Acyl-CoAs from Coenzyme Ribozymes†

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ABSTRACT: We describe in vitro selection of two novel ribozymes that mediate coenzyme reactions. The first is a *trans*-capping ribozyme that attaches coenzyme A (CoA) at the 5′ end of any RNA with the proper short terminal sequence, including RNAs with randomized internal sequences. From such a *trans*-capped CoA−RNA pool, we derive ribozymes that attack biotinyl-AMP using the SH group of CoA. These ribozymes, selected to acylate CoA with the valeryl side chain of biotin, also produce the crucial metabolic intermediates acetyl-CoA and butyryl-CoA with substantial velocities. Thus, we argue that RNAs might have used the chemical functionality offered by coenzymes to support an RNA world metabolism. In particular, we can combine our results with those of other labs to argue that simple chemistry and RNA catalysis suffice to proceed from simple chemicals to catalysis with acyl-CoAs. The *trans*-capping method can be generalized for production of varied coenzyme ribozymes using a single catalytic RNA subunit. Finally, the long-suggested RNA origin for CoA itself appears to be chemically feasible.

An RNA-like molecule may have been the major structural, genetic, and catalytic agent in the hypothetical "RNA world" era prior to the emergence of coded peptide synthesis (*1*, *2*). Small RNAs selected in vitro perform model reactions paralleling the essential biological information transfers: self-replication (*3*) and the four reactions of translation (*4*, *5*). However, replication and translation by macromolecular RNAs necessarily imply a diverse metabolism (*6*) for maintaining energy and mass flows required for these nonequilibrium reactions. Can RNA alone, using only four nucleotides, support this metabolic diversity? As an answer, we note that more than 52% of modern proteins depend on coenzymes (*7*), complex organic coreactants that are indispensable for cellular metabolism by proteins. In the RNA world, functionality from accessory molecules such as cofactors was probably at least as essential as it is for today's protein catalysts, with their 20 intrinsic side chains. The presence of ribonucleotides or heterocyclic bases in coenzyme structures has also long suggested their derivation from a previous generation of RNA enzymes (*7*, *8*). Here we report ribozymes that utilize CoA[1] in vitro for the synthesis of acyl-CoAs, mediated by reactions that generalize readily to ribozymes in an RNA world.

MATERIALS AND METHODS

RNA Library for Self-Capping Reselection. The oligonucleotides (Gibco-BRL) used for DNA library construction were as follows: PE1, 5′-GCT AAT ACG ACT CAC TAT AGA GGA GTA CGG GAG AGG ATA CTA CAC GTG; PE2, 5′-TTT AAG CTT CTG CTA CAT GCA ATA G; and DOPIS6, 5′-GCT AAT ACG ACT CAC TAT AGA GGA GTA CGG GAG AGG ATA CTA CAC GTG **TCC AUC CAC CGT TGT GAT CGT CTG AAG TCA ACC ACA ACA TGC CCT GAC TC**C TAT TGC ATG TAG CAG AAG CTA AA. The bold, underlined region is derived from isolate 6 and was synthesized with 30% mutagenesis (at each position, the ratio of parental nucleotide to the other three nucleotides was 7:1:1:1). Template DOPIS6 (100 pmol) was amplified by PCR and transcribed to obtain 1 nmol of gel-purified DOPIS6 RNA as the initial randomized pool (*9*).

Selection Strategy for Self-Capping Reselection. DOPIS6 RNA pool (1 nmol) in 40 μL of water was folded by heating at 70 °C for 2 min, and then cooled to room temperature in 15 min. Then 5 μL of selection buffer [final concentrations of 20 mM MES (pH 5.5), 20 mM $CaCl_2$, and 10 mM $MgCl_2$] and 5 μL of ^{4S}UMP were added, and the reaction mixture was incubated at room temperature. The selection stringency was increased by reducing the reaction time from 1 h in the first cycle to 5 min in cycle 10. Similarly, the ^{4S}UMP concentration was decreased from 1 to 0.05 mM. The reaction was stopped by addition of 20 μL of loading solution containing 0.1% BPB, 7 M urea, and 50 mM EDTA. Capped RNAs were separated from unreacted RNAs by 8% denaturing APM−PAGE (7 M urea and 80 μM APM). Isolate 6 RNA capped with 4-thioUMP was run in an adjacent lane as a capped RNA marker. The corresponding portion of the gel was excised and RNA eluted by crushing the gel in 100 mM DTT and 0.3 M NaOAc (pH 5.3). Eluted RNA was

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[1] Abbreviations: CoA, coenzyme A; tcr, *trans*-capping ribozyme; acs-RNA, acyl-CoA synthetase RNA; bio-AMP, *d*-biotinyl 5′-adenylate; ^{4S}UMP, 4-thiouridine monophosphate; AMP, adenosine 5′-monophosphate; DTT, dithiothreitol; MS, mass spectroscopy; APM, [(*N*-acryloylamino)phenyl]mercuric chloride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetate; MES, 2-(4-morpholino)ethanesulfonic acid; NADP, nicotinamide adenine dinucleotide phosphate; FMN, flavin mononucleotide.

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ethanol precipitated and dissolved in water, and then reverse transcribed followed by PCR and transcription as described previously. Tenth-round PCR DNA was cloned and sequenced as described previously (*9*).

