Cell
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A Promiscuous Ribozyme Promotes Nucleotide Synthesis in Addition to Ribose Chemistry

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SUMMARY

Here we report the in vitro selection of an unusual ribozyme that efficiently performs nucleotide synthesis even though it was selected to perform a distinctly different sugar chemistry. This ribozyme, called pR1, when derivatized with ribose 5-phosphate (PR) at its 3′ terminus and incubated with 6-thioguanine, produces two interconverting thiol-containing products corresponding to a Schiff base and its Amadori rearranged product. Consistent with this hypothesis, removing the 2-hydroxyl from the PR substrate results in only a single product. Surprisingly, as this was not selected for, switching the tethered PR substrate to 5-phosphoribosyl 1-pyrophosphate results in the synthesis of 6-thioguanosine 5′-monophosphate. The discovery that a ribozyme can promote such distinct reactions spontaneously demonstrates that an RNA-mediated metabolism early in evolution could have evolved important new functionalities via ribozyme promiscuity.

INTRODUCTION

The ability of RNA catalysts (ribozymes) to synthesize nucleotides as the basic building blocks for the assembly of RNA polymers is an essential aspect of the "RNA World" hypothesis (Fuller et al., 1972; Gilbert, 1986; Orgel, 1998; Joyce, 2002). In modern metabolism, there are various pathways by which nucleotides can be synthesized, nearly all of which utilize 5-phosphoribosyl 1-pyrophosphate (PRPP) as a substrate. In biological systems, PRPP is utilized by at least 16 different phosphoribosyltransferases to synthesize a range of purine, pyrimidine, and pyridine nucleotides together with several amino acids (Flaks, 1963; Unrau and Bartel, 1998, 2003). Previously, we isolated PRPP-dependent pyrimidine and purine nucleotide synthase ribozymes, which could synthesize either tethered uridine 5′-monophosphate ([4S]UMP) or 6-thioguanosine 5′-monophosphate ([6S]GMP), respectively (Figure 1A) (Unrau and Bartel, 1998; Lau et al., 2004). These findings indicate that both pyrimidine and purine nucleotides could have been synthesized by RNA catalysts using PRPP-dependent pathways early in evolution, laying the foundation for modern nucleotide synthesis.

The transition from the prebiotic to metabolic synthesis of nucleotides, however, remains a fundamental and unsolved problem. As PRPP hydrolyzes readily in aqueous solution (Trembacz and Jezewska, 1990; Dennis et al., 2000), it is difficult to imagine prebiotic levels of this important molecule ever being high. This presents a puzzle for models of early evolution, as nucleotide synthesis is essential for life as we know it and yet prebiotic sources of nucleotides were presumably scarce. An early RNA-based life form would therefore have needed to rapidly evolve the ability to synthesize nucleotides from some abundant prebiotically available material. The difficulty of synthesizing nucleotides prebiotically by dehydration from ribose and nucleobases (Fuller et al., 1972), combined with the poor yield of ribose from the formose reaction (Larralde et al., 1995), have led some to speculate that the origin of life did not start with nucleic acid but rather from some unknown information-coding polymer that presumably is prebiotically more plausible (Joyce, 2002; Orgel, 2004).

Recently, a novel potential abiotic synthesis of pyrimidine nucleotides has been demonstrated (Powner et al., 2009). This, together with work from the Benner laboratory showing that ribose can be synthesized prebiotically in high yield by the addition of borate to the formose reaction (Ricardo et al., 2004), suggests that activated RNA monomers might have existed transiently in localized regions of the early Earth where ribose had also accumulated abiotically. If such localized concentrations of nucleotides were able to give rise to evolving systems comprised of RNA, then it is plausible that abiotic sources of ribose might have been available to power an early metabolism.

In support of this hypothesis, we find that an in vitro-selected ribozyme can easily induce ribose 5-phosphate (PR) to react with 6-thioguanine ([6S]Gua), demonstrating the ability of RNA to chemically manipulate this important sugar (Figure 1B). Strikingly and unexpectedly, we find that substituting PR with the activated substrate PRPP causes the ribozyme to spontaneously produce tethered [6S]GMP, even though no selective pressure was applied to generate this particular nucleotide. This unexpected second chemistry indicates that ribozymes making use of simple abiotic compounds like ribose could have quickly switched to the use of higher energy metabolites such as PRPP by virtue of the intrinsic promiscuity of their active sites.

RESULTS

Selection of the pR1 Sequence

The initial goal of our work was to explore the potential of ribozymes to catalyze the synthesis of purine nucleotides starting from PR. We reasoned that a two step reaction where PR is first activated to PRPP using ATP and then forms a glycosidic bond with a nucleobase might be possible. This reaction is
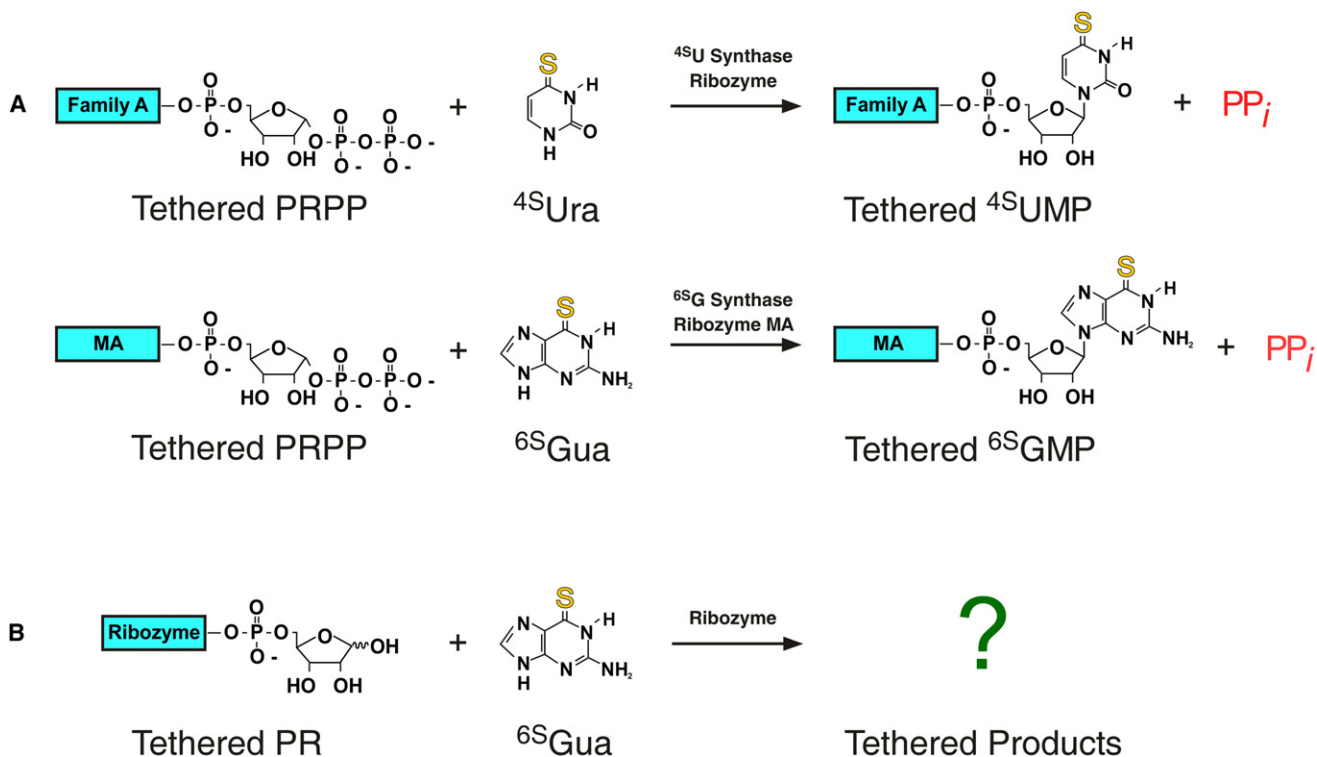
Figure 1. Ribozyme-Mediated Nucleotide Synthesis Pathways Studied using Thiol-Tagged Bases
(A) The family A pyrimidine synthase ribozyme catalyzes the synthesis of ^{4S}UMP using tethered PRPP and ^{4S}Ura as substrates, releasing pyrophosphate in the process (Unrau and Bartel, 1998). The purine synthase ribozyme MA synthesizes ^{6S}GMP from PRPP and ^{6S}Gua (Lau et al., 2004).
(B) In this study we isolate ribozymes that use ^{6S}Gua and tethered PR as substrates.

