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Debranchase-resistant labeling of RNA using the 10DM24 deoxyribozyme and fluorescent modified nucleotides

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Abstract

The 10DM24 deoxyribozyme can site-specifically label RNAs with fluorophore-GTP conjugates; however, the 2',5'-branched RNA linkage is readily cleaved by debranchase. To prevent loss of labels upon cleavage, we synthesized phosphorothioate-modified, fluorescent GTP derivatives and elaborated conditions for their incorporation by 10DM24. RNAs labeled with fluorescent derivatives of *Sp*-GTP^S were found to be resistant to debranchase.

Graphical Abstract

Despite the centrality of both coding and non-coding RNAs to life, few options exist for covalently labeling transcripts with fluorophores or other chemical tags.^{1–4} Deoxyribozymes (DNA catalysts) represent an attractive solution for chemically-modifying RNAs at specific locations that are not limited to the 3' - or 5' -termini or to specific sequences.^{5, 6} Exceptional labeling specificity can be achieved by base-pairing between the deoxyribozyme and the RNA target, the deoxyribozyme itself can be easily and cheaply made by solid-phase

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Conflicts of interest

There are no conflicts of interest to declare.

Electronic Supplementary Information (ESI) available: Fig S1–S4, Tables S1–S2, synthetic details and characterization of fluorescent GTP^S diastereomers, HPLC, ESI-MS, details of Dbr1 expression, See DOI: 10.1039/x0xx00000x

oligonucleotide synthesis, and DNA is chemically stable over a wide range of conditions. It has previously been shown that the 10DM24 deoxyribozyme can use GTP to site-specifically guanylate RNAs by creating a branched, 2',5'-phosphodiester linkage (Fig. 1A).^{6, 7} 10DM24 catalyzes this reaction rapidly, particularly in the presence of Mg^{2+} and Tb^{3+} , and it accepts 2' or 3' modified GTP molecules as substrates.⁶ Together these properties make 10DM24 an excellent tool for site-specific installation of fluorophores or other chemical groups onto RNA transcripts.

The 2',5'-phosphodiester linkage created by 10DM24 between the RNA and GTP derivative is the same as that created by the spliceosome during eukaryotic precursor mRNA splicing.⁸ The spliceosome removes introns by formation of RNA lariats containing these branched linkages. Accumulation of intron lariats in cells is prevented by debranching enzymes (*e.g.*, debranchase) that hydrolyze the 2',5'-phosphodiester to create linear, 5'-phosphorylated RNAs with a free 2'-hydroxy group at the branchpoint (Fig. 1B).⁹ The linearized introns can then rapidly be degraded.⁹ For using fluorescently labeled RNAs in bulk or single-molecule spectroscopic experiments, it is necessary that the covalent linkage generated by 10DM24 remains intact throughout the course of the investigation. We therefore investigated if RNAs labeled with fluorescent derivatives of GTP by 10DM24 would be stable towards debranching enzymes, or if the fluorophore-labeled single-nucleotide branch is recognized as substrate by debranchase (Fig. 1C).

We used 10DM24 to site-specifically incorporate a Cy3-labeled derivative of GTP into a 209-nt long, [³²P]-radiolabeled *UBC4* pre-mRNA transcript and then incubated the dual-labeled RNA with purified *Saccharomyces cerevisiae* debranchase (Dbr1). Fluorescence and phosphor imaging of the RNAs after Dbr1 incubation revealed loss of Cy3 fluorescence even though intact RNA was still detectable (Fig. 2A). Quantification of Cy3 fluorescence intensity and normalization to the amount of [³²P]-labeled RNA present in the sample indicated that debranching and loss of the GMP-fluorophore from the RNA occurred rapidly with a half-life of ~8 min (Fig. 2B). From this finding it became evident that further modification of the DNA-catalyzed labeling strategy is required for generating RNAs that can be used in experiments taking place in cellular extracts or inside cells including studies of pre-mRNA splicing.

It has previously been reported that lariats or Y-shaped RNAs containing guanosine or cytosine branchpoints are less susceptible to debranching.^{10–12} So, we next tested whether or not Dbr1 cleavage could be blocked simply by changing the nucleotide to which the GMP-fluorophore was attached. We prepared four 16-nt long, [³²P]-labeled RNAs that differed only by the identity of the branchpoint nucleotide and used 10DM24 to install GMP at each of these positions (sequences are in Table S1, ESI). The synthesis of 2',5'-branched RNAs at branch-sites other than adenosine had been reported using 10DM24 with 5'-triphosphorylated oligonucleotide substrates,¹³ but the 10–100-fold slower reaction rates prevented efficient installation of a fluorescent single-nucleotide branch. Taking advantage of the recent findings that lanthanide ions accelerate DNA-catalyzed 2',5'-phosphodiester bond formation,^{14, 15} and that branch-sites flanked by purine nucleotides are preferred substrates for 10DM24,⁶ GMP-branched RNAs with all four different branch sites were prepared in good yields using a combination of Mg^{2+} and Tb^{3+} . However, even in the

