RNAs synthesized using photocleavable biotinylated nucleotides have dramatically improved catalytic efficiency

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ABSTRACT

Obtaining homogeneous population of natively folded RNAs is a crippling problem encountered when preparing RNAs for structural or enzymatic studies. Most of the traditional methods that are employed to prepare large quantities of RNAs involve procedures that partially denature the RNA. Here, we present a simple strategy using 'click' chemistry to couple biotin to a 'caged' photocleavable (PC) guanosine monophosphate (GMP) in high yield. This biotin-PC GMP, accepted by T7 RNA polymerase, has been used to transcribe RNAs ranging in size from 27 to 527 nt. Furthermore we show, using an in-gel fluorescence assay, that natively prepared 160 and 175 kDa minimal group II intron ribozymes have enhanced catalytic activity over the same RNAs, purified via denaturing conditions and refolded. We conclude that large complex RNAs prepared by non-denaturing means form a homogeneous population and are catalytically more active than those prepared by denaturing methods and subsequent refolding; this facile approach for native RNA preparation should benefit synthesis of RNAs for biophysical and therapeutic applications.

INTRODUCTION

It is now clear that the cellular functions of RNAs rival those of proteins. Within the nucleolus, small nucleolar RNAs (snoRNAs) form complexes with proteins to remodel the pre-ribosomal RNAs through methylation and pseudouridylation (1). In the nucleus small nuclear RNAs, as ribonucleoprotein particles, catalyze and regulate pre-mRNA splicing (2,3); whereas in the cytoplasm, ribosomal RNAs catalyze and regulate protein synthesis (4–6). In addition, in bacterial cells (and some eukaryotic cells also), riboswitches regulate transcription or translation by directly sensing metabolite concentrations (7–10).

The fundamental ability of RNA to switch between structures is a great asset for signaling, yet it can also become a liability when studying complex RNAs *in vitro*. Standard methods for RNA preparation typically denature the RNA giving rise to heterogeneous populations of conformers (11–13). Preparation of RNA using these traditional protocols typically involves phenol–chloroform extraction of associated proteins away from the RNA, RNA concentration by ethanol precipitation, heating in urea or formamide and denaturing polyacrylamide gel electrophoresis (PAGE) fractionation. These established methods, while robust, may partially misfold the RNA and the resultant RNAs may retain only marginal activity (11–13).

Several methods recently proposed to overcome this problem have their own limitations (14–24). These are summarized in Supplementary Figure S1. The first group involves affinity capture-cleavage methods (15–20). The second group of methods uses traditional protein chromatography methods of either size exclusion (21,22) or weak anion exchange chromatography (23,24). The size-exclusion methods allow efficient recycling of unincorporated NTPs and separation of RNA oligomers. However, T7 RNA polymerase (T7 RNAP) needs to be removed prior to chromatography using tedious phenol–chloroform extraction and desalting (20–21). The weak anion exchange method dispenses with the phenol–chloroform step, but requires very high concentration of monovalent salts (500–1000 mM) for eluting high molecular weight RNAs (>400 nt) (23,24).

Development of new methods that facilitate the preparation of RNAs under native conditions would complement existing methods and aid in studies that investigate the impact of natively folded RNA structure on function. To this end, we have developed a new method that uses 'click' chemistry (25,26) to couple biotin (27–30) to a

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'caged' or photolabile (31) guanosine (hereafter referred to as biotin-PC GMP) in high yield and used this GMP analog as initiator nucleotide for T7 RNAP transcription (32) (Scheme 1). We demonstrate the methodology on four RNAs ranging in size from 27 nt RNA fragment (33) to a 527 nt ribozyme (34,35) (Figure 1). Importantly, we show that the biotin-PC GMP-synthesized ribozymes rapidly cleave fluorescently labeled substrates (in <0.5 h), whereas the same ribozymes prepared using the conventional methods and refolded are unable to cleave the substrate after 2 h. These results underscore an obvious but often overlooked fact: RNAs prepared using traditional denaturing methods can have reduced function making conclusions based on results from such preparations precarious.

MATERIALS AND METHODS

General procedures

Chemical synthesis procedures are shown in Supplementary Scheme S1 and details of the synthesis are provided in Supplementary Data.