thermodynamically much more favorable than a one step dehydration reaction between a ribose and a purine nucleobase (Fuller et al., 1972; Alberty, 2006). We therefore attempted to isolate ribozymes that, when derivatized with PR at their 3′ terminus, were capable of reacting with ^{6S}Gua in the presence of ATP (Figure 2). Isolation of reactive RNA species was made possible by utilizing an APM gel shift strategy, in which active ribozymes containing a thiol group from the ^{6S}Gua substrate were retarded in their migration through a mercury-containing polyacrylamide gel (Igloi, 1988; Unrau and Bartel, 1998; Lau et al., 2004). For the selection, a pool containing 95 random nucleotide positions flanked by constant primer regions at both the 5′ and 3′ ends was constructed, having a total diversity of ∼3 × 10^{14} distinct sequences (see Experimental Procedures). This pool was derivatized with PR at its 3′ terminus using T4 RNA ligase and adenylated PR (APPR), and subsequently incubated in a buffer containing 0.25 mM ^{6S}Gua and 10 mM ATP (see Experimental Procedures).

After six rounds of selection, catalytic RNAs were readily detectable using our N-acryloylaminophenylmercuric acetate gel (APM gel) shift assay. The shifted products of the same round were purified, cloned, and sequenced; out of 39 sequences analyzed, 22 sequences were identical. This dominant sequence, called pR1, was 127 nt in length. It did not share any obviously conserved sequence or helical elements with other purine and pyrimidine nucleotide synthase ribozymes that we have previously isolated (Unrau and Bartel, 1998; Chapple et al., 2003;

Lau et al., 2004) as judged by MFOLD (Zuker et al., 1999). Of the remaining 17 sequences, none occurred more than once or contained any conserved sequence elements relative to pR1 and were not subjected to further study.

Synthesis of Two Thiol-Containing Products Requires PR, but not Triphosphate

In order to examine the reactivity of pR1, the pR1-PR construct (dash indicates 3′–5′ phosphodiester linkage between pR1 and PR) was incubated with ^{6S}Gua as in the selection. Interestingly, pR1 produced two distinct but closely migrating thiol-containing products as indicated by APM gel shift analysis (Figure 3, lanes 1–4). To establish that the tethered PR was used as a substrate in catalysis, we incubated pR1 with ^{6S}Gua without prior ligation to APPR. We observed no product formation based on our APM gel shift assay, indicating that PR was required for the reaction to proceed. In addition, ATP was not required as the product yield and band shift pattern were not affected when ATP concentration was titrated from 0 mM to 100 mM (see Figure S1A available online). Therefore, ATP was not used in the rest of our experiments. To eliminate the possibility that the 5′ triphosphate of pR1 was involved in the reaction, we enzymatically removed these phosphates by treatment with calf alkaline phosphatase. The dephosphorylated RNA, after tethering with PR and reacting with ^{6S}Gua, again exhibited two shifted bands, confirming that triphosphate was not involved in producing the thiol-containing products (Figure S1B).
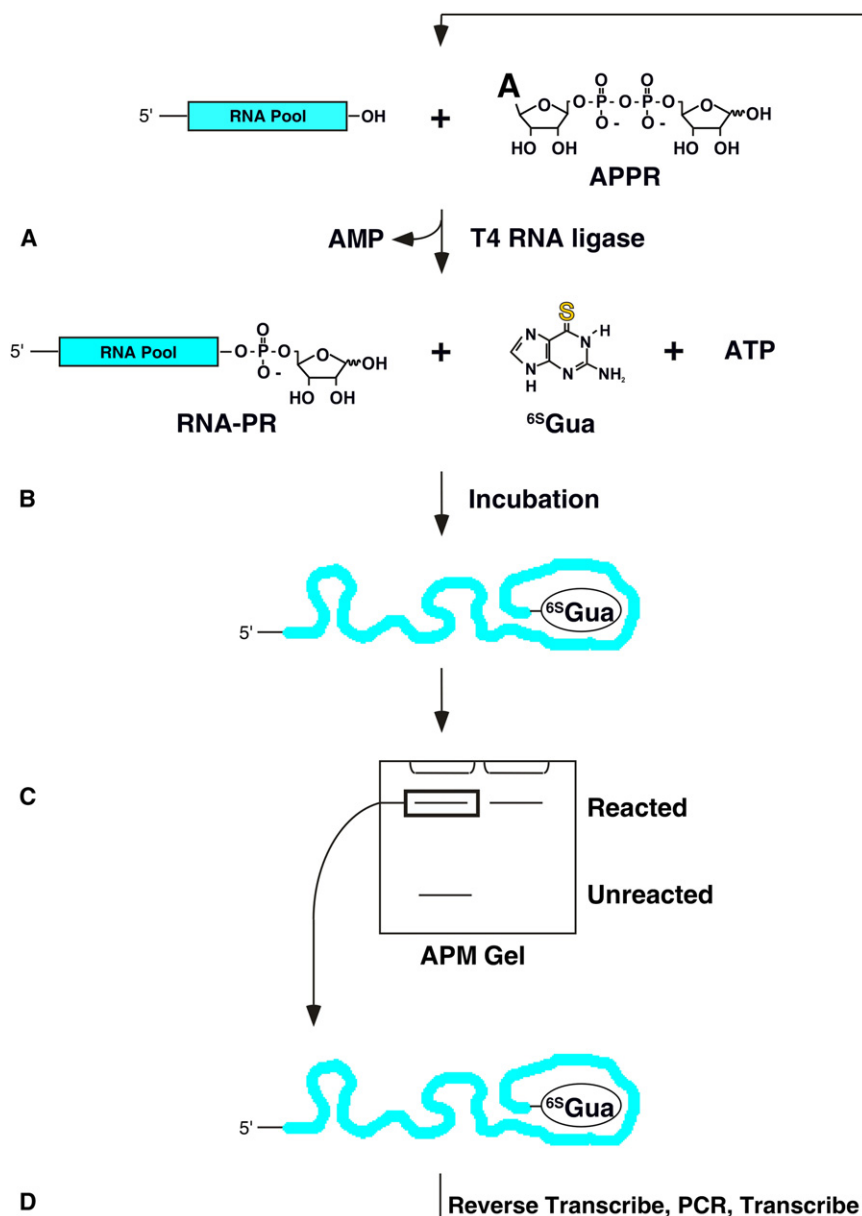
Figure 2. In Vitro Selection Scheme for the Isolation of Ribozymes that Utilize PR and [6S]Gua

(A) A random RNA pool was derivatized with PR by ligation with APPR using T4 RNA ligase, releasing adenosine 5′-monophosphate (AMP).

(B) RNAs derivatized with PR were then incubated with both [6S]Gua and ATP. ATP was added as a potential source of free energy for the ribozyme reaction. Ribozymes able to react with [6S]Gua are distinct from the unreactive RNA population by virtue of their thiol tag.

