Novel cyanine-AMP conjugates for efficient 5′ RNA fluorescent labeling by one-step transcription and replacement of [γ-32P]ATP in RNA structural investigation

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ABSTRACT

Two novel fluorescent cyanine-AMP conjugates, F550/570 and F650/670, have been synthesized to serve as transcription initiators under the T7 ϕ2.5 promoter. Efficient fluorophore labeling of 5′ RNA is achieved in a single transcription step by including F550/570 and F650/670 in the transcription solution. The current work makes fluorescently labeled RNA readily available for broad applications in biochemistry, molecular biology, structural biology and biomedicine. In particular, site-specifically fluorophore-labeled large RNAs prepared by the current method may be used to investigate RNA structure, folding and mechanism by various fluorescence techniques. In addition, F550/570 and F650/670 may replace [γ-32P]ATP to prepare 5′ labeled RNA for RNA structural and functional investigation, thereby eliminating the need for the unstable and radio-hazardous [γ-32P]ATP.

INTRODUCTION

Site-specific fluorescent labeling of RNA has many applications in biochemistry, molecular biology, structural biology and biomedicine (1–15). Fluorophores may be attached to RNA either by phosphoramidite chemistry during RNA synthesis (1–3,16), post-transcriptional fluorophore coupling with enzymatically prepared RNA (16,17), or direct fluorophore labeling during transcription (18,19). Phosphoramidite chemistry-based fluorescent labeling is commonly used to synthesize relatively small RNA molecules (<50–60 nt). When the size of RNA increases, such fluorescent-labeling procedures become impractical due to low yields of full-length RNA, high levels of impurities with short RNA fragments and high costs of chemical synthesis. On the other hand, transcription-based RNA synthesis and fluorescent labeling has no apparent RNA size restrictions; both purities and costs are essentially independent of RNA sizes. Post-transcriptional fluorescent labeling involves two essential steps: preparation of amino- or thio-derivatized RNA by transcription followed by fluorophore coupling via fluorophore-N-hydroxysuccinimide esters (NHS) or fluorophore-maleimides or fluorophore-bromides (16,17). The limitations of this method lie in (i) the scarcity of available amino- and thio-nucleotide precursors for the preparation of amino- and thio-derivatized RNA, respectively; (ii) the hydrolytic lability of fluorophore-NHS; (iii) high concentrations of fluorophore-NHS, fluorophore-maleimides or fluorophore-bromides that are required to achieve efficient fluorescent labeling of RNA; and (iv) multiple steps of manipulation of RNA samples, leading to laborious sample preparation and low RNA yields.

Direct RNA labeling at the 5′ end using the T7 ϕ2.5 promoter developed in our laboratory (18,19) only requires appropriate label-linker-AMP conjugates (adenosine 5′-monophosphate, AMP) to serve as transcription initiators. The newly developed in vitro transcription system recognizes the adenosine moiety and labels the 5′ end of RNA with high efficiency through transcription initiation (18,19). The only RNA sequence requirement is the 5′ AG. We have previously reported fluorescein-HDA-AMP (1,6-hexanediamin, HDA) for direct RNA labeling (19). For this new RNA-labeling method to be widely applicable, however, diverse fluorophores with better spectroscopic properties, such as the commonly used cyanine dye family, are highly desirable. Here, we describe the synthesis of two novel cyanine-AMP conjugates, F550/570 and F650/670, and their use to fluorescently label 5′ RNA in a single transcription step. Furthermore, we demonstrate one utility, among others, of F550/570 to replace the commonly used [γ-32P]ATP for 5′ RNA labeling in RNA structure/function/mechanism investigation.

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MATERIALS AND METHODS

Synthesis of cyanine-AMP conjugates

All reagents and chemicals were purchased from Aldrich and used as received. Synthetic procedures are shown in Scheme 1. Starting from 2,3,3-trimethyl-3H-indole-5-acetic acid \( \text{1} \) (20), the common intermediate 1,2,3,3-tetramethyl-indoleninium-5-acetate (2) was synthesized by methylation of \( \text{1} \) (20). Condensation of two molecules of 2 with one molecule of triethyl orthoformate (21) afforded the symmetrical red cyanine dye 3 with two free carboxyl groups that can be used for subsequent conjugation with AMP via a linker. Separately, condensation of one molecule of 1,3,3-triemethoxypropene (21) with two molecules of 2 produced another symmetrical blue cyanine dye 4 with two free carboxyl groups. The carboxyl groups of 3 and 4 were then activated by \( N,N' \)-dicyclohexylcarbodiimide (DCC) to form their corresponding NHS esters, 5 and 6. Finally, the intermediates 5 and 6 were individually coupled with 5\(^{-}\)-(6-aminohexyl) adenosine phosphoramidate (HDAAMP) (19) to afford a pair of novel symmetrical cyanine-AMP conjugates, \( \text{F550/570} \) and \( \text{F650/670} \). Detailed description of the syntheses of 2–6, \( \text{F550/570} \) and \( \text{F650/670} \) are given below.