Optimization of ribosomal A-site RNA and D5 RNA transcription

RNAs were synthesized by *in vitro* transcription (32) with T7 RNAP from synthetic DNA template (Integrated DNA Technologies). The T7 RNAP was expressed in *Escherichia coli* BL21 (DE3) and purified on a Ni-chelating Sepharose column (Pharmacia). The T7 RNAP promoter sequence was 5′-CTA ATA CGA CTC ACT ATA G-3′. The template strand of ribosomal A-site (33) was 5′-GmGmC GAC TTC ACC CGA AGG TGT GAC GCC TAT AGT GAG TCG TAT TAG-3′ and the template strand of the D5 RNA (34,35) was 5′-g AAC CGT ACG TGC GAC TTT CAT CGC ATA CGG CTC c TAT AGT GAG TCG TAT TAG-3′. Two terminal 2′-*O*-methyl modifications in the template strand indicated by 'm' were introduced to substantially reduce the amount of transcripts with extra nucleotides at the 3′-end (36). The lower case letters represent additional nucleotides introduced to improve transcription yield (37). All the purchased DNA strands were purified by denaturing PAGE. The transcription conditions for each RNA were optimized by varying the [A/C/UTPs] and ratio of [GTP]/[biotin-PC GMP] in
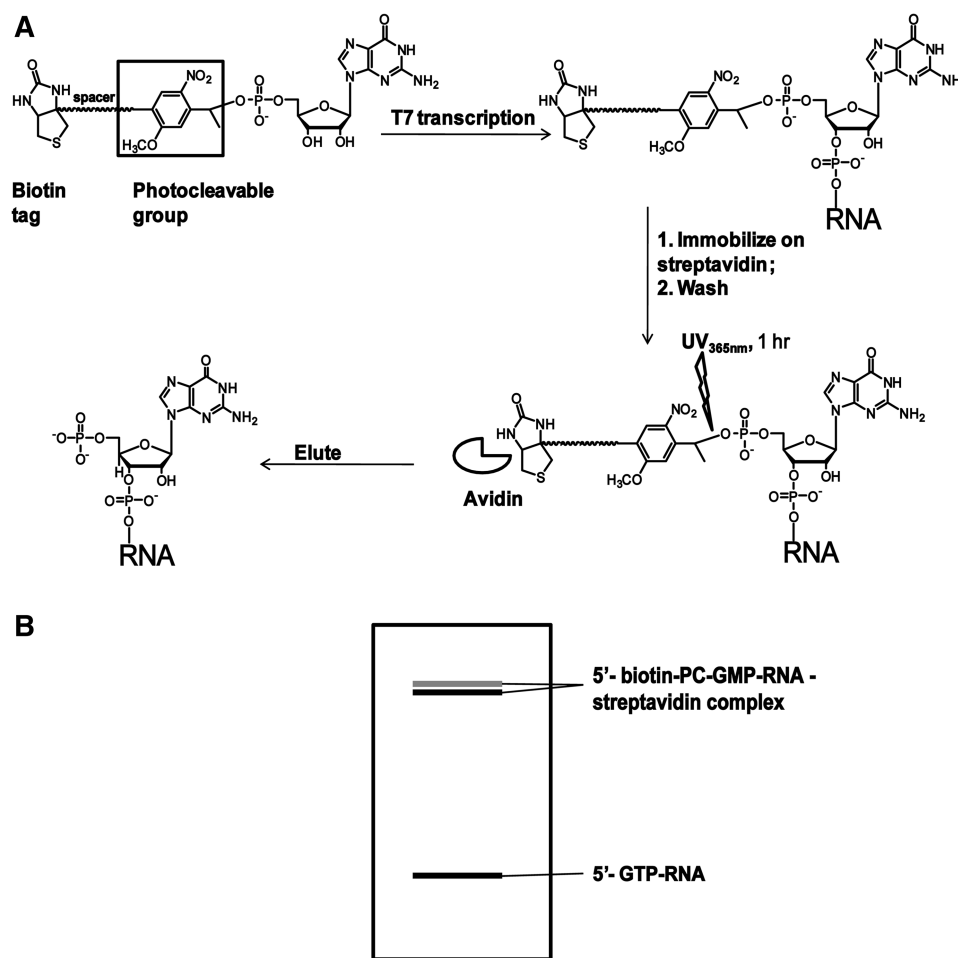
Scheme 1. Preparation of native RNA using biotin-PC GMP as an initiator. (**A**) Schematic procedure for biotin-PC labeling of RNA *in vitro* transcription. (**B**) Expected gel image for analysis of RNA labeling efficiency by PAGE (Two bands for biotin-PC-RNA–streptavidin complex shown are due to multivalent nature of streptavidin, i.e. one streptavidin molecule is able to bind to up to four biotinylated RNA) (41).
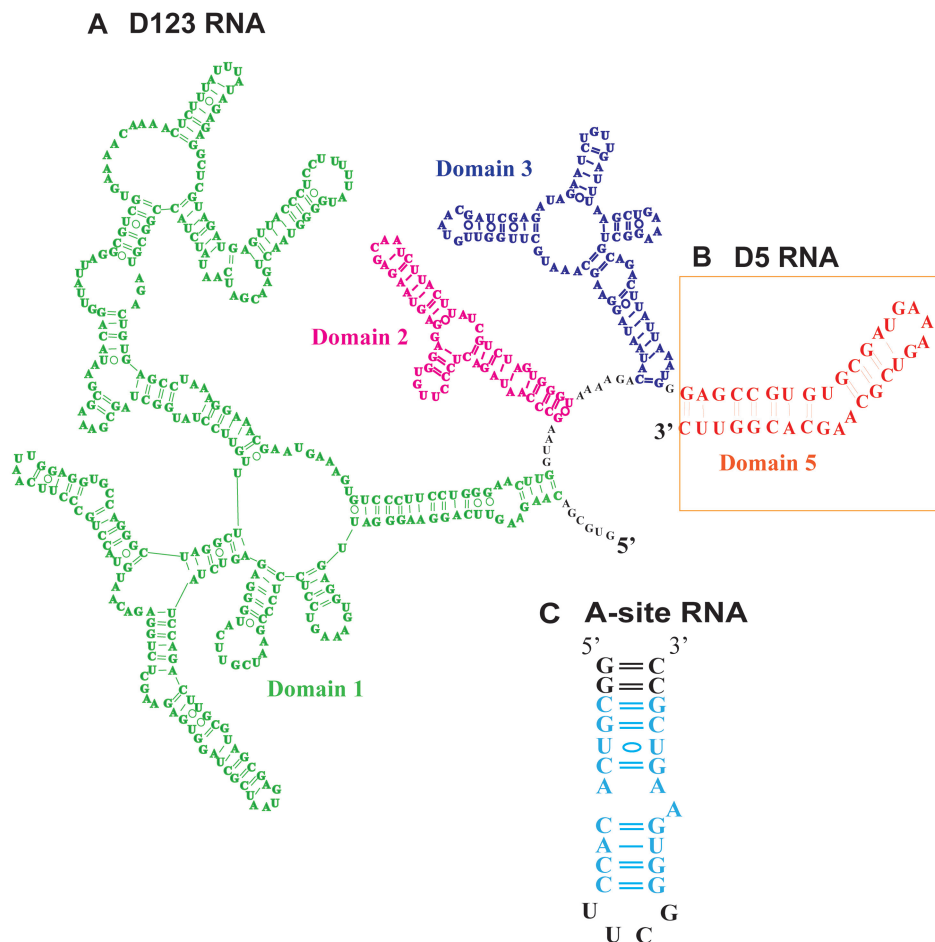
Figure 1. Secondary Structures for the four RNAs studied. (**A**) The 493 nt D123 RNA. (**B**) The 34 nt D5 RNA enclosed in the orange box. The 527 nt D1235 RNA comprises the 493 nt D123 RNA connected in *cis* to the 34 nt D5 RNA. (**C**) The 27 nt A-site RNA.

transcription buffer B [40 mM Tris–HCl (pH 8.1), 1 mM spermidine, 10 mM dithiothreitol (DTT), 0.01% Triton X-100, 80 mg/ml PEG 8000, 0.2 U of RNase inhibitor (New England Biolabs), 0.2 U of inorganic pyrophosphatase (New England Biolabs)] containing 15 mM Mg^{2+}, 300 nM of each DNA strand and 1 μl of 4 mg/ml T7 RNAP per 20 μl of transcription volume. The reactions were incubated for 4 h at 37°C. After 4 h, 0.2 U of Turbo DNase (Ambion) were added and the samples were incubated for another 15 min. The RNA transcripts were immediately analyzed on 12% denaturing PAGE/8 M urea/1× TBE gel and stained with ethidium bromide (EtBr).

Streptavidin–biotin-PC D5-RNA gel shift experiment

After the removal of unused biotin-PC GMP, the purified biotin-PC D5-RNA was incubated with 3-fold molar excess of streptavidin (Promega) (with respect to RNA) at 37°C for 1 h. After incubation, the sample was split into two equal parts, one was irradiated with UV light at 365 nm for 1 h and the other half was left on the benchtop for 1 h. Each set was loaded into a tube containing a 0.5 ml ultra spin filter with a molecular weight cut-off (MWCO)

of 10 kDa (Millipore-Amicon), followed by washing with 500 μl of double-distilled water. This wash step was repeated three times; the flow-throughs were combined, concentrated and analyzed through a 12% denaturing PAGE.

Purification of biotinylated RNA with Neutravidin-agarose resin and photocleavage

A 20 μl transcription mixture was loaded into a tube containing a 0.5 ml ultra spin filter with a MWCO of 3 kDa (Millipore-Amicon), followed by washing with 500 μl of potassium phosphate buffer (0.1 M phosphate, 0.15 M potassium chloride, pH 6.4). This wash step was repeated three times to completely remove unincorporated free biotin-PC GMP from the transcription reaction.

The transcription mixture was added to a suspension of NeutrAvidin Agarose Resin solution (Thermo Scientific, IL, USA) and pre-equilibrated in potassium phosphate buffer (0.1 M phosphate, 0.15 M potassium chloride, pH 6.4). After 1 h incubation, the column was washed with 8–10 column volumes of potassium phosphate buffer to remove all unbound molecules. The bound biotin-PC RNA on the resin was subsequently irradiated with UV light at 365 nm for 1 h. After irradiation, the sample in the

column was eluted with 8–10 column volumes of potassium phosphate buffer. All the fractions were pooled, concentrated and analyzed by UV absorption spectroscopy at 260 nm. The pooled fractions were then separated through 8 M urea denaturing PAGE.

Native D123 (GTP-D123, 493 nt) RNA sample preparation

D123 RNA (35) was transcribed from template DNA produced by PCR amplification from the original plasmid containing domains 1–3 (D1–D3). D123 template was amplified using an upstream primer containing the T7 RNAP promoter sequence and the first 19 nt from the 5′-end of the D1 sequence and a downstream primer containing 19 nt of the 3′-end of the intron domain 3. The RNA was transcribed in transcription buffer B containing 3.33 mM each ATP, CTP and UTP, 2 mM GTP, 8 mM biotin-PC GMP, 24 mM Mg^{2+}, 1.5 μg of gel-purified PCR template and 10 μl of T7 RNAP per 200 μl of reaction volume. After 4 h, 0.2 U of Turbo DNase (Ambion) was added and the samples were incubated for another 15 min. Then the D123 RNA was affinity purified as described above. UV absorbance at 260 nm was used to calculate the concentration based on the extinction coefficients of the individual nucleotides.