(C) Reactive sequences tethered to [6S]Gua were isolated from nonreactive sequences by separation through a thiol-sensitive APM gel (Igloi, 1988). A radiolabeled marker derivatized with [6S]GMP at its 3′ terminus was loaded in an adjacent lane to indicate the position of the gel-shifted ribozymes. The region of the gel containing the active species was excised, eluted, and recovered by ethanol precipitation.

(D) The enriched catalytic RNAs were then reverse transcribed to DNA, PCR amplified, and then transcribed back into RNA ready for the next round of selection.

Two Observed PR Products Are Not Caused by Sequence Inhomogeneity or Alternative Folding

During transcription by T7 RNA polymerase, additional untemplated nucleotides are often added to the 3′ end of the newly transcribed RNA (Milligan et al., 1987; Zaher and Unrau, 2004). To rule out the possibility that the two products resulted from transcriptional inhomogeneity, we purified the pR1 RNA through an 8% polyacrylamide sequencing gel, which allowed us to resolve RNA species with single nucleotide resolution. The size-purified ribozyme, after tethering with PR and incubation with [6S]Gua, again produced two shifted products. To positively confirm the sequence of the active ribozyme, we generated a precise pR1 3′ terminus defined by a series of enzymatic and deoxyribozyme reactions (see Experimental Procedures). Upon incubation with [6S]Gua, two APM-dependent bands were again observed, confirming that the two thiol-containing products were not due to

variation in length or terminal sequence of the ribozyme (Figure S2).

As stable alternative folds of the pR1 sequence could possibly account for the two observed gel mobilities, the pR1 products were digested with T1 ribonuclease (Pace et al., 1991). These fragments were then radiolabeled using T4 polynucleotide kinase and γ-[^{32}P] ATP before analysis through a 23% denaturing gel. As expected, a complicated spectrum of short radiolabeled RNA fragments was observed. However, when the sample was run into a 23% denaturing gel containing APM, two slower moving bands were observed to separate from the mixture (data not shown). These two bands presumably correspond to the two thiol-dependent products observed in Figure 3 and reinforce the interpretation that two distinct chemical species are produced by the pR1-PR system.

pR1 Uses PRPP in Addition to PR and Specifically Uses [6S]Gua

To explore the chemistry mediated by the pR1 ribozyme, the importance of the two substrates in the reaction, [6S]Gua and PR, were examined. pR1-PR was shown to be highly specific toward [6S]Gua, as no activity was observed when incubated with six different sulfur-containing purine compounds (6-thiopurine, 6,8-dithiopurine, 2-methyl-6-thiopurine, 6-thioxanthine, 6-thio-9-methylpurine, and 2-thiocytosine) even after incubation for up to 7 days (Figure S3). This is in contrast to two previously characterized purine synthase ribozymes that showed limited reactivity with 6-thiopurine in addition to [6S]Gua (Lau et al., 2004).

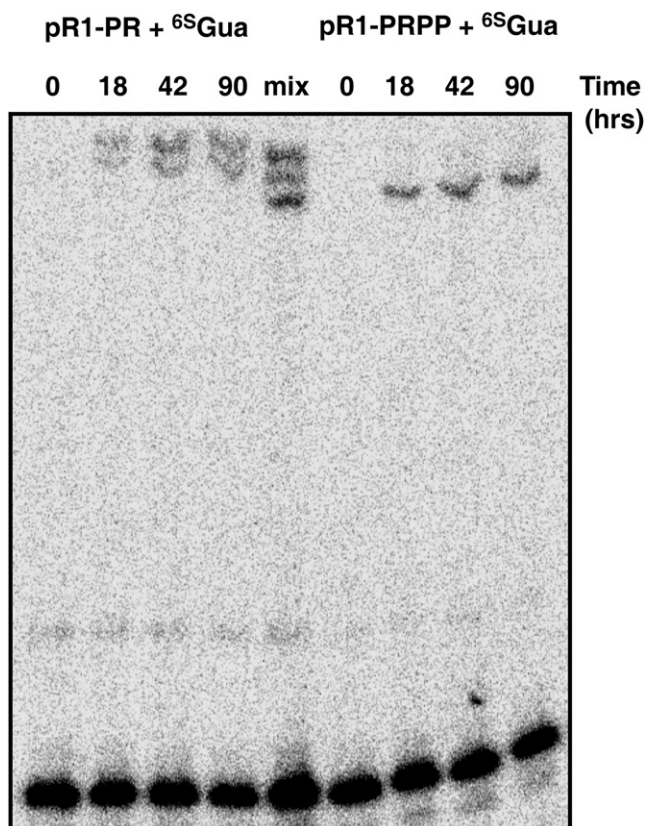**pR1-PR + ^{6S}Gua   pR1-PRPP + ^{6S}Gua**

Figure 3. Promiscuous Chemistry by the pR1 Ribozyme
Tethering pR1 with PR and reacting with 0.25 mM ^{6S}Gua produced two distinct bands as a function of time (pR1-PR + ^{6S}Gua lanes). Changing the tethered substrate to PRPP and reacting with ^{6S}Gua resulted in a single band that migrated slightly faster than the two pR1-PR product bands (pR1-PRPP + ^{6S}Gua lanes), consistent with the appearance of three distinct bands upon mixing the two 90 hr reaction mixtures (mix lane).

We next examined whether substituting PR with PRPP would give rise to reaction products. To our surprise, a new and distinct third product was observed (Figure 3, lanes 6–9), which did not comigrate with either of the two PR products (Figure 3, mix lane 5). Since previously isolated ribozymes from our laboratory also use PRPP as a substrate for ^{6S}GMP synthesis, it was possible that the PRPP product resulted from cross-contamination with these ribozymes. To eliminate this possibility, the pR1-PR products and pR1-PRPP product were purified separately from an APM gel, reverse transcribed, and subjected to sequencing. Thirteen sequences derived from the pR1-PR products and 13 sequences from the pR1-PRPP product were analyzed and all of them consisted of the pR1 sequence. This indicates that the pR1 ribozyme is indeed responsible for synthesizing all three distinct thiol-containing products.

The unusual ability of pR1 to react with both PR and PRPP prompted us to explore the possibility that our previously selected PRPP-dependent nucleotide synthase ribozymes might also be able to utilize both substrates. We derivatized four previously isolated purine nucleotide synthase ribozymes (RA, RE, MA, and ME) with PR (Lau et al., 2004), but observed no reactivity upon subsequent incubation with ^{6S}Gua for up to 4 days. This

demonstrates that the ability of pR1 to catalyze reactions with both sugar substrates is not a general property of all nucleotide synthase ribozymes.

Only the PRPP Product Is a Conventional Nucleotide
To give further insight into the three tethered products, we implemented an extension assay using α-[^{32}P] dATP and Poly(A) polymerase. Poly(A) polymerase requires the 3′ most nucleotide of the acceptor sequence to contain a free 3′ hydroxyl (Cao and Sarkar, 1992; Martin and Keller, 1998). Analysis by APM gel indicated that the PRPP product was labeled by the enzyme, whereas the two PR products were not (Figure 4A). As a positive control, the tethered ^{6S}GMP product of the purine synthase ribozyme MA (Lau et al., 2004) was shown to be a substrate for Poly(A) polymerase (Figure 4A). To reinforce these results, we performed another extension assay using T4 RNA ligase and 5′-[^{32}P] pCp. This enzyme, in contrast to Poly(A) polymerase, tolerates modifications at the 2′ position of the acceptor terminal nucleotide, but at the expense of ligation efficiency (Ebhardt et al., 2005). Consistent with our polymerase extension assay, we observed ligation only with the pR1-PRPP product and not with the pR1-PR reaction products (Figure S4A). To further examine if any of the three products synthesized by pR1 was ^{6S}GMP, we generated a 3′-[^{32}P] ^{6S}GMP marker by end labeling an RNA sequence containing a terminal 3′ ^{6S}GMP with 5′-[^{32}P] pCp, followed by digestion using T2 ribonuclease (see Experimental Procedures). Interestingly, when the pR1-PRPP product was end labeled and digested in the same manner it comigrated exactly with the 3′-[^{32}P] ^{6S}GMP marker when separated through an APM gel (Figure S4B). These results together indicate that the pR1-PRPP product is likely a conventional ^{6S}GMP nucleotide, whereas the pR1-PR products, which are not recognized by enzymes evolved to modify the termini of normal nucleic acids, are not.

