RNA Modifications

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Deoxyribozyme-Catalyzed Labeling of RNA**

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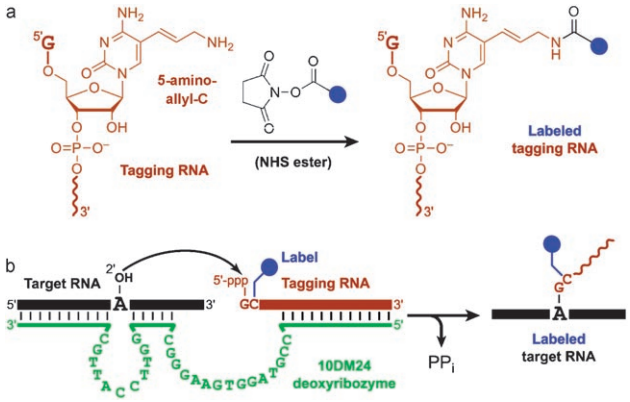
Site-specific covalent modification of RNA is important for enabling structure–function studies. For example, probes such as fluorescein are commonly used in fluorescence resonant energy transfer (FRET) investigations of RNA folding.[1] Biotin is used for immobilization during single-molecule analysis,[1] to enable RNA–protein cross-linking studies,[2] and as a key element of selection schemes in vitro.[3] The 5′ and 3′ termini of RNA may be derivatized,[4] but many experiments instead demand internal modification, and no direct methods are known for site-specific modification within an arbitrary RNA sequence. Therefore, covalent modifications are typically introduced by enzymatic splint ligation[5] in which a DNA template aligns oligoribonucleotide substrates that have modified nucleotides incorporated through solid-phase synthesis.[2,6] However, this approach often suffers from low yields and is unpredictable because identifying a high-yielding ligation site in the target RNA can be difficult without directly testing several possibilities. Non-natural nucleotides have recently been used to transcribe modified RNA.[7] Although this avoids the difficulties of splint ligation, extensive organic synthesis is required. As an alternative approach to RNA labeling, noncovalent Watson–Crick hybridization of a probe-labeled oligonucleotide has been used.[8] However, this is invasive because long stretches of nucleotides must be inserted within the RNA, and duplex formation involving these inserted nucleotides must be tolerated. These limitations have led us to develop a general deoxyribozyme-based strategy for site-specific RNA modification.

Deoxyribozymes are catalytic DNA molecules identified by in vitro selection,[9] and our laboratory has reported several deoxyribozymes that ligate two RNA substrates.[10] Herein, we have applied the 10DM24 deoxyribozyme[11] in a new approach for site-specific internal RNA modification that we term deoxyribozyme-catalyzed labeling (DECAL). A single 5-aminoallylcytidine nucleotide is incorporated at the second position of a short "tagging RNA" by in vitro transcription (see the Supporting Information for all experimental procedures). The aminoallyl-modified transcript is coupled with the amine-reactive form of a desired biophysical probe to form the labeled tagging RNA (Scheme 1 a). The tagging RNA is then attached by the deoxyribozyme to an internal 2′-hydroxy group of the target RNA (Scheme 1 b). This RNA modification approach avoids both solid-phase synthesis and organic synthesis; the 5-aminoallyl-CTP (CTP = cytidine triphosphate) necessary for in vitro transcription of the tagging RNA is commercially available. Furthermore, because the intact target RNA is derivatized directly with the tag, splint ligation is entirely obviated and no mutations are required in the target RNA to provide a modification site.



Scheme 1. DECAL of RNA. a) Coupling of the amine-reactive form of the label (blue) to 5-aminoallylcytidine, which was incorporated into the 19-nt tagging RNA (brown) by in vitro transcription. See the Supporting Information for details of all experimental procedures. b) Labeling of the target RNA. The 2′-OH group of a specific adenosine of the target RNA (black) attacks the 5′-triphosphate group of the labeled tagging RNA. NHS = *N*-hydroxysuccinimidyl. PP_i = inorganic pyrophosphate.

Previous studies indicated that the 10DM24 deoxyribozyme has considerable sequence tolerance with respect to its RNA substrates.[11] We tested the ability of 10DM24 to use a tagging RNA derivatized with biotin, dabcyl (4-(4'-dimethylaminophenylazo)benzoic acid; a quencher), fluorescein, or tamra (tetramethylrhodamine) as representative biophysical probes. 10DM24 catalyzed tag attachment to a comprehensive set of short target RNA substrates with promising generality (see Figure S1 in the Supporting Information).

