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5′-RNA Self-Capping from Guanosine Diphosphate†

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ABSTRACT: A selected RNA (isolate 6) efficiently catalyzes a self-capping reaction with free GDP, yielding the same 5′-capped structure as is formed by protein GTP:RNA guanylyltransferase. This unexplored RNA-catalyzed reaction type involving nucleophilic attack on phosphate by phosphate adds to the variety of possible postsynthetic RNA-catalyzed RNA modifications. The selected RNA requires only Ca^{2+} for activation and has a broad active pH range of 4.5−9.0. The RNA also has a 5′-pyrophosphatase activity.

Capping and methylation of the 5′-termini of mRNAs, small nuclear RNAs, and heterogeneous nuclear RNAs are important steps in eukaryotic RNA processing. The 5′-capped structure plays important roles in stabilizing eukaryotic mRNA and is required for translation (Muthukrishnan et al., 1975). The methylated cap is formed by a series of reactions catalyzed by RNA triphosphatase (Moss et al., 1976; Venkatesan et al., 1980), GTP:RNA (GTP, guanosine 5′-triphosphate) guanylyltransferase (Martin et al., 1975a; Wei & Moss, 1977; Mizumoto & Lipmann, 1979), and RNA (guanine-7) methyltransferase (Martin et al., 1975b; Ensinger & Moss, 1976), respectively:

$$\text{pppRNA} \rightarrow \text{ppRNA} + \text{P}_\text{i} \quad (1)$$

$$\text{G(5′)ppp} + \text{ppRNA} \rightarrow \text{G(5′)pppRNA} + \text{PP}_\text{i} \quad (2)$$

$$\text{AdoMet} + \text{G(5′)pppRNA} \rightarrow \text{m}^7\text{G(5′)pppRNA} + \text{AdoHcy} \quad (3)$$

We now report that an RNA derived by *in vitro* selection can efficiently catalyze a self-capping reaction with GDP to form a *bona fide* unmethylated cap G(5′)pppRNA.

As an extension of previous work on self-aminoacylation (Illangasekare et al., 1995), we designed a selection for release of 5′-terminal pyrophosphate from RNA transcripts in the presence of amino acids. Such release might signal reaction of the carboxyl of amino acids with the α-phosphate of 5′-pppRNA to yield a mixed carboxylate−phosphate anhydride, that is, to give the analogue of the aminoacyl adenylate formed by protein aminoacyl-tRNA synthetases. During the selection, however, we discovered a new $^{32}PP_i$ labeling activity that coevolved with 5′-pyrophosphatase activity of the pool RNA. On the basis of this $^{32}PP_i$ labeling, which is closely related to capping, we then purposefully used a new selection method to enhance 5′-capping. These nucleophilic attacks on the 5′-triphosphate seem to have been easily selected.

MATERIALS AND METHODS

Selection Procedure. Two different methods were used for the selection of self-capping RNA. The first selection method was based on 5′-pyrophosphate release and subsequent circularization of RNA, designed originally for enriching amino acid-activating RNA (Figure 1A,B). The selection was started with 1.7×10^{15} (2×10^{14} unique sequences) molecules of gel-purified 104mer RNA: 5′-pppGGGGAGUACGGGAGAGGAUACUACACGUG-$(N)_{50}$CCA UUGCAUGUAGCAGAAGCUUAAA-3′. N indicates random nucleotides. Before being incubated with

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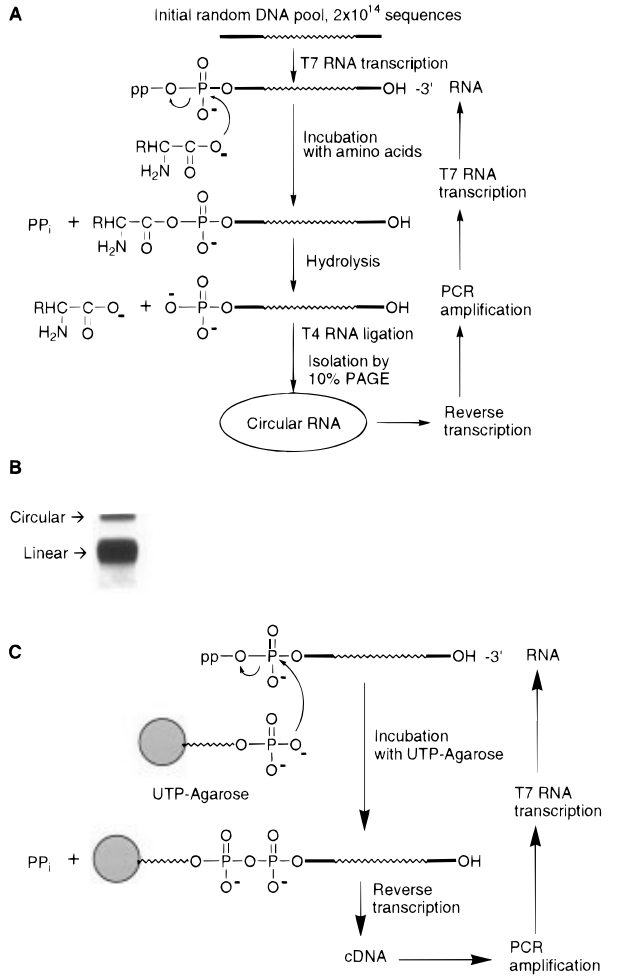