Further Characterization of the PRPP Product Nucleotide
To determine whether the product of the PRPP reaction was ^{6S}GMP, we utilized a three step labeling, digestion, and thin layer chromatography (TLC) analysis strategy that has been used previously to characterize the products of nucleotide synthase ribozymes (see Experimental Procedures) (Unrau and Bartel, 1998; Lau et al., 2004). This technique results in the incorporation of a 3′-[^{32}P] phosphate group on the 3′ terminal nucleotide. Analysis by two-dimensional TLC revealed a single spot (Figure 4B, bottom left panel). To determine whether the observed spot corresponded to 3′-[^{32}P] ^{6S}GMP, a reference was generated containing radiolabeled 3′-[^{32}P] AMP, GMP, CMP, UMP, and ^{6S}GMP (Figure 4B, top right panel) (Lau et al., 2004). When the digested pR1-PRPP product was mixed with the reference, it comigrated with the ^{6S}GMP standard when separated through both a two-dimensional TLC system (Figure 4B, bottom right panel) and a high percentage APM gel (data not shown), further substantiating the claim that pR1-PRPP synthesizes ^{6S}GMP when incubated with ^{6S}Gua.

Reduction of Ribose Prevents Formation of PR Products
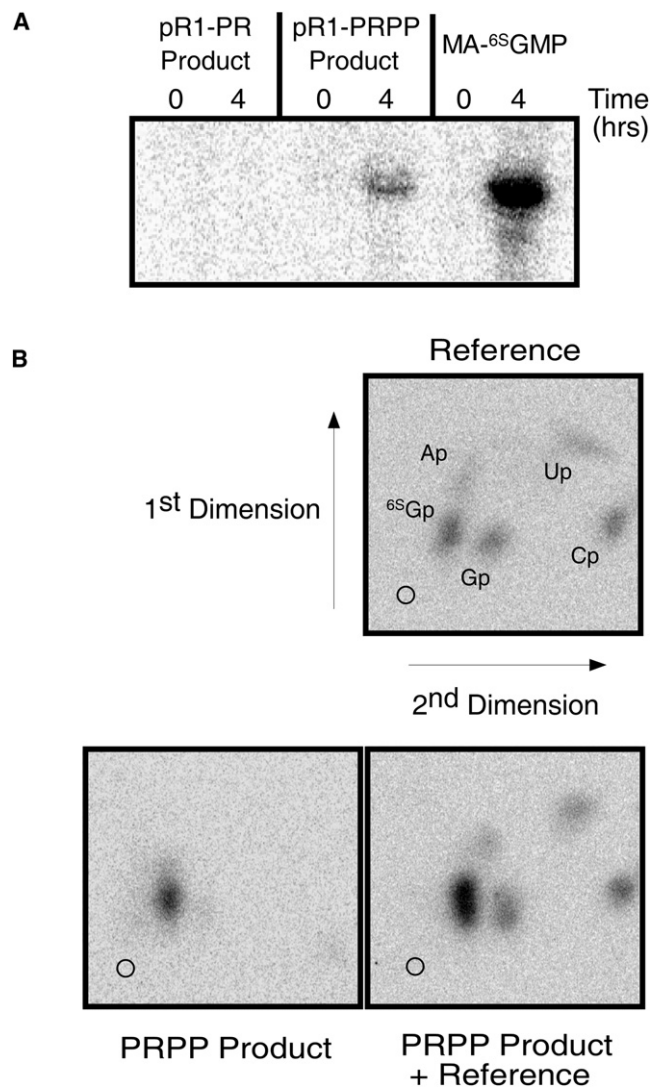Since the ribose sugar of PR is capable of interconverting between a closed ring conformation and an open aldehyde

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P R E S S

A

pR1-PR Product | pR1-PRPP Product | MA-⁶ˢGMP

B

Reference

1st Dimension → 2nd Dimension

Ap Up ⁶ˢGp Cp Gp

PRPP Product

PRPP Product + Reference

Figure 4. The pR1-PRPP, but not the pR1-PR, Reaction Products Are Enzymatically Recognized and Comigrate with ⁶ˢGMP Standards

(A) pR1-PR, pR1-PRPP, and MA-PRPP (used as a control) ribozymes were incubated for 90 hr with 0.25 mM ⁶ˢGua. RNA was separated and purified through an APM gel, followed by incubation with α-[^{32}P] dATP and Poly(A) Polymerase. After 4 hr these reactions were loaded on an APM gel. Only the region of the gel corresponding to the thiol-dependent shift is shown.
(B) Purified pR1-PRPP reaction products were 3′ end labeled with 5′-[^{32}P] pCp, followed by a second APM gel purification step. The resulting thiol-containing RNA was digested to mononucleotides using ribonuclease T2 and analyzed by two-dimensional TLC (lower left panel; see Experimental Procedures). A reference mixture containing five radiolabeled 3′ monophosphate nucleotides was also created (upper right panel, see Experimental Procedures). This set of standards was then mixed with the pR1-PRPP product digest. The ribozyme product comigrated with 3′-[^{32}P] ⁶ˢGMP as judged by this analysis (lower right panel). The black circles indicate the TLC origins.

form (>99% in furanose structure and <1% as aldehyde at equilibrium; Angyal, 1969), we hypothesized that perhaps it was the acyclic form that was responsible for the two observed products due to the intrinsic reactivity of aldehydes with nitrogenous compounds. To partially address this hypothesis, we reduced

the tethered PR to ribitol 5-phosphate by treatment with the strong reducing agent sodium borohydride (NaBH$_4$) (Ricardo et al., 2004). This results in the reduction of the aldehyde group to a terminal alcohol. Interestingly, reduction of pR1-PR with NaBH$_4$ prior to reaction with ⁶ˢGua resulted in no product formation (Figure 5, lanes 1–4, pR1-PR reaction without NaBH$_4$ pretreatment, and lanes 5–8, with NaBH$_4$ pretreatment). This is consistent with the interpretation that the aldehyde functionality is the reacting species. As a control, the same experiment was then carried out with pR1-PRPP (Figure 5, lanes 9–16). Satisfyingly, this reaction was unaffected by pretreatment with NaBH$_4$. This can be explained by the fact that the 1-pyrophosphate of PRPP prevents formation of the linear aldehyde form and consequently its reduction. In the pR1-PRPP reactions, with or without prior reduction, we observed the formation of a low yield of the two PR-dependent products. This is likely due to the fact that PRPP is not particularly stable and hydrolyzes over time to either PR or 5-phosphoribosyl-1,2 cyclic phosphate (Dennis et al., 2000). Since NaBH$_4$ reduction is a rapid process, it should therefore only reduce the PR initially present in the reaction mixture, but not the PR formed over time by PRPP hydrolysis. Work from Sandwick's group has shown that PRPP breaks down at an average rate of $\sim$0.001 hr^{-1} at neutral pH and this rate increases by $\sim$140-fold with the addition of 32 mM Mg^{2+} at neutral pH (Meola et al., 2003). Since our ribozyme was incubated for 96 hr in the presence of 75 mM Mg^{2+}, it is expected that a significant portion of the PRPP would degrade to PR, giving rise to the observed PR-dependent products.

