3′-End labeling of nucleic acids by a polymerase ribozyme

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ABSTRACT

A polymerase ribozyme can be used to label the 3′ end of RNA or DNA molecules by incorporating a variety of functionalized nucleotide analogs. Guided by a complementary template, the ribozyme adds a single nucleotide that may contain a fluorophore, biotin, azide or alkyne moiety, thus enabling the detection and/or capture of selectively labeled materials. Employing a variety of commercially available nucleotide analogs, efficient labeling was demonstrated for model RNAs and DNAs, human microRNAs and natural tRNA.

INTRODUCTION

A common procedure in nucleic acids research is the installation of a functional group, for example bearing a fluorescent reporter, onto a target RNA or DNA molecule. This is often accomplished by enzymatic modification of the target molecule using a kinase, ligase or polymerase enzyme. Several RNA and DNA enzymes have been explored for this purpose. For example, Müller and co-workers used a twin ribozyme with concurrent RNA cleavage and ligation activity to exchange a short patch of a target RNA with a 20-nt RNA containing an amino-modified deoxythymidine residue, which subsequently could be derivatized with a probe [1]. Baum and Silverman used a deoxyribozyme with RNA ligase activity to attach a 19-nt RNA, decorated with either a fluorophore or biotin, to an internal adenosine of the target RNA [2]. Subsequently, Höbartner and co-workers improved this method to allow the installation of a single modified guanosine nucleotide [3]. Heemstra and co-workers isolated a self-alkylating ribozyme that can react with fluorescein iodoacetamide to label a fusion molecule containing both the ribozyme and a target RNA [4]. Liu and co-workers identified a small catalytic RNA from a pool of genome-encoded RNAs that also has self-alkylating activity and can react with substituted epoxides to label a fusion RNA containing both the ribozyme and target [5].

Surprisingly, despite being one of the best studied classes of nucleic acid enzymes, polymerase ribozymes have been largely unexplored for their application in labeling target nucleic acids. Polymerase proteins are often used for this purpose, although special precautions must be taken if the goal is to add only a single modified nucleotide [6]. Holliger and co-workers, when investigating the chemical fidelity of an evolved form of the class I polymerase ribozyme, tested a variety of modified forms of uridine 5′-triphosphate (UTP) in the RNA-templated extension of an RNA primer. They found that the polymerase could accept many different nucleotide analogs, including 2′-azido-UTP, which would seem promising for RNA labeling [7]. Thus far, however, there has been no clear effort to apply a polymerase ribozyme as a general tool for the selective labeling of nucleic acids. This study reports the efficient 3′-end labeling of either RNA or DNA by a polymerase ribozyme, employing 50 different commercially available nucleoside 5′-triphosphate (NTP) analogs, including all four nucleobases.

RNA polymerase ribozymes are *in vitro* evolved RNA molecules that extend an RNA primer on a complementary RNA template using NTP substrates. Since the first reported polymerase ribozyme [8], several research groups have worked to improve both the efficiency and sequence generality of RNA-catalyzed polymerization [9–13]. Currently, the most advanced RNA polymerase ribozyme is the '24-3' polymerase, which has an extension rate of 1.2 nt/min and can operate on most template sequences [13]. Using specially designed templates, the polymerase can generally be limited to the addition of only a single modified nucleotide, thus enabling efficient 3′-end labeling of a target RNA or DNA using various commercially available NTP (and dNTP) analogs. This approach provides a broadly applicable tool for the labeling of target nucleic acids.

MATERIALS AND METHODS

Material

The polymerase ribozyme and all RNA templates were prepared by *in vitro* transcription, as described previously [14]. Target DNAs were purchased from IDT. Most target RNAs were prepared by solid-phase synthesis employing phosphoramidites and other reagents from Glen Research (Sterling, VA, USA), with the exception of yeast tRNA[Phe], which was purchased from Sigma-Aldrich (St. Louis, MO, USA). All modified NTPs and dNTPs were purchased from either