Synthesis of compound 2. To a 25 ml Schlenk tube was added 0.5 g (2.3 mmol) of compound 1, prepared from the published procedure (20), 1.2 ml (19.3 mmol) of iodomethane and 10 ml of acetonitrile. The reaction mixture was degassed with argon for 30 min and the tube was sealed with a cap and heated in an oil bath at 80°C for 1 h. After cooling, the reaction mixture was transferred into a flask and concentrated under vacuum to give 0.78 g (94%) of product 2.

Synthesis of compound 3. The literature procedures (20,21) were used to prepare compound 3. To 1.0 g (2.8 mmol) of compound 2, 20 ml of dry pyridine was added. While the reaction mixture was refluxing, 1.4 ml (8.4 mmol) of triethyl orthoformate was added slowly (0.4 ml per 15 min). After completion of addition, the reaction mixture was refluxed for another 2 h. Solvent was removed and the red residue was dissolved in 40 ml of methanol, followed by adding 200 ml of ethyl acetate. After concentrating to \(~50\) ml, another 100 ml of ethyl acetate was added, and concentrated to \(~50\) ml. To this suspension, 100 ml of ethyl acetate was added. The top solvent was decanted and the red residue was dried over under vacuum to give 0.81 g (96%) of the compound 3. Mass spectrometry (MS) analysis gave the following results: \( \text{C}_{29}\text{H}_{33}\text{N}_{2}\text{O}_{4}^{+} \), calcd, 473.24, found 473.2 (\( \text{M}^{+} \)).

Synthesis of compound 4. The literature procedure (21) was used to prepare compound 4. To 1.0 g (2.8 mmol) of compound 2, 28 ml of dry acetonitrile, 0.6 ml of triethylamine (TEA) and 0.2 ml of acetic acid were added. While the reaction mixture was refluxing, a solution of 1.0 g (7.6 mmol) of 1,3,3-triemethoxypropene in 4.0 ml of acetonitrile was slowly added (0.5 ml per 15 min). After completion of the addition, the reaction mixture was refluxed for another 2 h. Solvent was removed and the blue/purple residue was dissolved in 40 ml of methanol, followed by adding 200 ml of ethyl acetate. After concentrating to \(~50\) ml, another 100 ml of ethyl acetate was added, and concentrated to \(~50\) ml. To this suspension, 100 ml of ethyl acetate was added. The top solvent was decanted and the red residue was dried over under vacuum to give 0.84 g (96%) of the compound 4. The molecular peak found by MS, 499.2 (\( \text{M}^{+} \)), is consistent with the expected formula \( \text{C}_{31}\text{H}_{35}\text{N}_{2}\text{O}_{4}^{+} \), 499.26.

Synthesis of 5 and 6. To 100 mg (0.16 mmol) of compound 3 or 4, 90 ml of \( \text{CH}_{2}\text{Cl}_{2} \), 100 mg (0.48 mmol) of DCC, 55 mg (0.48 mmol) of NHS and 50 mg of 4-(dimethylamino)pyridine
were added. The reaction mixture was stirred for 2 h. The urea-derivative precipitate was formed and filtered off. The filtrate was concentrated to dryness and used for the next step of coupling reaction without further purification. MS analysis gave the following results:

5 \text{C}_3\text{H}_{39}\text{N}_4\text{O}_8^+\text{, calcd, 667.28, found 667.2 (M}^+\text{)}; 6 \text{C}_3\text{H}_{41}\text{N}_4\text{O}_8^+\text{, calcd, 693.29, found 693.2 (M}^+\text{)}.

**Synthesis and purification of F550/570 and F650/670.** To a 300 \text{ml aqueous solution of 370 mM HDAAMP (19), 150 \text{ul TEA, 750 \text{ml N,N-dimethylformide (DMF) and 20 mg of 5 or 6, which were dissolved separately in 150 \text{ml DMF, were added. After 30 min of reaction, 1.2 ml of water was added to the sample. The resulting solution was filtered with a 0.2 \text{um syringe filter. Purification of F550/570 and F650/670 was achieved by semi-preparative reverse phase high-performance liquid chromatography (HPLC) (Figures 1 and 2). Isolated yields for F550/570 and F650/670 were ~20%. High-resolution MS analysis gave excellent results: F550/570—C_{63}H_{87}N_{16}O_{14}P_{2}^+, \text{calcd, 1353.6057, found 1353.5998 (M}^+\text{)}; F650/670—C_{63}H_{87}N_{16}O_{14}P_{2}^+, \text{calcd, 1353.6057, found 1353.5998 (M}^+\text{).}

**Spectroscopic properties of F550/570 and F650/670.** UV absorbance spectra and molar extinction coefficients of F550/570 and F650/670 were obtained from a JASCO spectrometer (V-530) in 20 mM phosphate, pH 7.0. Fluorescence emission spectra were measured with an ISS PC1 fluorometer (Champaign, IL) in 20 mM phosphate, pH 7.0, under the

