and 5′ biotin-GCGACTGGACATCACGAG). Double stranded DNA was purified by native acrylamide electrophoresis to remove the excess biotinylated primer. After extraction from the gel, the double stranded PCR product was immobilized on streptavidin agarose and the single-stranded, iodinated strand was eluted with 0.2 N NaOH.[8] The eluate was immediately neutralized by the addition of 3 M NaOAc pH 5.2, and precipitated with ethanol.

Selection for catalysts

Eleven rounds of selection were performed according to a procedure originally described by Bartel and Szostak[1] and adapted by our laboratory[11] with some modifications (Fig. 1c). In short, the single stranded DNA pool was annealed to a biotinylated DNA primer (5′

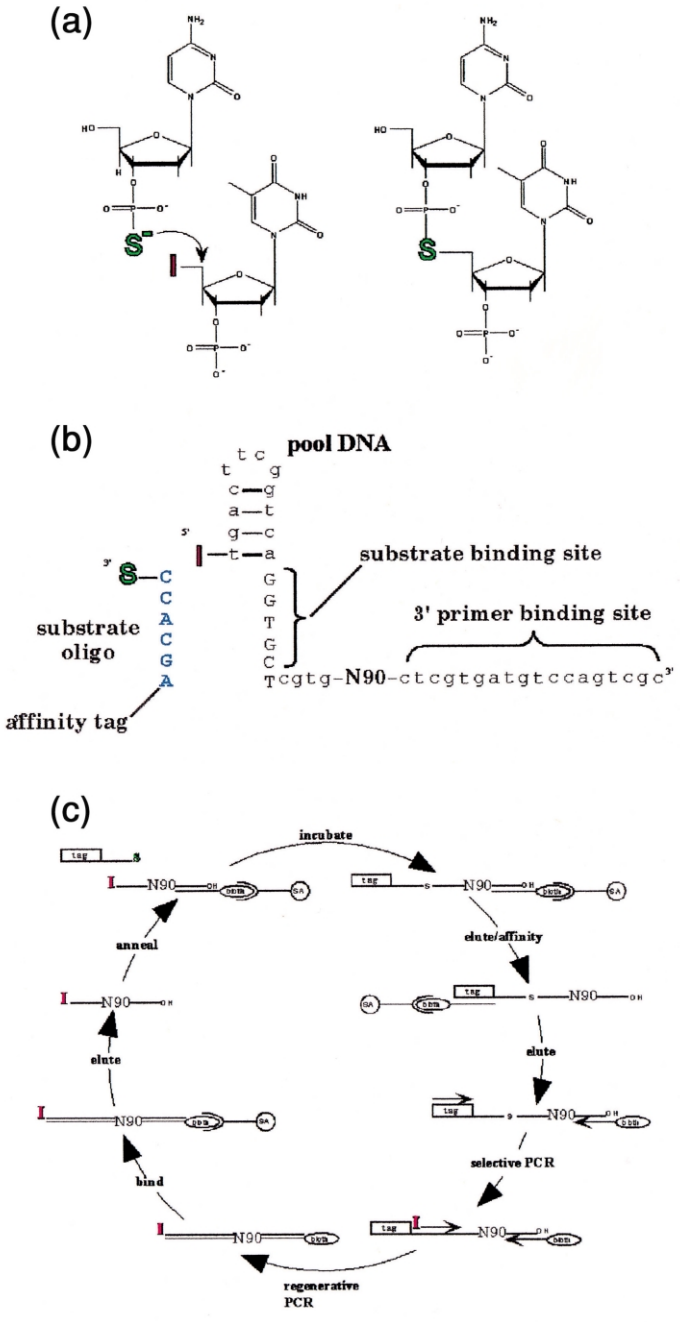

Figure 1. Pool and selection scheme. (a) Formation of a bridging 5′-phosphorothioate internucleotide linkage at the ligation junction. (b) 5IN90 pool. Constant sequence regions are shown in lowercase. The common substrate binding site is capitalized. The 5′ iodine is indicated by a capital I (red) and the 3′ phosphorothioate by a capital S (green). The hexanucleotide common to all substrates is shown in blue. (c) Selection scheme used to isolate deoxyribozyme ligases. The selection protocol is described in greater detail in Materials and Methods.

