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RNA-Catalyzed Thioester Synthesis

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Summary

A series of efficient ribozymes with thioester synthetase activities have been isolated from CoA-linked RNA libraries containing four different lengths (30N, 60N, 100N, and 140N) of random nucleotide regions. Competitive evolution of these size-heterogeneous CoA-RNA libraries resulted in an RNA size population in the order of 30N > 60N >> 100N > 140N. From isolated clones in the 30N and 60N size groups, two predominant RNA sequences, TES1 (30N) and TES33 (60N), have been shown to catalyze the synthesis of different thioesters using various acyl adenylates as the substrates. Together with our previous findings, the current results demonstrate a CoA thioester synthetic pathway catalyzed by individual metabolic ribozymes, and suggest a likely mechanism for thioester synthesis and utilization in an RNA world.

Introduction

Thioesters of coenzyme A (CoA) play a prominent role in living cells by serving as essential intermediates in numerous metabolic processes. With its "high-energy" thioester bond yet sufficient chemical stability, a thioester is an excellent intermediate for acyl group transfer reactions. In contrast, oxygen esters are more stable and have lower intrinsic chemical reactivity. Nature has fully exploited the properties of thioesters and has evolved efficient biotic systems for thioester synthesis and utilization. Acetyl CoA, a common thioester, is central to numerous complex metabolic pathways. Other thioesters of CoA are involved in the tricarboxylic acid cycle, lipid metabolism, amino acid metabolism, and pyrimidine nucleotide catabolism [1–3]. In addition, thioesters of fatty acids and amino acids are the key intermediates during the assembly of a structurally diverse group of bioactive polyketides and nonribosomal polypeptides catalyzed by the multifunctional protein complexes, polyketide synthases (PKS), and nonribosomal polypeptide synthetases (NRPK) [4–7].

The synthesis and utilization of CoA thioesters as active intermediates in broad biological processes by all three kingdoms of living organisms suggest an ancient origin of CoA thioesters, tracing at least back to the last common ancestor of life on Earth [8]. Furthermore, the inclusion of a ribonucleoside residue adenosine in the structure of CoA has led to the hypothesis [9, 10] that CoA and its thioesters might have functioned in a similar capacity to facilitate acyl group transfer reactions in an RNA world [11]. Recently, we have isolated RNA sequences that can synthesize CoA from its precursor phosphopantetheine [12], providing strong experimental evidence of plausible CoA synthesis and thereby its availability in the RNA world.

In our continuing effort to understand the origin and evolution of common coenzyme structures and functions, we have been conducting experiments to demonstrate the syntheses and functions of RNA-linked coenzymes [12–15]. We report here newly isolated RNA sequences of different lengths that are capable of catalyzing the efficient synthesis of thioesters of RNA-linked CoA using acyl adenylates as substrates. Combined with our previous work, the current findings not only add to the ribozyme repertoire with new catalytic RNA sequences but also link different ribozyme activities through the connecting intermediates between reactions in synthetic pathways (i.e., from phosphopantetheine to CoA to its thioesters). Therefore, multiple chemical steps of metabolite synthesis can in theory be realized by successive actions of a series of metabolic ribozymes.

Results

Selection of RNA with Thioester-Synthesizing Activity

In our search for ribozymes that can synthesize thioesters, we used random RNA libraries with covalently linked CoA at the 5′ end. Preparation of CoA-RNA libraries was based on a new in vitro transcription method by which RNA was initiated with 3′-dephospho coenzyme A (DePCoA) [15]. Selection procedures are shown schematically in Figure 1. Briefly, transcription in the presence of DePCoA leads to the synthesis of CoA-RNA (step 1), which is purified by thiopropyl affinity chromatography. CoA-RNA can react (step 2) with the selection substrate biotinyl adenylate (Biotin-AMP) by one of the two kinds of nucleophilic groups: the sulfhydryl group (−SH) (reaction A) from RNA-linked CoA, and the 2′- or 3′-hydroxyl group (−OH) (reaction B) of RNA ribose sugars. That is, both thioester synthesis and oxygen ester synthesis by CoA-RNA are likely to occur under the selection conditions. To eliminate the ester synthesis activity (reaction B) of RNA pools, substrate-reacted CoA-RNA was passed through a thiopropyl column (step 3), which, by virtue of a disulfide bond exchange reaction, binds biotinyl esters of CoA-RNA (with free −SH group) as well as unreacted CoA-RNA. Unbound RNA from the thiopropyl column was then applied to a second affinity column packed with Neutravidin beads (step 4), which retains any biotinylated RNA. Because biotinyl esters of CoA-RNA had already been removed by the previous thiopropyl column, the Neutravidin affinity column selected only biotinyl thioesters of CoA-RNA. Direct reverse transcription on the beads and PCR amplification completed the selection cycle.

To optimize the random nucleotide size of RNA pools in the search for thioester synthetase activity, we utilized

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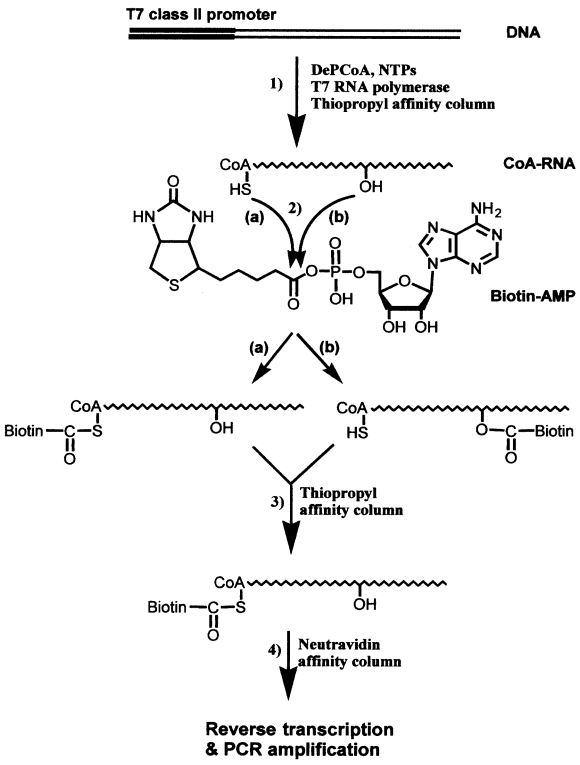


Figure 1. Selection Scheme for Isolating RNA with Thioester Synthetase Activity

(1) CoA-RNA library preparation by DePCoA-initiated transcription [15].

(2) Two possible reactions of CoA-RNA with the substrate Biotin-AMP: (a) desired thioester formation through the nucleophilic attack by the sulfhydryl group of CoA-RNA and (b) undesired oxygen ester synthesis from the 2′ hydroxyls or the terminal 3′ hydroxyl group within the RNA.

(3) Deselection of ester activity by thiopropyl affinity chromatography based on the free sulfhydryl group of the biotinyl ester.

(4) Capture of biotinyl thioester-RNA by Neutravidin affinity chromatography. Reverse transcription is then performed directly on the Neutravidin beads, followed by PCR amplification.