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**Figure 1.** Purification and subsequent analysis of F550/570 by HPLC. (A) F550/570 sample was injected onto a Delta Pak C18 column, 7.8 × 300 mm², pre-equilibrated in 20 mM phosphate buffer, pH 7.0, flow rate 5 ml/min. The mobile phase was manually changed in the following order: 100% water at 7 min → 20% MeOH/80% water at 9 min → 30% MeOH/70% water at 16 min → 40% MeOH/60% water at 23 min → 50% MeOH/50% water at 30 min → 60% MeOH/40% water at 38 min → 100% MeOH at 45 min. The 25–30 min fraction (marked as F550/570) has the desired UV spectrum from an online photodiode array detector. (B) Analysis of the 25–30 min fraction by an Econosphere C18 column, 4.6 × 50 mm², in 60% MeOH/40% 20 mM phosphate, pH 7.0, flow rate 0.5 ml/min. The insert shows the UV–visible spectrum of the peak.

**Figure 2.** Purification and subsequent analysis of F650/670 by HPLC. (A) F650/670 sample was injected onto a Delta Pak C18 column, 7.8 × 300 mm², pre-equilibrated in 20 mM phosphate buffer, pH 7.0, flow rate 5 ml/min. The mobile phase was manually changed in the following order: 100% water at 11 min → 20% MeOH/80% water at 18 min → 30% MeOH/70% water at 23 min → 40% MeOH/60% water at 29 min → 50% MeOH/50% water at 35 min → 60% MeOH/40% water at 43 min → 100% MeOH at 52 min. The 41–45 min fraction (marked as F650/670) has the desired UV spectrum from an online photodiode array detector. (B) Analysis of the 41–45 min fraction by an Econosphere C18 column, 4.6 × 50 mm², in 60% MeOH/40% 20 mM phosphate, pH 7.0, flow rate 0.5 ml/min. The insert shows the UV–visible spectrum of the peak.
excitation of 510 and 610 nm for F550/570 and F650/670, respectively. Quantum yields (Φ) of 3, 4, F550/570 and F650/670 were determined by using reference fluorophores according to the following equation:

\[ Φ_X = \Phi_R (SL_X / SL_R)(η_X / η_R)^2, \]

where X and R stand for the sample and the reference, respectively. SL is the slope of integrated fluorescence area versus absorbance and η is the refractive index of the solvent. The reference for 3 and F550/570 was rhodamine 101 (ΦR = 1; Fluka) (22). Zinc phthalocyanine (ΦR = 0.3; Aldrich) (23) was used as the reference for quantum-yield measurements of 4 and F650/670. Fluorescence was measured at the excitation of 490 nm (for 3 and F550/570) or 600 nm (for 4 and F650/670).

RNA 5’ labeling by F550/570 and F650/670

Fluorescent labeling of 5’ RNA by F550/570 and F650/670 was performed under normal in vitro transcription conditions (18,19) with slight modifications: changing [ATP] from 1 to 0.25 mM and adding 2 mM of F550/570 or F650/670 to the transcription solution. The final transcription solution contained 40 mM Tris–HCl, pH 8.0, 5 mM dithiothreitol, 6 mM MgCl2, 2 mM spermidine, 0.01% Triton X-100, 0.25 mM ATP, 1 mM each of UTP, GTP and CTP, 2 mM MgCl2, 2 mM spermidine, 0.05–0.5 mM dNTPs, 0.01% Triton X-100, 0.25 mM ATP, 1 mM each of UTP, GTP and CTP, 2 mM F550/570 or F650/670. 0.05–0.5 µM dsDNA containing the T7 promoter (18,19), 500 U of T7 RNA polymerase per 100 µl reaction and 10–20 U of RNase inhibitor per 100 µl reaction. Also included was 2 µl of [α-32P]ATP per 100 µl reaction as a tracer for RNA analysis. The labeling reaction was carried out at 37°C for 2 h before analysis by denaturing PAGE. Product detection and quantification were achieved by phosphorimaging/fluorimaging under the excitation by 32P, a 532 nm excitation of 510 and 610 nm for F550/570 at 37°C and 100 U of RNase inhibitor per 100 µl reaction. Also included was 2 µl of [α-32P]ATP per 100 µl reaction as a tracer for RNA analysis. The labeling reaction was carried out at 37°C for 2 h before analysis by denaturing PAGE. Product detection and quantification were achieved by phosphorimaging/fluorimaging under the excitation by 32P, a 532 nm green laser, and a 633 nm red laser (Typhoon 9400; Amersham Biosciences). For Figure 4, the RNA was a 92-nt ribozyme (TES33) with the sequence of AGGGAAGUCUACCA-CUUGCUGGUAUGCGCCUUCCCUUGCUGAUCUGCC-CUUCGGGCUCUCGCCCUGCACGGGCAUGCGGCA-GCCA, which was previously isolated in our laboratory (24). In addition, to test transcription-labeling yields and PAGE separation of varying RNA, we prepared five different RNA sizes of 100, 200, 300, 400 and 500 nt using self-constructed DNA templates from Epicentre’s control DNA for AmpliScribe™ system. The sequences of transcribed RNAs are AGAAAUUCAAGGGGAGAUCCGUCAGUUAUUAA-ACUAUUGCUCCGGACAUUCUCUGAGCCAUAUA-AAGAUAAUUGUUCUCCUUUCUGGGGACAGGUUGU-CUUCUUAGGGAGAGUAAGAUCCAAUAGCACA-AAGCAGAUCGAACGCUCAAAAUUCCCCUUUGG-GGAUCUUUCUGACUAAGUCUAUUUUAACAGAGAAAAAUUCAUUGUUUUCUGGGGUGGUGAU-UGCCACAAUCUAAUUGCUAAUUGUGUUGUUUCC-CCACCAUCAUCCGGCGCAUAAGAAGCAUGAU-GUCGGUCGGGCAUGAAGAUAUACCGUCACCUCAA-AGGUAUAGUUAACUAUCGAAUCCGGAGCCACUU-UUUCUUAUAAGAAAGUGGAACAUUCGAACAUU-CUGGCAACAUUUUAACACUGGCGAAAUCUGC-CCAGUAUUCGAGGAGUACCCCCUAAGAUAAGUGGAGGUUGAAUUGGAAGAGAGCCUCUCAUUU, where each boldface nucleotide represents the 3’ end of an RNA sequence from the beginning A.