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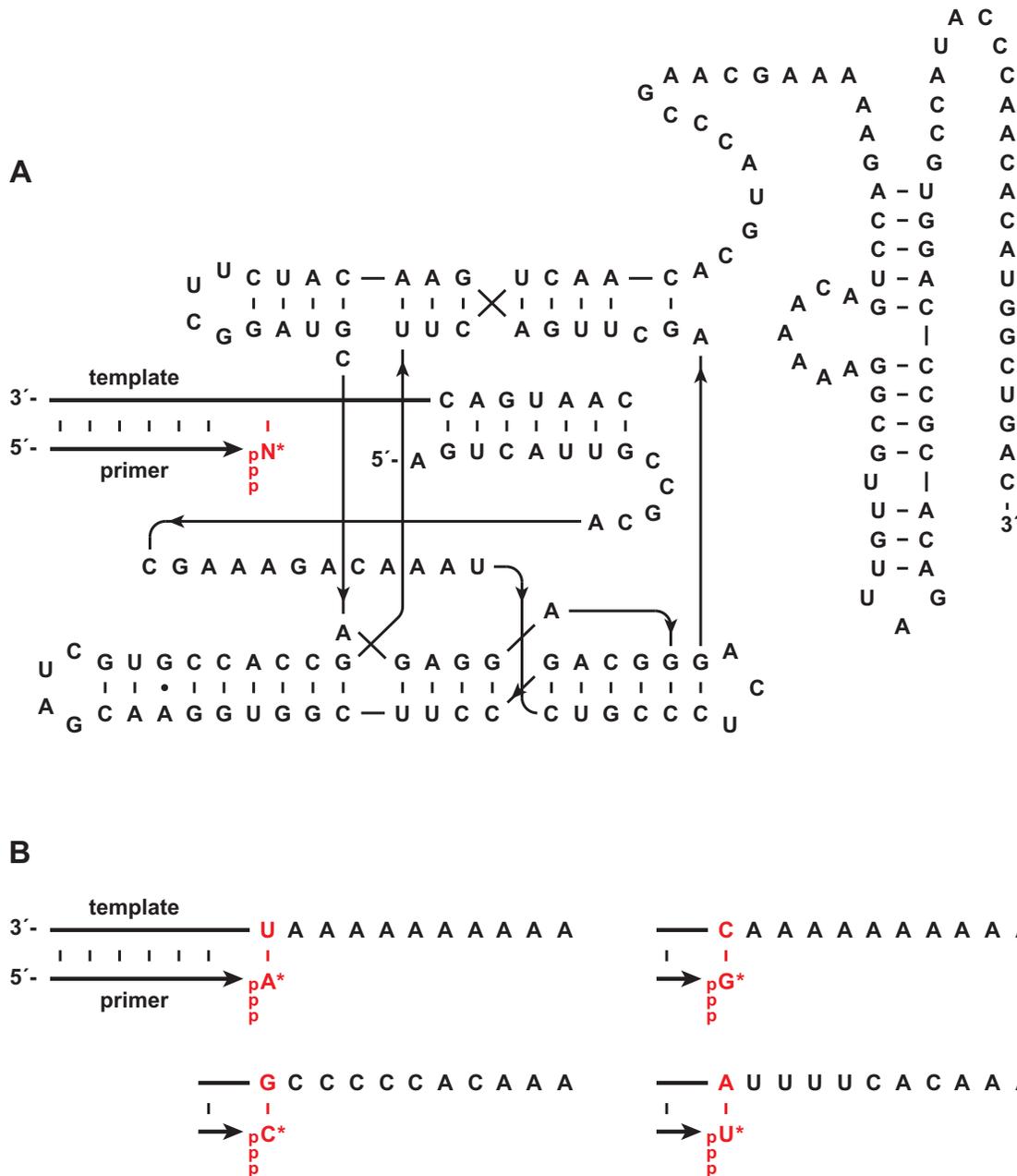
Figure 1. 3′-End labeling of nucleic acids by the 24-3 polymerase ribozyme. (**A**) Sequence and secondary structure of the ribozyme, bound to a template RNA that also binds the target nucleic acid and directs incorporation of a single NTP analog (red). (**B**) Four different template RNAs were prepared, each with a different templating nucleotide (red), followed by several non-complementary nucleotides.

Jena Bioscience (Jena, Germany) or TriLink BioTechnologies (San Diego, CA, USA). Streptavidin C1 Dynabeads were purchased from ThermoFisher (Grand Island, NY, USA).