FIGURE 1: Selection protocol. (A) First selection method for evolving a hypothetical amino acid-activating RNA based on pyrophosphate release and subsequent circularization. (B) Separation of circular RNA from linear RNA by 10% denaturing PAGE. (C) Second selection method for enriching self-capping RNA based on covalent linkage of UTP−agarose to 5′-RNA via a phosphate−phosphate anhydride bond.

the amino acid mixture, RNA was renatured by heating at 80 °C for 3 min and cooling to room temperature over 10 min. Selection buffer, containing 20 mM MES (pH 5.5), 20 mM $MgCl_2$, 10 mM $CaCl_2$, 5 μM $ZnCl_2$, 100 μM $MnCl_2$, 100 mM NaCl, and 40 mM KCl, was added when the RNA solution temperature reached 60 °C during renaturation. Renatured RNA (2 μM) and a mixture of amino acids, consisting of Gly, Ala, Val, Leu, Ile, His, Arg, Lys, Gln, Met, Pro, Phe, and Try each at 1 mM, was cycled for 3 h between 15 and 45 °C to encourage reaction by varying the conformation of the pool RNA.

After incubation with amino acids, RNA was EtOH precipitated and washed with 7:3 EtOH/H_2O. The RNA was denatured by heating at 95 °C for 2 min in 2 mM EDTA at pH 8. The amino acid−RNA mixed anhydride should be hydrolyzed under the above conditions (Mullins et al., 1984; Lacey et al., 1984). Circularization of pRNA was carried out by incubating denatured RNA (5 μM) with T4 RNA ligase (1.25 units/μL) in ligation buffer [50 mM TRIS (pH 7.5), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.2 mM ATP, 0.1 μg/mL BSA, and 15% DMSO] for 1 h at 37 °C. Reverse transcription was performed directly with the circular RNA isolated by 10% PAGE. PCR amplification and T7 RNA transcription were performed as described (Ciesiolka et al., 1996).

In the second selection method (Figure 1C), RNA molecules reacting with nucleotide phosphate were selected by their ability to link covalently to the terminal phosphate of UTP−agarose resin through a phosphate−phosphate anhydride bond. The pool RNA (5 μM, 100 μL) after cycle 20 of the first selection method and 100 μL UTP−agarose (Sigma; UTP linked via ribose hydroxyls to agarose with adipic acid dihydrazide) were rocked in the selection buffer at 37 °C for 1 h and then washed three times with 5 mL of following solutions: (A) 600 mM NaCl, 400 mM KCl, and 40 mM EDTA; (B) 1000 mM NaOAc (pH 5.2) and 40 mM EDTA; (C) 10% solution A; and (D) 10% solution B. Reverse transcription was performed directly with the washed UTP−agarose resin in the presence of 1 mM dNTP, 1 μM primer, and 1 unit/μL AMV reverse transcriptase for 30 min at 42 °C. cDNA was then recovered in supernatant by spinning the UTP−agarose resin for 2 min in a microcentrifuge. PCR amplification and T7 RNA transcription completed the selection cycle (Ciesiolka et al., 1996).

After 24 cycles of selection (20 cycles of the first method and 4 cycles of the second method), the RNA pool was reverse transcribed, PCR amplified, cloned, and sequenced as described (Ciesiolka et al., 1996).

Determination of RNA Pyrophosphatase Activity Dependence on Amino Acids. During the first selection, pool RNA was assayed for pyrophosphate release (with or without amino acids) by two different methods. (a) After uniformly ^{32}P-labeled RNA was incubated with selection buffer in the absence or presence of the amino acid mixture, RNA was circularized by T4 RNA ligase. Circular RNA was separated from linear RNA by 10% PAGE and quantitated by a phosphorimager. (b) 5′-γ-^{32}P-Labeled *pppRNA (prepared by transcription in the presence of [γ-^{32}P]GTP; the asterisk indicates the position of ^{32}P) was incubated with selection buffer in the absence or presence of the amino acid mixture, then separated from pyrophosphate by 8% PAGE, and quantitated as above.

Estimation of the Increase in $^{32}PP_i$ Labeling Activity of Selected RNA. The progress of the selection was also monitored by $^{32}PP_i$ labeling activity, which is closely related to the self-capping activity (see Results and Discussion). Gel-purified cold 5′-pppRNA (2 μM in 5 μL) from different cycles was incubated with 25 μCi $^{32}PP_i$ (1 nmol) in selection buffer for 1 h at 37 °C. $^{32}PP_i$-labeled RNA was then separated from $^{32}PP_i$ by 8% denaturing PAGE and visualized on a phosphorimager (BioRad, GS525).