Characterization of the PR-Dependent Products

To further characterize the pR1-PR reaction, we excised the two product bands separately from an APM gel (Figure S5A). Due to their close mobility in the mercury gel, it was difficult to ensure cross-contamination was completely avoided between the two bands. To address this issue, we loaded two lanes and excised RNA from the top half of the topmost (slower moving) product (Figure S5A, leftmost panel, left lane) and RNA from the bottom half of the lower (faster moving) product (Figure S5A, leftmost panel, right lane). The resulting top and lower band samples were then eluted and incubated in buffer with or without ⁶ˢGua for a further 2 days. Surprisingly, both the excised upper and lower products reassorted back to two bands (Figure S5A, T$_1$ and L$_1$ panels, reassortment with ⁶ˢGua data shown). To rule out possible contamination between bands this whole process was repeated for a total of three complete cycles. Since the maximum possible contamination was judged to be $\sim$20% for each excision, less than 1% contamination (0.2^3) seemed possible. In all cases (with or without ⁶ˢGua), the observed reassortment favored formation of the upper band (Figure S5B), along with a notable accumulation of a third unshifted band over time (Figure S5A). Since this unshifted band was more prominent in the absence of ⁶ˢGua, it suggests that at least one of the two interconverting products can liberate ⁶ˢGua so as to regenerate pR1-PR.

Deoxyribose Is an Alternative Sugar Substrate for pR1

Incubation with analogues of ⁶ˢGua, as described before, demonstrated that pR1 is highly specific toward ⁶ˢGua, but did not reveal further details regarding the mechanism of the
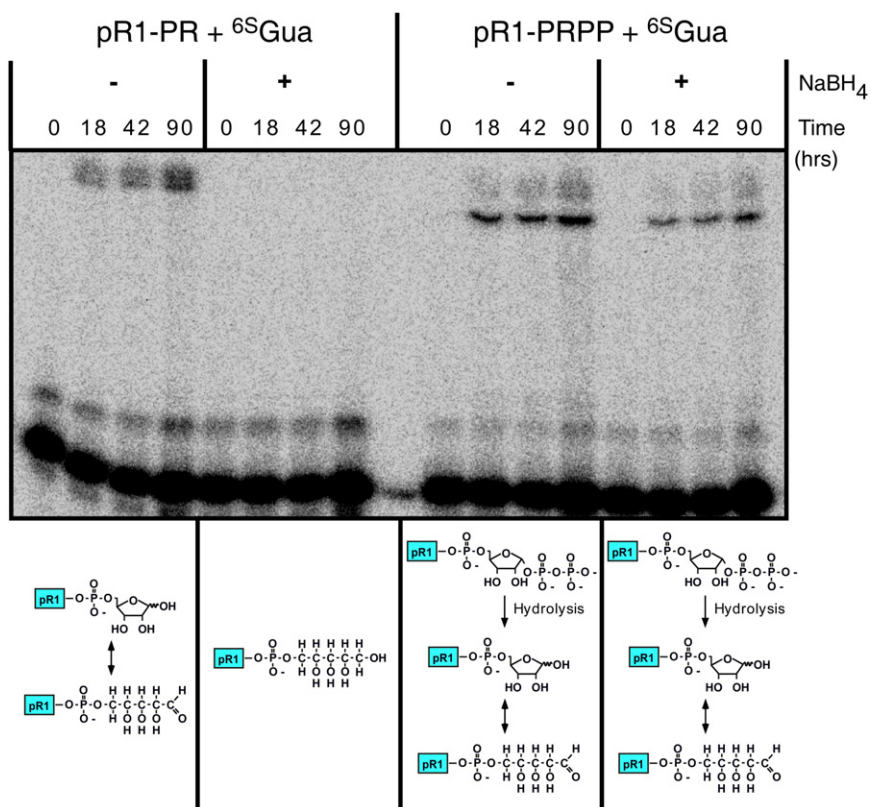
Figure 5. Reduction of Ribose Prevents the Synthesis of PR Products

pR1-PR and pR1-PRPP ribozymes were preincubated with (+ columns) or without (− columns) NaBH$_4$. These ribozymes were then incubated with 0.25 mM ^{6S}Gua for 0, 18, 42, and 90 hr. The diagrams below each time course series represent the state of the tethered substrates prior to incubation with ^{6S}Gua. As pR1-PRPP is not reduced by NaBH$_4$, but can slowly hydrolyze to pR1-PR after the reduction treatment, pR1-PR-dependent bands are found in both the + and − columns for the pR1-PRPP sample.

twice the background intensity when exposing on a phosphorimager screen, the complete absence of an APM-dependent gel shift implied an uncatalyzed rate of less than 2.5×10^{-6} hr^{-1} at 0.25 mM ^{6S}Gua concentration. The catalytic rate enhancement by pR1 for either the PR- or PRPP-dependent chemistry is therefore is at least 10^4-fold faster than the uncatalyzed rate. We next examined the magnesium requirement for pR1 by performing a magnesium titration experiment ranging from 0 to 75 mM (Figure S7). Interestingly, the PR products were detectable at 1 mM Mg^{2+} concentration, whereas 10 mM was required for the formation of the PRPP product. The finding that pR1-PR was less Mg^{2+} dependent than pR1-PRPP suggests the possibility that metal ions are utilized differently in each reaction. In both cases, however, the optimal Mg^{2+} concentration appears to plateau at 10 mM Mg^{2+} and increasing Mg^{2+} beyond 10 mM did not significantly enhance the rate or the fraction of products synthesized for either reaction.

DISCUSSION

The PR-Dependent Chemistry

The two thiol-containing reaction products formed by pR1-PR are consistent with Schiff base chemistry involving the open chain aldehyde form of ribose. This chemistry is observed between reducing sugars, including glucose, ribose, and PR, and amino acids in the Maillard reaction, a process commonly observed in the browning of foods at high temperatures (van Boekel, 2006; Munanairi et al., 2007). The first step is a condensation reaction, in which an amine group acts as a nucleophile, reacting with a sugar carbonyl to form a carbon-nitrogen bond. This leads to the reversible formation of an imine, which can undergo isomerization (Amadori rearrangement) with a proximal hydroxyl group to form a ketosamine product (Smith et al., 1994). In our reequilibration experiment with pR1-PR, we observed similar rearrangement occurring between the thiol-dependent product bands (Figure S5). While we are unsure as to the absolute identity of either shifted band, their ability to interconvert and to revert to the unreacted state is compatible with Schiff base chemistry

catalyzed chemistry involving PR. We therefore manipulated the tethered PR substrate and examined whether 2-deoxyribose 5-phosphate (PdR) was a viable substrate. We used acid depurination to synthesize pR1-PdR (Figure S6A). In brief, a RNA-DNA hybrid sequence, which contained a terminal deoxyguanosine nucleotide (CAAGCdG), was first phosphorylated with γ-[^{32}P] ATP, and then briefly depurinated in hydrochloric acid. Since purine deoxyribonucleotides depurinate at a rate of ~520-fold faster than purine ribonucleotides (Zoltewicz et al., 1970), only the guanine of dG was removed upon acid treatment, resulting in 5'-[^{32}P] pCAAGC-PdR. This construct was then ligated onto a truncated pR1 ribozyme RNA resulting in full-length pR1-PdR (see Experimental Procedures). Interestingly, pR1-PdR was found to be active when incubated with ^{6S}Gua, but in contrast to pR1-PR, only a single mercury-dependent product was produced. Both pR1-PR and pR1-PdR product formation was sensitive to initial reduction by NaBH$_4$ (Figure S6B).