Native D1235 (GTP-D1235, 527 nt) RNA sample preparation

D1235 RNA was transcribed from template DNA produced by PCR amplification from the original plasmid containing domains 1, 2, 3 and 5 as described (24). The RNA was transcribed and affinity purified as described above. RNAs produced from PCR transcripts were determined to be identical to those prepared from plasmids and the two methods gave similar RNA yields.

Gel-purified D5 RNA sample preparation

The D5 RNA was synthesized as described above in transcription buffer B containing 10 mM total NTPs and 15 mM Mg^{2+} and was purified by 12% denaturing PAGE. The product band was detected by brief UV shadowing, excised and electro-eluted in an Elutrap electro-separation system (Schleicher and Schuell). The purified D5 RNA was precipitated with three volumes of absolute ethanol and 0.3 M sodium acetate pH 5.2. The RNA pellet was then dissolved in water and dialyzed in a Biodialyzer (Nestgroup) with a 500 Da MWCO membrane (Nestgroup). Each dialysis step was performed for 12 h against dialysis buffer A (100 mM potassium phosphate at pH 6.4, 10 mM EDTA, 0.5 M KCl), B (100 mM potassium phosphate at pH 6.4, 1 mM EDTA, 0.1 M KCl) and C (100 mM potassium phosphate at pH 6.4, 0.1 mM EDTA, 0.1 M M KCl) and finally against two changes of double-distilled H_2O. Note that the denaturing preparation method just described may take about 2–3 days which contrasts with our native preparation method described earlier that is completed 4–6 h after the transcription reaction. After dialysis, the RNA was lyophilized.

Gel-Purified D123 RNA (GTP-D123 RNA) and D1235 RNA (GTP-D1235 RNA) sample preparation

D123 RNA (35) was transcribed from DNA produced by PCR amplification as described above using transcription buffer B containing 15 mM total NTPs and 24 mM Mg^{2+}. Similarly D1235 RNA was transcribed from DNA produced by PCR amplification as described above using transcription buffer B containing 10 mM total NTPs and 18 mM Mg^{2+}. Each resulting RNA was gel purified as described above for D5 RNA, except that a denaturing 6% gel was used.

Anion exchange purified D123 RNA (GTP-D123 RNA) and D1235 RNA (GTP-D1235 RNA) sample preparation

The *in vitro* transcription reaction mixture was incubated with 0.2 U of Turbo DNase at 37°C for 0.5 h. A 1 ml reaction mixture was pelleted to remove inorganic pyrophosphates and the supernatant was loaded into a 1 ml loop of an AKTA FPLC purifier system (GE Healthcare) connected to a 5 ml HiTrap Q column (GE Healthcare). The column was equilibrated with 4 column volumes of buffer A1 (20 mM KH_2PO_4, 25 mM KCl, 100 μM EDTA, pH 6.50). The anion exchange chromatography was performed with buffer A2 (20 mM KH_2PO_4, 2 M KCl, 100 μM EDTA, pH 6.50), generating a gradient by simultaneously decreasing the concentration of buffer A1 and increasing the concentration of buffer A2. The separations were collected as 5 ml fractions with the following gradients of elution: 0–30 ml for injection and loop washing (0% A2), 30–80 ml for washing off rNTPs and proteins (0–17% A2 at 2 ml/min), 80–110 ml for washing off small abortive transcripts (17–27% A2 at 2 ml/min), 110–175 ml for eluting RNA off the column (27–60% A2 at 2 ml/min). The column was further washed with 50 ml of buffer A2 for the next cycle of purification. Fractions were analyzed by 8% PAGE containing 8 M urea.

Labeling E1E2 substrate (22 nt) RNAs with Alexa 647 fluorophore

For the fluorescence studies, Alexa 647 carboxylic acid succinimidyl ester (Invitrogen/Molecular Probes Inc.) was site-specifically incorporated at the 5′-end of E1E2 RNA, which comprises the last 16 nt of the 5′-exon (E1) and the first 6 nt of the 3′-exon (E2), via a primary amine C-6 linker [A647-C6 linker-5′-GAC UGU UUA UUA AAA A↕CAC AGG-3′ (Dharmacon Inc); the cleavage site is indicated by ↕] (35). This labeled RNA is hereafter referred to as A647-E1E2 RNA. The RNA was labeled using 10–15 M excess of the dye in 0.1 M sodium tetraborate (pH 8.5) at room temperature for 12 h with gentle mixing. The resulting A647-E1E2 RNA was purified using a denaturing 15% gel prior to electroelution. The eluted RNA was ethanol precipitated and dissolved in 500 μl of MOPS buffer (40 mM MOPS at pH 7.5, 100 mM KCl) and dialyzed extensively against buffers A, B and C at pH 6.0 as described above and several times in double-distilled H_2O before lyophilizing and dissolving in MOPS storage buffer (40 mM MOPS at pH 7.5, 100 mM KCl).

RNA refolding protocol

Previous work on group II introns indicated the importance of properly folding D123 and D1235 RNAs to obtain efficient catalytic cleavage (24,35) as described above. For each model system, the following RNA refolding protocol was used as described previously (24,35): each component RNA in buffer D (40 mM MOPS-K, pH 7.5 and 100 mM KCl) was heated to 90°C for 1 min, allowed to cool to 42°C for 0.5 h, pre-folded for various times (0, 5, 10, 20, 30, 60, 120 min) in 1 M KCl and 50 mM MgCl$_2$ and then the separately folded RNAs were combined to initiate the SER reaction (24,35). We found that 2 h was necessary for pre-folding and so all subsequent experiments were performed using 2 h of folding. Another set of SER reactions were carried out without this elaborate heat denaturation and refolding protocol. Instead, each component RNA in buffer D was incubated at 42°C for 0.5 h and then all the RNA components were combined in high salt buffer (40 mM MOPS-K, pH 7.5, 1 M KCl and 50 mM MgCl$_2$) to initiate the SER reaction.

Cleavage of A647-E1E2 RNA substrate using gel, anion exchange or affinity-purified D123 RNAs monitored by an in-gel shift experiment

The spliced exon reopening reaction (SER) (35,38) was carried out at 42°C with D123 RNA purified by three different methods: denaturing gel, anion exchange and biotin-PC purified. The reaction used 1.46 μM D123, 2 μM D5 and 16 nM A647-E1E2 RNA in 50 mM MgCl$_2$, 40 mM MOPS-K (pH 7.5) and 1 M KCl. For one set of experiments, GTP-D123, anion exchange purified GTP-D123 and biotin-PC GMP-D123 RNAs were pre-folded using the RNA refolding protocol described above (with heating and cooling) in 50 mM MgCl$_2$ at 42°C for 2 h before adding D5 and substrate RNAs to initiate the SER reaction. For another set of experiments, GTP-D123, anion exchange purified GTP-D123 and biotin-PC GMP-D123 RNAs were not pre-folded (i.e. no heating and cooling), but allowed to incubate at 42°C for 30 min before adding D5 and substrate RNAs to initiate the SER reaction. In either case, each reaction was incubated at 42°C for various times (0, 2, 4, 8, 30, 60, 120, 240 min) and 5 μl of the reaction mixture was aliquoted into a stop buffer (50% glycerol and 100 mM EDTA) and loaded onto a denaturing 12% polyacrylamide gel. The Alexa-Flour 647 RNA band was then quantified using a fluorescence scanner (Molecular dynamics Storm 860) equipped with 635- and 450- nm diode pumped solid-state lasers.