Capping Reaction Assay. Isolate 6 RNA from cycle 24 was transcribed in the presence of [α-^{32}P]GTP and purified to single-nucleotide resolution by 8% denaturing PAGE. Gel-purified RNA was incubated with 1 mM GDP for 1 h at 37 °C in various solutions, including selection buffer. The reaction product (5′-capped RNA as described below) was separated from reactant RNA by 8% denaturing PAGE and analyzed by a phosphorimager.

Identification of Reaction Products. Three different methods were used to identify the reaction products of GDP with isolate 6 RNA (the most abundant sequence selected from the pool, see below). In the first method, isolate 6 RNA was labeled at 5′-γ-phosphate (*pppRNA) by transcription in the presence of [γ-^{32}P]GTP and purified to single-nucleotide resolution by 8% denaturing PAGE. After the

*pppRNA (0.1 μM in 5 μL) was incubated with 1 mM GDP for 1 h at 37 °C in the selection buffer, the reaction mixture was chromatographed along with standard $^{32}P_i$ and $^{32}PP_i$ on a cellulose PEI TLC plate with 20% methanol and 80% 0.5 M potassium phosphate at pH 6.3. ^{32}P was visualized by autoradiography.

The second method was based on the resistance and susceptibility of gel-purified reaction product (5′-capped RNA, here referred to as G′pppRNA adduct) to calf intestinal alkaline phosphatase (CIAP, NEB) and tobacco acid pyrophosphatase (TAP, Epicentre). The pure G′pppRNA adduct of isolate 6 RNA was prepared by (1) 8% denaturing gel purification of isolate 6 RNA, internally labeled by ^{32}P during transcription in the presence of [α-^{32}P]GTP, to single-nucleotide resolution, (2) incubation of the isolated pure RNA with 1 mM GDP in the selection buffer for 1 h at 37 °C, and (3) 8% denaturing gel purification of the G′pppRNA adduct to single-nucleotide resolution. Authentic gel-purified 5′-capped isolate 6 RNA [G(5′)pppRNA, internally ^{32}P-labeled], prepared by transcription in the presence of [α-^{32}P]-GTP and unmethylated cap G(5′)pppG (Pharmacia) with a ratio GTP:G(5′)pppG of 1:10, was used as a reference for the AP and TAP digestion analysis. The G′pppRNA adduct and authentic 5′-capped RNA (both 1 μM in 5 μL) were incubated for 1 h at 37 °C with 20 units of CIAP and 5 units of TAP, respectively, in 1× supplied buffers. The digestion mixtures were analyzed using an 8% denaturing gel.

The structure of the G′pppRNA adduct was further determined by TLC analysis of complete hydrolysis of gel-purified RNA with NaOH and RNases. Internally [α-^{32}P]-GTP-labeled pppRNA, G′pppRNA adduct, and authentic G(5′)pppRNA (10 μM in 30 μL) were treated with 50 units of CIAP for 1 h at 37 °C, followed by gel filtration through a G-25 microspin column (Pharmacia). The CIAP-treated RNAs were hydrolyzed to completion after 20 h at 37 °C with 0.5 N NaOH and 20 units each of RNases B, Phy M, and T1 (Pharmacia) in 35 mM sodium citrate at pH 5, respectively. The completely hydrolyzed solutions were analyzed by TLC under the same conditions as above.

The ^{32}P-labeled 2′(3′)-NMP markers, Ap*, Gp*, Up*, and Cp*, were prepared as follows. (1) Homooligonucleotides, $(Np)_n$ (N = A, G, U, or C; $n = 20-40$), were obtained by NaOH hydrolysis of homopolynucleotide poly(N) for 3 min at 95 °C in 50 mM NaOH, followed by 8% gel purification. (2) The 3′-phosphate of the isolated homooligonucleotides was removed by incubating with 20 units of polynucleotide kinase (Cameron & Uhlenbeck, 1977) for 1 h at 37 °C in 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 20 mM MES at pH 5.5. The 3′-dephosphorylated homooligonucleotides were isolated by 8% gel electrophoresis. (3) The 3′-dephosphorylated homooligonucleotides were ligated to *pCp by T4 RNA ligase (under the same conditions as RNA circularization in the first selection method) to yield 3′-^{32}P-labeled oligonucleotides $(Np)_{n-1}N$*pCp. (4) Complete hydrolysis (20 h at 37 °C with 0.5 N NaOH) of the labeled $(Np)_{n-1}N$*pCp by NaOH produced ^{32}P-labeled 2′(3′)-NMPs, Np*.

Pyrophosphatase Activity Assay. The pyrophosphatase activity (pyrophosphate release in the absence of GDP) of isolate 6 RNA was assayed by both TLC as described above and 8% denaturing PAGE after incubating *pppRNA (0.1 μM in 5 μL) in the selection buffer for different times at 37 °C.