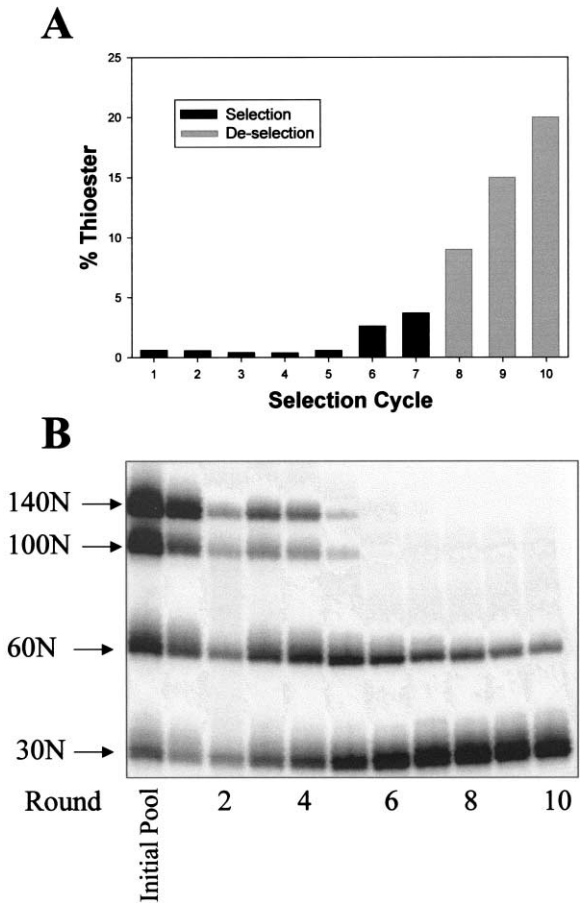


Figure 2. Selection Progression

(A) The amount of biotinylated RNA increases during the selection. Thioester yields are for total RNA (pppRNA and CoA-RNA) and rounds 8–10 incorporate the deselection of ester-forming RNA.

(B) RNA library size distribution changes during the selection. Internally ^{32}P-labeled RNA from different rounds is shown after gel electrophoresis. The initial RNA pool (lane 1) contains approximately equal moles of all four sizes. The intensity differences are attributed to incorporation of different amounts of the radiolabel. At the end of selection, the 30N size group dominates the pool and the two larger sizes (100N and 140N) have virtually disappeared.

heterogeneous lengths (30N, 60N, 100N, and 140N, N = the number of random nucleotides) of random RNA sequences. Such random RNA libraries are not conventional, but we have found them to be successful in isolating new RNA activities [12]. Inclusion of different sizes of random nucleotide regions in the same RNA initial pool increases the chances of isolating RNA sequences with desired activity without significantly burdening the selection process. During the selection, different sizes of RNA compete under the exact same conditions. Only the best fits between high catalytic efficiency and reproductive efficiency survive the selection processes. This type of in vitro selection therefore mimics natural selection, in which heterogeneous species compete for survival under the same conditions.

Selection progression was monitored by the yield of RNA retained by Neutravidin beads at each round of selection, shown in Figure 2A. In the early rounds of selection (rounds 1–7), RNA from transcription was used directly to react with the substrate. In addition, deselection (step 3 in Figure 1) was not performed due to low RNA yields from the Neutravidin affinity column. There was essentially no change in RNA yields from rounds 1–5. However, rounds 6 and 7 brought retained RNA to 4%–5% (based on total RNA after transcription), a 10-fold increase over the background. At this point, we performed an analysis of RNA pool activities, i.e., thioester synthesis versus oxygen ester synthesis. Using the same PCR DNA from the 7th round, two internally radiolabeled RNA libraries were prepared separately in the absence or presence of 4 mM DePCoA, resulting in normal RNA (pppRNA) and mixed RNA (pppRNA and CoA-RNA) libraries. If ester-forming RNA had been coselected, incubation of the pppRNA library with Biotin-AMP under the selection conditions would lead to the synthesis of RNA biotinyl esters, while both biotinyl esters and thioesters would be expected from the mixed RNA library. A streptavidin gel shift assay [16] was then used to determine the

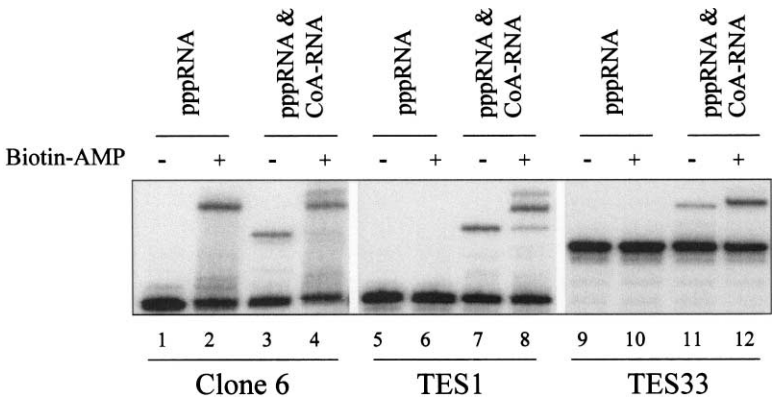


Figure 3. Thioester and Oxygen Ester Synthesis Activity Screening of Isolated Clones

Results from 3 representative clones (clone 6, TES1, and TES33) are shown. For each clone, two ^{32}P-labeled RNA preparations, pppRNA and mixed RNA (pppRNA and CoA-RNA), are reacted with the substrate Biotin-AMP. Biotinylation yields of RNA are then determined by a streptavidin gel shift assay [16]. Comparison of biotinylation yields of the pppRNA and mixed RNA reveals RNA catalytic activity. Clone 6 (lanes 1–4) has an oxygen ester-forming activity because both pppRNA and mixed RNA give similar biotinylation yields (lanes 2 and 4). On the other hand, TES1 and TES33 are thioester synthetase ribozymes, since only the mixed RNAs yield biotinylated products (lanes 8 and 12). Slower migrating bands in the control lanes (3, 7, and 11) are oxidized CoA-RNA dimers, and the slowest migrating bands in lanes 4 and 8 are attributed to streptavidin multimers [35].

biotinylated RNA yields. The results revealed that the normal RNA library produced 12% RNA products, while 22% biotinylated products were observed from the mixed library (results not shown). Therefore, approximately 50% of the RNA pool after 7 rounds of selection had biotinyl ester synthesis activity.

Deselection (step 3 in Figure 1) was then applied in the subsequent three rounds in order to eliminate the ester-forming RNA sequences. Biotinylated RNA yields continued to rise, and reached 21% of total RNA (Figure 2A) after 10 rounds. Recovered RNA from round 10 was then reverse-transcribed and PCR-amplified. For the reason described in the following section, only the 30N and 60N sizes of PCR DNA were purified by nondenaturing PAGE, cloned, and sequenced.