To implement the DECAL strategy with a large RNA molecule, we chose ten sites within the 160-nucleotide (nt) *Tetrahymena* group I intron P4–P6 domain,[12] an often-studied model RNA.[12–17] Target sites were selected on the basis of 2′-OH group accessibility of adenosines in the X-ray crystal structure[13] because 10DM24 prefers adenosine 2′-OH groups.[11] We specifically included target sites that would be useful in FRET studies if they were successfully derivatized.

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P4–P6 labeling was tested with a tagging RNA that lacks the aminoallyl group as well as with tags incorporating aminoallyl, biotin, fluorescein, and tamra (Figure 1).

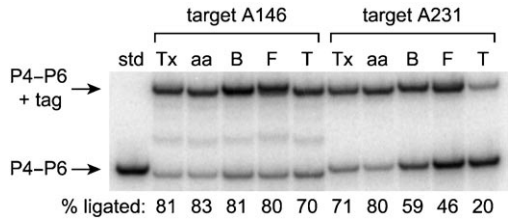

Figure 1. Covalent modification of P4–P6 RNA as an implementation of DECAL. The P4–P6 RNA was targeted at positions A146 and A231 for attaching different forms of the tagging RNA. Tx denotes the unmodified tagging RNA transcript, whereas aa denotes the tag incorporating a single 5-aminoallylcytidine at the second position. Biotin (B)-, fluorescein (F)- and tamra (T)-modified tags were also tested. Attachment of the tag to P4–P6 adds 19 nucleotides, resulting in the observed gel shift. See the Supporting Information for details and results for all ten P4–P6 target sites.

Six of the ten tested P4–P6 sites were derivatized in greater than 50% yield by using a tag that only has the aminoallyl modification (see Figure S2 in the Supporting Information). The same six locations were labeled with biotin in greater than 40% yield. The fluorescein tag was appended to five sites with greater than 40% yield, whereas the tamra tag was attached at one location with greater than 50% yield. On the basis of these results, two sites (A231 and A146; Figure 2a) were chosen for preparative labeling of P4–P6 with the FRET pair fluorescein and tamra. The fluorescein tag was attached to A231, and the singly labeled product was purified by PAGE. The tamra tag was then appended to A146, leading to the doubly labeled P4–P6. Owing to the gel shift upon each tag addition, the final PAGE-purified product is homogenous with respect to the two labels.

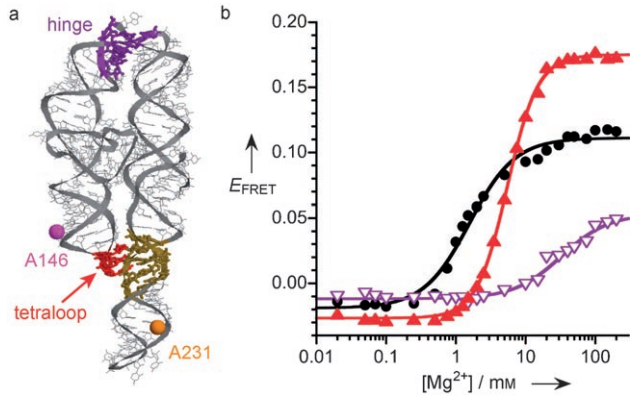

Figure 2. Folding of doubly tagged P4–P6 assayed by FRET. a) P4–P6 with the two tag locations marked (A231-fluorescein, orange; A146-tamra, pink). The tetraloop–receptor interaction is colored red and gold. b) Mg^{2+} ion dependence of FRET efficiency (E_{FRET}) for wild-type P4–P6 (P4–P6-wt ●), the nonfoldable mutant (P4–P6-bp ▽), and the P4–P6 tetraloop mutant (▲). E_{FRET} was determined by the $(ratio)_A$ method.[20] See the Supporting Information for assay details and data analysis methods. Representative fluorescence spectra are shown in Figure S5 in the Supporting Information.