Synthesis of d-Biotinyl 5′-Adenylate. Bio-AMP was synthesized as described previously (*10*) and purified on a C18 Sep-Pak cartridge (Waters) by washing with water (40 mL) followed by elution with pure acetonitrile. The product was dried in a Speed Vac (Savant). Purity was confirmed by mass analysis as well as resistance to shrimp alkaline phosphatase (due to blocked 5′-phosphate).

Preparation of EXT-RNA Pool. Synthetic DNA oligos (Gibco-BRL) used for preparing the full-length EXT−DNA duplex by PCR were as follows: primer PS1, 5′-TAA TAC GAC TCA CTA TAG AGG AGT ACG GGA GAG GAT AC; primer PS2, 5′-CTG ACT CAT GCA ATG GAG; and template EXT50N, 5′-CTG ACT CAT GCA ATG GAG-N_{50}-GTA TCC TCT CCC GTA CTC C. EXT50N template (100 pmol) was amplified by PCR and transcribed to obtain 3 nmol of the gel-purified EXT−RNA as the initial randomized pool.

Selection Procedure for Bio-CoA−RNA Formation. The selection method has three steps. In the first step (negative selection), uniformly [α-^{32}P]GTP-labeled 5′ppp-EXT−RNA (3 nmol) was treated with bio-AMP (3 mM) in selection buffer [50 mM Hepes (pH 7.25), 50 mM NaCl, 5 mM $CaCl_2$, 10 mM $MgCl_2$, 100 μM $MnCl_2$, and 5 μM $ZnCl_2$]. After the mixture had been shaken for 1 h at 25 °C, the reaction was stopped with 1 volume of 20 mM EDTA (pH 8.3). Excess bio-AMP was removed by gel filtration through Microspin P6 columns (Bio-Rad). The eluted RNA pool was treated with an equal volume of immobilized neutravidin slurry (Pierce) for 30 min. Unreacted RNA was recovered by a quick centrifugation, while biotinylated RNA remained bound to neutravidin. Unreacted RNA was ethanol precipitated and used in the second step. In this second step, the 5′-ppp-EXT−RNA pool was capped with CoA using the tcr1 ribozyme. The 5′-ppp-EXT−RNA (25 μM) and tcr1 (75 μM) were heated together in water at 75 °C for 20 s, and then cooled. At 60 °C, 20 mM MES (pH 5.5) was added, followed by 5 mM CoA and 10 mM $CaCl_2$ at 42 °C. After the mixture had been kept for 38 min at 42 °C, the reaction was terminated by addition of EDTA to a final concentration of 10 mM. Excess CoA was removed by gel filtration, and CoA−EXT−RNA conjugate was attached to thiopropyl-Sepharose via disulfide linkage, followed by elution with 100 mM DTT (*5*). Recovered CoA−EXT−RNA conjugate was ethanol precipitated and immediately used for the third step. In the third step of a round, selection for bio-CoA−EXT−RNA formation used the CoA−EXT−RNA conjugate (1 nmol) and bio-AMP (3 mM) under conditions described for negative selection. Biotinylated RNA was captured on immobilized neutravidin and extensively washed to remove unreacted RNA: 3 × 300 μL of 50 mM Hepes (pH 7.25), 0.5 M NaCl, and 1 mM EDTA; 12 × 300 μL of 20 mM MES (pH 5.5), 0.5 M NaCl, 7 M urea, and 1 mM EDTA; and then 5 × 300 μL of water wash. Biotinylated RNA was reverse transcribed on the neutravidin beads, followed by PCR amplification and transcription (*9*). In later rounds, the amount of the CoA−EXT−RNA conjugate was reduced to ~0.4 nmol.

Ribozyme Assays. Uniformly [α-^{32}P]GTP-labeled *trans*-capped RNAs (1 μM) and excess bio-AMP were incubated in 50 mM HEPES (pH 7.25) and 5 mM $CaCl_2$ at 25 °C. Aliquots were quenched with 10 mM EDTA (pH 8.3). Excess bio-AMP was removed by gel filtration. After 20 min with 1 μg/μL neutravidin, 0.5 volume of gel loading buffer (0.1% bromophenol blue, 7 M urea, and 1× TBE) was added. Biotinylated RNAs were resolved by electrophoresis on 8% polyacrylamide gels. Product and total RNA were quantified after phosphorimaging using Molecular Analyst (Bio-Rad).

Product Characterization. HPLC utilized a Waters 996 liquid chromatography system with a Microsorb-MV C18 (5 μm, 100 Å) reverse phase column. Elution was achieved with a gradient of 100% 0.1 M ammonium acetate (pH 4.3) at 0 min to 50% each acetonitrile and 0.1 M ammonium acetate in 35 min, and then to 100% acetonitrile in 40 min. Product peaks were analyzed by MALDI-TOF using a Perceptive Biosystems Voyager-DE STR instrument.

Kinetic Analysis. The scheme shown in Figure 6 was simulated in Windows using 20-sim 2.3 Pro (Controllab Products). The mechanism was integrated numerically using a Gear adjustable-step, adjustable (first to fifth)-order integrator. Integrated kinetics were fit to all five kinetic courses simultaneously. With decapping for E, ES, and P set at 9.4×10^{-3} min^{-1} (as measured for CoA−acs1−RNA) or zero, a Polack−Ribiere gradient algorithm varied K_M and k_{cat} to minimize the least-squares difference between integrated kinetics and observed points. This particular fitting protocol was very robust, giving the same K_M and k_{cat} (to seven significant figures) with widely varied initial estimates.