During the selection, the size distribution of the RNA pool changed quite dramatically (Figure 2B). The smallest RNA group (30N) progressively gained representation and became predominant, while the two RNA groups with large random nucleotide regions (100N and 140N) steadily lost their presence and virtually disappeared in later rounds of selection. The decrease of the 100N and 140N size groups accelerated precipitously at rounds 6 and 7, coinciding with the emergence of catalytic activity (compare Figures 2A and 2B). The 60N size group slowly decreased its population relative to the 30N group. At round 8 the 30N and 60N size groups represented 82% and 18% of the total RNA sequences, and this ratio remained roughly the same until the end of selection. Because of the apparent disappearance of 100N and 140N size groups at the end of the selection (Figure 2B), no effort was made to recover RNA sequences from these two groups.

Isolated RNA Sequences and Synthesis of Biotinyl CoA-RNA

Thirty clones from each of the 30N and 60N size groups were isolated. The presence of inserted 30N or 60N DNA in the clones was verified by PCR amplification and nondenaturing gel analysis. For each positive clone, two separate RNA (internally radiolabeled) preparations, pppRNA and mixed RNA (pppRNA and CoA-RNA), were transcribed from PCR DNA. Both oxygen ester and thioester activities were screened by reacting with the substrate Biotin-AMP according to the previously described procedure. Figure 3 shows representative screening results from three clones. For clone 6 (lanes 1–4), approximately equal amounts of biotinylated RNA product (lanes 2 and 4) were observed from both pppRNA and the mixed RNA, indicating that clone 6 RNA possesses only oxygen ester-forming activity. On the other hand, both TES1 and TES33 produced biotinylated RNA products from the mixed RNAs (lanes 8 and 12), but not from the pppRNAs (lanes 6 and 10). Therefore, TES1 and TES33 represent two thioester-synthesizing (TES) ribozymes. For several clones, no biotinylated RNA product could be detected from either pppRNA or the mixed RNA after reaction with Biotin-AMP (not shown). These RNA sequences thus have neither oxygen ester- nor thioester-forming activity. Twenty clones with thioester-synthesizing activity from each of the 30N and 60N size groups were sequenced, and their sequences are listed in Figure 4.

In both 30N and 60N size groups, the diversity of the isolated sequences is quite limited. Within the 30N group, 17 of the 20 clones (TES1-28) can be classified into a single family, and the remaining three have a single appearance. Alignment of the RNA sequences in the single family of the 30N group revealed a consensus continuous tract of 20 nucleotides (with 14 uninterrupted nucleotides completely conserved), acUuyCCGGCUU UAAUACGu, where upper case letters represent completely conserved nucleotides and lower case letters indicate 1 or 2 nucleotide substitutions. The letter y at the fifth nucleotide position specifies a pyrimidine (C or U). It is unlikely that all the isolated sequences in this family descended from one original sequence. Evidently, TES1 had a different origin from the other members of the family, while it is not clear how the other members are evolutionarily related to each other. It is possible that mutations during the selection contributed to the nucleotide changes among various clones in the family. However, an initial RNA library with short random nucleotide regions (i.e., 30 random nucleotides) might well have contained these sequences as isolated. Unlike the 30N group, no consensus nucleotide sequences are evident among the isolated sequences in the 60N group.

Clone	Sequence	Repeats
	30N size group	
TES1	**AUUUCCCGGCUUUAAUACGU**UAUUGACUAA	9
TES2	AAUUC**ACUUCUCGGCUUUAAUACGU**GUAAA	2
TES4	AACU**ACUUUCCGGCUUUAAUACGC**CUCAAA	
TES10	UAUUC**ACUUCCCGGCUUUAAUACGG**GCAAC	
TES17	UUAC**ACUUUCCGGCUUUAAUACGU**CUCAGA	
TES22	AUCGC**CCUUUCCGGCUUUAAUACGU**GCAAA	
TES25	AUCGU**CCUUCCCGGCUUUAAUACGU**GCAAA	
TES30	UUAG**ACUGUCCGGCUUUAAUACGU**CUCAGA	
TES12	UUGAAAGGCCACAUUUCCUGCCCGGUACCU	
TES16	UUGAAAUGAGCACAGGUCCGAGUCACAU	
TES28	UUAAUGCACUUUAACUUCGCGCCCGACCCU	
	60N size group	
TES31	ACACUUGCUGGUGUACGCGCCCCCUUGCGUACUCUGCCCUUCCGCGUCUACCCCGUCCAA	
TES33	ACACUUGCUGGUGUACGCGCCCCCUUGCGUACUCUGCCCUUCCGCGUCU-CCCCGUCCAA	8
TES35	GGAUACGCGAGCACCUUCUGGCUUAUUAGACUUUCUAAUAUCUGCCCUUCGAGCCCCCAU	
TES37	GGAUA**U**GCGAGCACCUUCUGGCUUAUUAGACUUUCUAAUAUCUGCCCUUCGAGCCCCCAU	2
TES40	**AGU**UACGCGAGCACCUUCUGGCUUAUUAGACUUUCUAAUAUCUGCCCUUCGAGCCC**U**CAU	
TES43	**AGC**UACGCGAGCACCUUCUGGCUUAUUAGACUUUCUAAUAUCUGCCCUUCGAGCCC**UCAA**	
TES44	GGAUACG**A**GAGCACCUUCUGGCUUAUUAGACUUUCUAAUAUCUGCCCUUCGAGCCCC**AA**U	
TES45	G**AA**UACGCGAGCACCUUCUGGCUUAUUAGACUUUCUAAUAUCUGCCCUUCGAGCCCCCAU	
TES34	UACUCUAAGCACCUGCAGUAAAGUAGUUGCGGCACUCCCGGCUCUAAUACGGACACCAUC	
TES36	UACUC**C**AAGC**U**CC**G**GC**G**GUAAAGUAGU**C**GCGGCACUCCCGGCU**U**UAAU**G**CGGACA**U**CAUC	
TES47	UACUCUAA**U**CU**C**C**G**GCACUAAAGUAGU**C**GCGGCACUCCCGGCUCUAAUACGGACA**U**CAUC	
TES48	UUCACCCACUCCGGGUGCGGUGUGCCCAAACCUCUUCCUACGCCGCAUUAGCCUAAGCAU	

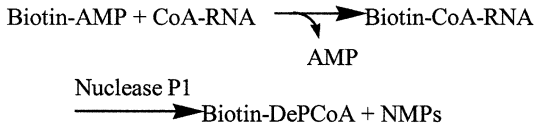
Figure 4. Isolated RNA Sequences from the 30N and 60N Size Groups

(A) The 30N group contains one family and three individual sequences. The sequences of the single family are aligned to reveal a consensus sequence (in bold).

(B) Four distinct sequences are isolated from the 60N group. Mutations during the selection generate minor variations (in bold).

The conserved 14 continuous nucleotides from the 30N group do not appear in the 60N group. All the isolated clones (TES31–48) from the 60N group contain four original RNA sequences. Point mutations during the selection resulted in slight variations from the original sequences.