RESULTS AND DISCUSSION

Design of Selection Methods. The selection was designed originally for a possible amino acid-activating RNA on the basis of the release of 5′-terminal pyrophosphate from RNA transcripts in the presence of amino acids (Figure 1A,B). In principle, such a selection could enrich pppRNA that would react via its 5′-triphosphate with the carboxyl of amino acids to yield a mixed carboxylate−phosphate anhydride, that is, to give the analogue of the aminoacyl adenylate formed by protein aminoacyl-tRNA synthetases. The planned selection of amino acid-activating RNA anticipated the initial formation of an activated amino acid:

$$\text{R-C(O)-O}^- + \text{pppRNA} \rightarrow \text{R-C(O)-O-pRNA} + \text{PP}_i \quad (4)$$

followed by the rapid base-catalyzed hydrolysis of the unstable anhydride:

$$\text{R-C(O)-O-pRNA} \rightarrow \text{R-C(O)-O}^- + \text{pRNA} \quad (5)$$

This RNA product (pRNA) can be circularized by T4 RNA ligase and isolated from linear unreacted RNA by 10% denaturing PAGE (Figure 1B). A circularization test of initial pool pRNA, transcribed in the presence of 12:1 GMP/GTP, gave a 73% yield of circularization under ligation conditions. Ligation tests with a random pppRNA transcript gave a background circularization yield of 0.5−1%.

Circular RNA increased from 1.3% (background for the first three cycles) to 15% at selection cycle 5, suggesting (ligation yield is <100%) that >15% of the pool pppRNA was converted to pRNA under the selection conditions. However, no difference in either circularization yield or 5′-PP_i release yield was observed in the absence and presence of amino acids; that is, the pool in aggregate acts as a simple pyrophosphatase activity. The eventual appearance of pyrophosphatase activity of RNA was expected since such an activity converts pppRNA into pRNA that could be circularized and thus selected (Figure 1A). However, a major pyrophosphatase activity at a stage that was so early (cycle 5) was not anticipated.

Further selection−amplification of other PP_i-releasing activities requires suppressing of this easily selected pyrophosphatase. To counterselect against pyrophosphatase activity, the reactants of subsequent cycles of selection were first incubated with the selection buffer in the absence of any added nucleophile, including amino acids, followed by RNA ligation. Unreacted linear pppRNA, that is, RNA unaffected by pyrophosphatase activity, was reisolated by 10% PAGE and then used for selection.

During the selection, pools were checked for both pyrophosphatase activity and $^{32}PP_i$ RNA labeling activity, reasoning that the formation of a mixed anhydride between an amino acid and an RNA might promote $^{32}PP_i$ exchange:

$$\text{R-C(O)-O-pRNA} + {}^{32}\text{PP}_i \rightarrow \text{R-C(O)-O}^- + {}^{32}\text{pppRNA} \quad (6)$$

Neither amino acid-dependent pyrophosphatase activity nor amino acid-dependent $^{32}PP_i$ labeling activity was observed after 20 cycles of selection/counterselection−amplification. However, amino acid-independent $^{32}PP_i$ la-

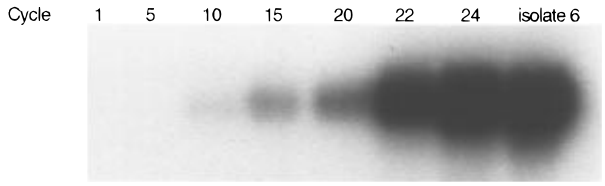

FIGURE 2: Progress of $^{32}PP_i$ labeling activity of pool pppRNAs from different selection cycles and isolate 6 RNA. The first five lanes (from cycles 1 to 20) represent an increase of $^{32}PP_i$ labeling activity from the first selection method. Lanes 6 and 7 are the result of the second selection method. Lane 8 represents the activity of isolate 6 RNA from the cycle 24 pool.

beling appeared at cycle 10 and increased progressively over subsequent selection cycles (lanes 1−5 in Figure 2).

Selection of Self-Capping RNA. Because our selection required pyrophosphate release, we reasoned that pyrophosphatase activity (attack by H_2O) and $^{32}PP_i$ labeling (attack by $^{32}PP_i$) were probably the same RNA activity acting at the same pppRNA 5′-α-phosphate. Therefore, such a $^{32}PP_i$ labeling activity could also self-cap the RNA if $^{32}PP_i$ was replaced with a nucleoside 5′-diphosphate or -triphosphate.

To enrich self-capping activity from the pool, a second selection method (reaction with UTP−agarose) was used (Figure 1C). $^{32}PP_i$ labeling activity increased 10-fold during the first two cycles of this UTP−agarose selection (lane 6 in Figure 2), and the next two cycles increased activity 2-fold further.

RNA molecules from cycle 24 (the net result of both selections) were converted to cDNA, cloned, and sequenced (Ciesiolka et al., 1996). There were 13 independent sequences out of 30 sequenced clones; isolate 6 was recovered 12 times, with a sequence of 5′-UCCAUCCAC-CGUUGUGAUC GUCUGAAGUCAACCACAACAUGC-CCUGACUC-3′ in the random 50N region. Because isolate 6 is the most abundant selected sequence, all the studies below were carried out with isolate 6 RNA.