Cleavage of A647-E1E2 RNA substrate using gel, anion exchange or affinity-purified D1235 RNAs monitored by an in-gel shift experiment

The SER reactions (35,38) were carried out at 42°C with gel, anion exchange or biotin-PC-purified D1235 (1.5 μM) using 16 nM A647-E1E2 RNA substrate in buffer D. The A647-E1E2 RNA substrate was heated at 90°C for 2 min separately and incubated at 42°C in a water bath for 30 min (35). Before adding the substrate to initiate the SER reaction, GTP-D1235 and biotin-PC GMP-D1235

RNAs were either pre-folded using the RNA refolding protocol or not refolded as described above for D123. Again the reaction was incubated at 42°C for various times (0, 2, 4, 8, 30, 60, 120, 240 min), 5 μl of the reaction mixture was aliquoted into a stop buffer (50% glycerol and 100 mM EDTA) and loaded onto a denaturing 12% polyacrylamide gel. The Alexa-Flour 647 RNA band was then quantified using a fluorescence scanner as described above. The fraction of the A647-E1E2 RNA substrate cleaved at each time point was quantified and fitted to an exponential function using SigmaPlot 11.0.

D123 RNA analyzed on native gel

The gel purified, anion exchange purified and biotin-PC-purified D123 were analyzed on 6% native PAGE with 0.5× TME (12.5 mM Tris/MOPs, 0.1 mM EDTA) as the running buffer at 4°C. To ensure the accurate determination of the RNA concentrations loaded on the gel, a small amount of sample was completely hydrolyzed in 1.0 M sodium hydroxide at 65°C for 2 h and the UV absorbance was used to calculate the concentrations. To rule out overloading on the gel, two concentrations of RNAs were used: 1 μM and 3 μM for each RNA.

RESULTS AND DISCUSSION

General concept

The traditional method of synthesizing RNAs *in vitro* typically involves denaturation during purification followed by a refolding step. This method implicitly assumes these RNAs are properly folded and that they retain their function. While several methods have been developed recently to purify RNAs without denaturation, very few studies have examined how the manner of RNA preparation affects both the folding and the function of the RNA. To show that the manner of RNA preparation can remarkably affect function such as catalysis, we proceeded to develop a simple strategy to make biotinylated GMP (Supplementary Scheme S1), which can be used to prime RNA synthesis. This strategy also allows for complete removal of the biotin moiety without leaving any non-natural chemical entity appended to the RNA. Importantly, we show for the first time that large complex RNAs that are prepared by denaturing methods, and subsequently refolded, are catalytically less active than those prepared using native methods.

Synthesis of biotin-PC GMP

Rationale. Results from previous studies have indicated that nucleoside monophosphates modified with an affinity label such as biotin can be used to prime RNA synthesis (39–42). Once made, the RNA has to be cleaved from the biotin label. None of these previous methods provide a means to remove the biotin tag without leaving any trace. Therefore, we have devised a facile strategy that allows efficient biotinylation of RNA, provides a traceless means to remove the biotin after the purification and allows preparation of natively folded RNAs.

The synthesis of the biotin-PC GMP 6 is depicted in Supplementary Scheme S1 (See 'Materials and Methods' section in the Supplementary Data for more details). The photosensitive precursor 1 was coupled with propargylamine to produce the PC-alkyne 2. Then compound 2 and protected GMP 3 were reacted to produce an intermediate phosphite and oxidation of the resulting phosphite yielded the phosphate. The PC-alkyne GMP 5 was then coupled to biotin-dPEG^{TM} azide 4 through azide alkyne Huisgen cycloaddition 'click' chemistry using Cu(I) catalyst (25,26), and the final product biotin-PC GMP 6 was obtained in 85% overall yield starting from 5. The polyethylene glycol (PEG) spacer was chosen to increase the water solubility of biotin-PC GMP and also to position the RNA further away from the biotin as the RNA could interfere with the biotin-neutravidin interactions during the immobilization step.

T7 RNAP accepts biotin-PC GMP

Biotin-PC GMP 6 was designed for direct incorporation into RNA by *in vitro* transcription catalyzed by T7 RNAP. To demonstrate that T7 RNAP accepts biotin-PC GMP, we transcribed four different RNAs of varying size: a 27 nt A-site RNA fragment (33) as well as three group II intron-derived RNAs (24,34,35), i.e. a 34 nt D5 RNA, a 493 nt D123 RNA comprising domains 1–3 and a 527 nt D1235 RNA comprising domains 1, 2, 3 and 5 (Figure 1).

Visualization of the reaction product by 12% denaturing PAGE demonstrated that the initiator GMP was incorporated into the 34 nt D5 RNA. Lane 1 (Figure 2A) shows the D5 RNA synthesis without 6 and Lane 2 (Figure 2A) shows the transcription with 4 mM of initiator 6, demonstrating that T7 RNAP is able to initiate transcripts with the photolabile biotinylated nucleotide monophosphate (Figure 2A, Lanes 2) to make the photolabeled D5 RNA. A maximum incorporation of ~17% was achieved using 4 mM of the initiator 6 and 1 mM GTP. Interaction of this photolabeled D5 RNA with the streptavidin column and its photocleavage were evaluated by mixing streptavidin agarose beads with the RNA, separating the beads from solution by filtration and irradiating the beads with UV light at 365 nm. Lane 3 (Figure 2A) demonstrates that the wash from the streptavidin column is identical to 5'-GTP-RNA loaded on the column (Figure 2A, Lane 1). Lane 4 indicates that the 5'-biotin-PC GMP-RNA is retained on the column (cf. Lane 2) and only the unlabeled 5'-GTP-RNA is washed off the column (Figure 2A, Lanes 4). The 5'-biotin-PC GMP-RNA bound to the column (Figure 2A, Lanes 4) was released in the wash following photocleavage (Figure 2A, Lane 6). As expected, 5'-GTP-RNA was not present in the wash following photocleavage (Figure 2A, Lane 5). These results also show that photocleavage does not degrade the RNA (Figure 2A, Lanes 6 and Figure 2B, Lane 4). To rule out any subtle UV damage effects, we compared the FPLC UV chromatogram of a mixture of all four nucleotide triphosphates that were either irradiated with UV light at 365 nm or left on the desktop for 2 h. The FPLC traces for the irradiated and non-irradiated nucleotide mixture are completely superimposable with identical retention times, lineshapes and intensity (Supplementary Figure S2A). As a second test to rule out UV induced cleavage, we compared the mass spectrum of an RNA tetramer that was either irradiated with UV light or left on the desktop for 2 h; no change in the mass of the tetramer was observed (Supplementary Figure S2B and C).

To further ascertain that the D5 RNA was indeed labeled with biotin-PC, the fractions were complexed to streptavidin and analyzed by denaturing PAGE (Figure 2B). Given the rather strong affinity between biotin and avidin complexes, one of the strongest known in nature (K_d of ~10^{-15} M), denaturing PAGE is often used to visualize RNA–biotin–Avidin complexes (39–41). As expected, only the 5'-biotin-PC labeled RNA underwent a gel shift after incubation with streptavidin (Figure 2B, compare lanes 2 and 3). After photocleavage of these streptavidin–biotin-PC GMP D5 RNA complexes (irradiated for 1 h using UV light at 365 nm), the band migrated at almost the same position as the unmodified 5'-GTP-D5 RNA (Figure 2B, Lane 4).

Transcription optimization of ribosomal A-site RNA and D5 RNA using unmodified GTP and biotin-PC GMP

As the modified GMP competes with unmodified GTP for transcription initiation, it is important to optimize the different concentrations of ATP, CTP and UTP, as well as the different ratios of biotin-PC GMP 6 to unmodified GTP in order to increase the transcription efficiency of biotin-PC GMP 6. To quantify the incorporation efficiency of 6 by PAGE, a 27 nt A-site RNA and a 34 nt D5 RNA were used, as we had earlier observed that the electrophoretic mobility of the 5'-biotin-PC-RNA transcripts differs from that of the unmodified 5'-GTP-RNA transcripts of these RNAs. Therefore, we reasoned that it would be straightforward to quantify the amounts of 5'-biotin-PC GMP-RNA and the unlabeled RNA (i.e. 5'-GTP-RNA) using these two RNAs. As the concentration of the biotin-PC GMP was increased, a new band corresponding to the 5'-biotin-PC RNA appeared that exhibited decreased mobility on PAGE. For quantification, the volumes of each of the peak positions on the gel were measured using the Quantity One software (Bio-Rad). Global background correction was applied using similar-sized rectangles for regions devoid of signal. The results of the 27 nt ribosomal A-site transcription priming efficiency trials are shown in Figure 3 and Supplementary Figure S3 and those for the optimization of 34 nt D5 RNA are shown in Figure 4.