Reaction products from the substrate Biotin-AMP, catalyzed by the dominant CoA-RNA sequences (TES1 and TES33) in each of the 30N and 60N size groups, were analyzed to confirm their identity as biotinyl thioesters. First, blocking the free thiol group of both TES1 and TES33 CoA-RNA with Ellman's reagent (5,5′-dithio-bis-[2-nitrobenzoic acid], DTNB) resulted in the complete inhibition of thioester product formation (Figure 5A), indicating that the thiol group of CoA is required for the reaction. Second, after incubation of Biotin-AMP with CoA-RNA, purified RNA-linked products were completely digested with nuclease P1 to release biotinyl dephospho-CoA (Biotin-DePCoA) and nucleoside monophosphates (NMPs). For both TES1 and TES33, reverse phase HPLC separation of Biotin-DePCoA from NMPs revealed a distinct product peak with a retention time around 4 min (Figure 5B) and a characteristic shoulder in the UV spectrum at 230–240 nm (Figure 5C). In contrast, neither nuclease P1-digested CoA-RNA itself nor similarly treated pppRNA incubated with Biotin-AMP exhibited the same peak. Furthermore, both the HPLC retention time and the UV spectrum of the RNA-synthesized products matched those of chemically synthesized Biotin-DePCoA standard (not shown). In addition, the peak at 4 min in Figure 5B was collected and subjected to mass spectrum analysis. MALDI-TOF MS gave a single molecular peak of 912.5 mass units (Figure 5D), in excellent agreement with the calculated molecular mass of 912.9 for the Biotin-DePCoA anion $(M\text{-}H)^-$. In summary, the ribozyme-catalyzed synthesis and identification of Biotin-DePCoA can be described by the following equation:



Alternate Substrate Utilization

To explore the possibility that the isolated RNA sequences (TES) may use alternate substrates other than Biotin-AMP, thereby producing a broad spectrum of thioesters, we chemically synthesized two other acyl adenylates: an alkyl adenylate acetyl AMP (Ac-AMP) and an aminoacyl adenylate L-phenylalanyl AMP (Phe-AMP). A substrate competition assay was used to quickly assess whether Ac-AMP and Phe-AMP could act as substrates for TES1 and TES33 CoA-RNA. Both CoA-RNAs were reacted with Biotin-AMP (0.5 mM) for 10 min in the absence or presence of 2 mM Ac-AMP or Phe-AMP. Biotinylated RNA yields were then determined by a streptavidin gel shift assay. The competition assay was based on the assumption that, if these substrates were utilized by the ribozymes, the thioesterification reaction with the substrate Biotin-AMP would be inhibited. For TES1, Phe-AMP displayed inhibition of biotinyl CoA-RNA synthesis, while Ac-AMP exerted no detectable influence. However, both Phe-AMP and Ac-AMP reduced the biotinylated product synthesis of TES33 CoA-RNA (results not shown). These results suggested that both TES1 and TES33 could use Phe-AMP as an alternate substrate, while Ac-AMP could only be used by TES33.

To confirm that different thioesters were indeed produced from alternative substrates by TES1 and TES33 CoA-RNA, both Ac-AMP and Phe-AMP were reacted with each of the two CoA-RNAs. The resulting RNA products were purified by ethanol precipitation, digested by nuclease P1, and analyzed by HPLC and mass spectroscopy. Figure 6 shows the analysis results for TES33. HPLC elution profiles revealed distinctive product peaks

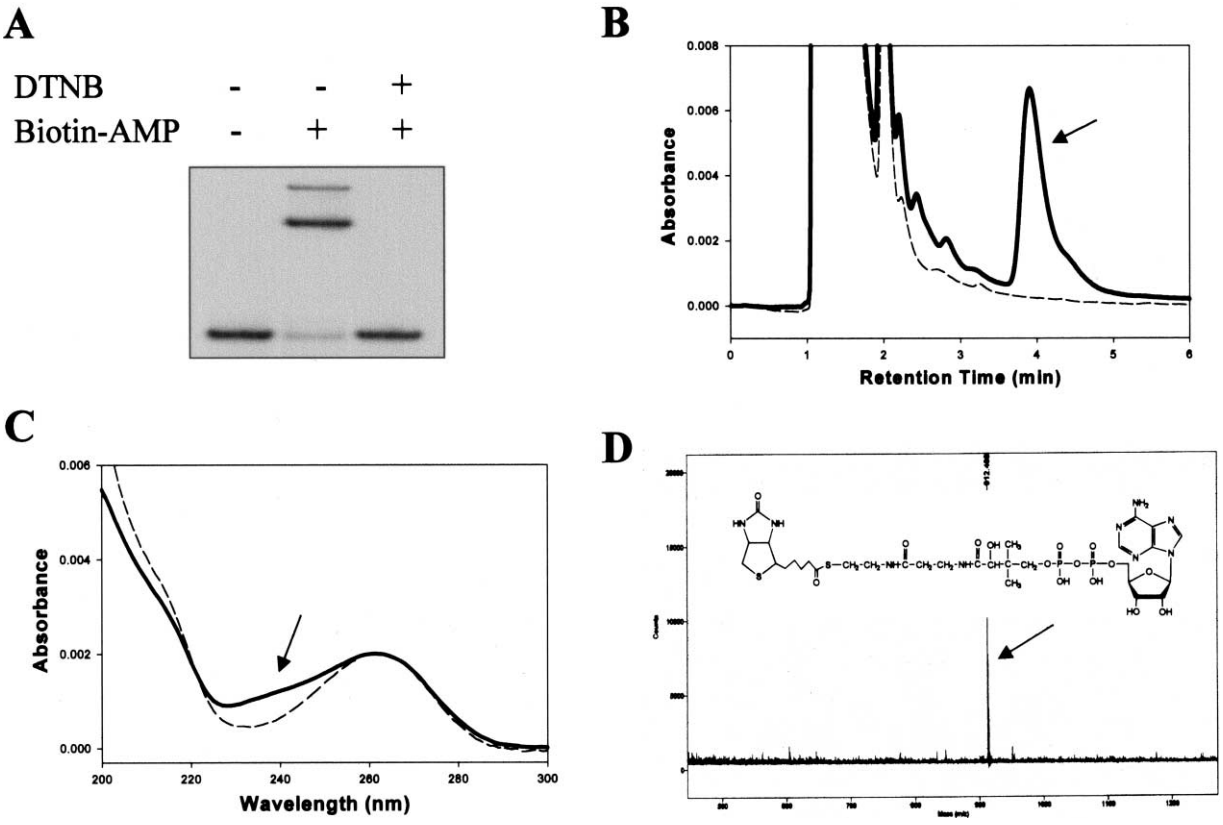


Figure 5. Confirmation of Biotinyl Thioester Synthesis by TES33

(A) Comparison of the standard reaction (lane 2) with that following blocking of CoA-RNA with Ellman's reagent (lane 3).

(B) HPLC separation of nuclease P1-digested products for both CoA-RNA (dashed line) and substrate-reacted CoA-RNA (solid line). A product peak is observed in the reacted RNA at a retention time of 4 min.

(C) UV spectral comparison of standard DePCoA (dashed) with the 4 min peak (solid) in Figure 5B reveals a characteristic thioester shoulder within 230–240 nm. The spectra are collected by an online HPLC photodiode array detector.