Replacement of [γ-32P]ATP by F550/5570 in RNA reaction site mapping

F550/570-labeled RNA (prepared as described above) was used to investigate the reaction site of an in vitro isolated ribozyme ACT3 that catalyzes self-aminoacylation from biocytinyl CoA (biocytin-CoA) (25). Gel-purified dye-labeled ACT3 RNA was reacted for 10 min at 25°C with 1 mM biocytinyl CoA in the selection buffer containing 20 mM HEPES (pH 7.4), 200 mM KCl, 100 mM NaCl, 30 mM MgCl2 and 10 mM CaCl2 (25). After purification by membrane filtration using a microcon M30 (Millipore), the RNA was partially hydrolyzed by lead under the following conditions: 2 mM lead acetate in 7 M urea and 50 mM HEPES, pH 5.5, 40 s at 80°C, followed by quenching with 50 mM EDTA. The resultant RNA fragments were loaded onto a Neutralvadin column (Pierce). Following washing with 4 M NaCl, 3 M sodium acetate and water, RNA fragments were eluted by 20 mM biotin and 5 M urea at 95°C for 10 min. The eluted RNA was EtOH-precipitated and the recovered RNA was analyzed by 8% denaturing PAGE, followed by phosphorimaging under the excitation of a 532 nm green laser.

An RNase T1 ladder of the F550/570-labeled RNA was prepared by digestion (10 min at 50°C) of F550/570-labeled RNA with 2 µg of carrier tRNA, 1 U of RNase T1 in 7 M urea, 2 mM EDTA and 0.1 M sodium acetate, pH 5.2. The ladder served as RNA size markers in RNA fragment analysis by PAGE (Figure 4B).

RESULTS

Synthesis of F550/570 and F650/670

Cyanine dyes (26) display some excellent fluorescent properties, including their high molar extinction coefficients and improved resistance to photobleaching (in contrast to the commonly used fluorescein). In addition, the color and solubility of cyanine dyes can be modulated by the number of double bonds between the two indole rings and by changing the attached chemical groups. Having successfully demonstrated 5’ fluoroscein labeling of RNA by direct transcription (19), we sought to develop similar methods to site-specifically label 5’ RNA by cyanine dyes with the intention of bringing this simple and efficient RNA-labeling technique to broad applications in biosciences and biomedicine.

After two synthetic steps (Scheme 1), the two fluorescent cyanine dyes 3 and 4 were obtained in high yield (91%). After activation by DCC and NHS, the NHS esters 5 and 6 were readily coupled with the amino group of HDAAMP (19). Purification by reverse phase HPLC (Figures 1A and 2A) yielded pure (>96% purity) fluorescent dyes F550/570 and F650/670 (with ~20% total yields) as shown in Figures 1B and 2B. In addition to F550/570 and F650/670, there were other peaks containing cyanine dyes (Figures 1A and 2A). Their identities were not further investigated.