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 a 1.5-fold excess of a substrate oligonucleotide bearing a 3′ phosphorothioate. Incubation times and reactant concentrations are as shown in Figure 2. 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 base. After neutralization and precipitation the eluted population was amplified using a 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 PCR product served as a template for regenerative PCR with the 5′ primer containing iodine and the 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 (~10^{15} sequences). Five different substrate oligonucleotides were employed during the selection: KSS1 5′ AAAAAAAAAAAAAAAAAA AAAGCACC; KSS2 5′ TACATGTCTATCGA TCT GACTAAGCACC-S; KSS3 5′ GATCTAGTCGATCG TAGAGCACC-S; KSS6 5′ GCATACGACAAACTGG ACCTACTGAAGCACC-S; and KSS7 5′ TCTACGCT GATCTGTACTGAATGGCAGCACC-S. 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 μM.

Cloning deoxyribozymes

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

Kinetic assays

All assays 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. The single-stranded DNA was 3′ end-labeled using terminal deoxynucleotide transferase (Gibco BRL, Gaithersburg, MD) and dideoxyadenosine 5′-[α-^{32}P]-triphosphate (Amersham Parmacia Biotech, Piscataway, NJ). DNA was heat denatured at 70 °C and cooled to 25 °C in selection buffer prior to addition of DTT-treated substrate. Assays done in the absence of Mg^{2+} or in the presence of EDTA were adjusted to constant ionic strength with 1 M NaCl. Reactions were stopped by the addition of 95% formamide gel-loading buffer. Analysis was conducted on a 8% denaturing acrylamide gel and quantitated using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Site-directed mutagenesis

Deoxyribozymes containing mutations in the designed substrate-binding site were generated by PCR with a mismatched, iodinated 5′ primer (I-TGACTTCGGTC A*C*GTGCTCGTG; italics indicate mismatch). Deoxyribozymes containing mutations in the evolved substrate-binding site were generated by PCR with mismatched, non-iodinated 5′ primers (C14 5′ TGACTTCGGTCA GGTGCTCGTGCATGAATCGAAGACA*C*GTTGCC GAGT, and C15 5′ TGACTTCGGTCAGGTGCTCG TGACGCGTACGAACGGAGCCGTGTCA*C*GTTT GCTCGC; italics indicate 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 (TACATGTCTATCGAT CTGACTAAGCAC*G*-S; italics indicate the mutation).

Results and Discussion

Selection of deoxyribozyme ligase

In order to select a deoxyribozyme ligase capable of enhancing the formation of a bridging 5′ phosphorothioester linkage we employed a strategy similar to

(a)

Round	Pool Concentration (uM)	Substrate Concentration (uM)	Substrate	Time
1	1.0	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

(b)

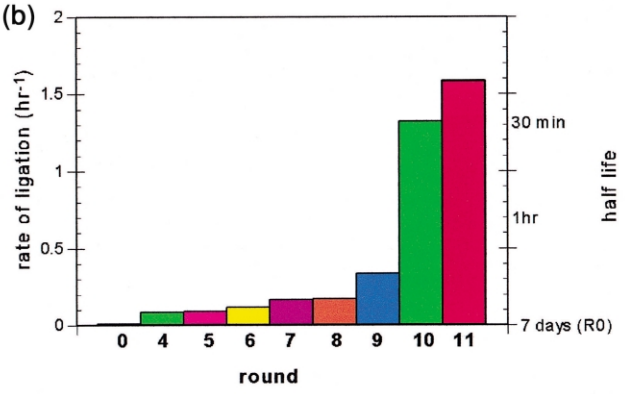

Figure 2. Selection of deoxyribozyme ligases. (a) Reaction conditions and substrates used during each round of selection. (b) Enhancement in ligation activity with successive rounds of selection.

that originally developed by Bartel and Szostak (1993) and successfully adapted by our lab[11] (Fig. 1b,c). The starting pool contained 90 random sequence positions. The 5′ constant region was designed to form a hairpin structure and contained a 5′I-dT. A phosphoramidite containing the iodinated residue was incorporated into a primer and subsequently incorporated into the pool via the polymerase chain reaction. In order to guard against the accumulation of ligases that merely developed extensive templating abilities, a total of five different substrates were employed during the course of the selection. All contained a 3′phosphorothioate and the terminal hexanucleotide sequence 5′ TGCACC-ps, but otherwise differed extensively in their sequences. Ligation products accumulated upon incubation of substrates with the iodinated pool and could be readily amplified via PCR, indicating that the unnatural linkage does not impede DNA polymerases, as Kool and coworkers have previously observed.[15] Following amplification, single-stranded DNA was again isolated and the selection repeated. The overall stringency of the selection was increased by decreasing both the substrate concentration and the time allowed for ligation (Fig. 2a).