RESULTS AND DISCUSSION

Principle of Acyl-CoA−RNA Selection Design. Acetyl-CoA (Figure 1A inset, R = CH_3) is a crucial reactant in modern metabolism. It participates in numerous universal metabolic pathways, suggesting that it has acted in similar capacities since early in the evolution of life (*11*, *12*). Approximately 4% of all protein enzymes act on CoA (red and blue, Figure 1A) or a thioester of CoA (*13*). To obtain RNAs catalyzing acyl-CoA synthesis, we emulated ATP-dependent fatty acyl synthetase protein enzymes (*14*). These react in two steps, the first being activation of fatty acid carboxylates by formation of an acyl adenylate (mixed phosphoanhydride; eq 1). In a second step, this adenylate is attacked by the sulfhydryl of CoA to form a thioester (eq 2). We have already shown that step 1 (eq 1) is within the RNA catalytic repertoire (*5*).

$$\text{fatty acid} + \text{ATP} \leftrightarrow \text{fatty acyl-AMP} + \text{PP}_i \quad (1)$$

$$\text{fatty acyl-AMP} + \text{CoA} \leftrightarrow \text{fatty acyl-CoA} + \text{AMP} \quad (2)$$

To determine if RNA can accelerate acyl-CoA (Figure 1A inset) synthesis (eq 2), we performed selection-amplification in two phases. In the first phase, a *trans*-capping ribozyme (tcr) was used to specifically attach CoA to randomized RNA (eq 3).

$$\text{ppp-RNA} + \text{CoA-p} \rightarrow \text{CoA}_{(3'-5')}\text{pp-RNA} + \text{PPi} \quad (3)$$

In the second phase, a purified CoA−RNA pool was reacted

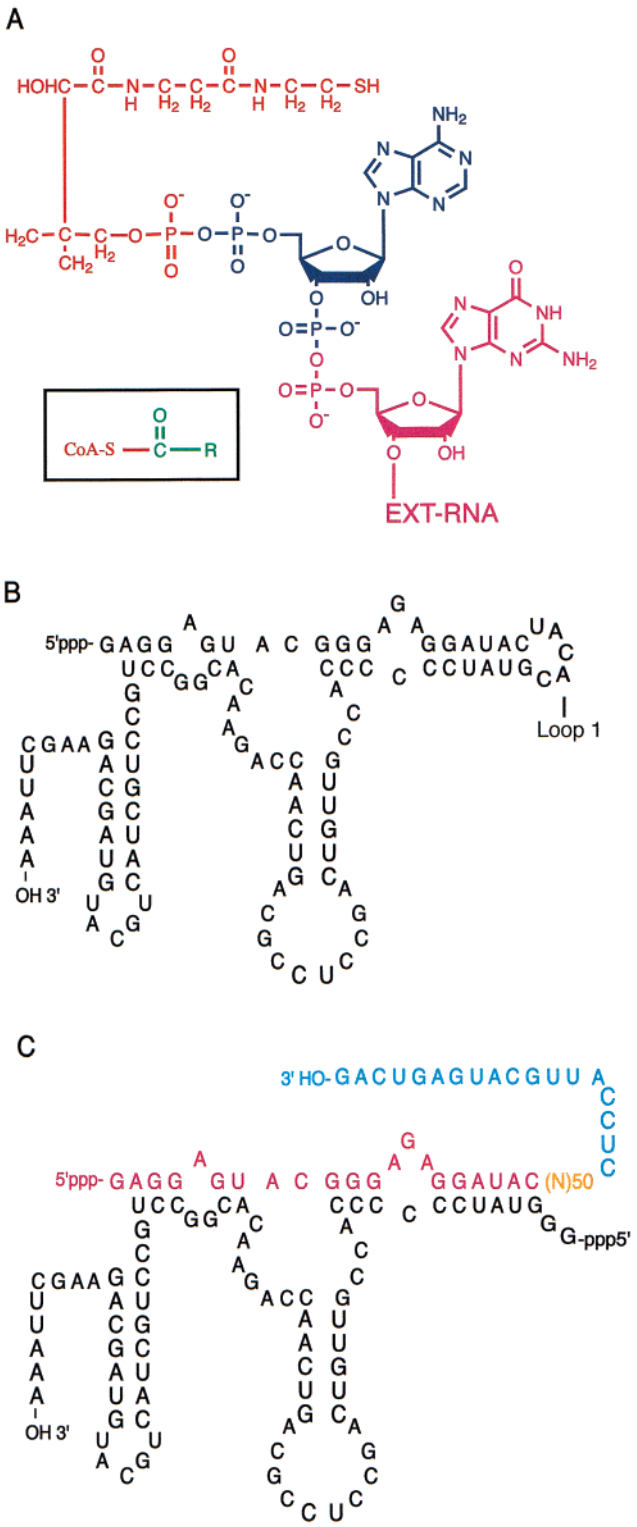
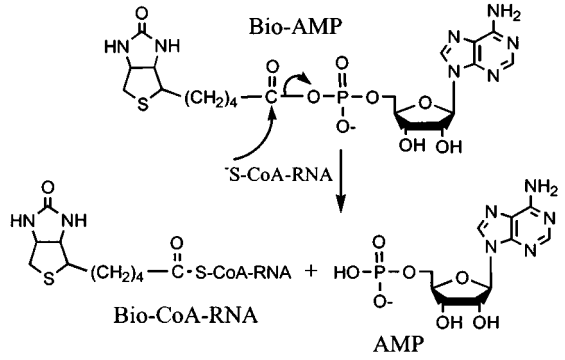