UV absorbance and fluorescent properties of F550/570 and F650/670

Ultraviolet and visible spectra of F550/570 and F650/670 (Figure 3, Ab lines) show additive contribution from both the adenosines and the cyanine cores. For both cyanine-AMP conjugates, the absorbance within 220–300 nm is
contributed mainly by the adenosine moieties. The absorbance between 450 and 580 nm of F550/570, with $\lambda_{\text{max}}$ at 550 nm, is purely due to the cyanine dye 3 core. Similarly, for F650/670, the absorbance between 580 and 700 nm ($\lambda_{\text{max}} = 650$ nm) originates from the cyanine dye 4 core. Molar extinction coefficients measured in 20 mM phosphate buffer, pH 7.0, are $\varepsilon_{550} = \sim 130 000 \text{ M}^{-1} \text{ cm}^{-1}$ and $\varepsilon_{650} = \sim 210 000 \text{ M}^{-1} \text{ cm}^{-1}$ for F550/570 and F650/670, respectively. Fluorescence emission spectra of F550/570 and F650/670 are marked as Em curves in Figure 3. F550/570 fluoresces between 550 and 700 nm, with emission $\lambda_{\text{max}} = 570$ nm. Under excitation, F650/670 emits fluorescence within 640–780 nm ($\lambda_{\text{max}} = 670$ nm). Within the visible range (440–780 nm), both the absorption spectra and fluorescence emission spectra of F550/570 and F650/670 are similar to those of common cyanine dyes, Cy3 and Cy5, respectively (26). The quantum yields (from three sets of measurements) of F550/570 and F650/670 are 0.11–0.16 for Cy3 and 0.03–0.27 for their corresponding precursors F650/670 and F550/570, respectively. Therefore, the attachment of two adenosines and HDA linkers at the opposite ends of the cyanine cores increases their quantum yields, but has no apparent effects on other spectroscopic properties of the cyanine dye within the visible range.

Fluorescent labeling of 5’ RNA by F550/570 and F650/670

Fluorescent labeling of 5’ RNA is achieved by simply including F550/570 or F650/670 in transcription solutions under the T7 class II promoter $\phi$2.5 (18,19). One of the two adenosines within F550/570 or F650/670 initiates transcription, resulting in 5’ RNA labeling by the cyanine dyes. Although there are two identical adenosines within F550/570 or F650/670, the probability of both adenosines initiating transcription to produce head-to-head joined RNA via F550/570 or F650/670 is low due to the high concentration ratios of F550/570 (or F650/670) over transcribed RNA molecules (i.e. mM versus $\mu$M). To confirm the prediction, purified F550/570-RNA (TES33, 32P-labeled) was added to the transcription solution in the absence of [\(\alpha\)-32P]ATP and F550/570. No head-to-head joined RNA dimer was observed by phosphorimaging after PAGE. Because neither F550/570 nor F650/670 contains a nucleoside 5’-triphosphate, the cyanine dyes cannot be incorporated into internal RNA positions by T7 RNA polymerase. Figure 4 shows 5’ RNA (TES33) labeling by F550/570 and F650/670. Three parallel transcription experiments (with [\(\alpha\]-32P]ATP as the internal radiolabel) were carried out in the absence of the cyanine dyes (lane 1) or in the presence of F550/570 (lane 2) or F650/670 (lane 3). Phosphorimaging based on 32P (Figure 4A) revealed an additional slower RNA band in lanes 2 and 3. Fluorescence scanning of the same gel under the excitation with the 532 nm green laser (Figure 4B) shows only a single RNA band in lane 2, whose location overlaps with that of the upper band of lane 2 in Figure 4A. Under the excitation of a 633 nm red laser (Figure 4C), scanning of the same gel displays another single RNA band in lane 3, whose location superimposes with that of the upper band of lane 3 in Figure 4A. Taken together, the three different scannings of the same gel based on excitation by 32P (Figure 4A), 532 nm photons (Figure 4B) and 633 nm photons (Figure 4C) indicate fluorescent labeling of RNA by F550/570 (lane 2) and F650/670 (lane 3) during transcription. The labeling yields were 60 ± 5% and 35 ± 5% for F550/570 and F650/670, respectively. Total RNA yields for F550/570- and F650/670-labeled RNA were 110 ± 30% and 90 ± 30% of the control RNA (in the absence of dye-AMP). In a different set of labeling experiments with varying RNA sizes (100–500 nt), 15 independent transcriptions gave labeling yields of 78 ± 3% and 55 ± 3% for F550/570 and F650/670, respectively. The respective total RNA yields relative to the control RNA were 150 ± 30% and 110 ± 30%. Therefore, F550/570 and F650/670 appear to stimulate transcription initiation under the transcription.

Figure 3. UV–visible absorption spectra (A) and fluorescence emission spectra (Em) of F550/570 and F650/670. The spectra were measured in 20 mM phosphate buffer, pH 7.0. All spectra were normalized to 1 at their $\lambda_{\text{max}}$. The $\lambda_{\text{max}}$ difference between excitation and emission is 20 nm for both F550/570 and F650/670.

Figure 4. RNA fluorescent labeling by F550/570 and F650/670 under the T7 $\phi$2.5 promoter (18,19). All RNA was also internally 32P-labeled by [\(\alpha\]-32P]ATP. After transcription, RNA samples were fractionated by PAGE. Lane 1, normal transcription; lanes 2 and 3, transcription in the presence of F550/570 and F650/670, respectively. (A) 32P-phosphorimaging reveals total RNA bands in different transcription experiments. (B) Scanning of the same gel under the excitation of a 532 nm laser shows only F550/570-labeled RNA. (C) Under excitation with a 633 nm laser, only F650/670-labeled RNA is visible. The RNA sequence was that of a thioester-synthesizing ribozyme TES33, 92 nt (24). RNAs from ~5 μL transcription were used for the gel.
conditions. In a typical experiment, ~20 µg of dye-labeled RNA can be prepared from 100 µl transcription.