3′-End labeling reaction

Unless otherwise stated, the reaction mixture contained 1 μM polymerase ribozyme, 1 μM RNA template and 0.8 μM target RNA (or DNA), which were first heated at 90°C for 2 min, then cooled to 17°C over 5 min. The reaction then was initiated by adding 200 mM MgCl$_2$, 50 mM Tris (pH 8.3), 0.05% TWEEN20, and 0.5 mM NTP analog. Following incubation at 19°C for 1 h, the reaction was quenched by adding ethylenediaminetetraacetic acid (EDTA) in 1.25-fold excess over Mg^{2+}. For analytic purposes, the target RNA was modified at its 5′ end with both 6-carboxyfluorescein and biotin. Unreacted and labeled target RNAs were captured on streptavidin C1 Dynabeads, washed twice with alkali (25 mM NaOH, 1 mM EDTA and 0.05% TWEEN20) and once with TE-urea (10 mM Tris (pH 8.0), 1 mM EDTA, 0.05% TWEEN20 and 8 M urea), then eluted with 98% formamide and 10 mM EDTA (pH 8.0) at 95°C for 15 min. The reaction products were analyzed

Table 1. Incorporation of functionalized nucleotides by the 24-3 polymerase ribozyme

NTP analog	Source	Yield %	NTP analog	Source	Yield %
2′-azido-dATP	T	90	2′-azido-dGTP (**3**)	T	91[a]
8-azido-ATP	J	55[a]	2′-amino-dGTP	T	70[a]
2′-amino-dATP	T	85	7-propargylamino-dGTP (**4**)	T	89[a] ± 1.3
N6-methyl-2-amino-ATP	T	49[a]	thieno-GTP	T	50[a]
biotin-11-dATP (**8**)	J	57[a]	α-thiol-dGTP	T	86[a]
α-thiol-ATP	T	70	7-deaza-GTP	T	83[a]
2-aminopurine-riboside-TP	J	81[a]	1-borano-dGTP	T	37[a]
2′-azido-dCTP (**5**)	T	86 ± 1.4	ganciclovir-TP	T	73
5-azido-PEG$_4$-CTP	J	86	2′-azido-dUTP	T	55
5-azido-PEG$_4$-dCTP	J	82	azide-PEG$_4$-aminoallyl-dUTP	J	32
C8-alkyne-dCTP (**9**)	J	85	C8-alkyne-dUTP	J	19
5-DBCO-PEG$_4$-CTP	J	73[a]	5-ethynyl-UTP (**1**)	J	54 ± 2.0
5-DBCO-PEG$_4$-dCTP	J	58[a]	5-ethynyl-dUTP	J	61
2′-amino-dCTP	T	80[a]	5-DBCO-PEG$_4$-dUTP	J	28[a]
5-aminoallyl-CTP	T	67[a]	2′-amino-dUTP	T	50
biotin-16-propargylamino-dCTP	J	81	5-aminoallyl-UTP	J	54[a]
Cy5-aminoallyl-CTP (**7^T**)	T	47[c]	biotin-16-aminoallyl-dUTP	T	28
5-propargylamino-CTP-Cy5 (**7**)	J	55[c]	fluorescein-12-UTP (**2**)	J	50[c]
5-propargylamino-CTP-TAMRA	J	42[c]	aminoallyl-UTP-Cy5	J	26[b]
5-propargylamino-dCTP-TXRed	J	71[b]	thieno-UTP	T	25[b]
5-propargylamino-dCTP-Rhod (**6**)	J	88[c]	α-thiol-UTP	T	11
thieno-CTP	T	78[b]	2-thio-UTP	T	68
α-thiol-CTP	T	84	4-thio-UTP	T	36
5-formyl-CTP	T	85[a]	5-formyl-UTP	T	58
1-borano-dCTP	T	12	pseudo-UTP	T	66

Chemical structures of numbered compounds (**1**–**9**) are shown in Figures 2 and 3. The source of compounds was either Jena Bioscience (J) or TriLink BioTechnologies (T). Yield is the percentage of RNA primer extended by incorporation of the modified NTP in 1 h. Reactions were performed in quintuplicate for compounds **1**, **4** and **5**, reporting the mean and standard deviation.
[a] Multiple product bands were observed, but yield reflects only the single-addition product.
[b] Because both the primer and modified nucleotide are fluorescent, yield was calculated based on the amount of primer that was not extended.
[c] Multiple product bands were observed and the modified nucleotide is fluorescent; thus yield reflects only the single-addition product, but with the caveat that overlap of fluorescence excitation/emission for the two fluorophores may alter the apparent yield.

by denaturing polyacrylamide gel electrophoresis (PAGE). When labeling with biotinylated nucleotides or when natural tRNA was the target, the quenched reaction mixture was ethanol precipitated and analyzed directly by PAGE. Some labeled RNAs were purified by PAGE and analyzed by electrospray ionization LC/MS, performed by Novatia LLC (Newtown, PA, USA) on the Oligo HTCS platform.