$^{32}PP_i$ labeling increased dramatically during selection (Figure 2). From cycle 10 to isolate 6 RNA, this activity increased 120-fold. While $^{32}PP_i$-labeled cycle 10 pool RNA was detectable after 30 s by a phosphorimager, an equivalent amount of cycle 5 RNA gave no signal after 34 h of exposure. In both assays, background was subtracted from the signals through Molecular Analyst (BioRad) volume analysis. Therefore, the $^{32}PP_i$ labeling activity of pool RNA increased at least 4080-fold from cycle 5 to cycle 10 and at least 500 000-fold from cycle 5 to isolate 6. The increase in $^{32}PP_i$ labeling activity of earlier cycles (1−5) could not be estimated by the same method because reaction was undetectable in the initial pool.

Self-Capping of Isolate 6 RNA by GDP. As might be expected from the role of the nucleotide in the UTP−agarose selection method and from $^*PP_i$ labeling of selected RNA, isolate 6 also reacts rapidly with GDP under selection conditions. The reaction product of GDP and isolate 6 RNA (5′-capped RNA, see below) can be well resolved from unreacted RNA by 8% gel electrophoresis (Figure 3). The reaction yield in selection buffer approaches 50% within 1 h at 37 °C in the presence of 1 mM GDP (though these conditions are not optimal; see below). From Figure 3B, a pseudo-first-order rate constant of 0.03 min^{-1} for capping with 1 mM GDP in the selection buffer was estimated.

Structure of Capped Isolate 6 RNA. To locate the reaction site of isolate 6 RNA with GDP, we incubated 5′-γ-^{32}P-labeled RNA with GDP and assayed the product(s) by TLC. As shown in Figure 4A, $^{32}PP_i$ was the only ^{32}P-labeled product of a nearly complete reaction. Therefore, GDP reacts

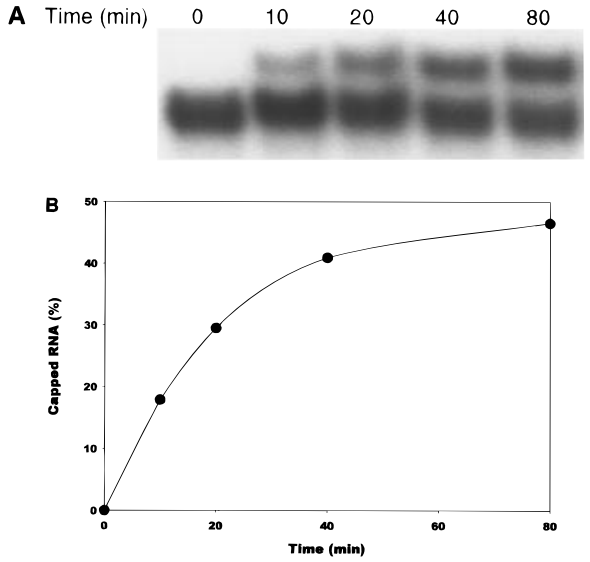

FIGURE 3: Self-capping reaction of GDP with isolate 6 pppRNA in selection buffer at 37 °C. (A) The reaction product (5′-capped RNA) is well resolved from the reactant RNA by 8% denaturing PAGE. (B) Reaction yield time course.

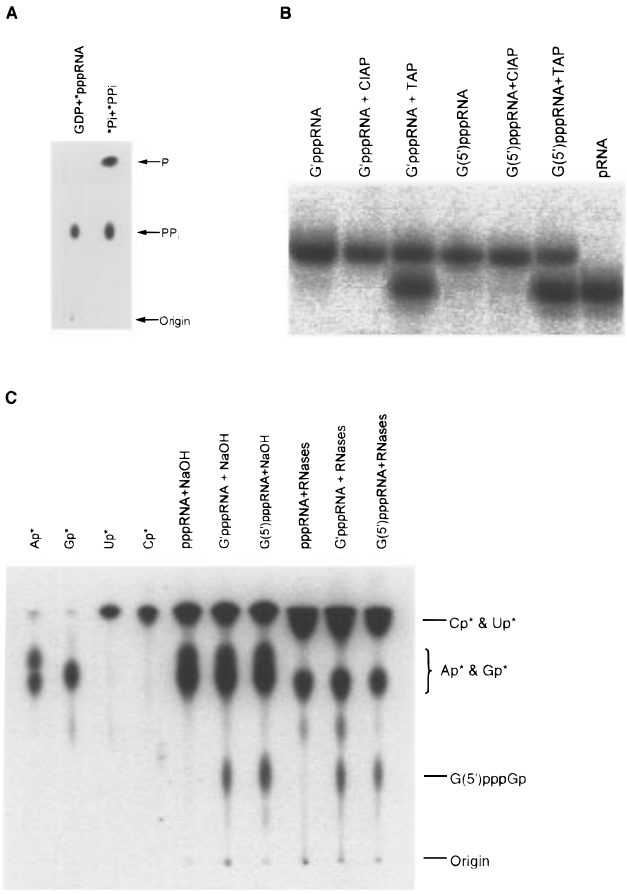

FIGURE 4: Identification of reaction products (G′pppRNA adduct) of GDP with isolate 6 pppRNA. (A) TLC analysis of reaction between 1 mM GDP and 5′-γ-^{32}P-labeled isolate 6 *pppRNA for 60 min at 37 °C in selection buffer. (B) PAGE analysis (8%) of CIAP and TAP digestion of the G′pppRNA adduct and authentic 5′-capped RNA [G(5′)pppRNA]. (C) TLC analysis of complete hydrolysis products of isolate 6 RNA, the G′pppRNA adduct, and authentic 5′-capped G(5′)pppRNA.