The overall yield of transcribed 'biotin-PC primed' A-site RNA was higher (~16 % incorporation yield) at 5 mM total NTP (ATP, CTP and UTP) concentration (Figure 3) than at 3.5 mM (~11 % incorporation yield) or 10 mM (~10% incorporation yield) total [NTP] concentrations (Supplementary Figure S3). At the optimal 5 mM total concentration of NTP for 5'-biotin-PC GMP-RNA incorporation, 28% of the transcribed RNAs were full-length 5'-GTP-RNA, whereas abortive transcripts accounted for ~29% (Supplementary Figure S2C).
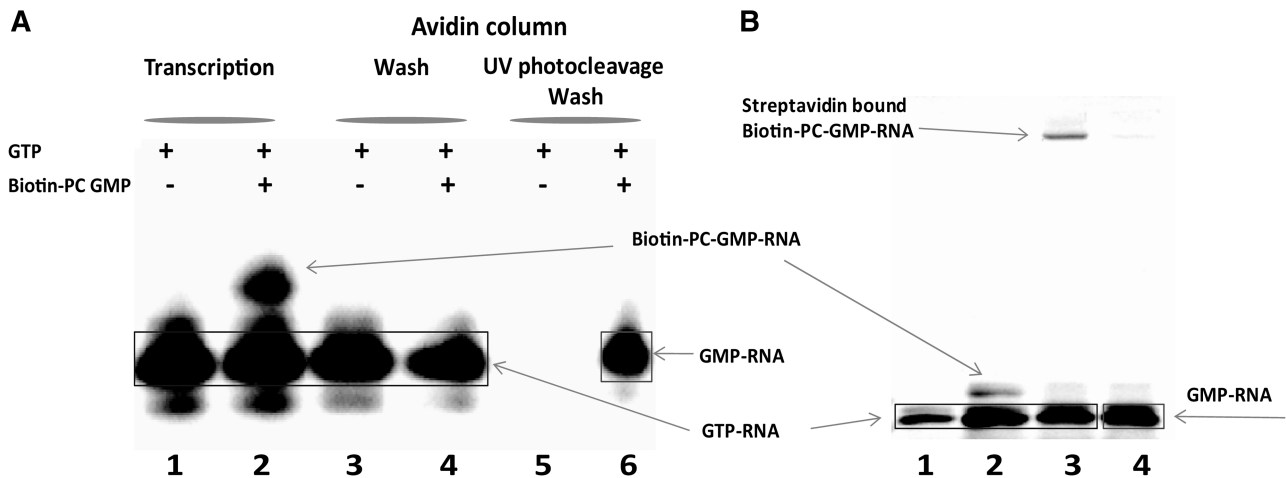
Figure 2. Denaturing PAGE shift assay for biotin-PC-modified D5 RNA. (**A**) Transcription of D5 RNA with (+) or without biotin-PC GMP (−) followed by purification of the D5 RNA (GTP-D5 or biotin-PC GMP-D5 RNA) with an avidin column after photocleavage. Lane 1: Transcription of D5 with unmodified GTP; Lane 2: Transcription of D5 with biotin-PC GMP/GTP mixture; Lane 3: Streptavidin column flow-through of unlabeled GTP-D5 RNA; Lane 4: Streptavidin flow-through of biotin-PC labeled D5 RNA. Lane 5: Elution of the RNA on the streptavidin column following photocleavage of unlabeled D5 RNA. Lane 6: Elution of the RNA on the streptavidin column following photocleavage of biotin-PC labeled D5 RNA. The RNAs are depicted as: GTP transcribed RNA, biotin-PC transcribed RNA. (**B**) RNA biotinylation assay with streptavidin. Lane 1: Unlabeled D5 RNA; Lane 2: Biotin-PC labeled D5 RNA; Lane 3: Biotin-PC labeled D5 RNA incubated with large excess streptavidin (three times number of moles to RNA) for 1 h. Lane 4: GMP-RNA, flow-through using 10 K MWCO spin filter after photocleavage of streptavidin–biotin-PC RNA.
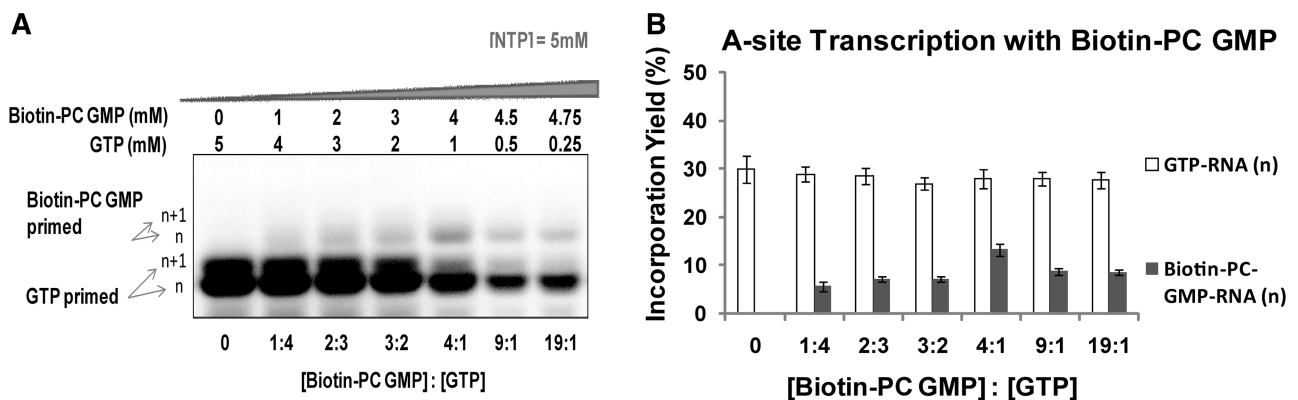

Figure 3. Enzymatic incorporation of the initiator biotin-PC GMP with different ratios of biotin-PC GMP: unmodified GTP using the ribosomal A-site DNA template. (**A**) Dependence of transcription priming of A-site RNA with biotin-PC GMP on the ratios of biotin-PC GMP: GTP in the presence of a total NTP concentration of 5 mM (i.e. 1.67 mM each ATP, CTP and UTP); n and $n+1$ products appeared for transcripts initiated with GTP and biotin-PC analog. (**B**) Relative amounts of GTP- and biotin-PC-primed A-Site RNA products transcribed in the presence of different ratios of biotin-PC GMP: GTP at 1.67 mM each ATP, CTP and UTP. The incorporation yield is the amount of initiated transcripts divided by the total amount of transcripts for each band (see Supplementary Figure S3 for details of incorporation yield for $n+1$ RNA).

Note that the $n+1$ transcripts for the 5′-GTP-RNA and the 5′-biotin-PC GMP-RNA were 20 and 7%, respectively. The efficiency of transcription initiation was also analyzed using the DNA template for 34 nt D5 RNA. Again, as the concentration of biotin-PC GMP was increased, a new band corresponding to 5′-biotin-PC GMP D5 RNA appeared and migrated with decreased mobility on PAGE. A 17% yield (incorporation into the transcripts) of 5′-biotin-PC GMP D5 RNA transcripts was obtained at (biotin-PC GMP): (unmodified GTP) ratio of 4:1 and at 5 mM total NTP concentration (Figure 4). The results for A-site and D5 RNA transcription suggest that it is essential to optimize the NTP concentrations and

to include a 4-fold excess of biotin-PC GMP over unmodified GTP in order to maximize analog incorporation.