FIGURE 1: (A) CoA−RNA conjugates. Phosphorylated pantetheine is in red. It is attached to 3′,5′-ADP (blue) to form CoA. CoA (red and blue) is attached through its 3′-phosphate to the 5′-α-phosphate of the first nucleotide (G) of the EXT−RNA (magenta). The inset shows the terminal SH group of CoA as a thioester with the COOH groups of fatty acids (green), forming acyl-CoA. (B) The predicted secondary structure of efficient self-capping isolate 6-1 RNA. The loop 1 sequence can be deleted to create a *trans*-capping ribozyme. (C) The bimolecular *trans*-capping ribozyme system based on reselected self-capping isolate 6-1 RNA. The *trans*-capping ribozyme (tcr1) sequence of 80 nt is shown in black letters. The 89 nt extended substrate RNA (EXT−RNA) contains a 5′ 21 nt tract (magenta) from the original 5′ end of isolate 6-1 RNA. This sequence is extended at its 3′ end by 50 randomized nucleotides (yellow) followed by an 18 nt 3′ constant region (light blue).

with bio-AMP to select for biotinylated RNAs that catalyze acylation of the SH group of CoA:



Here biotin mimics a fatty acid via its valeryl side chain [$(CH_2)_4COO$], and also enables selection using its affinity for neutravidin.

First Phase: Self-Capping Ribozyme Reselection. To obtain a *trans*-capping ribozyme that would attach CoA to the 5′ end of randomized RNAs, we began with a previously isolated self-capping RNA termed "isolate 6" (*15*). It attaches varied phosphorylated molecules to its 5′ end, making a phosphate−phosphate linked cap. However, isolate 6 RNA gives low capping yields with monophosphates (*16*), particularly CoA (ca. 8%). A *trans*-capping ribozyme based on isolate 6 gave even poorer yields (<2%) of the CoA-capped RNA. Hence, reselection was used to find more efficient self-capping ribozymes (see Materials and Methods). With ^{4S}UMP as the capping ligand, rapidly reacting RNAs were enriched from a 30% doped (10% for each mutation) isolate 6 progenitor, using APM gel electrophoresis. This technique exploits a strong interaction between the ^{4S}UMP thione and immobilized mercury ions (*17*). Increasing selection stringency through 10 rounds gave a highly active pool. Many cloned and characterized RNAs had k_{cat}/K_M values with ^{4S}UMP ≥100-fold higher than did the parent (not shown). The yield of CoA-capped RNA was also improved to ∼40%.

First Phase: Design of a trans-Capping Ribozyme. The predicted secondary structure of isolate 6-1 RNA (one of the reselected clones) by energy minimization (supported by lead cutting and S1 digestion, not shown) showed an interrupted helix that included part of the 5′ constant region (Figure 1B). As the capping reaction occurs at the 5′-triphosphate, we envisaged the 5′ sequence as a substrate and the rest of the reselected RNA as a *trans*-acting ribozyme. Hence, we cut 6-1 RNA in two parts within loop 1. Figure 1C shows the resulting system in which a larger RNA (Figure 1C, black) acts as a *trans*-capping ribozyme (tcr1) for the second RNA, which retains a small 5′ complement to it (Figure 1C, magenta). The 21 nt 5′ fragment was extended at its 3′ end by 50 randomized nucleotides, and then an 18 nt 3′ constant region. This extended substrate RNA (EXT−RNA) carries the randomized tract; in addition, the 5′ constant sequence serves for both amplification and the *trans*-capping interaction. The EXT−RNA complex with tcr1 yielded up to 35% of the CoA−EXT−RNA conjugate. Such a CoA−EXT−RNA conjugate can be purified to homogeneity and freed of the *trans*-capping ribozyme by disulfide formation with thiopropyl-Sepharose, washing, and then DTT elution (*5*).

The 3′−5′ linkage between CoA and RNA (blue and magenta, Figure 1A) might have been considered stable once the *trans*-capping ribozyme was removed. However, the purified CoA−EXT−RNA conjugate undergoes decapping, releasing 2′,3′-cyclic phosphate CoA and 5′-pRNA (data not shown). The 2′-OH of CoA apparently attacks the adjacent, newly formed 3′−5′ diphosphate linkage between CoA and RNA (blue and magenta, Figure 1A), which would free 2′,3′-cyclic phosphate CoA and 5′-p-RNA. As decapping is observed with a randomized RNA pool, it does not seem to be specifically catalyzed, explaining why it continues in the absence of the *trans*-capping ribozyme. In selection buffer, CoA release from the EXT−RNA is a first-order reaction at 9.4×10^{-3} min^{-1} ($t_{1/2}$ = 74 min; not shown). To compensate for decapping, our initial CoA−EXT−RNA pool contained 10^{13} unique sequences in ≈10 copies each. Thus, even with decapping, the true representation was 10^{13} sequences during a second selection for autonomous acyl-CoA synthetase RNAs (acs-RNAs).