RESULTS

The 24-3 polymerase ribozyme is a highly evolved form of the class I polymerase ribozyme that exhibits substantially less sequence preference compared to earlier forms of this enzyme (13). Employing either NTP or dNTP substrates, it is able to extend either an RNA or DNA primer on an RNA template to generate complementary RNA or DNA products (14). The polymerase contains 180 nt (Figure 1A; for the sequences of all nucleic acids used in this study see Supplementary Table S1). There is a short tag sequence at the 5′ end of the polymerase that is complementary to a sequence at the 5′ end of the template. Otherwise the template sequence is not constrained. The primer, which corresponds to the target nucleic acid, binds to the template through Watson–Crick pairing and is extended by the polymerase to achieve 3′-end labeling.

Although the polymerase ribozyme can add multiple successive NTPs to the 3′ end of a template-bound primer, the reaction can mostly be restricted to the addition of a single NTP by choosing an appropriate template and providing only one of the four nucleobase substrates. Four templates were constructed, each with a different templating nucleotide at the first position of primer extension, followed by several non-complementary nucleotides (Figure 1B). Self-complementary and oligo(G) sequences were avoided within the non-complementary region to prevent formation of secondary structure. Together this set of templates enabled the testing of NTP analogs containing each of the four nucleobases.

Although a great variety of functionalized nucleotides can be prepared by chemical synthesis, this study focused on commercially available NTP analogs, including sugar, nucleobase and backbone modifications, to demonstrate the utility of the approach for general users. Fifty different analogs were tested in a reaction employing 0.8 μM RNA primer (target RNA) having the sequence 5′-UUGCUACUACACGAC-3′, together with 1 μM ribozyme and 1 μM RNA template. The reactions were carried out in the presence of 200 mM MgCl$_2$ and 0.5 mM NTP analog at pH 8.3 and 17°C for 1 h, then the yields of the single-nucleotide extension products were determined by PAGE. Yields averaged 61%, with >80% yield for 16 of the analogs (Table 1). The yields tended to be lower with UTP analogs, reflecting the known propensity of the polymerase to be somewhat less efficient in incorporating UTP compared to the other three NTPs, attributed in part to the lower template occupancy of UTP (13).