Since no transcription-based methods would produce 100% labeled RNA, isolation of labeled RNA from unlabeled (normal) RNA may be required for its applications. Owing to their relatively large sizes, F550/570- and F650/670-labeled RNA displays significant migration retardation (5–6 nt difference) by PAGE. This added property of F550/570- and F650/670-labeled RNA may be exploited to achieve high purity levels of fluorescent RNA by PAGE. To establish the RNA size-PAGE resolution relationship, five different sizes of RNA (100, 200, 300, 400 and 500 nt) were labeled by both 32P and fluorescent RNA by PAGE. To establish the RNA size-PAGE resolution relationship, five different sizes of RNA (100, 200, 300, 400 and 500 nt) were labeled by both 32P and fluorescent RNA by PAGE. To establish the RNA size-PAGE resolution relationship, five different sizes of RNA (100, 200, 300, 400 and 500 nt) were labeled by both 32P and fluorescent RNA by PAGE. To establish the RNA size-PAGE resolution relationship, five different sizes of RNA (100, 200, 300, 400 and 500 nt) were labeled by both 32P and fluorescent RNA by PAGE. To establish the RNA size-PAGE resolution relationship, five different sizes of RNA (100, 200, 300, 400 and 500 nt) were labeled by both 32P and fluorescent RNA by PAGE.

Replacement of [γ-32P]ATP by F550/F570 in RNA reaction site mapping

Investigation of RNA structure/function/mechanism frequently requires the use of [5'-32P]RNA (27–29). However, it may be difficult to phosphorylate some RNA by [γ-32P]ATP and polynucleotide kinase (PNK) due to the structure of RNA. As an example, a recently isolated ribozyme (ACT3) from our laboratory poses such a problem. ACT3 is a ribozyme that catalyzes the formation of aminocatalyzed RNA from aminoacyl thioesters of CoA (25). Attempts to label the 5'-HO-ACT3 by [γ-32P]ATP and PNK gave very poor (<2%) labeling yields due to the 5' recessed structure (Figure 6A). Here, we demonstrate that 5' F550/570-labeled RNA can be used to map the reaction site within the ribozyme.

BiocytinCoA-reacted RNA, which was 5'-end-labeled by F550/570, was first treated with lead acetate to generate all possible single-cut RNA fragments. The resulting RNA sample contained both biocytin-tagged RNA fragments and normal RNA fragments. After Neutravidin affinity chromatography, only biocytin-tagged RNA fragments would be retained on the column. Analysis of the eluted RNA sample by PAGE and F550/570-based fluorimaging should reveal the location of the reactive site within the ribozyme. Figure 6B shows the fluorescence scanning image of RNA fragments eluted from the Neutravidin column. Figure 6C is the profile of F550/570 fluorescence intensity from Figure 6B. The gel indicates that RNA fragments shorter than C51 (from the 5' end) were completely missing from the affinity column, while all the fragments longer than C51 were present. The result is schematically interpreted in Figure 6D. The C51 RNA fragment (with 5' F550/570 as well) thus contained the reaction site at a 2' OH group. Since lead-induced RNA hydrolysis requires the presence of a free 2' OH group, the actual reaction site is the 2' OH group of U50, which agrees with the results obtained by reverse transcriptase-catalyzed primer extension, HPLC analysis and MS analysis (25).

DISCUSSION

Our approach for direct fluorescent labeling of RNA by F550/570 and F650/670 during transcription offers the following distinct advantages over the phosphoramidite chemistry method and post-transcriptional fluorescent labeling. First, site-specific RNA fluorescent labeling is achieved in a single step of transcription, greatly reducing the RNA sample handling time and RNA loss. Typically, fluorophore-labeled RNA can be made available in common laboratories within 2–4 h. Second, there is no apparent RNA size restriction; fluorescently labeled small and large RNAs can be prepared by the same transcription method with similar yields, purities and costs. The commonly used in vitro transcription under the T7 66.5 promoter produces G-initiated RNAs. The same RNA sequences can be used for fluorescence labeling under
The T7 62.5 promoter by adding an extra A before the first G. Third, F550/570 and F650/670 are chemically stable, allowing laboratories to maintain steady stocks without constant purchase of fresh supplies. The current finding will make site-specifically fluorophore-labeled RNA readily available for a variety of applications in biochemistry, structural biology and nucleic acids-based clinical diagnostics. In particular, fluorophore-labeled large RNAs prepared by the current method may be appealing to biochemists and structural biologists to investigate RNA structure, folding and mechanism by various fluorescence techniques such as fluorescence resonance energy transfer (6–9,13) and single-molecule kinetics (10,15,30). Without an apparent restriction on RNA sequence (except for the 5' AG) and size, the described fluorescent labeling by F550/570 and F650/670 can be easily applied to large RNAs such as the group I and group II introns, the RNase P RNA, spliceosomal RNAs, ribosomal RNAs and artificially selected functional RNAs (aptamers and ribozymes).

