Non-canonical 3′-5′ Extension of RNA with Prebiotically Plausible Ribonucleoside 2′,3′-Cyclic Phosphates

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Supporting Information

ABSTRACT: Ribonucleoside 2′,3′-cyclic phosphates (N>p)s) are generated by multiple prebiotically plausible processes and are credible building blocks for the assembly of early RNA oligomers. While N>p's can be polymerized into short RNAs by non-enzymatic processes with variable efficiency and regioselectivity, no enzymatic route for RNA synthesis had been described. Here we report such a non-canonical 3′-5′ nucleotidyl transferase activity. We engineered a variant of the hairpin ribozyme to catalyze addition of all four N>p’s (2′,3′-cyclic A-, G-, U-, and CMP) to the 5′-hydroxyl termini of RNA strands with 3′ nucleotide addition enhanced in all cases by eutectic ices formation at −7 °C. We also observed 5′ addition of 2′,3′-cyclic phosphate-activated β-nicotinamide adenine dinucleotide (NAD>bp) and ACA>p RNA trinucleotide, and multiple additions of GUCCA>p RNA pentamers. Our results establish a new mode of RNA 3′-5′ extension with implications for RNA oligomer synthesis from prebiotic nucleotide pools.

The conjecture of an “RNA world”, a primordial biology preceding our own in which RNA was the central biomolecule for both catalysis and information storage, is supported by growing evidence. A recent key advance has been the establishment of a prebiotically plausible synthesis for the pyrimidine nucleotides. Ribonucleoside 2′,3′-cyclic phosphates (N>p’s) are not only the main products of said synthesis but also of prebiotic nucleoside phosphorylation and iterative degradation of RNA through transesterification. Furthermore, continuous N>p regeneration from the 2′- and 3′-monophosphates (resulting from, e.g., N>p hydrolysis and/or RNA degradation) is possible by activation with simple prebiotically plausible electrophiles. The sustainable and varied prebiotic supply routes thus point to N>p’s as plausible building blocks for early RNA oligomer synthesis (Chart S1). However, the 2′,3′-cyclic phosphate is a poor activating group, and polymerization of N>p’s under aqueous conditions is thermodynamically disfavored. Therefore, RNA oligomer formation requires high concentrations of N>p’s such as might have arisen by evaporation or eutectic freezing. Here, we sought to explore if small catalytic RNAs (ribozymes) could have promoted incorporation of >p-activated substrates into RNA under favorable conditions. We describe the engineering and characterization of variants of the small hairpin ribozyme (HPz) to catalyze RNA 5′ addition of >p-activated mononucleotides.

A minimal hairpin ribozyme–substrate complex comprises a two-way junction that forms a docking complex through RNA tertiary interactions (Figure 1a). Reversible cleavage of substrate RNA results from a general acid−base mechanism that catalyzes nucleophilic attack of the 2′-oxygen atom from position A-1 upon the scissile phosphorus between A-1 and G+1, leaving G+1 with a 5′-OH and A-1 with a 2′,3′-cyclic phosphate (Figure 1a). In the active complex, A-1 is positioned by a single hydrogen bond from A9 and a cross-strand base stack to G8. This binding mode is compatible with all four bases, and the HPz tolerates any base at position -1.

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Figure 1. Design of a 5′-nucleotidyl transferase for N>p’s. (a) Two-way junction HPz, which catalyzes reversible RNA ligation using a 2′,3′-cyclic phosphate. (b) Redesign of HPz into 5NTz. (c) Structural model of the substrate-binding pocket of 5NTz (based on PDB 1MSV). The ribozyme is shown as surface; A>p and the acceptor strand (AS) are shown in stick models. (d) 5NTz catalyzes 5′-adenylation in ice (2 mM A>p, 2 μM 5NTz, 1 μM 3′-FITC-labeled AS, 72 h in ice at −7 °C).
during substrate cleavage. With the goal to create a rudimentary but general binding pocket for N\textsuperscript{-}p's, we connected the \textsuperscript{5}' fragment of the pre-ligation substrate strand lacking A-1 with the unpaired A residue at the 3'-end of helix H3 by a short \textsuperscript{3}G linker (Figure 1b). We reasoned that the resulting ribozyme construct (SNTz) would now present a major groove cavity able to bind N\textsuperscript{-}p's in an orientation suitable for \textsuperscript{5}'-nucleotidyl transfer to the acceptor strand (AS, Figure 1c).