with isolate 6 RNA by attacking the 5′-α-phosphate, releasing $^{32}PP_i$:

$$GDP + *pppRNA \rightarrow GDP{-}pRNA + *PP_i \quad (7)$$

To determine how GDP is linked to the 5′-α-phosphate of isolate 6 RNA, we gel purified the reaction product (G′pppRNA adduct). The G′pppRNA adduct was incubated with CIAP and TAP, respectively, and analyzed by 8% gel electrophoresis (Figure 4B). No effect of CIAP on the adduct could be observed, indicating that either there was no terminal phosphate in the adduct or less likely in that the CIAP digestion product of the adduct was not resolved from the adduct by 8% gel electrophoresis. However, the G′pppRNA adduct clearly could be digested by TAP, resulting in a product with the migration rate of pRNA. Complete digestion of the capped RNA into pRNA was not achieved due to contaminating nuclease activity in TAP, which degraded the RNA when a higher enzyme concentration or a longer digestion time was used. The combined results of CIAP and TAP digestion of the G′pppRNA adduct suggest that there is no terminal phosphate from GDP in the G′pppRNA adduct. Instead, the terminal phosphate of GDP is linked to the 5′-α-phosphate of isolate 6 RNA to form a phosphate anhydride, which can be cleaved by TAP.

This argument is strengthened by a parallel standard. The same behavior was observed for authentic capped isolate 6 RNA, G(5′)pppRNA, prepared by transcription in the presence of authentic G(5′)pppG as the transcription initiator. This parallel behavior toward CIAP and TAP by our product and *bona fide* G(5′)pppRNA strongly supports a structure containing GDP linked to isolate 6 RNA through its pyrophosphate.

To further investigate the product, we performed TLC analyses (Figure 4C) of complete phosphodiester hydrolysis products from the unreacted RNA, the G′pppRNA adduct, and the authentic G(5′)pppRNA transcript, by both NaOH and a mixture of RNases. All RNAs were CIAP treated to remove free terminal phosphate before the complete hydrolysis. The RNA was labeled by [α-^{32}P]GTP during transcription; thus, all nucleotides 5′ to guanosines (G) in the RNA sequence would be labeled by ^{32}P after hydrolysis by either NaOH or RNAses. The ratio of four ^{32}P-labeled nucleoside 3′(2′)-monophosphates, Ap*, Cp*, Gp*, and Up*, from complete NaOH hydrolysis agreed with the theoretical value for (A + G):(C + U) of 12:12 based on the sequence of isolate 6 RNA. Migrating much slower than Gp* and Ap*, an extra band appeared in both G′pppRNA adduct and standard G(5′)pppRNA lanes, but not in the RNA lane. Because it was the only new band in the standard G(5′)-pppRNA lane, the extra band can only be the capped structure, G(5′)pppGp*. The band accounted for 3.4% of total ^{32}P activity, comparing well with the expected value of 4%. Thus, the slower-migrating band is also stoichiometrically consistent with capping at a unique 5′-terminus. The discrepancy of 15% between the experimental and theoretical values for standard G(5′)pppRNA likely came from <100% incorporation of G(5′)pppG at the first position during transcription (10:1 G(5′)pppG/GTP in transcription solution). Finally, comigration with G(5′)pppGp* in the standard G(5′)pppRNA lane supports the conclusion that the extra band below Ap* and Gp* in the G′pppRNA adduct lane has the structure G(5′)pppGp*.

Complete digestion with RNases was consistent with complete NaOH hydrolysis (Figure 4C, lanes 8−10). By comparing the hydrolysis products of the G′pppRNA adduct with those of the RNA and the authentic G(5′)pppRNA, we again deduce that the band below Ap* and Gp* in the G(5′)-pppRNA lane is the cap G(5′)pppG(3′)p*, which was also present in the G′pppRNA adduct lane. However, the ratio of nucleoside 3′-monophosphates did not agree with the theoretical value, though NaOH hydrolysis had given the expected values from the same RNAs. The Gp* band had much lower ^{32}P activity than expected, suggesting RNase contamination with a phosphatase, perhaps a phosphatase especially active on Gp*.