(D) MALDI-TOF analysis of the sample collected from the 4 min peak in Figure 5B shows a single molecular peak of 912.5 mass units for the negative ion $(M–H)^-$.

Similar results were obtained for TES1 (not shown).

at around 5 min for Ac-DePCoA (Figure 6A) and 4.5 min for Phe-DePCoA (Figure 6B). Both peaks possessed characteristic thioester UV absorption shoulders (not shown) and had the same retention times as those of the chemically synthesized Ac-DePCoA and Phe-DePCoA standards. MALDI-TOF MS analysis of the RNA-synthesized Ac-DePCoA and Phe-DePCoA (collected from HPLC) identified single-molecular peaks of 728.1 (Figure 6C) and 833.7 (Figure 6D) mass units, respectively, matching calculated molecular weights of the negative ions $(M-H)^-$ (728.6 and 833.7). Similar HPLC and MS analysis on TES1 confirmed Phe-DePCoA product formation. However, no Ac-DePCoA was detected, indicating that TES1 exhibits a certain degree of substrate specificity. In summary, isolated thioester-forming RNA sequences are able to use various adenylates, such as alkyl adenylates and aminoacyl adenylates, in addition to the biotinyl adenylate used during the selection.

Ribozyme Properties

After thioester formation by TES1 and TES33 CoA-RNA had been confirmed, further analysis was carried out to determine their metal ion dependencies for catalytic activity. Since the selection buffer contained only Ca^{2+} and Mg^{2+} as the divalent metal ions, we tested ribozyme activity under different combinations of Ca^{2+} and Mg^{2+}. Both TES1 and TES33 absolutely require Ca^{2+} for activity, while Mg^{2+} does not appear to have an appreciable effect on catalysis. Reduction in both Ca^{2+} and Mg^{2+} concentrations from 20 mM (selection conditions) to 5 mM results in decreased activity.

Kinetic analysis of the thioester-synthesizing activity for both TES1 and TES33 was conducted to determine their catalytic efficiencies. The apparent rate constants (k_{obs}) of RNA-catalyzed thioester formation at different Biotin-AMP concentrations were first obtained by fitting experimental data to a first-order reaction equation (Figure 7A). Both enzymatic parameters, k_{cat} and K_M, were then derived from fitting the acquired k_{obs} data to the Michaelis-Menten equation (Figure 7B). TES1 has k_{cat} and K_M values of 0.29 ± 0.07 min^{-1} and 1.3 ± 0.5 mM, respectively, while the corresponding values for TES33 are 0.40 ± 0.10 min^{-1} and 0.35 ± 0.14 mM. Catalytic efficiencies (k_{cat}/K_M) for these two ribozymes are there-

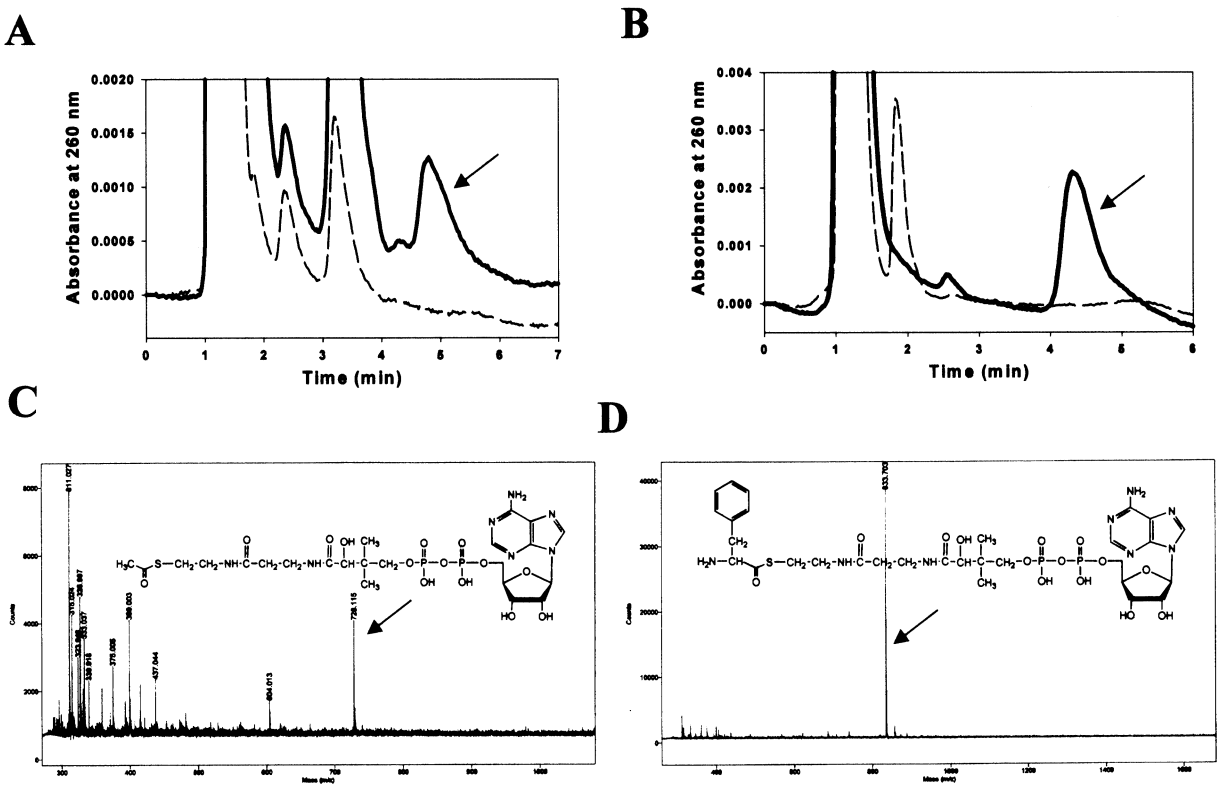


Figure 6. HPLC and MS Analysis of Other Thioesters Made by TES33 Ribozyme

(A) HPLC profiles of nuclease P1-digested CoA-RNA and Ac-CoA-RNA, displaying a thioester (Ac-DePCoA) peak at 5 min.

(B) A distinct Phe-DePCoA peak from Phe-AMP reacted CoA-RNA after nuclease P1 digestion.

(C and D) MS analysis of the samples collected from the two thioester peaks in Figures 6A and 6B, respectively. Negative ions for both Ac-DePCoA and Phe-DePCoA are clearly displayed with the correct molecular masses of 728.1 and 833.7, respectively. HPLC and MS results for both RNA-made Ac-DePCoA and Phe-DePCoA are identical to those of chemically synthesized standards.

fore 220 ± 10 $M^{-1} \cdot min^{-1}$ for TES1 and 1100 ± 50 $M^{-1} \cdot min^{-1}$ for TES33.

Discussion

Using coenzyme A-linked random RNA libraries of heterogeneous lengths, we have isolated a series of RNA sequences with general thioester-synthesizing activities. Although RNA sequences were isolated using Biotin-AMP as the substrate, various acyl adenylates including alkyl adenylates and aminoacyl adenylates can also serve as substrates, leading to the synthesis of a variety of RNA-linked CoA thioesters.