The efficiency of ligation was assayed at each round, and the progress of the selection is shown in Figure 2b. After 11 rounds of selection the ligation rate of the selected pool was 1.6 h^{-1}, a 400-fold increase over the starting pool (0.004 h^{-1}). The final, selected pool was as fast as that selected by Cuenoud and Szostak (1.7 h^{-1}).[2] However, given the fact that the initial rate of phosphorothioester bond formation is much faster than the initial rate of phosphodiester bond formation the overall improvement of the pool was much less. Additional rounds of selection did not greatly increase the speed of the pool. It is unlikely that the lack of improvement reflected constraints on the selection experiments themselves; the final rounds of selection were carried out with incubation times on the order of 5 seconds.

Characterization of selected ligases

Deoxyribozymes from Rounds 10 and 11 were cloned and sequenced. Individual clones from Round 11 were assayed for their ability to catalyze phosphorothioester bond formation. The speeds of individual deoxyribozymes varied from 1–2/h, consistent with the speed of the pool as a whole. While some sequences appeared multiple times in each round, a great deal of diversity remained in the population. However, most of the ligases could be grouped into a single major class (Fig. 3) based on shared sequence motifs. An octamer sequence motif (5′ TGCTTTTT, blue) was found near the 3′ end of the deoxyribozymes. This octamer was frequently (although not always, see ligases No. 7 and 18 from Round 11) accompanied by an adjacent pentamer motif (5′ GACGG, purple). The major class of deoxyribozymes also possessed a similar octamer motif nearer the 5′ end (5′ G[A/T]CAGGTT, red). Conserved motifs that occupied different locales in different clones were also a hallmark of the deoxyribozyme ligases selected by Cuenoud and Szostak.[2]

In order to determine if these sequence motifs might be presented in similar structural contexts, the secondary structures of individual clones were predicted using the program RNAStructure 3.5[10,12] Two putative structures for the major clone (No. 5) from Round 11 are shown in Figures 4a and 4b; the substrate KSS2 is shown in green. In one structure, the 5′-most octamer motif (red) pairs with the 5′ end of the substrate oligonucleotide, while in the other it templates the ligation junction itself. Functional analysis of site-directed, sequence covariations (Fig. 5) suggests that the octamer motif does template the ligation junction. The wild-type deoxyribozyme preferentially uses a substrate that terminates in C. A variant of Clone No. 5 (C14.m1) in which the designed substrate binding site was mutated still preferred the wild-type substrate. In contrast, a variant (C14.m2) in which the hypothesized internal substrate binding site was similarly mutated strongly preferred the mutant substrate. Once this interaction is fixed, RNAStructure strongly prefers the structure shown in Figure 4b. Similar structures in which the octamer motif participates in the ligation junction can also be drawn for other ligators, including another prominent clone (No. 15, from Round 10; Figure 4c). However, in this clone neither the designed nor the internal binding site is supported by covariation analysis (Fig. 5),

Round 10

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

Round 11

clone			rate(hr⁻¹)
	5 (7)	CATGAATCGAAGACAGGTTGCCGAGTCTCTCGTAGCGAGCAAGGATCGCTGCGATTGGGGCGAGGCTGCTTTTTGACGGCCAGATCGGTG	1.4
	7 (2)	TAACGCAGAAAACGAAGTGCCTGCAACCACAGTCAGGTTCCCCCATCCCGACCTACGGGCGAATTGGGGTGCTTTTTTTAGATCGTTGAC	1.0
	1	GTCCCAGGAAGCGAAGTCAGGTTTGCCGGGCTTGGCAAGCGCTTAGCTTGCAGCACCCGGATGCTTTTTTAGACGATTAGTACAACAGG	2.0
	3	TTCAGGTCGAAAACTCACGCAGTTTCGTATTTTATTTTTTGCATTTGTAATTTTTCTGCTGGCGATTGTCTGAGCGGGCTCTTGTTGTGCC	0.1
	9	GATCAGATCATGAATGACCAATTTTTTTTTTGCGTTACGGCGGCCTCGTAATGCGAGATCGGGACGCCCACAGGGACAGGCTTGTTCAGC	0.1