Among the compounds tested were a variety of fluorophores and affinity probes, including fluorescein-labeled UTP, Cy5-labeled CTP, rhodamine-labeled dCTP, 5-ethynyl-UTP, 2′-azido-dCTP, 2′-azido-dGTP, 7-deaza,7-propargylamino-dGTP and biotin-modified dATP (Figure 2). This suite of compounds provides broad opportunities for the facile 3′-end labeling of RNA. The incorporation of an alkyne, azide or propargylamino moiety enables sub-
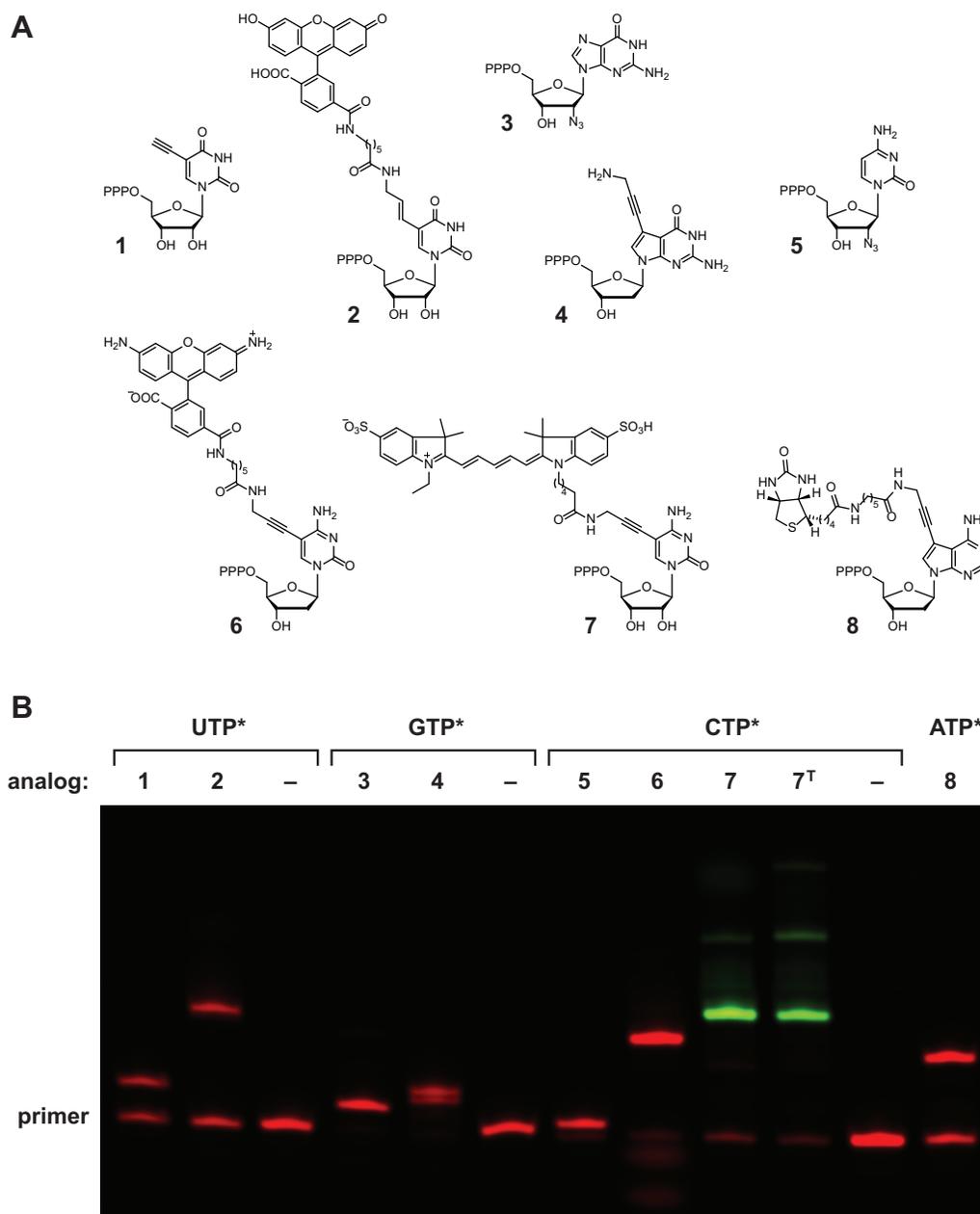
Figure 2. Addition of various NTP analogs to a target RNA. (**A**) Chemical structures of 5-ethynyl-UTP (**1**), fluorescein-12-UTP (**2**), 2′-azido-dGTP (**3**), 7-deaza,7-propargylamino-dGTP (**4**), 2′-azido-dCTP (**5**), 5-propargylamino-dCTP-rhodamine (**6**), 5-propargylamino-CTP-Cy5 (**7**) and biotin-11-dATP (**8**). Each of these NTP analogs was added to the 3′ end of a template-bound RNA primer having the sequence 5′-UUGCUACUACACGAC-3′. The template was chosen based on the nucleobase component of the NTP analog (Figure 1B). (**B**) Analysis of the reaction products by 20% PAGE. The primer was 5′-labeled with fluorescein (red). The concentration of NTP analog was 0.5 mM for compounds **1**, **3**, **4** and **5** and 0.25 mM for compounds **2**, **6**, **7** and **8**. The addition of compound **4** or **5** results in a very slight mobility shift, requiring the gel to be run for longer times to obtain the data shown in Table 1. The additional lower-mobility band observed with compound **7** (green) was likely due to an impurity. This compound was obtained from both Jena Bioscience (**7**, with 5-propargylamino linker) and TriLink Biotechnologies (**7ᵀ**, with 5-aminoallyl linker).

sequent chemical modification with a variety of commercially available reagents. Notably, a 'clickable' group, either an alkyne or azide, can be installed for each of the four nucleobases (Table 1). One can also install a variety of post-transcriptional modifications, including pseudouridine, 5-formyl uracil and 5-formyl cytosine, as well as fluorescent isomorphic nucleotide analogs derived from thieno[3,4-*d*]pyrimidine (15). The 3′-labeled products containing 5-ethynyl-U, 7-deaza,7-propargylamino-dG and 2′-azido-dC were analyzed by electrospray ionization LC/MS to confirm the identity of these materials (see examples in Supplementary Figure S1).