Both A231 and A146 are part of canonical helical regions and are not involved in tertiary interactions. Therefore, no perturbation of the native P4–P6 RNA folding was expected upon attachment of the two tagging RNAs. To verify this experimentally, we assayed Mg^{2+}-ion-dependent folding by nondenaturing PAGE.[14–16] Attachment of the fluorescein and tamra tags to P4–P6 caused almost no shift in Mg^{2+} ion dependence (see Figure S3 in the Supporting Information; $\Delta\Delta G^\circ = 0.5\ kcal\,mol^{-1}$). Although the tags do not perturb folding of P4–P6, other RNA targets could be more sensitive. To address this, we have shown that the RNA-cleaving 10–23 deoxyribozyme[18] can truncate each tag efficiently, leaving only eight tag nucleotides at each labeling site (see Figure S4 in the Supporting Information). Unfortunately, incorporation of one or more phosphorothioates into the tagging RNA cannot be used to cleave the majority of the tag after labeling of the target RNA because phosphorothioate cleavage induced by iodine or other reagents is not preparatively useful (data not shown).[19]

The Mg^{2+}-ion-dependent folding of doubly tagged P4–P6 was investigated by steady-state FRET. When P4–P6 is unfolded (at low Mg^{2+} ion concentrations), the tagged A231 and A146 sites are relatively far apart owing to opening of the "hinge" region (Figure 2a), and the observed FRET efficiency (E_{FRET}) is approximately 0 (Figure 2b). When the Mg^{2+} ion concentration is raised, folding of the RNA brings the two tagged sites closer together,[12,13] which is expected to increase E_{FRET}. In addition to wild-type P4–P6 (P4–P6-wt), two mutant forms of P4–P6 were each doubly tagged with fluorescein and tamra. "Nonfoldable" P4–P6 (P4–P6-bp) contains base pairs in the hinge that disrupt folding.[12,14,21] The second P4–P6 mutant has two adenosine residues inserted into the tetraloop, which was previously shown to increase the Mg^{2+} ion dependence considerably.[16]

E_{FRET} is observed to increase at higher Mg^{2+} ion concentrations for the doubly tagged P4–P6-wt, with a $[Mg^{2+}]_{1/2}$ value of 1.6 mM (Figure 2b). As expected, P4–P6-bp has essentially no change in E_{FRET} at low Mg^{2+} ion concentrations (< 10 mM). At higher Mg^{2+} ion concentrations, the E_{FRET} increases, indicating that the fluorophores can come closer together because of RNA folding or compaction. Also as expected, the tetraloop mutant P4–P6 has its $[Mg^{2+}]_{1/2}$ value shifted considerably to the right. The higher E_{FRET} observed for the tetraloop mutant at greater than 10 mM Mg^{2+} ion concentration suggests a folded structure that allows the two fluorophores to come closer together than was possible in P4–P6-wt. The E_{FRET} values for all three P4–P6 RNA molecules are similar (≈ 0) at very low Mg^{2+} ion concentrations, suggesting similar unfolded states. FRET provides information about P4–P6 folding that cannot be obtained by native PAGE or single-fluorophore methods,[14–16] thereby demonstrating the utility of the DECAL approach. Despite the widespread use of P4–P6 as a model system for studying RNA structure and folding in many laboratories,[12–17] FRET studies have not been reported previously owing to the synthetic difficulties inherent to incorporating two internal labels by splint ligation. Single-molecule RNA folding experiments with our labeled P4–P6 and FRET[1] should now be possible.

In summary, we have developed DECAL as a general strategy for direct site-specific internal RNA modification. The 10DM24 deoxyribozyme tolerates different biophysical labels on the tagging RNA, which suggests the versatility of DECAL for applications that require covalent RNA modification. Many different labels can be tested at a single site in the target RNA by using a single deoxyribozyme and varying the label on the tagging RNA. Testing a particular label at different target sites simply requires the same tagging RNA and deoxyribozymes with binding arms that correspond to each new target site. Because the target RNA itself has no sequence modifications, many sites can be tested with a single target sequence. This is particularly important for large RNA targets for which preparation of mutants is relatively cumbersome. By varying the location of the aminoallyl nucleotide in the tagging RNA, the DECAL approach should permit adjusting of the distance of the label from the target RNA, which may be important for various applications. In a specific application of DECAL, two fluorophores were successfully attached to P4–P6 and enabled the first reported FRET assays of this RNA.[22] The DECAL strategy should be applicable to essentially any RNA, including challenging targets such as large catalytic RNA molecules and the ribosome. Efforts are currently underway to identify improved deoxyribozymes with even broader RNA-target and label tolerance. The ability to covalently modify RNA at internal positions in a site-selective manner without splint ligations or organic synthesis should make the DECAL approach immediately useful in many contexts.

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[22] Note added in proof. After the final version of this manuscript was submitted, a report described the use of FRET with P4–P6, see: T.-H. Lee, J. L. Lapidus, W. Zhao, K. J. Travers, D. Herschlag, S. Chu, *Biophys. J.* **2007**, DOI: 10.1529/biophysj.106.094623.