PNK-catalyzed phosphorylation by [γ-32P]ATP is a common procedure to prepare [5',32P]RNA that is required in various studies of structure/function/mechanism (27–29). However, such a 32P-based RNA labeling method may present a series of problems for the experimenter. First, 32P radioactivity quickly diminishes with time due to its short half-life. In addition, RNA labeling efficiency by [γ-32P]ATP actually decreases much faster than the radiodecay of 32P, probably due to ATP damage caused by strong 32P radioactivity. Therefore, usable [γ-32P]ATP has an even shorter half-life (<14 days). Frequent supply of fresh [γ-32P]ATP is necessary for efficient 5' RNA labeling by 32P and PNK. Unless it is used by several experimenters in a sizable laboratory or shared among different laboratories, a substantial amount of [γ-32P]ATP is wasted due to its fast radiodecay. Second, [5',32P]RNA may slowly lose its function during storage as a result of structural damage by its 32P radioactivity. Accordingly, it may be necessary to frequently prepare freshly 32P-labeled RNA before a previous preparation is fully available.

Figure 6. Ribozyme reaction site mapping by F550/570-labeled RNA. RNA from 10 μl transcription was used for the analysis. (A) The secondary structure of ACT3 (25). It is difficult to label the 5' end by [γ-32P]ATP and PNK due to its recessed 5' end. (B) PAGE analysis of Neutravidin column-eluted RNA fragments (lane 1), along with a RNase T1-digested ladder of the same F550/570-labeled RNA. RNA fragments were generated by partial lead hydrolysis of biocytinCoA-reacted RNA (F550/570-labeled). After electrophoresis, the gel was scanned directly by an Amersham Typhoon phosphorimager under the excitation of a 532 nm laser. (C) Fluorescence intensity profile of lane 1 in (B). (D) An illustration of the reaction site mapping. Controlled lead-induced RNA hydrolysis randomly cuts the RNA to all possible fragments, except for the reactive site where the 2' OH is blocked by biocytin. The eluted RNA sample from Neutravidin chromatography contains all biocytin-tagged fragments, C51 and above. None of the untagged RNA fragments (U49 and below) is retained by the column. Therefore, PAGE analysis and F550/570-based phosphorimaging can detect all RNA fragments equal to or longer than C51. The reaction site is one nucleotide below C51, i.e. U50.
consumed. Third, some RNA (such as the one shown in Figure 6A) may present difficulties for efficient labeling by PNK and [γ-32P]ATP. Finally, 32P is a source of radio-hazard. Its use requires user training and strict workplace safety regulations.

Our newly developed F550/570 and F650/670 offer solutions to the above [γ-32P]-ATP-based RNA labeling problems. Regardless of RNA structure, the 5' end of RNA can be readily labeled by F550/570 and F650/670, if the RNA has AG at its 5' end. If the subject RNA does not possess a 5' AG sequence, AG can be added to the 5' end for the purpose of facilitating 5' fluorescent labeling by F550/570 or F650/670. With phosphorimagers/fluorimagers becoming widely accessible, the RNA-labeling advantages offered by F550/570 and F650/670 over traditional 32P-based methods may be fully realized.

The cyanine dye-AMP conjugates F550/570 and F650/670 described in this report have been made available by AdeGenix Inc. (Monrovia, CA). Detailed application notes can be found at www.adegenix.com.

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REFERENCES

1. Tuschl,T., Gohlke,C., Jovin,T.M., Westhof,E. and Eckstein,F. (1994) A three-dimensional model for the hammerhead ribozyme based on fluorescence measurements. Science, 266, 785–789.

2. Qin,P.Z. and Pyle,A.M. (1997) Stopped-flow fluorescence spectroscopy of a group II intron ribozyme reveals that domain 1 is an independent folding unit with a requirement for specific Mg2+ ions in the tertiary structure. Biochemistry, 36, 4718–4730.

3. Walter,N.G., Hampsel,K.J., Brown,K.M. and Burke,J.M. (1998) Tertiary structure formation in the hairpin ribozyme monitored by fluorescence resonance energy transfer. EMBO J., 17, 2378–2391.

4. Singh,K.K., Purwaresch,R. and Koup,G. (1999) Rapid kinetic characterization of hammerhead ribozymes by real-time monitoring of fluorescence resonance energy transfer (FRET). RNA, 5, 1348–1356.

5. Walter,N.G. and Burke,J.M. (2000) Fluorescence assays to study structure, dynamics, and function of RNA and RNA–ligand complexes. Methods Enzymol., 317, 409–440.

6. Klostermeier,D. and Millar,D.P. (2002) Time-resolved fluorescence resonance energy transfer: a versatile tool for the analysis of nucleic acids. Biopolymers, 61, 159–179.

7. Klostermeier,D. and Millar,D.P. (2001) RNA conformation and folding studied with fluorescence resonance energy transfer. Methods, 23, 240–254.