Employing a DNA rather than RNA primer generally resulted in lower yield (Supplementary Table S2). This may be partially due to the lower stability of the RNA–DNA heteroduplex, which might be compensated by increasing the
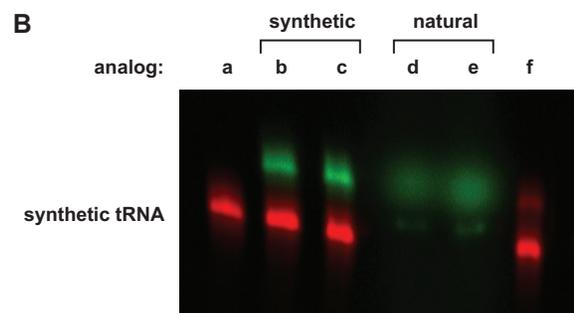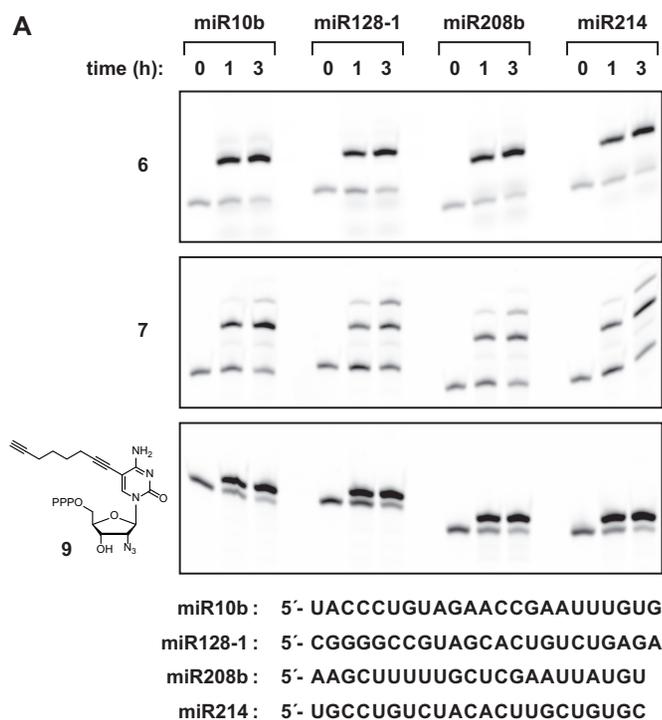
Figure 3. 3′-End labeling of biological RNAs. (**A**) Four different microRNAs were used as the primer for labeling by 0.1 mM 5-propargylamino-dCTP-rhodamine-12 (**6**), 0.05 mM 5-propargylamino-CTP-Cy5 (**7**) or 0.5 mM C8-alkyne-dCTP (**9**). The microRNAs were 5′-labeled with fluorescein. The reactions were sampled at 1 and 3.5 h and the products were analyzed by 15% PAGE. See Supplementary Table S1 for sequences of templates. (**B**) Both synthetic (lanes b and c) and natural (lanes d and e) forms of yeast tRNA^{Phe} were labeled with 0.1 mM 5-propargylamino-CTP-Cy5 (**7**) in a 1-h reaction. Prior to the reaction, synthetic tRNA was 5′-labeled with fluorescein (red) and natural tRNA was unlabeled. Upon reaction with **7**, both were 3′-labeled with Cy5 (green). Natural tRNA is from a biological source and was not purified prior to analysis, resulting in a more diffuse product band. Two different templates were used, forming either 15 (lanes b and d) or 20 (lanes c and e) base pairs with the 3′ end of the tRNA (for sequences see Supplementary Table S1). Lane a shows the reaction with synthetic tRNA and no NTP; lane f shows the reaction with synthetic tRNA and 0.1 mM 5-propargylamino-dCTP-rhodamine-12 (**6**).