Second Phase: Selection Conditions for Bio-CoA Forming RNAs. Selection for acs-RNA using bio-AMP and the CoA−EXT−RNA conjugate employed three steps per round (Figure 2). To minimize CoA-independent biotinylation, the first step in each round was a negative selection; the 5′-ppp-EXT−RNA (no CoA) was incubated with bio-AMP. Spurious biotinylated EXT−RNA complexes were removed on neutravidin beads, and unreacted 5′-ppp-EXT−RNA was recovered by centrifugation (Figure 2A). In the second step, unreacted 5′-ppp-EXT−RNAs were capped with CoA using tcr1 to give CoA−EXT−RNA conjugates. Such CoA-capped RNAs were purified by capture on thiopropyl-Sepharose and DTT elution (Figure 2B). This purified RNA was immediately used in the third step, the actual selection, by incubating with bio-AMP. Resulting bio-CoA−EXT−RNA conjugates were captured on immobilized neutravidin (Figure 2C), reverse transcribed on the beads, then amplified by PCR, and transcribed (*9*). This completes one round of selection.

Sequenced Clones. The level of formation of the bio-CoA−EXT−RNA conjugate increased to 1.4% of total RNA in seven rounds, and then jumped to 19% in the next round. This signal was 100-fold higher than with 5′-ppp-RNA from the same pool; biotinylation therefore depends completely on CoA. The round 9 pool RNAs were similar in activity, and were cloned and sequenced. They are still diverse in origin; there are 10 different families containing two or more sequences and four unique sequences among 40 clones (Figure 3). Kinetic analysis of individual clones by a neutravidin gel shift assay (*18*) showed that all are biotinylated at similar rates, varying only 4-fold. Individual transcripts also gave no detectable activity without CoA capping. Negative selection may therefore have been effective in eliminating CoA-independent biotinylation of 5′-ppp-RNA (but see below!).

Analysis of the acs1-Catalyzed Reaction. Bio-CoA−RNA formation by the most frequently recovered sequence, acs1, was studied by a neutravidin gel shift assay (Figure 4). No biotinylation was observed with acs1 as 5′-ppp- or 5′-p-RNA (lanes 1 and 2), suggesting that the acs1-catalyzed reaction occurs only in the CoA−RNA form. Activated biotin as bio-AMP is essential as there is no reaction with free biotin (lane 3). Except for Ca^{2+}, no other metal ions present in selection buffer are required (lanes 4 and 5). When *trans*-capped to make the desulfoCoA−RNA (lane 6) or acetyl-CoA−RNA conjugate (blocked SH group, lane 7), the acs1 biotinylation signal is not observed. Thus, a free SH group on CoA is essential. The retarded gel band is specific to biotin−neutravidin interaction as excess bio-AMP (saturating for neutravidin, lane 8) and omission of neutravidin (lane 9) gave no signal. Therefore, acs1-RNA appears to mediate a Ca^{2+}-dependent reaction between the SH group of the acs1 CoA−RNA conjugate and the activated carboxyl of biotin, as expected.

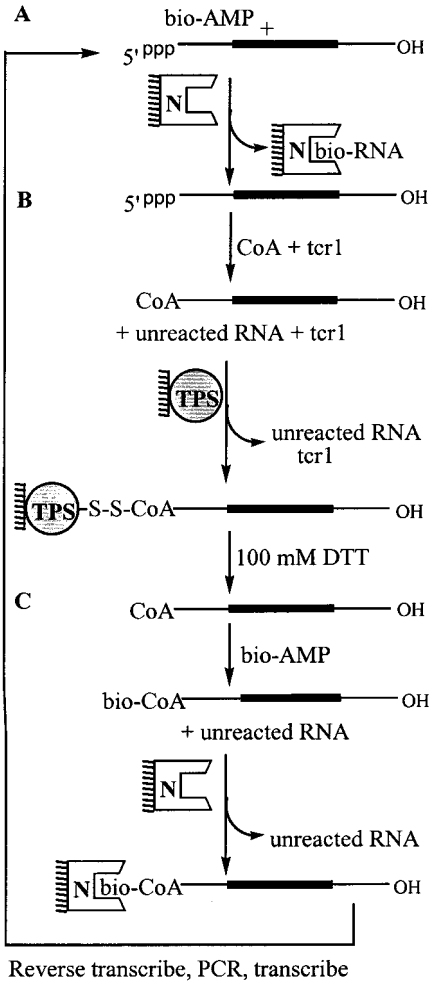


FIGURE 2: In vitro selection scheme for bio-CoA forming RNAs, where N represents immobilized neutravidin and TPS represents thiopropyl-Sepharose. (A) Negative selection reaction, where pool RNAs supporting CoA-independent biotinylation are eliminated by capture on immobilized neutravidin. (B) CoA capping of the RNA pool by tcr1 and purification of the CoA−RNA conjugate on thiopropyl-Sepharose by elution with 100 mM DTT. (C) Bio-CoA−RNA formation using bio-AMP, and purification on immobilized neutravidin.