optimal sequence context and reaction conditions, the single nucleotide 2',5'-linkage at branchpoint adenosines was generated ~280-fold faster than at branchpoint cytidines (Fig. S1, ESI). Incubation of the branched RNAs with debranchase revealed that yeast Dbr1 rapidly cleaved the 2',5' linkage and released GMP in each case with the preference A>G>U>C (Fig. 2C, D). Thus, changing the branchpoint identity alone is not sufficient for preventing cleavage of fluorophore-modified RNAs. Moreover, the adenosine preference for yeast Dbr1 catalysis is identical to that for 10DM24, which means that while labeling of RNAs with fluorophores at cytidine will result in slower cleavage by debranchase, this may be accompanied by poor labeling by the deoxyribozyme itself. Consequently, we sought to develop an alternative labeling strategy using modified nucleotide substrates that provided resistance to debranchase.

Like many phosphodiesterases, yeast Dbr1 is a metal-ion dependent enzyme that is unable to hydrolyze phosphorothioate linkages of a particular configuration (*Rp* in the case of Dbr1).^{16, 17} Therefore, we reasoned that if 10DM24 could incorporate fluorescently modified GMP moieties with the proper phosphorothioate stereochemistry, then the resulting labeled RNAs should be stable to cellular debranching activities. We synthesized α -S-*Sp*- and α -S-*Rp*-diastereomers of GTP (also known as *Sp*-GTP^S and *Rp*-GTP^S, respectively) and resolved the two diastereomers by RP-HPLC according to known procedures.¹⁸ Simultaneously, we prepared 2'-*O*-propargyl-substituted derivatives of *Sp*- and *Rp*-GTP^S (Fig. S2, ESI) that were in turn converted into fluorescent GTP^S derivatives by Cu(I)-catalyzed 1,3-dipolar cycloaddition with fluorophore azides.¹⁹

We used 10DM24 to label RNA oligonucleotides with either *Sp*- or *Rp*-GTP^S, which resulted in an inversion of the phosphorothioate configuration (Fig. 3A). In comparison with GTP, the reaction rate for both diastereomers was very low, but the *Sp*-GTP^S diastereomer reacted more quickly than corresponding *Rp*-GTP^S diastereomer (Fig. 3B), resulting in phosphorothioate interference effects (also known as thio effects), represented as k_O/k_S , of ca. 400 for *Sp*-GTP^S and ca 10000 for *Rp*-GTP^S. In analogy to ribozyme catalysis, we reasoned that sulfur substitution could be interfering with metal ion coordination by the 10DM24/RNA/GTP^S ternary complex and that the addition of thiophilic metals such as Cd²⁺ or Mn²⁺ could rescue 10DM24 activity.^{20–23} Indeed, addition of 1 to 5 mM Cd²⁺ greatly increased the rate and yield of 10DM24 labeling. Both *Sp*-GTP^S and *Rp*-GTP^S reacted 10–50-fold faster with 5 mM Cd²⁺ than in its absence, but the rescue effect decreased again at 10 mM Cd²⁺ (Fig. 3C, S3, Table S2, ESI). The observed thio effect and its partial rescue with Cd²⁺ suggest that the α -phosphate of GTP is coordinating a metal ion within the active site of 10DM24 and that there is a preferred geometry for this interaction that promotes catalysis. Using the optimized reaction conditions, we used 10DM24 to label RNAs with *Rp*- or *Sp*-2',5'-linked GMP or alkynyl- and Cy5-GMP moieties (Fig. 3B, D). The rates of RNA labeling were similar for each *Sp*-GTP^S and *Rp*-GTP^S derivative, with all *Sp* derivatives reacting faster than their *Rp* counterparts (Fig. 3C, D). Even for RNA substrates with a suboptimal sequence context around the labeling site (R5, ESI), reactions with the *Sp*-GTP^S derivatives neared completion after 2h at 37°C, approximately 10-fold slower than when RNAs are labeled with GTP under similar conditions.⁶