Purification of biotin labeled RNA with affinity avidin column and photocleavage

To utilize the biotin-PC RNA in functional or biophysical studies, the tag needs to be removed. To prevent the free biotin-PC GMP from competing with the biotin-labeled RNA for the Neutravidin-agarose resin binding, the unincorporated free biotin-PC GMP was removed from the mixture using 3 kDa MWCO spin filters (For details, see 'Materials and Methods' section). The 5′-tagged RNA was
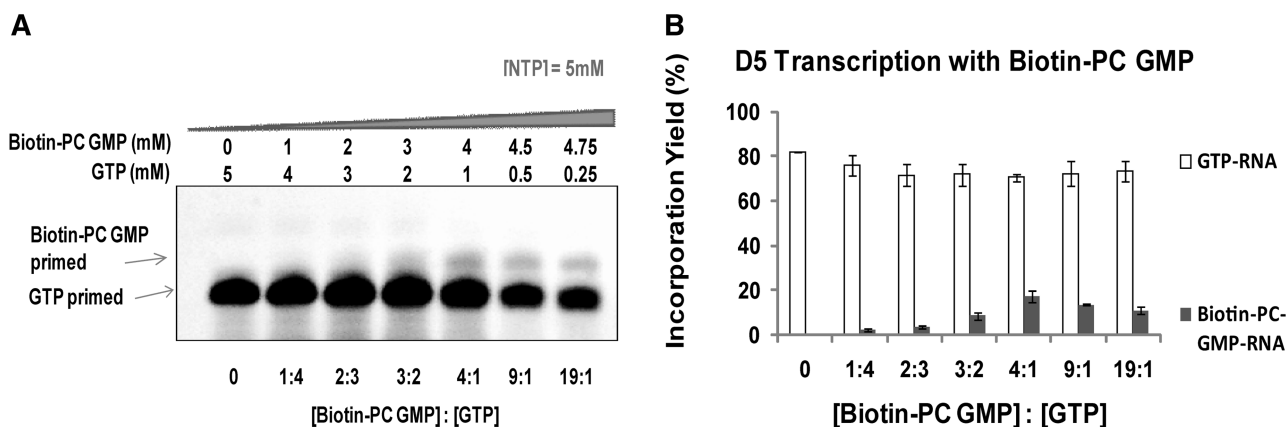
A

B

Figure 4. Enzymatic incorporation of the initiator biotin-PC GMP with different ratios of biotin-PC GMP: unmodified GTP using the D5 template. (**A**) Dependence of transcription priming of D5 RNA with biotin-PC GMP on the ratio of biotin-PC GMP: GTP in the presence of 1.67 mM each ATP, CTP and UTP; (**B**) Relative amounts of GTP- and biotin-PC-primed D5 RNA products transcribed in the presence of different ratios of biotin-PC GMP : GTP at 1.67 mM each ATP, CTP and UTP. The incorporation yield is the amount of initiated transcripts divided by the total amount of transcripts for each band.

then immobilized on the Neutravidin-agarose resin and the unbound RNA (primarily unlabeled 5′-GTP-RNA) was washed off with phosphate buffer. The RNA, bound to the resin, was then irradiated with UV light at 365 nm for 1 h to liberate the bound RNA (Figure 2A, Lane 6).

Biotin-PC synthesized minimal group II intron ribozyme has enhanced catalytic activity compared to gel-purified GTP-RNA in the SER reaction

While a number of methods have been proposed to synthesize RNA under native conditions, it remains unclear how native RNA preparations affect function. To test the hypothesis that natively prepared RNA are more catalytically active than RNA prepared using denaturing methods that require heat-cooling refolding cycles, two variants of the minimal group II intron ribozyme splicing model system from *Pylaiella littoralis* (PL) were employed (24,35). The first variant is a three-piece ribozyme system comprising D123, D5 and a substrate reflecting the product of exon ligation (A647-E1E2 RNA, 22 nt) (35). In contrast, the second variant is a two piece ribozyme system comprising D1235 and the same substrate used with the three-piece system (24). The bipartite model is expected to be more catalytically active than the tripartite model because D5 is connected *in cis* rather than added *in trans*. For each model system, denatured GTP-RNA, anion-exchanged purified RNA and 'natively' prepared biotin-PC GMP-RNA were used in the spliced exon reopening reaction (24,35,38) to test catalytic cleavage of the A647-E1E2 RNA (24,35).

The effect of heat denaturation and refolding on the D123 RNA to cleave the A647-E1E2 RNA substrate in the presence of D5 RNA were tested first (Figure 5A). In the absence of heat annealing, the natively prepared biotin-PC GMP-D123 RNA was able to catalyze cleavage of the A647-E1E2 RNA substrate to near completion in ~30 min (Figure 5B). Similarly, natively prepared GTP-D123 RNA purified using anion exchange was able to

catalyze near complete cleavage of the substrate in ~30 min (Figure 5C), again underscoring the importance of native RNA preparation. In contrast, gel-purified GTP-D123 RNA required at least 180 min for complete cleavage (Figure 5D and H). These results suggest that the method of the RNA preparation makes a dramatic difference in D123 RNA ribozyme catalytic function; whereas cleavage by natively purified RNA was detectable within 2 min and was complete in <60 min, substantial cleavage by RNA purified by denaturing PAGE was not observed before 30 min and only reached completion after ~240 min (Figure 5H).

After heat annealing and refolding, the natively prepared biotin-PC GMP-D123 RNA cleaved the substrate in <60 min (Figure 5E), slightly more slowly than without the heat annealing procedure (Figure 5B and H). Similarly natively prepared GTP-D123 RNA purified using anion exchange cleaved the RNA substrate in ~60 min (Figure 5F). Here, again the gel-purified GTP-D123 RNA required at least 2 h for complete cleavage (Figure 5G and I). In this case, cleavage began at ~8 min instead of 30 min. These findings suggest that under conditions typically used in making folded RNAs, even heat annealing does not completely rescue the catalytic activity of the D123 RNA ribozyme. As shown below, folding is incomplete for the gel-purified RNA.