Figure 3. Selected sequences from Round 10 and 11. The number of times a clone appears is shown in parentheses. The 3′ conserved motif is shown in blue with accompanying conserved pentamer in purple. The 5′ conserved motif is shown in red. Rates for individual clones from Round 11 are shown at right.

Figure 4. Putative secondary structures for selected Round 11 clones. Substrate KSS2 is shown in green. Other colors correspond to the sequence data in Figure 3. (a,b) Two potential structures for the major clone (No. 5) from Round 11. Mutational and other analyses suggest that the structure in (b) is correct. (c) Potential structure for Clone No. 15 from Round 10.

indicating either that this deoxyribozyme has an undiscovered substrate binding site, or more likely that the sequence as well as the complementarity of the deoxyribozyme is important for catalysis (note the loss of activity in variant C15.m2).

As in many previous selections for ligases,[4,5,7,11] it appears as though the selected catalysts have eschewed the substrate binding site provided for them in favor of creating their own unique substrate binding site from random sequence. Taken together, these results suggest that a ligase junction may be as much a part of the evolution of an enzyme active site as is the so-called catalytic core of the enzyme, and correspondingly that it may be difficult to develop nucleic acid ligases or replicases that can act generally on a variety of ligation junctions or base pairs.[3]

Metal independence of selected ligases

Almost all natural and selected nucleic acid enzymes have proved to be metalloenzymes.[6,9,13] This is not surprising, given the high nascent affinities of metal cations for nucleic acid polyanions and the fact that metals represent a wealth of novel chemistries not found in the five canonical nucleotides. Previous experiments with this ligation chemistry have typically included magnesium, and it was possible in the current instance that a metal ion would contribute to the active structure of the deoxyribozyme. We examined the metal dependence of the dominant ligase, Clone No. 5, 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 show <2-fold difference in rates. Replacing Mg^{2+} with the much more thiophilic Mn^{2+} had little effect on the reaction rate. It appears as though the fact that much of the ligation chemistry was 'pre-encoded' in the phosphorothioate nucleophile and iodine leaving group made it possible for selected deoxyribozyme ligases to avoid or ignore the generic chemical advantages that metal ions provide for most catalysts.