Kinetics

To examine the catalytic rate of pR1, we performed separate time course experiments for pR1-PR and pR1-PRPP reactions with 0.25 mM ^{6S}Gua in the presence of 75 mM MgCl$_2$. The first order rate constants were obtained by plotting the fraction reacted versus time for four independent time courses (see Experimental Procedures). The average measured first order rate of synthesis for pR1-PR (~0.04 ± 0.03 hr^{-1}) was ~2-fold slower than the synthesis rate for pR1-PRPP (~0.07 ± 0.04 hr^{-1}). To examine the uncatalyzed rates, a 5 nt and a 125 nt RNA were ligated to either APPR or APPRPP. These RNAs were then incubated with ^{6S}Gua for 7 days. Assuming that we could detect a peak
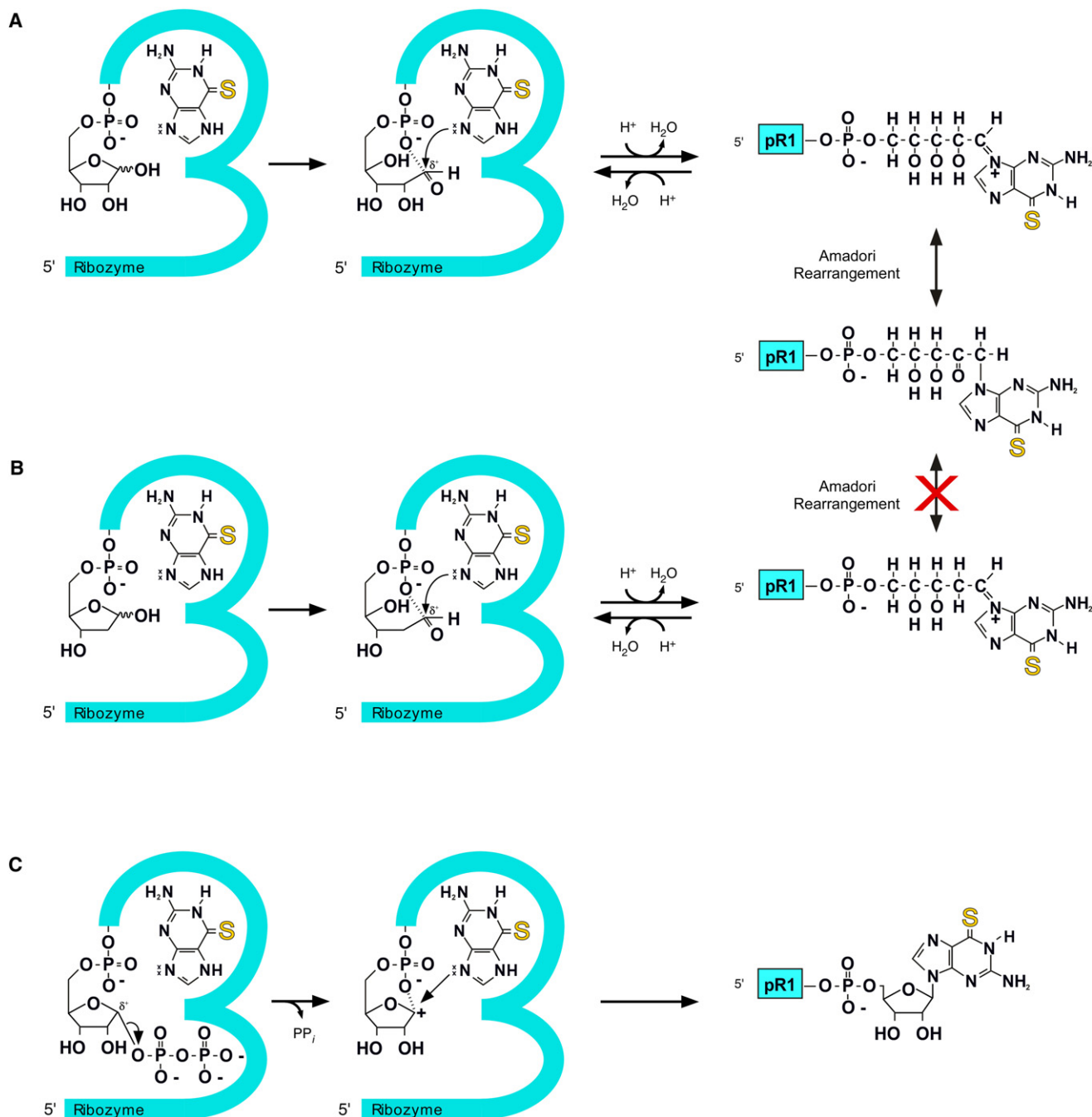
Figure 6. Proposed Reaction Mechanisms for the Synthesis of Distinct pR1 Products

(A) In the pR1-PR reaction, the negative charge present in the phosphate group from the phosphodiester backbone can stabilize the carbonyl group present in the acyclic form of ribose. This would encourage nucleophilic attack from a [6S]Gua nitrogen (assumed here to be N9), resulting in the formation of a Schiff base. The resulting product can then undergo an Amadori rearrangement to give a ketosamine product.

(B) In the pR1-PdR reaction, the deoxyribose sugar can similarly undergo acyclization and react with [6S]Gua to form a Schiff base. However, since PdR does not have a 2-hydroxyl group, it cannot form the enaminol intermediate and fails to isomerize to the more stable Amadori product.

(C) In the pR1-PRPP reaction, phosphate groups can similarly stabilize the carbocation of the ribose that is formed upon displacement of the pyrophosphate, resulting in formation of the glycosidic bond.

involving a [6S]Gua nitrogen (Figure 6A, assuming N9 of [6S]Gua). Consistent with this idea, removing the 2-hydroxyl from the PR substrate produces only a single reaction product (Figure S6b).

This is expected as the imine cannot undergo further Amadori rearrangement in this configuration (Figure 6B) (Munanairi et al., 2007).

While DNAzymes modified to contain primary amines and imidazole functionalities have been shown to promote cleavage at an apurine/apyrimidinic sites via a Schiff base intermediate (May et al., 2004), there are currently no ribozymes that are known to mediate this chemistry. Schiff base chemistry plays an important role in glycolysis and the pentose phosphate pathway and enzymes such as fructose 1,6-biphosphate aldolase and transaldolase utilize such transient intermediates to manipulate their high energy substrates (Sygusch et al., 1987; Hall et al., 1999). The pR1 ribozyme extends our knowledge of ribozyme-mediated sugar chemistries beyond the previously characterized Zn^{2+}-dependent (class II) aldolase ribozyme to include a sugar reaction with a Schiff base (Fusz et al., 2005). This ribozyme chemistry allows for the possibility that such reactions could have been used early in evolution to harness chemical energy in a fashion analogous to modern metabolism.

Ribozyme Promiscuity

More intriguingly, pR1 demonstrates an unusual catalytic promiscuity (O'Brien and Herschlag, 1999; Forconi and Herschlag, 2005) that might have played an important role in the early evolution of small molecule ribozyme function. The pR1-PR aldehyde-dependent chemistry is in distinct contrast to the reaction by pR1-PRPP, which generates a nucleotide glycosidic linkage based on our TLC and enzymatic recognition data (Figure 4). The unanticipated PRPP-dependent chemistry is surprisingly efficient ($\sim$5 M^{-1} min^{-1}), and is $\sim$2-fold faster than the PR-dependent chemistry. This nucleotide synthesis rate is equal to the activity of the pyrimidine nucleotide synthase ribozymes selected from a random sequence pool ($\sim$4 M^{-1} min^{-1}) (Unrau and Bartel, 1998) and is only $\sim$50 times slower than the PRPP-dependent purine nucleotide synthase ribozymes selected directly for ^{6S}GMP synthesis ($\sim$250 M^{-1} min^{-1}) (Lau et al., 2004). This similarity in rates was quite unexpected, particularly given that ribozymes selected directly for purine or pyrimidine nucleotide synthesis do not promote PR-dependent chemistry.