The effect of heat denaturation and refolding on the D1235 RNA to cleave the A647-E1E2 RNA substrate was employed as a second example of an RNA-mediated catalysis. For this second model system, the catalytic co-factor D5 RNA was connected *in cis* and thus it was anticipated that this ribozyme would be catalytically more active and more robust in response to heat denaturation and refolding than D123. As expected, in the absence of heat annealing and refolding, the natively prepared biotin-PC GMP-D1235 RNA was able to catalyze complete cleavage of the substrate in almost 30 min (Figure 6A). As observed for D123 RNA, natively prepared GTP-D123 RNA purified using anion exchange completely
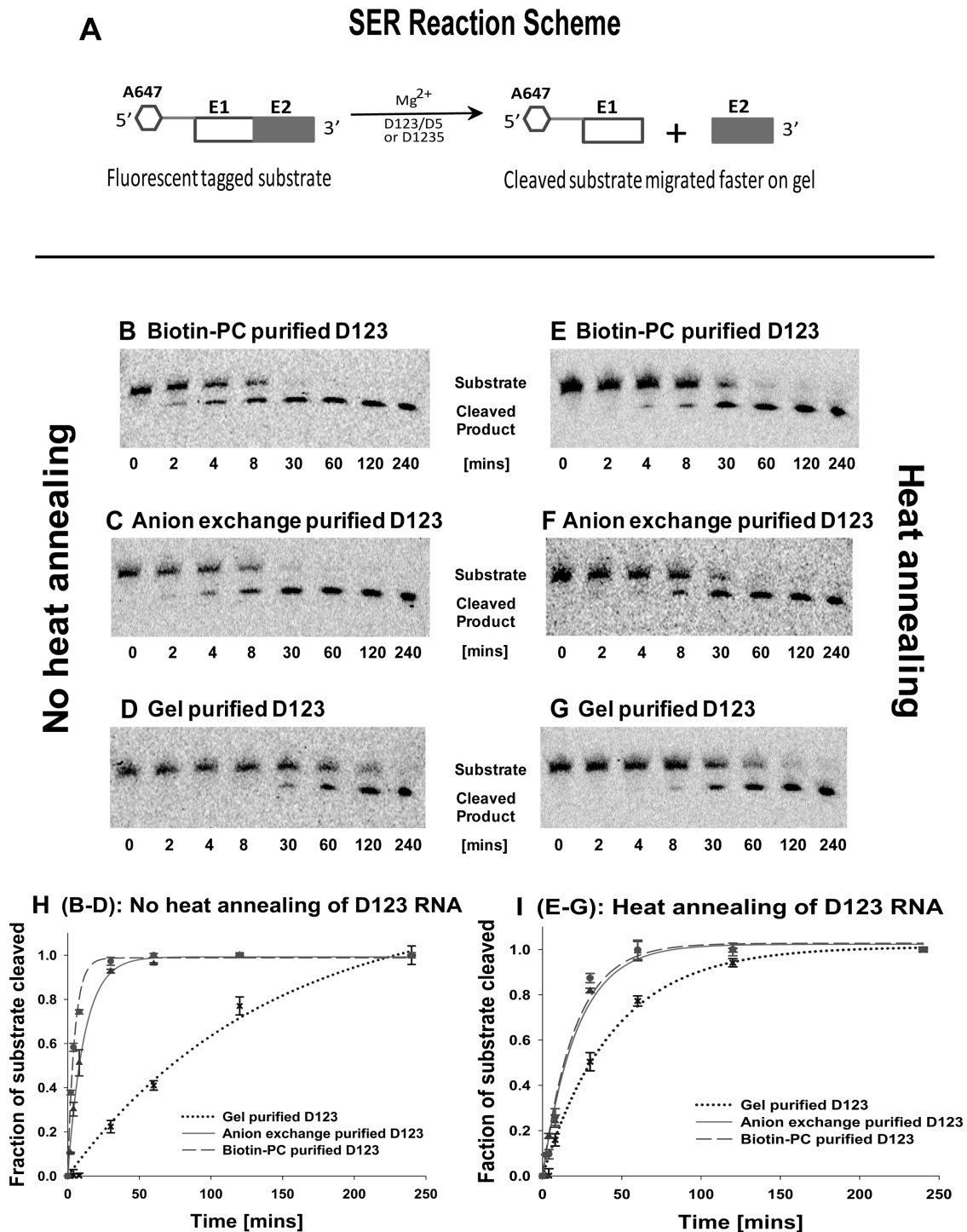
Figure 5. (**A**) In-gel fluorescent assay for catalytic cleavage of a 22 nt spliced exon substrate (A647-E1E2 RNA) by a minimal group II intron ribozyme comprising domains 1–3 (D123) and domain 5 (D5) or a ribozyme comprising domains 1–3 and 5 (D1235) in the presence of 50 mM Mg^{2+} at 42°C (SER reaction, for details, see 'Materials and Methods' section); (**B–D**) Comparison of the catalytic activity of gel purified, anion exchange purified and photocleaved biotin-PC-purified D123 RNA under conditions of no heat annealing (native); (**E–G**) Comparison of the catalytic activity of gel purified, anion exchange purified and photocleaved biotin-PC-purified D123 RNA under conditions of heat annealing (refolding). (**H**) Exponential fit to the buildup of cleaved substrate product under conditions of no heat annealing using nonlinear least square minimization in SigmaPlot 11.0. (**I**) Exponential fit to the buildup of cleaved product under conditions of heat annealing. Error bars are based on standard deviation of 3 experiment repeats.

cleaved the substrate in ~1 h (Figure 6B). Once again, the gel-purified GTP-D1235 RNA that had been heat denatured and refolded for 2 h now required ~4 h for complete cleavage (Figure 6F). When the heating step was omitted, the endpoint of the reaction was reached at a comparable time period of ~4 h, but unlike the gel-purified GTP-D123 RNA the onset of the reaction was only slightly retarded (Figure 5D, G, H, I versus Figure 6C, F, G, H). Altogether, these results suggest that conditions that are traditionally used in folding RNAs may not be as effective as envisioned especially in rescuing the catalytic activity of ribozymes. As shown below, folding is also incomplete for the gel-purified RNA.

The population of biotin-PC synthesized minimal group II intron ribozyme conformers is more homogeneous than that of gel-purified GTP-RNA

Using the native preparation method, the question of how different RNA preparation methods affect folding was examined. To test the hypothesis that natively prepared RNA should be more conformationally homogeneous than RNA prepared using denaturing methods that require heat-cooling refolding cycles, samples of D123 RNA prepared by urea PAGE (denatured and heat annealed as described earlier), anion-exchanged purified D123 RNA and 'natively' prepared biotin-PC GMP-D123 RNA were analyzed by non-denaturing PAGE. Strikingly, the denaturing preparation had a significant
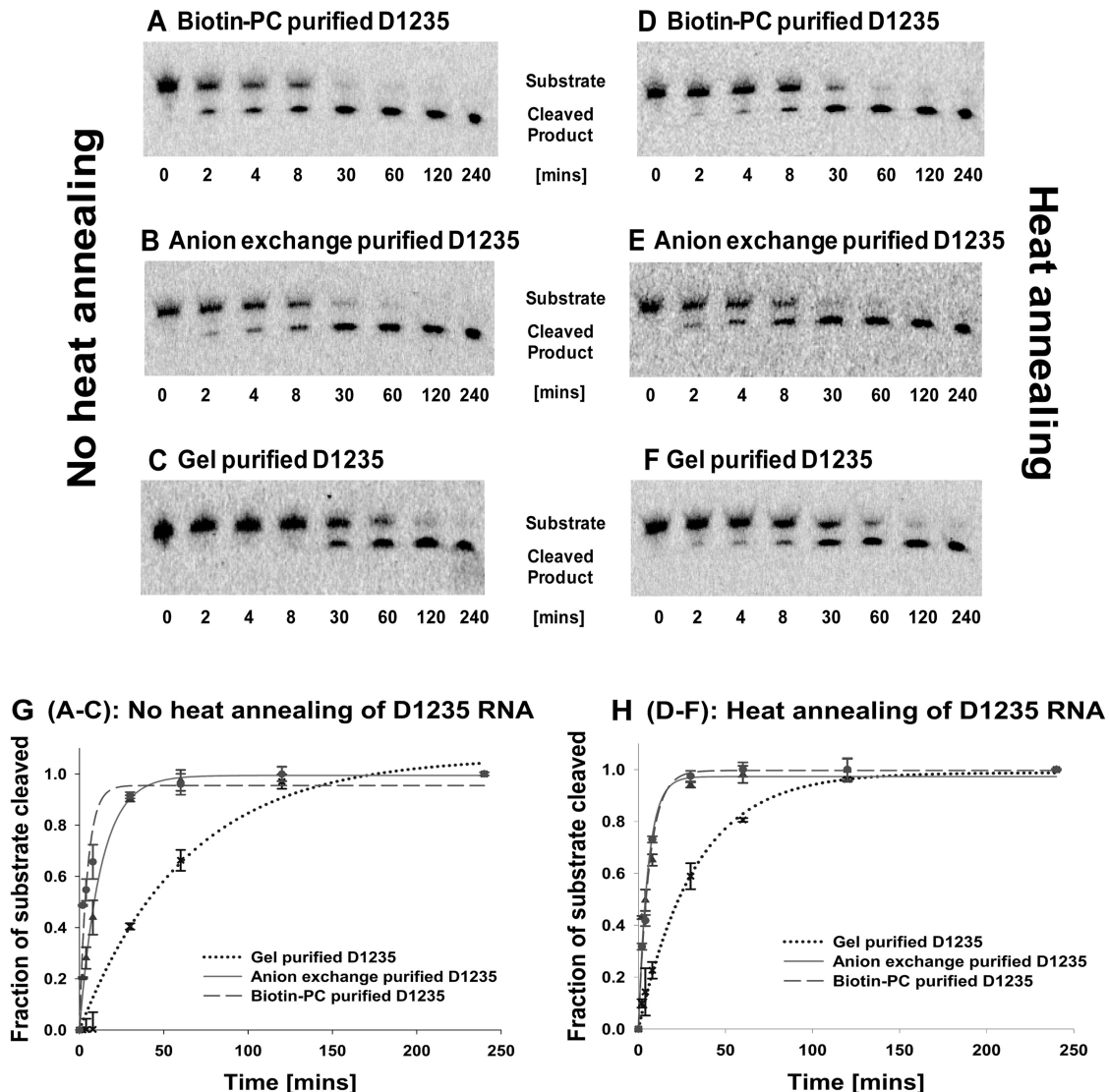