Analysis of Reaction Products by HPLC and MS. To confirm that acs1-RNA carried out acyl-CoA synthesis, products were characterized by HPLC and mass spectroscopy (Figure 5). Large-scale CoA−acs1-RNA reactions with acetyl-AMP and butyryl-AMP were carried out separately. Acyl-CoA products were released from RNA by P1 nuclease, which yields dephospho acyl-CoAs (lacking the blue 3′-phosphate, Figure 1A). In both cases, RNA products had the same retention time on HPLC as corresponding standards (17.9 min for dephosphoacetyl-CoA and 22.8 min for dephosphobutyryl-CoA). They also showed the characteristic

Number of sequences in family	Clone acs #	Family/Sequence
5	1	AUUCGUCGAGGAGCUCACCAGGACUUAAUAAGUGCCAGUGCGCCGCUUCC
1	10	----------G----------
1	15	UAUUUCGUCGAGGACCAUAGCAUGUCGUAAAACAAUGACAAGGCGCUUCC
3	6	CUCCGUCGAGGAACGAUGCAUCGAACAUAGAUUAGACAUCGUCGCUUCCC
2	4	AGAGAUCCGUCGAGGACAGUUGUAUACCAGAGUGAGCAGCUGCGCUUCC
1	7.3	----------A----------
2	31	ACUGGCAUAACUCUUUGGGCAUGUGCGUCAGACCACGUGUUACCGCCAGC
2	29	AAUAAAGGCAAUGGACAUAUCCAUCCCAGGAAGCCCCUGCGCCUCCUUGC
1	24	AAAGAAAAUUCAAAGACAGGGCGUGGAGGAAUAUCCUGGAACUCUUUGCC
3	34.1	UCCCGAUUGCAAUGACCUGCUCAUGGGCUAAACCCAAUUUUAGCUCGCG
1	22.2	UCAGUGAAAGGUACCUCUCAAAUGUGAUCGAGGCAUUGUUUAAUGCAGGC
4	26	ACAGAUACUCAAACGAAUAGUCUUAGCAAUUGGAACUUUAUAUACUCCG
1	22.1	GUACGGAUCAGAAAAUGAAGAAACAUCCUCCGAUGGGGUGCAUAAUCUGC
4	18	UCAGCCCCCAUUACAUCGAUAUGCAAAUCACUUGAGGGUCUUAAGUCGUG
5	11	AACUACUAAAUGCGUUUCCGUCGAGGAUAUUCAGAAUCGAAUACGCUUCC
4	12	GAAAAAUAACCAUAUCUUCCAAGAAUGCAAUCAGGGCUCAUUACAUUUGG

FIGURE 3: Randomized regions of sequences obtained from bio-CoA−RNA selection. The common flanking 5′ and 3′ constant regions are 5′-GAG GAG UAC GGG AGA GGA UAC-3′ and 5′-CUC CAU UGC AUG AGU CAG-3′, respectively.

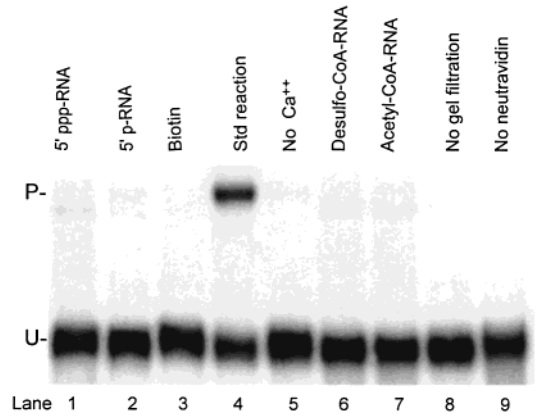


FIGURE 4: Formation of bio-CoA by acs1-RNA. Standard (std) reaction mixtures contained 1 μM CoA−acs1-RNA conjugate and 2 mM bio-AMP at 25 °C for 1 h. After gel filtration, analysis was achieved by a neutravidin gel shift assay. The essential quality of each reaction is shown at the top of the gel: U, unreacted RNA; and P, bio-CoA−RNA product.

230−240 nm UV thioester absorption shoulder of acyl-CoAs. Mass spectroscopy with the products showed a mass peak (Figure 5) identical to authentic dephosphoacetyl-CoA (728.2, $[M - H]^-$) and dephosphobutyryl-CoA (756.2, $[M - H]^-$). These results, showing chromatographic, spectroscopic, and mass identities between standards and ribozyme products, confirm that acs1-RNA catalyzes nucleophilic attack by the SH group of CoA on varied acyl adenylates to form acyl-CoAs.

Michaelis−Menten Kinetics. The kinetic analysis of bio-CoA−acs1-RNA formation gave Michaelis−Menten behavior with bio-AMP. Bio-AMP is very stable under our conditions ($k_{hydrolysis} = 9.3 \times 10^{-5}$ min^{-1}, $t_{1/2} \approx 7400$ min). However, decapping of the CoA−acs1-RNA conjugate, the (bio-AMP) CoA−acs1-RNA intermediate, and the acyl-CoA−acs1-RNA product (at 9.4×10^{-3} min^{-1}, $t_{1/2} = 74$ min) may slightly affect a typical 25 °C incubation. Therefore, kinetic data were fit to the numerically integrated scheme in Figure 6, where the three CoA decapping reactions are explicit. The slightly curved least-squares lines in the figure are the calculated kinetics, including decapping, and fit very well; for acs1-RNA at 25 °C, $K_M = 2.2$ mM and $k_{cat} = 0.026$ min^{-1}. Fitting with a k_{decap} of 0 (normal Michaelis−Menten analysis) yielded a K_M of 2.2 mM and a k_{cat} of 0.025 min^{-1}. Thus, correction for decapping increased k_{cat} by only 4%, and the true k_{cat}/K_M (second-order rate constant for the bio-AMP and CoA−acs1-RNA reaction) is 12 M^{-1} min^{-1}. The uncatalyzed rate was estimated by incubating bio-AMP with the randomized CoA−EXT−RNA conjugate. This uncatalyzed reaction to give a neutravidin-shifted RNA band has a rate of $\leq 3.6 \times 10^{-3}$ M^{-1} min^{-1} (not shown). The CoA−acs1-RNA conjugate therefore accelerates its reaction at least 3400-fold.