However, \textsuperscript{5}'-nucleotidyl transferase activity was only weak (although detectable) at ambient temperatures (Figure S1). We speculated that the entropically disfavored \textsuperscript{5}' addition might be enhanced by the high substrate concentrations and low temperatures effected by eutectic ice-phase formation. Indeed, we found that \textsuperscript{5}' addition of 2',3'-cyclic adenosine monophosphate (A\textsuperscript{p}) proceeded with enhanced efficiency in ice (−7 °C) (Figure 1d), yielding >10\% single-nucleotide addition. \textsuperscript{5}' extension was 10-fold lower in unfrozen, supercooled samples and 20-fold lower at 17 °C (Figure S1), demonstrating that eutectic conditions promote N\textsuperscript{-}p addition in spite of the unfavorable entropy term. No (or very weak) strand extension was observed with SNTz variants lacking the N\textsuperscript{-}p binding pocket or catalytic features of the parental HPz (Figure S2).

Next we investigated whether SNTz could catalyze nucleotide transfer of the three other canonical N\textsuperscript{-}p's. Indeed, we observed ribozyme-dependent addition of G\textsuperscript{p}, U\textsuperscript{p}, and C\textsuperscript{p} (Figure 2). Strikingly, \textsuperscript{5}' extension was already visible at an apparent N\textsuperscript{-}p concentration as low as 10 \muM for purine and 100 \muM for pyrimidine nucleotides. The preference of SNTz for purines may be due to their stronger stacking interactions with G8 (Figure 1b). A further increase in \textsuperscript{5}'-nucleotidyl transfer efficiency at high millimolar nucleotide concentrations was impeded by substrate inhibition, which was observed for all N\textsuperscript{-}p's except C\textsuperscript{p}. Inhibition was most severe for G\textsuperscript{p} (Figure 2), possibly due to competition of exogenous G\textsuperscript{p} with the internal G-1 base for pairing to C25 (Figure 1a), an interaction required for loop-loop docking during active-site assembly. In-ice \textsuperscript{5}'-adenylation was nearly independent of bivalent metal ions, and we observed nucleotidyl transfer even in the absence of magnesium ions (Mg\textsuperscript{2+}) (Figure S3a). As metal ions are also potent catalysts of RNA degradation,\textsuperscript{3}d this would boost RNA synthesis efficiency by this route. Eutectic \textsuperscript{5}' nucleotidyl transferase activity was also largely temperature independent (Figure S3b) above −28 °C, the eutectic freezing point, suggesting that the interstitial liquid brine is a prerequisite for ribozyme catalysis.\textsuperscript{1a,12}

To further characterize SNTz catalysis, we investigated the kinetics of \textsuperscript{5}'-adenylation under pseudo-first-order conditions using excess nucleotide (Figure 3). Time courses of \textsuperscript{5}'-adenylation were monophasic (Figure 3a and S4) but showed a biphasic concentration dependency (Figure 3b): The observed rate \(k_{\text{obs}}\) increased from 2.4 \times 10^{-2} h^{-1} (\(T_{1/2} = 29\) h) at 0.1 mM A\textsuperscript{p} to 3.7 \times 10^{-2} h^{-1} (\(T_{1/2} = 18.6\) h) at 0.8 mM A\textsuperscript{p} but decreased at higher A\textsuperscript{p} concentrations. These rates are comparable to template-dependent \textsuperscript{5}'-3' elongation rates of non-enzymatic (\(\sim 1 \times 10^{-2}\) h\textsuperscript{-1} for adenosine \textsuperscript{5}'-monophosphomimidazole at −18.4 °C)\textsuperscript{13} and enzymatic (\(\sim 0.1\) h\textsuperscript{-1} for \textsuperscript{5}'-triphosphates)\textsuperscript{14} primer extension reactions under eutectic conditions. The rate inhibition observed at high nucleotide concentrations complicates a detailed analysis of the reaction mechanism. However, from the fitted extension amplitudes, we estimate an apparent (aqueous) \(K_{d}^{\text{app}} = 7.2\) mM of SNTz for A\textsuperscript{p} at ~25\% extension and a maximal extension level (\(E_{\text{max}}\)) of ~51\% (Figure 3c). It is illustrative to compare these numbers with the average equilibrium constant for N\textsuperscript{-}p addition by ribonucleases (\(K_{d} = 2.2\) M\textsuperscript{-1} at 0 °C),\textsuperscript{5} which predicts that ~150 mM N\textsuperscript{-}p would be required for 25\% \textsuperscript{5}' extension under aqueous conditions. Similarly, the \(K_{d}^{\text{app}}\) of SNTz for A\textsuperscript{p} increases to ~170 mM at 0 °C (Figure S5). Altogether, these data imply that improved \textsuperscript{5}' extension in ice is predominantly a result of the concentrating effect of eutectic phase formation rather than low temperature or product stabilization.