The selection scheme used to isolate these thioester-forming ribozymes incorporated several unique features. First, we utilized a new DePCoA-initiated transcription method developed in our laboratory to covalently link CoA onto the 5′ ends of RNA transcripts [15]. Covalent coupling of CoA to RNA in this manner effectively eliminates the requirement for ribozymes to first bind the coenzyme and then use the thiol nucleophile to perform unique chemistry. The same principle is applied by acyl carrier proteins (ACP) through covalent coupling of the phosphopantetheinyl group to a serine residue [17]. Secondly, because some oxygen ester-synthesizing RNA activity was coisolated during the selection, we used a distinctive deselection method to eliminate RNA with this undesired activity. A combination of thiopropyl and Neutravidin affinity chromatography was used for deselection and selection, based on the unique chemistries (i.e., free sulfhydryl group versus protected thioester group) of oxygen ester- and thioester-linked RNA. Although the nucleophilic reactivity of a sulfhydryl group is about three orders of magnitude higher than that of a hydroxyl group [18], it should be noted that an RNA molecule contains a large number of hydroxyl groups depending on the size of the RNA. In addition, the free energies for thioester and ester formation from an acyl phosphate are −11.8 kJ/mol and −23.7 kJ/mol, respectively [19]. To successfully isolate RNA catalytic activities other than ester-synthesis using acyl adenylates, an effective deselection procedure must therefore be incorporated into a selection scheme.

The third unique aspect of the selection was the use of size-heterogeneous RNA pools with completely random regions of 30N, 60N, 100N, and 140N. We used the differently sized libraries in our selection in an attempt to investigate size versus activity relationships in catalytic RNA, because all four RNA sizes were allowed to compete under identical conditions throughout the selection. The ultimate result is the "survival of the fittest" where the optimal sizes predominate in the pool. It has

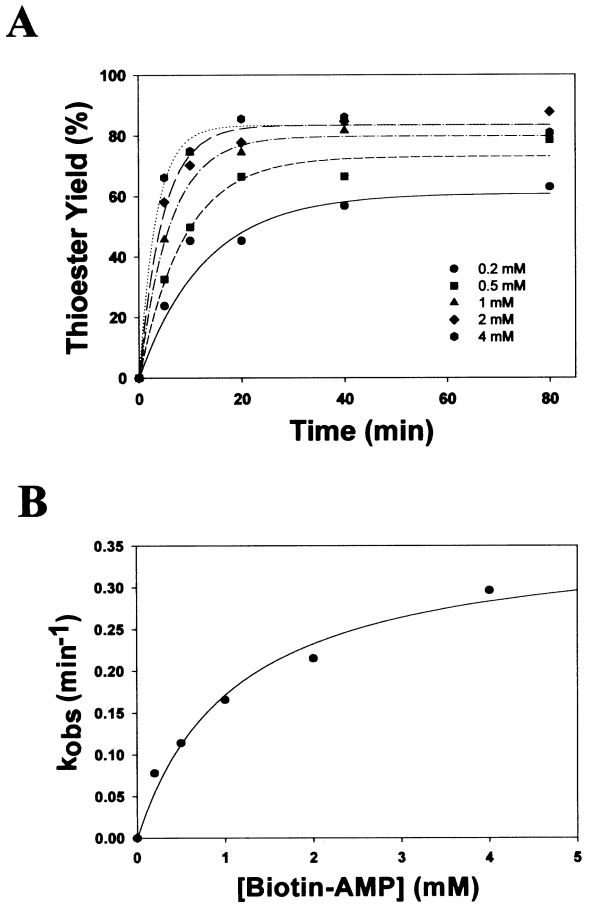


Figure 7. Representative Kinetic Analysis of TES1 Ribozymes

(A) Thioester product yields determined by streptavidin gel shift are plotted versus time for various Biotin-AMP concentrations. Curves are fitted to an apparent first-order kinetic equation to derive k_{obs} values under each Biotin-AMP concentration.

(B) Enzymatic parameters k_{cat} and K_M are then obtained by fitting k_{obs} according to the Michaelis-Menten equation.

been argued that longer random regions are superior to shorter RNA sizes because longer RNA sequences can potentially support more complex secondary and tertiary structures and therefore catalytic capabilities [20]. However, it is also true that longer random regions are at a disadvantage because of their inherent relative inefficiency of replication, especially under the conditions of the RNA world. In order to minimize the difference in replication efficiency of varying sizes, we thoroughly investigated, prior to this selection, the factors influencing the efficiency of individual selection steps (transcription, reverse transcription, and PCR amplification). From simulated competitive selection cycles with the four sizes, we were able to derive the optimal conditions (used for this selection) under which the bias against the longer sequences was reduced to the minimum. Starting with an equal molar ratio of random DNA templates, five rounds of simulated selection resulted in a size distribution of 40%(± 4%), 34%(± 3%), 15%(± 2%), and 11%(± 1%) for 30N, 60N, 100N, and 140N, respectively (unpublished results). Through extrapolation, the relative sizes after ten cycles were estimated to be 55%, 36%, 6%, and 3%. In contrast, the size distribution after 10 rounds of selection for thioester synthesis was 81% (± 8%), 18% (± 2%), 1% (± 0.5%), and 0.06% (± 0.03%).

While the 60N size group population was somewhat below the simulated results (36% versus 18%), the selection strongly discriminated against the two largest size groups (6-fold and 50-fold lower than the simulated size distribution). The 2-fold difference between the selection and simulation of 60N over 10 rounds does not necessarily indicate that the 60N size group is selected against relative to the 30N group. In fact, kinetic results from the two predominant sequences (TES1 and TES33) seem to suggest the 60N size is superior to 30N (a detailed kinetic analysis of all isolated individual RNA sequences from each size group should reveal the catalytic efficiency of different size groups, which is the subject of our next manuscript). This study agrees well with our previous work on isolation of coenzyme synthetase ribozymes from size-heterogeneous RNA libraries [12]. Only shorter RNA sequences (30N and 60N random regions) survived the selection conditions, while the longer tracts (100N and 140N) disappeared early in the selection. Therefore, there appears to be an optimal balance between RNA replication efficiency and catalytic efficiency. Our results indicate that an optimal random RNA size is centered around 60N. Any potential advantages of longer random segments do not appear to overtake their replication inefficiency.

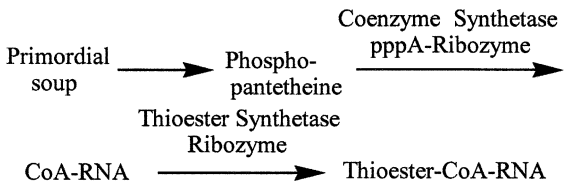
Our isolated thioester synthetase ribozymes perform well as judged by ribozyme catalysis standards. The catalytic efficiencies (k_{cat}/K_M) of TES1 and TES33 are 220 ± 10 $M^{-1} \cdot min^{-1}$ and 1100 ± 50 $M^{-1} \cdot min^{-1}$, respectively, which compare favorably with that (12 $M^{-1} \cdot min^{-1}$) of an independently isolated ribozyme with acyl CoA-RNA synthetase activity [21]. The background reaction rate of random RNA pools was estimated by incubating pure CoA-RNA with Biotin-AMP and analyzed by a gel shift assay. The uncatalyzed second order rate constant for both 30N and 60N initial pools was determined to be ~1 $M^{-1} \cdot min^{-1}$ under the selection conditions. Therefore, the two ribozymes (TES1 and TES33) accelerate thioester formation about 200-fold and 1000-fold over the background, respectively. On the other hand, second order rate constants for protein acyl-CoA synthetases are in the order of 10^6–10^8 $M^{-1} \cdot min^{-1}$ [22, 23].