A Selection Curiosity. Not all the selected RNAs were CoA-dependent for the obvious reason. Our selection found CoA-dependent acylation of a type we had not anticipated. As emphasized above, negative selection with 5′-ppp-RNA excluded CoA-independent biotinylation. Remarkably, however, we selected RNAs (acs18 and acs26) that catalyze their own biotinylation as 5′-p-RNA but not as 5′-ppp-RNA (intact transcripts). Such RNAs required CoA caps only because they subsequently formed decapped RNA (5′-pRNA), which was the true biotinyl acceptor.

$$5'\text{-p-RNA} + \text{bio-AMP} \rightarrow 5'\text{-p-RNA-bio} + \text{AMP}$$

However, neither of the most frequent isolates (acs11 or acs1, characterized above) exhibited this indirect CoA dependence (Figure 4).

Conclusions. First, this work demonstrates the first ribozyme making efficient use of a natural enzymatic cofactor, coenzyme A. These selected CoA−RNA conjugates self-acylate via a Michaelis complex with several acyl-AMPs. This activity is therefore an acyl-CoA synthetase, paralleling a prominent modern metabolic reaction by producing acetyl-CoA and butyryl-CoA as RNA-bound products. While substrate specificity has its uses, it has also been argued that ribozymes with broad specificity, as reported here, could participate in multiple pathways (*19*) and thus help sustain a complex metabolism.

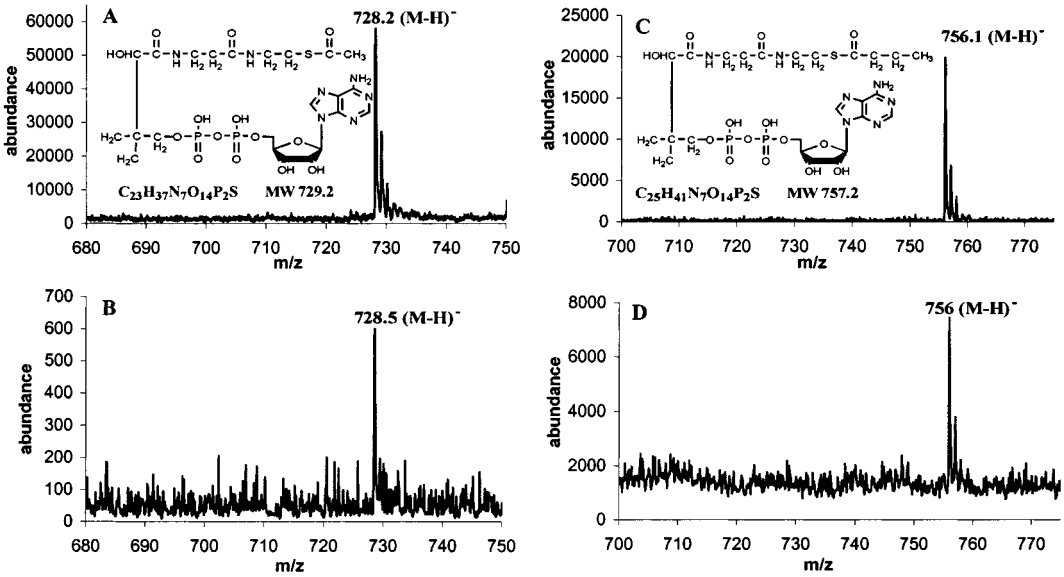


FIGURE 5: Confirmation of acyl-CoA formation by acs1-RNA using MALDI analysis. (A) Dephosphoacetyl-CoA was prepared by P1 digestion of authentic acetyl-CoA and purified by HPLC. The 17.9 min peak was submitted to MALDI analysis. (B) The acetyl-CoA−acs1-RNA conjugate was digested with P1 nuclease to release dephosphoacetyl-CoA that was resolved by HPLC. This peak was analyzed by MALDI-TOF. (C) Dephosphobutyryl-CoA was prepared by P1 digestion of authentic butyryl-CoA and purified by HPLC. The 22.8 min peak was submitted to MALDI analysis. (D) The butyryl-CoA−acs1-RNA conjugate was digested with P1 nuclease to release dephosphobutyryl-CoA, which was resolved by HPLC, and the peak was analyzed by MALDI.