We next incubated RNAs containing either a 2',5'-linked GMP, *Sp*-GMP^S, or *Rp*-GMP^S with yeast Dbr1. Dbr1 was able to debranch RNAs labeled with GMP and *Sp*-GMP^S but the *Rp*-GMP^S-labeled RNA was much more resistant to cleavage. The *Rp* phosphorothioate linkage resulted in an 8-fold increase in resistance to debranching (~96 vs. 12% debranched for *Sp*-P^S and *Rp*-P^S, respectively (Fig. 4A). This result is consistent with prior studies using chemically synthesized phosphorothioate-branched RNA.¹⁶ Finally, we used 10DM24 to prepare RNAs with debranchase-resistant fluorophores. We labeled RNAs with both *Rp* and *Sp* diastereomers of GMP^S linked to either the Cy5 or fluorescein (FAM) fluorophores (Fig. S4, ESI). RNAs containing the *Rp*-linked fluorophores were also resistant to debranching (Fig. 4B), showing again only 8–12% debranched product after 1 hour incubation with debranchase, while the fluorophores were largely released from both the *Sp*-GMP^S-modified RNAs. Under our optimized conditions, 10DM24 is thus able to label RNAs with stereospecific, 2',5' linked GMP-fluorophores that are not enzymatically debranched by Dbr1.

In conclusion, we have developed a system for site-specifically installing debranchase-resistant fluorophores into RNAs using a DNA catalyst and modified NTPs. We expect this approach to facilitate labeling and use of modified RNAs in complex environments such as unfractionated cellular extracts or *in vivo*. Further optimization of 10DM24 or the reaction conditions towards increased reactivity with *Sp*-GTP^S may allow the deoxyribozyme to use this nucleotide as efficiently as GTP or to selectively synthesize the *Rp* diastereomer of the labeled RNA from a diastereomeric nucleotide triphosphate mixture, eliminating the need for chromatographic separation of the diastereomers. Such developments could facilitate both fluorophore labeling of large RNAs as well as preparation of the necessary GTP^S substrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Overview of RNA labeling by the 10DM24 deoxyribozyme and Dbr1 debranching. (A) Reaction scheme depicting 2' OH guanylation and RNA labeling by 10DM24. RNAs can be labeled by use of fluorescent or other derivatives of GTP. (B) Debranchase (Dbr1) cleaves the 2',5'-linkage found in lariat introns. (C) Dbr1 debranching of RNAs labeled by 10DM24 with the same type of 2',5'-linkage as in lariat RNAs could result in release of the label.

Figure 2. Debranching of 10DM24 modified RNAs by Dbr1. (A) UBC4 pre-mRNA labeled by 10DM24 with Cy3-GTP is debranched and the Cy3 signal is lost in the presence of Dbr1. Shown are fluorescence (Cy3) and phosphor imaging (^{32}P) results from the same gel. (B) Quantification of the data shown in panel A. (C) Alternative branchsite nucleotides (BS A, G, U, or C) do not prevent Dbr1 debranching. Shown are phosphor imaging results using 5'- ^{32}P -labeled RNAs and in each subpanel the top bands represent RNA-GMP and bottom bands the debranched RNA. (D) Quantification of the data shown in panel C.

Figure 3.

Optimization of 10DM24 ligation of GTP^S diastereomers. (A) Scheme depicting inversion of the phosphorothioate stereochemical configuration upon 10DM24-catalyzed 2' OH guanylation. (B) Gel images showing labeling reactions of 5'-fluorescein-modified RNA with GTP^S or GTP^S derivatives. In each subpanel the top bands represent tagged RNA-GMP^S (or the corresponding GMP^S derivative) and bottom bands the unlabeled RNA. (C) Rates and yields of 10DM24-catalyzed RNA labeling with *Sp*- or *Rp*-GTP^S in the presence of Cd²⁺; a. Cd²⁺ concentration in mM, b. *k*_{obs}*10⁻³ min⁻¹, and c. yield after 7 h in %. (D) Quantification of RNA labeling results from panel B using *Sp*-GTP^S or its derivatives. Curve fits are for pseudo-first order reaction with $Y = Y_{\max}[1 - e^{(-k_{\text{obs}}t)}]$.

Figure 4.

Labeling of RNAs with debranchase-resistant fluorophores by 10DM24. (A) RNAs labeled by 10DM24 with a *Rp*-phosphorothioate linkage resist Dbr1 cleavage while those labeled with a *Sp*-phosphorothioate linkage do not. Shown are phosphor imaging results using 5'-[³²P]-labeled RNAs. (B) RNA labeling with fluorophores stable to debranchase using 10DM24. 10DM24 was used to prepare RNAs containing the indicated dye fluorophores with either *Rp* or *Sp* linkages. RNAs containing the *Rp* linkage remained fluorescent in the presence of debranchase. Shown are phosphor imaging results using 5'-[³²P]-labeled RNAs (top) and superimposed fluorescence scans of the same gel taken at different wavelengths (bottom). Debranching reactions were run for 1h at 23°C. (C) Mnemonic for debranchase-resistant RNA labeled with fluorescent guanyl phosphorothioates.