In addition to the selection substrate Biotin-AMP, both TES1 and TES33 have been shown to utilize the aminoacyl adenylate Phe-AMP as an alternate substrate. Because a phenylalanyl group was never used during the isolation of these ribozymes, we expect that other aminoacyl adenylates can also act as substrates for these thioester synthetase ribozymes to produce a whole class of aminoacyl CoA-RNA thioesters. This class of thioesters is of particular interest, since current biological systems use aminoacyl pantetheine-protein as the intermediate to synthesize small peptides based on nonribosomal thiotemplate systems [4–7]. The fact that our ribozymes utilize aminoacyl adenylate substrates with substantial catalytic efficiency lends credibility to thioester involvement in the origin and evolution of protein biosynthesis [24, 25]. Derivatives of our ribozymes specific for various L- and D-isomers of amino acids or amino acid derivatives might prove useful in

designing an artificial RNA-based in vitro peptide synthesis system.

From known activities of current ribozymes and protein enzymes, it is evident that RNA catalytic capabilities cannot reach those of proteins. RNA alone, with only four standard ribonucleotides, does not possess diverse chemical groups to support efficient catalysis. Our newly isolated thioester synthetase ribozymes provide a mechanism for RNA to expand its functional group diversity by covalently linking amino acids through thioesterification of CoA-RNA, which can itself be synthesized by catalytic RNA [12].

Thioesters of CoA play an important role in modern cellular metabolism. A plethora of evidence exists that suggests their involvement in the origin and early evolution of life [24]. We have previously characterized ribozymes that catalyze the formation of the coenzymes CoA, NAD, and FAD from their respective precursors [12]. Specifically, these coenzymes can be synthesized by RNA-catalyzed coupling of precursors (phosphopantetheine, NMN, or FMN) to the 5′ ATP of RNA. Coenzyme precursors such as pantetheine and nicotinamide could have been available through prebiotic synthesis [26–29]. Therefore, our current results, taken together with previous findings, suggest a plausible mechanism of coenzyme A synthesis and utilization in the RNA world according to the following scheme:



In this scheme, the multiple chemical steps required to synthesize thioester-CoA-RNA would have constituted a synthetic pathway, and individual reactions could have been catalyzed by a series of metabolic ribozymes, whose activities would have been associated through the intermediate CoA-RNA. When prebiotic synthesis of pantetheine and phosphopantetheine had gradually become inadequate to feed this pathway, it would have been necessary to evolve RNA activities to make pantetheine and phosphopantetheine from simpler precursors. A full ribozyme-based metabolic pathway could then have been evolved.

Significance

A series of efficient thioester synthetase ribozymes (TES) of different lengths have been isolated from size-heterogeneous random RNA libraries by a unique competitive selection method. Different RNA-linked CoA thioesters can be made by the TES ribozymes from a broad spectrum of acyl adenylates including alkyl and aminoacyl adenylates. In extant biology, thioesters are ubiquitous and play essential roles in cellular metabolism. Our findings reveal RNA's new catalytic capability of making the "high-energy" thioester bond, and suggest plausible synthesis and utilization of thioesters in an RNA world. In addition, our previously isolated CoA synthetase ribozymes and the new TES ribozymes catalyze consecutive chemical reactions that form a CoA thioester synthetic pathway, demonstrating RNA's capacity to function as metabolic ribozymes. Therefore, there might have existed metabolism based solely on ribozymes in a preprotein world. Furthermore, the ability of TES ribozymes to synthesize RNA-linked aminoacyl thioesters, an essential property of nonribosomal peptide synthetases, makes it possible for RNA to make antibiotic-like peptides. It is also feasible for RNA to expand its functional group diversity by recruiting amino acids through thioesterification. Finally, our previous and current work demonstrates the existence of optimal lengths (around 60 nucleotides) of random nucleotide regions in RNA libraries used for in vitro selection, which can prove to be valuable for other researchers in designing selection strategies.

Experimental Procedures

Substrate and Thioester Standard Preparation

Three adenylate compounds, biotinyl adenylate (Biotin-AMP), L-phenylalanyl adenylate (Phe-AMP), and acetyl adenylate (Ac-AMP), were used as substrates during the selection and subsequent analysis of ribozyme-catalyzed reaction products. These adenylates were chemically synthesized according to established procedures [30] with some modifications. Briefly, Biotin-AMP was prepared from biotin (Sigma) and adenosine monophosphate (AMP, Sigma) as follows. Biotin (0.5 mmol) and AMP (0.5 mmol) were dissolved in 610 μl of ice-cold water, 250 μl of 2 N HCl, and 2.6 ml of pyridine. Dicyclohexylcarbodiimide (DCC, 2.6 g, Sigma) was dissolved separately in 3 ml of pyridine and added to the above mixture. The reaction was allowed to proceed for 3 hr on ice with constant stirring. Cold acetone (38 mL, −20°C) was then added to precipitate reaction product Biotin-AMP along with residual byproducts. Following thorough washing with cold acetone and ethanol, the precipitate was dissolved in water and Biotin-AMP was purified by HPLC (Shimadzu) on a semipreparative column DeltaPak C18, 300 × 7.6 mm (Waters). The reaction mixture was loaded onto the column preconditioned in 100% water. After 10 min of washing with water, Biotin-AMP was eluted with 40% methanol and 60% water. Purified Biotin-AMP had similar UV spectrum as that of adenosine, and could be hydrolyzed readily to AMP by 20 mM hydroxylamine, indicating the presence of the mixed carboxyl-phosphate anhydride bond. Product purity was determined by HPLC to be 90%–95%. The other two adenylates, Phe-AMP and Ac-AMP, were prepared similarly except for using 0.5 mmol of L-phenylalanine (Sigma) and 0.5 mmol of glacial acetic acid (EM Science).

Three dephopho-CoA thioester standards, Biotin-DePCoA, Ac-DePCoA, and Phe-DePCoA, were prepared in small scale by incubating equal amounts (10 mM in 20 μL) of the appropriate adenylate and 3′ dephospho-CoA (DePCoA, Sigma) in 100 mM imidazole (pH 6.5) for 30 min at 25°C. We developed this procedure based on imidazole catalysis for acyl transfer reactions [31, 32]. The thioester standards were purified by HPLC with an Alltech Econosphere C18 column, 4.6 × 50 mm. Separation for Biotin-DePCoA and Phe-DePCoA was achieved in 30% MeOH and 70% 20 mM KH_2PO_4 while for Ac-DePCoA the same was achieved in 15% MeOH and 85% 20 mM KH_2PO_4. The acidity of HPLC-purified thioester standards was adjusted to around pH 3 to reduce hydrolysis during storage.