We hypothesized that \(E_{\text{max}}\) of SNTz may be limited by the presence of an inactive ribozyme fraction (as with the parental hairpin ribozyme).\textsuperscript{12,15} Indeed, only ~60\% of a fully adenylated \textsuperscript{5}'-RNA was deadenylated in presence of SNTz under quasi-reversible conditions (ice, 0 \muM A\textsuperscript{p}) (Figure 3d). Additionally, the end points of \textsuperscript{5}' extension diverged by ~35\% when the reaction in presence of 3 mM A\textsuperscript{p} was started from either the
substrate or product side (Figure S6), suggesting that 35–40% of ribozyme/AS complexes reside in an unproductive conformation.

We anticipated that additional interactions such as Watson–Crick base-pairing would allow $S'$ additions with enhanced efficiencies and at lower substrate concentrations compared to $N'>p$'s. We found that a truncated variant of SNTz that lacks C-2 and A-3 (SNTzΔ2A, Figure S7a) was able to catalyze $S'$ transfer of an ACA$p'>p$ trinucleotide with near-maximal yields even at low micromolar concentrations (Figure 4a). This trinucleotide can form two base pairs (in place of C-2 and A-3) as part of helix H2, resulting in an increase in affinity by ~3 orders of magnitude compared to mononucleotides. Thus, trinucleotides (as well as presumably di-, tetra-, pentanucleotides, etc.) resulting from polymerization or degradation of longer RNA oligomers are potential substrates for $S'$ addition by SNTz. Indeed, we were able to redesign HPz (Figure S7b,c) for iterative additions of GUCCA$p'>p$ pentamers (Figure 4b).

This prompted us to further explore $S'$ addition of other prebiotically plausible building blocks. SNTz was able to charge the $S'$-end of RNA with β-nicotinamide adenine dinucleotide 2',3'-cyclic phosphate (NAD$p'$) (Figure 4c). This ubiquitous cofactor of modern enzymes is widely considered a relic of early metabolism and has been shown to expand the catalytic repertoire of ribozymes. Additionally, the β-nicotinamide mononucleotide moiety has been shown to be an eutectic phase reaction in unrelated sequence motifs, which may likewise be amenable to reconfiguration for $S'$ addition. Random cleavage and ligation catalyzed by such ribozymes has been proposed to allow bootstrapping of more complex activities and, indeed, HPz self-replication from prefabricated oligonucleotides and self-processing into new topologies has been demonstrated. Our success in engineering HPz for multiple $S'$ addition suggests a route by which larger RNAs might have been assembled from the short oligonucleotides provided by non-enzymatic processes.

In conclusion, we describe a strategy for $S'$ addition of ribonucleotide substrates to RNA oligomers. Such 3'-S' nucleotidyl transfer reactions are unusual in modern biology with tRNA$^{\text{His}}$ guanylyl transferases the only known example. However, this mode of 3'-S' addition from prebiotically plausible building blocks, together with others, such as chemoselective acetylation, may have aided expansion of primitive RNA oligomer pools in both length and complexity. Early RNA oligomers may thus have been able to grow “from both ends” both by canonical 3' extension (by addition of 5'-activated monomers) as well as by non-canonical S' extension utilizing both non-enzymatic and enzymatic routes.

![Figure 4. Eutectic S' addition of other substrates activated by 2',3'-cyclic phosphates.](image)

### ASSOCIATED CONTENT

Supporting Information
Experimental procedures and supplemental Chart S1, Figures S1–S7, and Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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