8. Walter,N.G., Harris,D.A., Pereira,M.J. and Rueda,D. (2002) In the fluorescent spotlight: global and local conformational changes of small catalytic RNAs. Biopolymers, 61, 224–242.

9. Walter,N.G. (2001) Structural dynamics of catalytic RNA highlighted by fluorescence resonance energy transfer. Methods, 25, 19–30.

10. Kim,H.D., Nienhaus,G.U., Ha,T., Orr,J.W., Williamson,J.R. and Chu,S. (2002) Mg2+-dependent conformational change of RNA studied by fluorescence correlation and FRET on immobilized single molecules. Proc. Natl Acad. Sci. USA, 99, 4284–4289.

11. Sekella,P.T., Rueda,D. and Walter,N.G. (2002) A biosensor for theophylline based on fluorescence detection of ligand-induced hammerhead ribozyme cleavage. RNA, 8, 1242–1252.

12. Klostermeier,D., Sears,P., Wong,C.H., Millar,D.P. and Williamson,J.R. (2004) A three-fluorophore FRET assay for high-throughput screening of small-molecule inhibitors of ribosome assembly. Nucleic Acids Res., 32, 2707–2715.

13. Lilley,D.M. (2004) Analysis of global conformational transitions in ribozymes. Methods Mol. Biol., 252, 77–108.

14. Rueda,D., Bokinsky,G., Rhodes,M.M., Rust,M.J., Zhuang,X. and Walter,N.G. (2004) Single-molecule enzymology of RNA: essential functional groups impact catalysis from a distance. Proc. Natl Acad. Sci. USA, 101, 10066–10071.

15. Xie,Z., Srividya,N., Sosnick,T.R., Pan,T. and Scherer,N.F. (2004) Single-molecule studies highlight conformational heterogeneity in the early folding steps of a large ribozyme. Proc. Natl Acad. Sci. USA, 101, 534–539.

16. Qin,P.Z. and Pyle,A.M. (1999) Site-specific labeling of RNA with fluorophores and other structural probes. Methods, 18, 60–70.

17. Czerwinski,J., Odom,O.W. and Hardesty,B. (1991) Fluorescence study of the topology of messenger RNA bound to the 30S ribosomal subunit of Escherichia coli. Biochemistry, 30, 4821–4830.

18. Huang,F. (2003) Efficient incorporation of CoA, NAD and FAD into RNA by in vitro transcription. Nucleic Acids Res., 31, e6.

19. Huang,F., Wang,G., Coleman,T. and Li,N. (2003) Synthesis of adenosine derivatives as transcription initiators and preparation of 5' fluorescein- and biotin-labeled RNA through one-step in vitro transcription. RNA, 9, 1562–1570.

20. Southwick,P.L., Carins,J.G., Ernst,L.A. and Waggoner,A.S. (1988) One pot Fischer synthesis of (2,3,3-trimethyl-3H-indol-5-yl)-acetic acid. Derivatives as intermediates for fluorescent biolabels. Org. Prep. Proced. Int., 20, 279–284.

21. Southwick,P.L., Ernst,L.A., Tauriello,E.W., Parker,S.R., Mujumdar,R.B., Mujumdar,S.R., Clever,H.A. and Waggoner,A.S. (1993) Cyanine dye labeling reagents: sulfoindocyanine succinimidyl esters. Cytometry, 11, 418–430.

22. Karstens,T. and Kobs.K. (1980) Rhodamine B and Rhodamine 101 as reference substance for fluorescence quantum yield measurements. J. Phys. Chem., 84, 1871–1872.

23. Vincent,P.S., Voigt,E.M. and Rieckhoff,K.E. (1971) Phosphorescence of a group II intron ribozyme. Proc. Natl Acad. Sci. USA, 78, 1940–1943.

24. Coleman,T.M. and Huang,F. (2002) RNA-catalyzed thioester synthesis. Biochemistry, 41, 60–70.

25. Li,N. and Huang,F. (2004) Ribosome-catalyzed aminoacylation from CoA thiosteres. Biochemistry, in press.

26. Mujumdar,R.B., Ernst,L.A., Mujumdar,S.R., Lewis,C.J. and Waggoner,A.S. (1993) Cyanine dye labeling reagents: sulfoindocyanine succinimidyl esters. Bioconjug. Chem., 4, 105–111.

27. Celler,D.W. and Cech,T.R. (1991) Visualizing the higher order folding of a catalytic RNA molecule. Science, 251, 401–407.

28. Sclavi,B., Sullivan,M., Chance,M.R., Brennowitz,M. and Woodson,S.A. (1998) RNA folding at millisecond intervals by synchrotron hydroxyl radical footprinting. Science, 279, 1940–1943.

29. Swisher,J., Duarte,C.M., Su,L.J. and Pyle,A.M. (2001) Visualizing the solvent-inaccessible core of a group II intron ribozyme. EMBO J., 20, 2051–2061.

30. Zhuang,X. and Rief,M. (2003) Single-molecule folding. Curr. Opin. Struct. Biol., 13, 88–97.