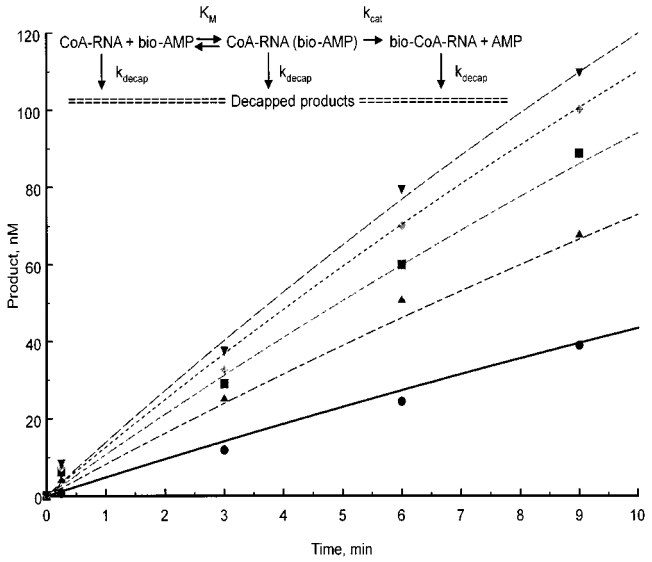


FIGURE 6: Kinetic analysis of bio-CoA formation by acs1-RNA (*25*). Points are bio-CoA−RNA concentration vs time at five concentrations of bio-AMP (0.5, 1, 1.5, 2, and 2.5 mM). The lines are numerically integrated using the mechanism at the top using a k_{decap} of 9.4×10^{-3} min^{-1}. A K_M of 2.2 mM and a k_{cat} of 0.026 min^{-1} (see the text) gave the best fit to the data.

Potential enhancement of the ribozyme repertoire by CoA is significant, because CoA-SH brings the unique nucleophilicity and redox properties of the sufhydryl group to RNA. These chemistries were previously native to protein enzymes (e.g., cysteine) but absent among ribozymes.

Second, this work completes a proof of principle showing that a practicable path links simple, prebiotically plausible chemicals to the ultimate use of CoA as an enzymatic cofactor in an RNA world. The only undemonstrated step is the appearance of an RNA-like molecule itself, to be sure a nontrivial requirement (*20*) that must yet be satisfied.

Some cofactor constituents are unexpectedly straightforward in origin. For example, a plausible prebiotic synthesis exists for pantetheine (*11*) and nicotinamide (*21*), the precursors for CoA and NAD, respectively. The only requirements for pantetheine are pantoyl lactone, β-alanine, and cysteamine, easily generated by early earth sources. Phosphorylation of prebiotic pantetheine is a difficult chemical step (*11*). However, the required phosphorylation at the CH_2OH group parallels the reaction of an already isolated polynucleotide kinase ribozyme (*22*). A previous CoA synthase RNA (*23*) creates CoA by adding phosphopantetheine (red, Figure 1A) to the 5′-ATP of an RNA (blue, Figure 1A). We have now extended this hypothetical pathway, showing here that CoA (perhaps released by spontaneous hydrolysis from CoA synthase RNA) can be added to other RNAs *in trans* and used as a cofactor in ribozyme reactions.

Thus, RNA catalysis and simple chemistry are together sufficient to invent CoA and initiate CoA-dependent metabolic reactions. By extension, many modern CoA reactions (transacylation, racemization, dehydration, reduction, carboxylation, condensation, thioester hydrolysis, and anaerobic carbon fixation) may have been available for constructing an RNA-based metabolism (*24*). If we add the use of other noncovalently bound small molecules such as amino acids as ribozyme cofactors (*25*), the gap between the diversity of protein functional groups (which indisputably support a complex biology) and RNA is notably narrowed.

Third, these experiments fulfill a prediction originally made from CoA structure (*7*, *8*). CoA likely formed within an RNA backbone, since it has both 3′- and 5′-phosphates on ADP (Figure 1A). However, while ADP ribonucleotide has no role in CoA cofactor chemistry, its presence implies that if CoA came from RNA, it was 5′ terminal. Only a 5′ terminal can accommodate CoA's active group, the phosphopantetheine SH group (Figure. 1A). By showing cofactor activity at its apparent initial 5′-RNA site, and by demonstrating a practical route to varied 5′-CoA−RNA conjugates using a single *trans*-capping catalyst, these results support a polymeric 5′-RNA origin for CoA.

Fourth, other missing reactions for an RNA world biota may plausibly come from cofactor-bound RNA. The RNA world arguably begins with RNA replicators, whose evolution will be accelerated for the first time by Darwinian selection for better replication, and subsequently for other types of catalysis. A model ribozymic RNA replicase has been selected (*3*). An RNA world that begins with replicators plausibly ends after the invention of RNA-mediated translation. Then large, accurately formed peptides appear and ultimately displace RNAs as principal biological agents. The four essential chemistries for translation have also been shown to be within the capability of RNAs (*4*, *5*). Between the beginning and the end of the RNA world, supporting metabolic roles can now be filled, at least in part, by cofactor-bound RNAs. Given that RNAs have membrane activities (*26*) potentially required to support protocells, we are surprisingly close to a guess about the nature of the ribocytes that were hypothetically our progenitors (*6*, *27*).

Our fifth conclusion is an experimental one. Covalent linkage between cofactors and randomized RNA sequencess is essential for selection−amplification protocols requiring self-modification (*9*), that is, for almost all ribozyme selections. There have been no general methods available for their synthesis (*28*), though noncovalent CoA binding sites on RNA are known (*29*). *trans* capping is unequivocally apt for an RNA world because it requires only known RNA catalyses. It is also advantageous for selection and amplification because it imposes no new molecular constraint; the requisite 5′ constant sequence was already required for RNA amplification. Finally, 5′ caps without a destabilizing 2′-OH group should be very stable once the *trans*-capping ribozyme is removed. *trans* capping generalizes to coenzymes such as NADP, thiamin pyrophosphate, FMN, pyridoxal phosphate, and biotin phosphate. Subsequent modern selections may therefore yield varied coenzyme-assisted group transfer ribozymes, justifying an RNA-based metabolism.

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