Mechanistically, the simplest hypothesis consistent with our data is that the pR1 ribozyme uses charge stabilization to enhance both reactions (Figure 6). The PR reaction likely involves the reactive carbonyl of the open chain ribose, which is consistent with the complete absence of activity observed upon its reduction. As charge stabilization is known to enhance Maillard chemistry, with PR reacting appreciably faster than ribose (Sandwick et al., 2005), it is plausible that the pR1 ribozyme utilizes the negative charge available from its phosphodiester backbone to stabilize the positive polarized charge at the carbonyl carbon, so as to encourage acyclic chemistry (Sandwick et al., 2005) with a nitrogen nucleophile from the ^{6S}Gua substrate (Figure 6a). Moreover, since the ribozyme utilizes the N9 of ^{6S}Gua for its PRPP-dependent chemistry, and if we assume that the ribozyme does not reorganize its active site when either PR or PRPP is utilized, it appears likely that the N9 of ^{6S}Gua is involved in both chemistries (as suggested by Figure 6). As charge stabilization of the reactive oxocarbenium ion intermediate found at the anomeric carbon of the furanose ring is well known to be utilized by glycosidic bond cleaving enzymes (Dinner et al., 2001) and is likely to be involved in ribozyme-mediated pyrimidine nucleotide synthesis (Unrau and Bartel, 2003), we propose that the catalytic promiscuity achieved by the bifunctional pR1 ribozyme

results from a unique pattern of charges that can stabilize both reactions. This active site, which accelerates both PR- and PRPP-dependent reactions, is sensitive to the additional negative charge from the pyrophosphate of the PRPP substrate and requires increased levels of magnesium to perform the nucleotide synthesis chemistry maximally (Figure 6C).

SIGNIFICANCE

Early in the evolution of life on this planet, RNA and not protein is thought to have been the dominant catalyst. In this scenario, both activated purine and pyrimidine ribonucleotides, as suggested most recently by Sutherland's laboratory, could have been initially synthesized by chemical means using plausible prebiotic molecules, giving rise to early RNA life (Powner et al., 2009). However, the exponential growth of ribo-based organisms, whose genomes and catalysts were built of RNA polymers, would place a heavy demand on abiotic supplies of ribonucleotides. A metabolic source of nucleotides would therefore have been required to quickly replace these dwindling supplies. The promiscuous chemistry of the pR1 ribozyme demonstrates how a ribozyme can immediately and efficiently switch from a PR-dependent Schiff base chemistry to nucleotide synthesis using PRPP without a single nucleotide change to its primary sequence. This suggests that promiscuous small molecule RNA catalysts may have played an important role in the rapid evolution of new and metabolically important chemistries early in RNA-based molecular evolution. This is particularly important as Schiff base chemistry plays a central role in the sugar metabolism of modern cellular biology and appears highly likely to have been of importance in an earlier "RNA world". If the synthesis of PRPP can also be simply catalyzed by RNA, then there is no reason why early RNA-based life could not have very rapidly evolved nucleotide synthesis pathways by promiscuous chemistry of the sort reported here. This promiscuity would allow early ribo-organisms powered by abiotic supplies of ribose to simultaneously wean themselves from a presumably scarce supply of abiotically synthesized nucleosides while rapidly evolving new enzymatic function early in the emergence of life on this planet.

EXPERIMENTAL PROCEDURES

Design of RNA Pool

A random sequence DNA pool was synthesized on an ABI 392 DNA synthesizer using 0.2 μM of 2000 Å control pore glass columns and standard cyanoethyl phosphoramidite chemistry. The DNA pool had the final sequence: 5'-***TTC TAATACGACTCACTATA***GGAGCGAAGTGCCC-N$_{95}$-**TCGCACCGCAGCAA GC** (95 random nucleotides, N$_{95}$, flanked by a constant 34 nucleotide 5' primer and a 16 nucleotide 3' primer region, in bold letters; T7 promoter sequence in italics). The 95 random nucleotide couplings were obtained by mixing of equiactive phosphoramidite stocks (ABI) in the molar ratios of 0.28:0.27:0.23:0.22 (dA/dC/dG/dT, respectively) (Zaher and Unrau, 2005). Sequencing of the synthesized pool revealed nucleotide frequencies of 25.8% A, 21.1% G, 28.7% C, and 24.4% T in the 95 nt random region. Large scale PCR was performed using a top strand primer, 5'-TTCTAATACGACTCACTATAGGAGC GAAGTGCCC, and bottom strand primer, 5'-GCTTGCTGCGGTGCGA.

Substrate Ligation and Product Marker Synthesis

Synthesis of adenosine 5′-diphosphoribosyl 1-pyrophosphate (APPRPP) and adenosine 5′-diphospho-6-thioguanosine (APP^{6S}G) was as previously described (Unrau and Bartel, 1998). Adenosine 5′-diphosphoribose (APPR) was purchased from Sigma-Aldrich and further HPLC purified through a reverse phase C18 column (Agilent Technologies). Ligations were performed typically using 2 μM RNA with 60 μM APPR or APPRPP for 4 hr at 23°C in ligation buffer (50 mM HEPES, 10 mM MgCl$_2$, 15 mM sodium phosphite, 10 μg/mL BSA, and 8.3% v/v glycerol [pH 8.0]) using 0.5 U/μL T4 RNA ligase (Wang and Unrau, 2002). Reactions were stopped by the addition of 12 mM EDTA and 300 mM NaCl, followed by a phenol-chloroform extraction and ethanol precipitation. Marker RNA was synthesized by ligation of APP^{6S}G (at 33 μM) to the end of a 125 nt long RNA having the 3′ terminal sequence of …UCAGAA GACAUCACAUUGC-3′, which shares the same last four residues as pR1, so as to generate a marker sequence containing a terminal ^{6S}GMP. The resulting marker was gel purified through an APM gel (Igloi, 1988) for use from rounds 1–6.

Selection

In the first selection round, ∼1.67 nM of DNA, containing ∼2.5 × 10^{14} distinct sequences, were transcribed in the presence of α-[^{32}P] UTP using T7 RNA polymerase and purified through a 6% denaturing polyacrylamide gel. A total of 2.5 nM of this initial RNA pool was ligated (at 5 μM concentration) with APPR as described above. RNA derivatized with PR at its 3′ terminus (RNA-PR, dash symbolizes 5′–3′ phosphodiester linkage) was incubated at 0.25 μM for 18 hr at 23°C in incubation buffer (50 mM Tris-HCl, 150 mM KCl, and 75 mM MgCl$_2$ [pH 7.5]) containing 0.25 mM ^{6S}Gua and 10 mM ATP. Reactions were terminated by the addition of 75 mM EDTA and then applied to 30 kDa MWCO centrifugal filters (Amicon) to concentrate RNA and remove free ^{6S}Gua. The resulting RNA, along with the marker RNA in an adjacent lane, was separated through a 6% denaturing PAGE containing 3.75 μM APM to isolate reactive (thiol-containing) from nonreactive RNA. The gel slice at the position of the marker was excised, eluted in salt solution (300 mM NaCl and 1 mM dithiothreitol [DTT]) overnight and recovered by ethanol precipitation. The eluted RNA was reverse transcribed for 1 hr at 48°C in RT buffer (50 mM Tris-HCl, 25 mM KCl, 3 mM MgCl$_2$, 10 mM DTT, and 560 μM of dNTPs [pH 7.5]) together with 5 μM of 3′ primer using 10 U/μL Superscript II (Invitrogen). The RNA was hydrolyzed using 100 mM KOH at 90°C for 10 min followed by neutralization to pH 8 by addition of HCl. cDNA was then PCR amplified and used for the next round of selection.