stability of the primer-template interaction, but may also reflect the intrinsically weaker interaction between the ribozyme and a template-bound DNA primer (16). No concerted effort was made to optimize the yield of the individual 3′-end labeling reactions for either RNA or DNA. Instead a generic set of conditions was chosen to con-

serve the use of NTP analogs, which are often expensive, and to provide a common starting point for optimization depending on the particular application. For three of the analogs, however, the RNA-primed reaction was carried out in quadruplicate using either 0.5 or 2 mM NTP analog, with a reaction time of either 1 or 16 h (Supplementary Figure S2). The addition of either 2′-azido-dCTP or 7-propargylamino-dGTP is already efficient under the standard conditions and the yield increased only slightly when either the substrate concentration or the reaction time was increased. In contrast, the addition of 5-ethynyl-UTP occurs with a yield of only $54 \pm 2.0\%$ under the standard conditions, and this was increased to $69 \pm 0.8\%$ using 2 mM substrate for 1 h and to $91 \pm 0.1\%$ using 2 mM substrate for 16 h. It is likely that the incorporation of other NTP analogs could be enhanced by optimizing these and other aspects of the reaction conditions.

The identity of the 3′-terminal nucleotide of the primer did not have a significant effect on the yield of the reaction, as tested with both standard and 2′-azido-modified NTPs (Supplementary Figure S3). For some NTP analogs there was a small amount of double-nucleotide addition, despite the presence of a non-complementary nucleotide at the second template position. In those cases the yield was calculated based on only the single-addition product (Table 1) because it is this material, following purification, that will be of interest to most users.

To explore the diversity of target RNAs that can be labeled using the polymerase ribozyme, various human microRNAs were used as the primer for the templated addition of Cy5-labeled CTP, rhodamine-labeled dCTP or alkyne-modified dCTP. MicroRNAs miR10b, miR128-1, miR208b and miR214 were prepared by chemical synthesis and tested under the reaction conditions as above. All were efficiently labeled with each of the three modified NTPs (Figure 3A and Supplementary Table S3). Note that each of these four microRNAs has a different 3′-terminal nucleotide. Lastly, an unmodified RNA corresponding in sequence to yeast phenylalanyl tRNA was prepared by chemical synthesis and natural yeast tRNA^{Phe} was obtained from a commercial source. Both were efficiently labeled using Cy5-labeled CTP (Figure 3B).

DISCUSSION

A highly efficient polymerase ribozyme can be used to install a wide variety of functionalized nucleotide analogs onto the 3′ end of a target nucleic acid molecule. The target nucleic acid acts as a primer that is bound to a complementary RNA template and is extended by addition of an NTP that carries the desired modification. The active site of the polymerase is highly tolerant of modified NTPs, including those that bear a fluorescent moiety, alkyne or azide modification, or pendant amino or biotin group, or that contain a natural or unnatural modification of the sugar, nucleobase or phosphate backbone. The simple, one-step installation of a fluorophore or affinity probe is likely to have the broadest application, offering an attractive alternative to 3′-end labeling using a polymerase protein such as poly(A) polymerase (6) or terminal transferase (17). These polymerase proteins operate in the template-independent manner, and

thus result in multiple successive additions, unless the NTP analog itself is a chain terminator.

The polymerase ribozyme is readily prepared by *in vitro* transcription and, in principle, could be expressed in cells to bring about *in situ* labeling of a target RNA. The latter would require cellular delivery of the NTP analog, which would raise additional challenges. Many functionally modified NTPs are commercially available and others can be prepared by a simple coupling reaction starting from commercially available materials. Some optimization may be necessary to achieve high yields with certain NTP analogs, especially those based on UTP. The conditions of pH and temperature are already optimized for ribozyme function, and thus are unlikely to provide an opportunity for enhancing yields. In any case, even the standard conditions will provide 3′-labeled products in sufficient yield for most practical purposes. In conclusion, the ribozyme-based 3′-end labeling strategy provides a new approach for installing specific modifications within a target nucleic acid molecule in a template-directed manner. With the opportunity for secondary modifications, a broad range of labeling reactions are accessible.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

FUNDING

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