Thus, isolate 6 RNA reacts with GDP to form a capped structure G(5′)pppRNA, the same capped structure formed by protein enzyme GTP:RNA guanylyltransferase. The mechanism involves the nucleophilic attack on the α-phosphate of the pppRNA by the oxygen of β-phosphate of GDP with subsequent release of PP_i:

$$G(5')p\mathbf{p} + pppRNA \rightarrow G(5')p\mathbf{p}pRNA + PP_i \quad (8)$$

The $^{32}PP_i$ labeling activity and self-capping activity of isolate 6 RNA from GDP are from similar reactions involving a terminal phosphate attack on the α-phosphate of the pppRNA. Although the self-capping rate of random ppRNA (background self-capping reaction) is difficult to estimate, the increase in $^{32}PP_i$-labeling activity of this selection can be used to calculate the upper limit of random RNA self-capping: $0.03\ min^{-1}/500000 = 6 \times 10^{-8}\ min^{-1}$. The actual rate of background self-capping reaction is likely to be much lower since a 500000-fold increase in $^{32}PP_i$ labeling activity covers only from selection cycle 5 to isolate 6 RNA.

The RNA capping reaction can be distinguished from the reaction catalyzed by the protein enzyme GTP:RNA guanylyltransferase from *Vaccinia* virus, reovirus, and eukaryotic cells, although the reaction products are the same (reactions 1 and 8). The protein enzyme-catalyzed reaction starts with GTP and ppRNA and proceeds through the formation of an enzyme−GMP intermediate EpG (E, the protein-capping enzyme) with simultaneous release of PP_i (Shuman & Hurwitz, 1981). In our RNA self-capping reaction, GDP is the nucleophile and the α-phosphate of the pppRNA is the center of nucleophilic attack. On the contrary, in the guanylyltransferase-catalyzed capping reaction, the nucleophile is ppRNA while the nucleophilic attack center is located on the α-phosphate of GTP. Interestingly, a closer parallel exists with RNA polymerase of vesicular stomatitis virus, which synthesizes capped mRNA that contains α- and β-phosphates of GTP and α-phosphate from the RNA (Abraham et al., 1975), but a different capping reaction mechanism has been proposed (Shuman, 1997).

Self-capping is an interesting RNA reaction involving nucleophilic attack on phosphate by phosphate oxygen to form a phosphate−phosphate linkage (phosphate anhydride). While a related reaction has been detected with artifically activated phosphate−imidazolide-terminated oligomers (Chapman & Szostak, 1995), this is the first demonstration that RNA catalysis can join phosphates utilizing natural PP_i leaving groups, and use a small molecule (e.g., GDP) substrate. Though such reactions are frequent in modern biology, capping requires a highly negatively charged

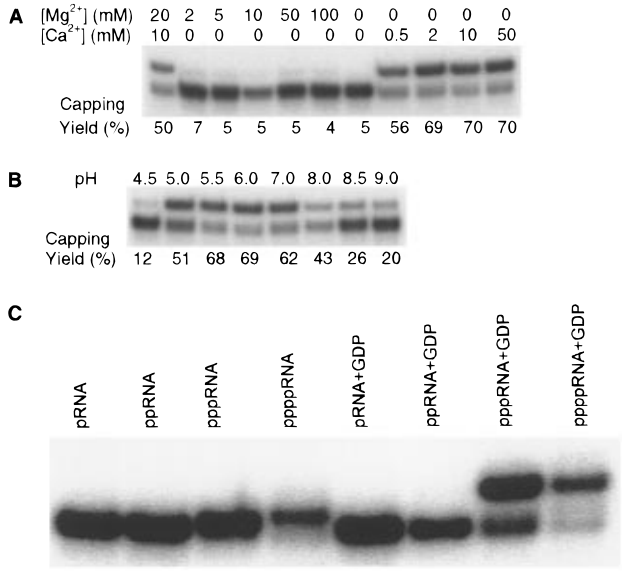

FIGURE 5: Capping activity of isolate 6 pppRNA under different conditions for 1 h at 37 °C. (A) Divalent metal ion dependence in 1 mM GDP and 20 mM MES at pH 5.5. (B) pH dependence of capping activity. The solution contained 1 mM GDP, 2 mM Ca^{2+}, and 20 mM buffer (sodium acetate for pH 4.5 and 5.0, MES for pH 5.5 and 6.0, HEPES for pH 7.0, and TRIS for pH 8.0−9.0). (C) Capping of isolate 6 RNA requires 5′-triphosphate or tetraphosphate RNA. 5′-Tetraphosphate RNA (ppppRNA) was transcribed in the presence of guanosine 5′-tetraphosphate (ppppG) (ppppG:GTP = 20:1).

nucleophile to attack a phosphate center also ringed with negative charge. The RNA may have a binding site for the nucleophile to overcome phosphate−reactant repulsions, and the divalent metal calcium may play its virtually unique role in this process (see below).

Conditions for Self-Capping of Isolate 6 RNA. Self-capping was assayed under different conditions to establish the requirements for activity (Figure 5). The capping reaction of isolate 6 does not require all the divalent metal ions present during selection. Instead, this RNA is calcium specific (Figure 5A). Magnesium ions (between 1 and 200 mM) alone do not activate the RNA. But calcium ions at a concentration as low as 500 μM support activity (lane 8). In fact, the yield of capping in the presence of 10 mM Ca^{2+} only (lane 10) is higher than that in 10 mM Ca^{2+} and 20 mM Mg^{2+} (lane 1), indicating that Mg^{2+} inhibits the reaction. Among the metal ions tested (divalent Sr^{2+}, Ba^{2+}, Zn^{2+}, and Mn^{2+}, 2−50 mM; and trivalent La^{3+}, Dy^{3+}, and Tb^{3+}, 0.005−10 mM tested), only calcium ions give efficient RNA capping activity. However, Mn^{2+} (2−50 mM) can also activate the RNA to yield 10−15% capped products in 1 mM GDP (pH 5.5, 60 min, 37 °C).