Synthesis of pR1 with a Precise 3′ End and Uniform Length

pR1 DNA was PCR amplified using the top strand selection primer and a extended bottom strand primer having sequence 5′-TGATCTCCGCTTGC TGCGGTGCGA. The amplified product was transcribed into RNA and digested using a 38 nt 10–23 DNAzyme having the sequence 5′-**TGATC TCCGGGC**TAGCTACAACGA**TTGCTGCGGTGCGA** (binding arms in bold) (Santoro and Joyce, 1997). This DNAzyme specifically cleaves between G$_{126}$ and C$_{127}$ of the RNA transcript, producing a full-length ribozyme minus its terminal cytidine residue. The resulting 126 nt RNA was purified by 6% PAGE and dephosphorylated for 1 hr at 50°C (50 mM Tris-HCl and 0.1 mM EDTA [pH 8.5]) using 0.5 U/μL alkaline phosphatase (Roche) to remove the terminal 2′–3′ cyclic phosphate that results from the DNAzyme-induced cleavage. The reaction was terminated by heating at 65°C for 20 min, followed by a phenol-chloroform extraction, ethanol precipitation, and purification by 6% PAGE. The purified RNA was 3′ extended with radiolabeled cytidine 5′-[^{32}P], 3′ bisphosphate (pCp) as previously described to regenerate the missing cytidine (Unrau and Bartel, 1998; Lau et al., 2004). The resulting radiolabeled RNA was finally dephosphorylated a second time to remove the 3′ phosphate added by the incorporation of 5′-[^{32}P] pCp and gel purified prior to ligation with APPR.

Kinetic Analysis of pR1

The pR1 isolate was transcribed and derivatized with either PR or PRPP as described above. These constructs were then incubated with constant 0.25 mM ^{6S}Gua and time points (0, 18, 42, and 90 hr) were taken. Time points were stopped by the addition of an equal volume of gel-loading buffer (formamide, 10 mM EDTA, and xylene cyanol). Four independent time courses

were performed and their fraction reacted was fitted to the equation $F = \beta(1 - e^{-k_{obs}t})$ using the program KaleidaGraph (Synergy Software), with F being the fraction reacted at time t, k_{obs} the first-order rate constant, and β the maximum fraction reacted. The overall reaction rate was determined from the average of the four k_{obs} values determined from each individual plot.

3′ Enzymatic Extension Assays

pR1 isolate was derivatized with PR or PRPP as described above and reacted for 96 hr with 0.25 mM ^{6S}Gua, followed by an APM gel purification. The pR1-PR and pR1-PRPP products were then subjected to 3′ ligation with radiolabeled 5′-[^{32}P] pCp in the presence of 2 μM ATP. Extension activity was determined using an APM gel shift assay to detect the amount of labeled 5′-[^{32}P] pCp incorporated. For Poly(A) extension assays, 2 μM of pR1-PR and pR1-PRPP reaction products were extended with 2 μM of α-[^{32}P] dATP in 1× PAP buffer (Ambion) containing 2.5 mM MnCl$_2$ using 0.2 U/μL E. coli Poly(A) polymerase (Ambion) for 2 hr at 37°C.

TLC Analysis of PRPP Product

Both unlabeled pR1-PRPP and internally labeled MA-PRPP were reacted to completion with ^{6S}Gua, ligated to 5′-[^{32}P] pCp and purified by an APM gel as described above. The recovered RNA products were digested to completion for 15 hr at 23°C (25 mM sodium citrate and 4 mM DTT [pH 4.5]) using 0.26 U/μL T2 ribonuclease (Sigma-Aldrich) (Lau et al., 2004). Digested pR1 products and MA products (reference) were analyzed using TLC by spotting on a 10 × 10 cm cellulose TLC plate (J.T. Baker) presoaked in 1:10 saturated (NH$_4$)$_2$SO$_4$: H$_2$O and developed in two dimensions (first dimension 80% ethanol and second dimension solvent contains 40:1 saturated (NH$_4$)$_2$SO$_4$: 2-propanol; both solvents contained 100 μM β-mercaptoethanol) (Lau et al., 2004). Samples were spotted 1 cm in from each edge at the bottom left corner of the TLC plates.

Purine Analogues

All purine analogues were obtained from Sigma-Aldrich and dissolved in 1.05× incubation buffer. 6-thioguanine ε (347 nm; pH 1) = 20,900 M^{-1} cm^{-1} (Elion and Hitchings, 1955), 6-thiopurine ε (325 nm; pH 1) = 20,500 M^{-1} cm^{-1} (Elion, 1962), 6,8-dithiopurine ε (358 nm; pH 1) = 27,800 M^{-1} cm^{-1} (Robins, 1959), and 2-methyl-6-thiopurine assumed ε (330 nm; pH 1) = 20,000 M^{-1} cm^{-1} were used at 0.25 mM concentration while analogues 6-thioxanthine (OD of 0.128 Å determined at 200-fold dilution, 340 nm, pH 1), 6-thio-9-methylpurine (OD of 0.206 Å determined at 200-fold dilution, 335 nm, pH 1), and 2-thiocytosine (OD of 0.451 A determined at 500 fold dilution, 270 nm, pH 8) were used at saturating concentrations.

Reduction of PR

NaBH$_4$ (EMD Chemicals) was freshly dissolved in water and immediately added at 10 mM concentration to pR1-PR or pR1-PRPP. The RNA was reduced for 1 hr (until bubbling of hydrogen gas ceased) before being incubated with ^{6S}Gua.

Derivatization of pR1 with PdR

pR1 DNA was PCR amplified using the top strand selection primer and a bottom strand primer having sequence 5′-CTCCGCTTGCTGCGGTGCGA, adding an additional 4 nt to the 3′ end of pR1. The amplified product was transcribed into RNA and then digested using a 34 nt 10–23 DNAzyme having the sequence 5′-**CTCCGCTTG**GGCTAGCTACAACGA**TGCGGTGCGA** (binding arms in bold), resulting in cleavage between G$_{122}$ and C$_{123}$ of the RNA construct (Santoro and Joyce, 1997). The 122 nt fragment was gel purified, treated with T4 kinase to remove the terminal phosphate, and gel purified a second time as described above. A mixed oligonucleotide having the sequence CAAGCdG (CAAGC being ribonucleotides; dG, deoxyguanosine; Dharmacon) was end labeled with T4 polynucleotide kinase and γ-[^{32}P] ATP. Depurination of the terminal dG was achieved by heating at 80°C for 2 min in the presence of 200 mM HCl followed by neutralization with NaOH. The resulting sample was separated through a 23% PAGE and the lower band product excised and purified. A similar RNA oligonucleotide pCAAGCdG (with a 5′ phosphate) was obtained from Dharmacon and subjected to the same depurination and gel purification procedures as described. The resulting sample was sent for mass spectrometry analysis (mass of the purified lower

product found: 1827.24 U, predicted for pCAAGC-PdR: 1827.17 U). To obtain the desired pR1 sequence with a terminal tethered PdR, 5 μM of the shortened pR1 RNA was ligated with 5 μM of purified 5′-[^{32}P] pCAAGC-PdR in the presence of 2 μM ATP using the ligation protocol described above. The resulting RNA was separated through 8% PAGE and the product corresponding to full-length pR1 was excised and eluted (with body-labeled pR1 RNA loaded in an adjacent lane as a marker). To enhance the radioactive signal, the RNA (pR1-PdR) was further radiolabeled by phosphorylation with γ-[^{32}P] ATP using T4 kinase and gel purified as described above, before incubation with ^{6S}Gua under selection conditions.

ACCESSION NUMBERS

The pR1 sequence has been submitted to GenBank with the accession number EU267931.

SUPPLEMENTAL DATA

Supplemental Data include seven figures and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00215-4.

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