Heterogeneous DNA/RNA Random Library Preparation

Four different DNA libraries with varying number of random nucleotides, 30N, 60N, 100N, and 140N (N = number of random nucleotides), were prepared individually by PCR amplification of DNA oligonucleotides (Operon Tech) as previously described [12]. The resulting DNA library contained the T7 class II promoter ϕ2.5 [33] where the initiating nucleotide was an adenosine. The starting DNA

pool was constructed from 100 pmol of each of the four random DNA libraries.

Size-heterogeneous RNA libraries that contain CoA at the 5′ end of RNA were prepared by a new in vitro transcription method [15]. The transcription conditions were the same as used previously [12] except for the inclusion of 4 mM 3′-dephospho Coenzyme A (DePCoA, Sigma). Under the transcription conditions, ~30% of RNA transcripts were initiated with DePCoA (i.e., 30% CoA-RNA and 70% pppRNA) [15]. RNA was radiolabeled internally with [α-^{32}P] ATP (NEN) and purified by membrane filtration through Microcon-10 (M10) filters (Amcon).

Selection and Deselection

RNA pools (containing both CoA-RNA and pppRNA for the first 7 rounds, 5 μM total) were allowed to react with 1 mM Biotin-AMP for 30 min at 25°C in a selection buffer containing 20 mM HEPES, 100 mM NaCl, 20 mM $MgCl_2$, and 20 mM $CaCl_2$ (pH 7.4). After incubation, RNA was purified by ethanol precipitation and membrane filtration through M10 filters. The RNA was then resuspended in a Neutravidin binding buffer (140 mM NaCl, 2 mM KH_2PO_4 [pH 6.6]) and loaded onto a Neutravidin affinity column (Pierce). To eliminate nonspecifically bound RNA, the column was washed extensively with one volume each of water, 3 M NaCl, and 2 M NaAc. The washing procedure was repeated three times. Finally, reverse transcription was performed directly on the Neutravidin beads, followed by PCR amplification and T7 transcription [13]. PCR conditions were modified to optimize the DNA amplification efficiencies of all size groups as follows: 0.1 u/μL *Taq* polymerase and 4 min for primer extension (our previous conditions were 0.05 u/μL *Taq* polymerase and 1 min).

During the selection, normal ester-synthesizing activity was coenriched. Such an activity was removed by incorporating deselection in the selection cycles. Pure CoA-RNA was used during the late rounds (8 through 10) of selection. Total RNA (CoA-RNA and pppRNA) from transcription was first purified by Microcon filtration, then loaded onto a thiopropyl sepharose 6B column (Amersham Pharmacia) in a thiol binding buffer (20 mM Tris, 100 mM NaCl, 5 mM EDTA [pH 7.4]). CoA-RNA was eluted from the column with 100 mM DTT and 20 mM Tris (pH 8.0), and recovered by ethanol precipitation. The RNA was then reacted with 1 mM Biotin-AMP for 30 min at 25°C in the selection buffer. After purification by two consecutive EtOH precipitations, the RNA was dissolved in the thiol binding buffer and passed through another thiopropyl sepharaose column to capture oxygen ester-forming RNA by way of free thiols (Figure 1B) that are available for the disulfide exchange reaction. The flow-through RNA contained the thioester Biotin-CoA-RNA because the free thiol of CoA-RNA was blocked (Figure 1A). The volume of the collected RNA was reduced through Microcon filtration and the RNA was resuspended in the Neutravidin binding buffer. Biotin-CoA-RNA was then purified by Neutravidin affinity chromatography. Finally, reverse transcription and PCR amplification completed the deselection cycle.

Activity Assay and Product Confirmation

All isolated clones were screened for thioester synthesis activity prior to sequencing. For each clone, PCR DNA was used to prepare internally ^{32}P-labeled mixed RNA (CoA-RNA and pppRNA) as described previously for the selection. In addition, internally ^{32}P-labeled pppRNA was prepared under standard transcription conditions [34]. Mixed RNA and pppRNA from each clone were incubated for 30 min in the selection buffer at 25°C in either the presence or absence of 1 mM Biotin-AMP. Reactions were quenched by ethanol precipitation. Recovered RNA was incubated with 1 μg streptavidin for 5 min, followed by adding an equal volume of gel loading buffer (0.05% bromophenol blue, 0.05% xylene cyanole, 7 M urea, 1× TBE). Streptavidin-bound biotinylated RNA was resolved from unreacted CoA-RNA and pppRNA by 8% denaturing PAGE.

In the first step to analyze TES1 and TES33 activity, we determined whether the free thiol of CoA was required for catalysis. The free thiol group of CoA-RNA (~0.1 μM) was blocked by incubating with 2 mM 5,5′-dithio-bis-[2-nitrobenzoic acid] (DTNB or Ellman's reagent, Pierce) in 20 mM Tris (pH 8.0), for 20 min at 25°C. Both unblocked and blocked CoA-RNA were then incubated with Biotin-AMP under the standard reaction conditions and analyzed by streptavidin gel shift.

Thioester products from the two most predominant RNA sequences (TES1 and TES33, one from each of the 30N and 60N size groups) were confirmed by digestion of substrate-reacted RNAs with 1 unit of nuclease P1 for 5 min at 25°C in a buffer containing 10 mM NaAc, 0.4 mM $ZnCl_2$ (pH 5.2). The thioester products were purified by HPLC under the following conditions: 12% 5 mM ammonium acetate and 88% MeOH were used for Biotin-DePCoA and Phe-DePCoA elution, whereas for Ac-DePCoA, 99% 5 mM ammonium acetate and 1% MeOH were used. Collected thioester fractions from HPLC were vacuum-dried to remove ammonium acetate and then analyzed with MALDI-TOF mass spectroscopy (Macromolecular Resources, Fort Collins, CO) using an α-cyano-4-hydroxycinnamic acid matrix.

Kinetic Analysis

Catalytic rates were determined for the two RNA sequences (one from each size group), TES1 (30N) and TES33 (60N), by incubating internally ^{32}P-labeled pure CoA-RNA (~0.1 μM) with various concentrations of the substrate Biotin-AMP in the selection buffer at 25°C. Aliquots were taken at various time points and quenched by ethanol precipitation. Biotinylated RNA was analyzed as previously described. RNA bands were visualized by phosphorimaging (BioRad, Molecular Imager). Quantitation by Molecular Analyst (BioRad) gave thioester yields at various reaction times. Observed first-order rate constants (k_{obs}) were derived from nonlinear fitting (Sigma Plot, Jandell Scientific). Pairs of k_{obs} and [Biotin-AMP] were then plotted and the ribozyme kinetic parameters k_{cat} and K_M were obtained by fitting to the Michaelis equation. Uncatalyzed thioesterification rates for both the random 30N and 60N were determined by incubating pure CoA-RNA with 4 mM Biotin-AMP for 3 and 6 hr under the selection buffer and temperature conditions, then analyzed as described.

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