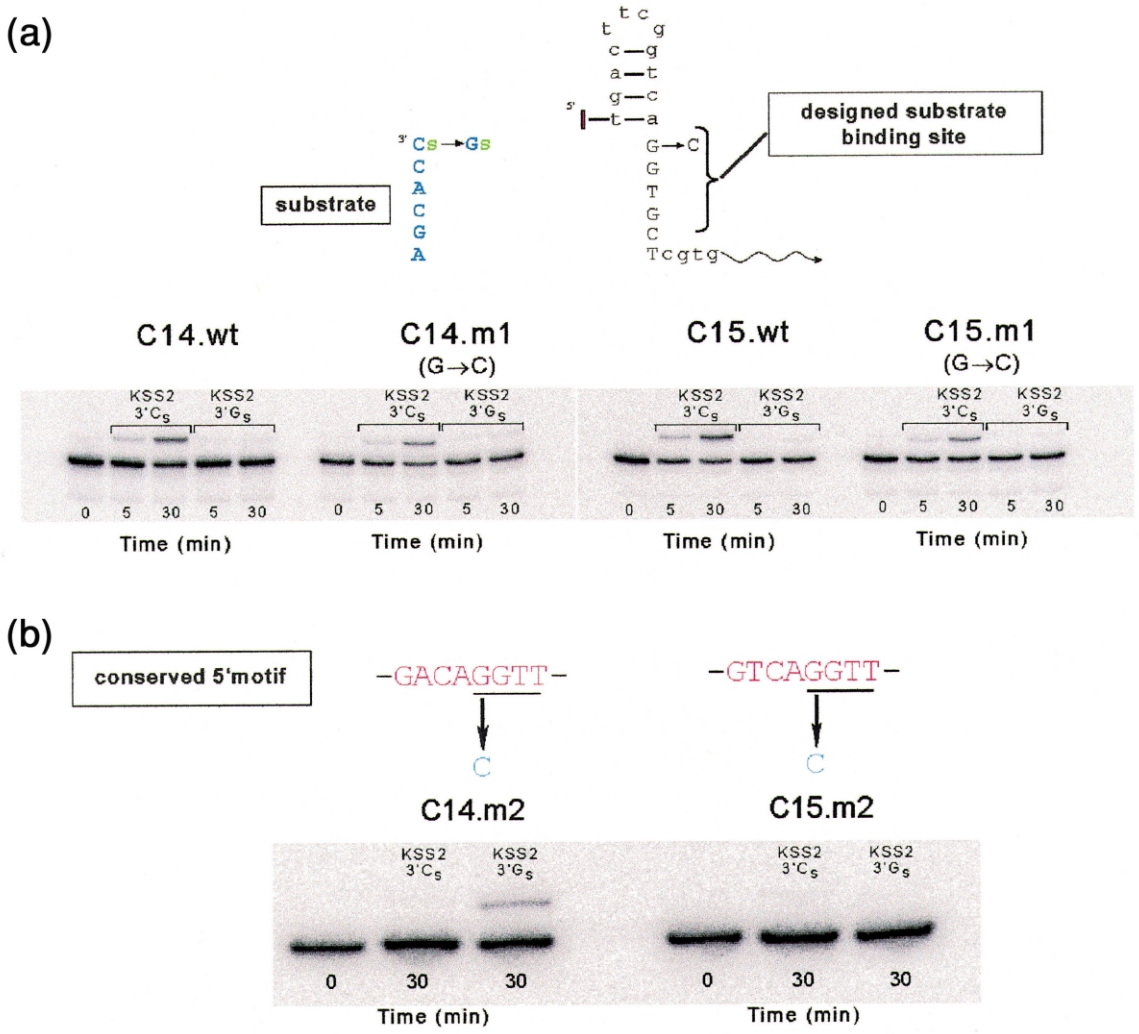

Figure 5. Identification of substrate binding site. (a) Deoxyribozyme C14 is Clone No. 5 from Round 11, while deoxyriboyzme C15 is Clone No. 15 from Round 10. Deoxyribozymes C14.m1 and C15.m1 contain a G to C mutation in the 5′ portion of the designed primer binding site. KSS2 3′ Cs is the wild-type substrate, while KSS2 3′ Gs contains a guanosine at its 3′ termini. (b) Deoxyribozymes C14.m2 and C15.m2 contain a G to C mutation in the 5′ portion of the hypothesized, internal primer binding site.

The metal-independence of the deoxyribozymes may be a result of the fact that no improvements in catalysis could be garnered by localizing magnesium near the phosphorothioester ligation junction. However, it is somewhat remarkable that no magnesiums apparently stabilized the active structure of the deoxyribozyme. An alternative explanation for inability to use metals is that the rate enhancements observed are primarily the result of the deoxyribozymes acting as complex 'templating elements' for oligonucleotide substrates. This conjecture may also explain why short sequence motifs were scattered throughout the primary and secondary structures of different deoxyribozyme ligases, and why the rate improvements observed in selected catalysts were modest. In this respect, it is interesting to note that the rate of ligation when the substrate was extensively paired (28 base-pairs) with a hairpin stem was 0.1/h, considerably faster than the nascent pool, but not as fast as the selected deoxyribozymes.

In order to better determine whether the selection of metal independent catalysts was the result of the evolutionary primacy of phosphorothioate ligation chemistry or the evolutionary primacy of templating, we are repeating these selection experiments with a variety of metal ions. For example, it is possible that the presence of thiophilic metals in the selection buffer may greatly improve the rates and catalytic improvement of selected deoxyribozymes. Alternatively, if templating is the primary constraint on the catalytic improvement of deoxyribozyme ligases then it is likely that sequences and structures similar to those observed here will again be selected.

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