The particular requirement of Ca^{2+} for the activity of isolate 6 RNA may be significant. Even at twice the concentration, Mg^{2+} cannot replace Ca^{2+}. Though closely related to Ca^{2+}, neither Sr^{2+} nor Ba^{2+} can play the role of Ca^{2+}. It has been suggested that Ca^{2+} plays a special role in a self-aminoacylating RNA (Illangasekare et al., 1995), implying that Ca^{2+} may have an essential function in both RNA-catalyzed acyl and phosphoryl transfer reactions. Understanding this role may help in the understanding and expansion of RNA catalysis in general (Yarus, 1993; Pyle, 1993).

Figure 5B shows that isolate 6 RNA is active over a broad pH range. Substantial yields of capped RNA were detectable

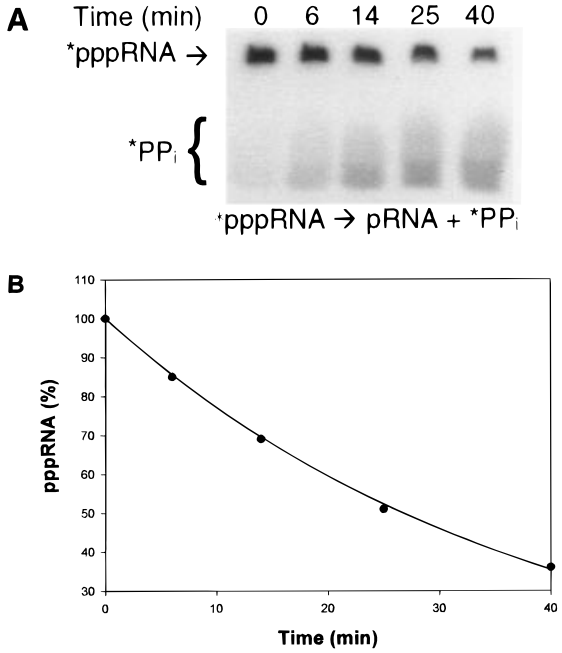

FIGURE 6: Pyrophosphatase activity of isolate 6 RNA. (A) 5′-γ-^{32}P-labeled *pppRNA was incubated with the selection buffer and assayed by 8% PAGE. (B) Time course of $^{32}PP_i$ release of isolate 6 RNA.

over the entire pH range tested (4.5−9.0). The peak yield occurs at pH 5.5−6.0, where the RNA was selected.

The capping reaction requires the 5′-triphosphate or 5′-tetraphosphate of the RNA (Figure 5C). When the 5′-β- and/or -γ-phosphates were removed, there was no detectable capping activity. Within 1 h at 37 °C in 1 mM GDP and 2 mM Ca^{2+} at pH 5.5, 5′-triphosphate- and 5′-tetraphosphate-terminated isolate 6 RNA yielded 70 and 80% capped product G(5′)pppRNA, respectively.

Pyrophosphatase Activity of Isolate 6 RNA. Isolate 6 RNA also retains a 5′-pyrophosphatase activity (Figure 6). In selection buffer, 5′- PP_i is released with a first-order rate constant of 0.026 min^{-1} at 37 °C (in the absence of GDP).

Both self-capping and pyrophosphatase reactions of isolate 6 RNA are centered at 5′-α-phosphate of pppRNA, but they are two distinct activities. The pyrophosphatase activity results from nucleophilic attack on the 5′-α-phosphate of pppRNA by water, while the nucleophilic attack by the terminal phosphate of GDP on the 5′-α-phosphate of pppRNA leads to self-capping. Although the relationship between the two activities has yet to be defined, the coexistence of self-capping and pyrophosphatase activities in isolate 6 RNA was required, on the basis of a selection for PP_i release and subsequent circularization.

CONCLUSION

A self-capping RNA (isolate 6) has been selected by a uniquely designed procedure. The RNA reacts with GDP to form 5′-capped RNA, the same structure formed by protein enzyme GTP:RNA guanylyltransferase. This demonstrates an unexplored reaction type of RNA catalysis that joins phosphates utilizing natural PP_i leaving groups and small nucleophiles (pyrophosphate and GDP). The RNA requires only Ca^{2+} for activity and is active at pH 4.5−9.0.

Our current finding adds to the observed catalytic versatility of RNAs, supporting an RNA world (Gilbert, 1986) with

a prominent role for RNAs catalyzing a variety of reactions. Particularly, it suggests that postsynthetic RNA modifications (as in modern mRNAs, tRNAs, and rRNAs) may have arisen initially from ancient RNA-catalyzed reactions. Such modifications could have helped diversify reactive groups in primordial ribozymes.

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