Figure 6. (**A–C**) Comparison of the catalytic activity of gel purified, anion exchange purified and photocleaved biotin-PC-purified D1235 RNA under native conditions of no heat annealing. (**D–F**) Comparison of the catalytic activity of gel purified, anion exchange purified and photocleaved biotin-PC-purified D1235 RNA under denaturing conditions of heat annealing and refolding. (**G**) Exponential fit to the buildup of cleaved substrate product under conditions of no heat annealing using nonlinear least square minimization in SigmaPlot 11.0. (**H**) Exponential fit to the buildup of cleaved product under conditions of heat annealing. Again error bars are based on standard deviation of 3 experimental repeats.

impact on the migration pattern of the gel-purified D123 RNA: even after refolding, >86% of the RNA was in alternative conformations (Figure 7). In contrast, ~93% of the 'natively' prepared biotin-PC GMP-RNA, which had not been denatured, was in a homogeneous conformation (Figure 7). Similarly ~85% of natively prepared GTP-D123 RNA purified using anion exchange was in homogeneous conformation with ~15% of the population in alternative slower migrating conformations (Figure 7). To rule out overloading of the gel-purified D123 RNA, the experiment shown in Figure 7 was executed on 6% native PAGE using two different dilutions of all three RNAs tested. The concentrations of the RNAs were determined by UV absorbance measurements of RNAs hydrolyzed using NaOH to remove any hyperchromicity effects. As can be seen in Figure 7, the gel-purified D123 RNA was consistently smeared, while the native preparations consistently produce yields of >90% as a single band.

Altogether, these results suggest that although the D123 or D1235 RNAs purified by denaturing PAGE conditions were heat annealed under conditions typically used in making folded RNAs, their catalytic activities were not completely rescued and most of the RNA existed in alternative conformations. These findings are in agreement with previous observations that RNA can be kinetically trapped in misfolded states which are not easy to efficiently correct, even by heat annealing (11,20,43).

CONCLUSION

Experimental evidence is presented demonstrating that RNA enzymes not only fold differently but also have compromised catalytic activities depending on whether they were prepared using denaturing or non-denaturing purification methods. Although, the RNA community has believed that large RNAs can become trapped into what has been described as the 'alternative conformer hell' (11,43), no systematic investigations to reveal the

true extent of the problem have been heretofore performed. Standard methods for RNA preparation, while robust and well-established, can create not only heterogeneous populations of conformers (11,20), but also produce RNAs that may become marginally active. The goal of this work was to develop a photolabile biotinylated nucleotide monophosphate strategy to facilitate native RNA preparation and to address the question of how the nature of the preparation can affect RNA function. This designed and synthesized biotin-PC GMP was shown to be utilized by T7 RNAP as an initiator nucleotide to produce RNA labeled at the 5′-end with biotin-PC.

This photoremovable affinity tag confers several desirable advantages for use in numerous RNA biophysical applications. First, the ability to prepare 'native' RNA without having to refold should minimize misfolding and thereby preserve the functionality of these RNAs. As proof of concept, we have shown that the two ribozymes prepared by our new method retain greater catalytic activity than when they were prepared by denaturing methods and have to be refolded. To determine if other native preparation methods would also give enzymatically more proficient RNAs, anion exchange was used to purify the RNAs under native conditions. As anticipated, RNAs purified via the native anion exchange method were almost as active as those prepared via the biotin-PC GMP method. The biotin-PC GMP native preparation is therefore a nice complementary method to other native RNA preparation methods such as anion exchange FPLC and there is no panacea for preparing native RNAs. Importantly, this biotin-PC GMP method does not require expensive instrumentation and is amenable to high throughput native RNA preparation. Our results are also comparable to those reported recently which showed quite unambiguously that heating and subsequent cooling of a 164 nt Varkud satellite (VS) ribozyme led to ~70% slowly migrating non-native RNA folds, whereas a VS ribozyme purified under nondenaturing conditions appeared to be ~93% fast migrating single band representing the native
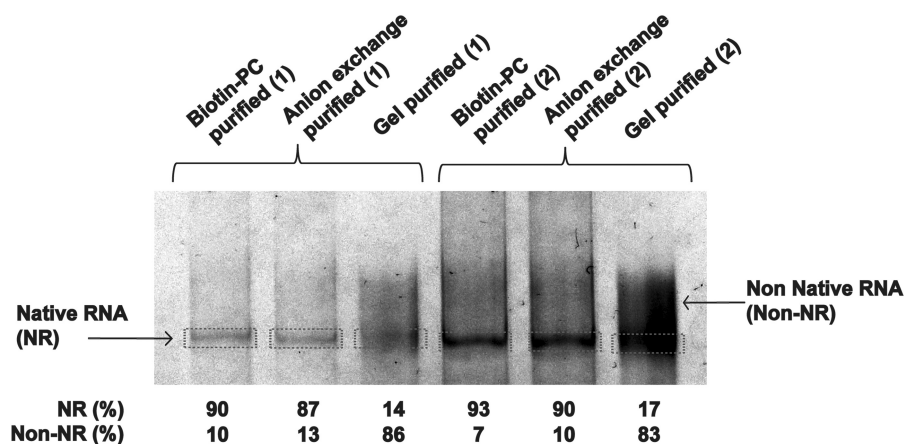

	Biotin-PC purified (1)	Anion exchange purified (1)	Gel purified (1)	Biotin-PC purified (2)	Anion exchange purified (2)	Gel purified (2)
NR (%)	90	87	14	93	90	17
Non-NR (%)	10	13	86	7	10	83

Figure 7. Comparison of gel purified, anion exchange purified and biotin-PC-purified D123 on the 6% native PAGE gel with 0.5 × TME (12.5 mM Tris/MOPs, 0.1 mM EDTA) as the running buffer at 4°C. The boxed bands represent the native RNAs and the region of smearing represents the non-native RNAs with heterogeneous conformations. To rule out overloading the gel-purified RNA, a small amount of all three RNAs were hydrolyzed in 1.0 M NaOH and the concentration calculated using the measured UV absorbance. Two concentrations of RNAs were loaded on the native PAGE gel: (2) was three times more than (1), and (1) is ~250 ng.

RNA fold. These observations are consistent with the expectation that heat annealing cannot adequately guide RNAs across their rugged free energy folding landscape toward the native states (20). Rather native purification methods are increasingly vital to perform meaningful RNA research in general and specifically in *in vitro* RNA folding and structural RNA studies. Our work argues for caution in interpretation of kinetic data involving RNA prepared using heat-denaturation/refolding protocols. It also provides an important framework for future studies regarding RNA catalysis and recognition.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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