RNA Detection

DOI: 10.1002/anie.201104425

Fluorescence Detection of Intron Lariat RNA with Reduction-Triggered Fluorescent Probes**

Kazuhiro Furukawa, Hiroshi Abe, Yasutsugu Tamura, Rei Yoshimoto, Minoru Yoshida, Satoshi Tsuneda, and Yoshihiro Ito**

Lariat RNAs (LaRNAs) with 2′,5′-branched linkages are well-recognized intermediates by both the group II introns and the spliceosomes of higher organisms, and play an important role in biological RNA splicing.[1] In this process, the maturation of mRNA is achieved through a series of transesterification reactions that ultimately lead to the excision of noncoding regions (introns) from the pre-mRNA in the form of RNA lariats. The branch point of these molecules is a highly conserved adenosine residue that is connected to the loop and tail extensions through vicinal 2′–5′ and 3′–5′ phosphodiester linkages, respectively (Figure 1 a). Some group II intron LaRNAs, which are referred to as mobile group II introns, can be found in bacterial and organellar genomes and can function as parasitic RNAs or mobile genetic elements that migrate within a genome or into new genomes.[2] In addition, intron LaRNAs cause gene silencing by behaving as microRNA.[3] LaRNA was found not only in introns but also in mature mRNA. Most mRNAs are capped at their upstream terminus with a modified guanosine residue that protects the mRNA against degradation, however Nielsen et al. reported that an mRNA referred to as I-*Dir* I is capped by a circularized RNA.[4] The upstream terminus of mature I-*Dir* I mRNA is a circle that results from a branching reaction in which a 2′-hydroxy group near the beginning of the mRNA reacts with a nearby phosphodiester linkage. Thus, LaRNAs potentially have a large number of functions in biological processes.

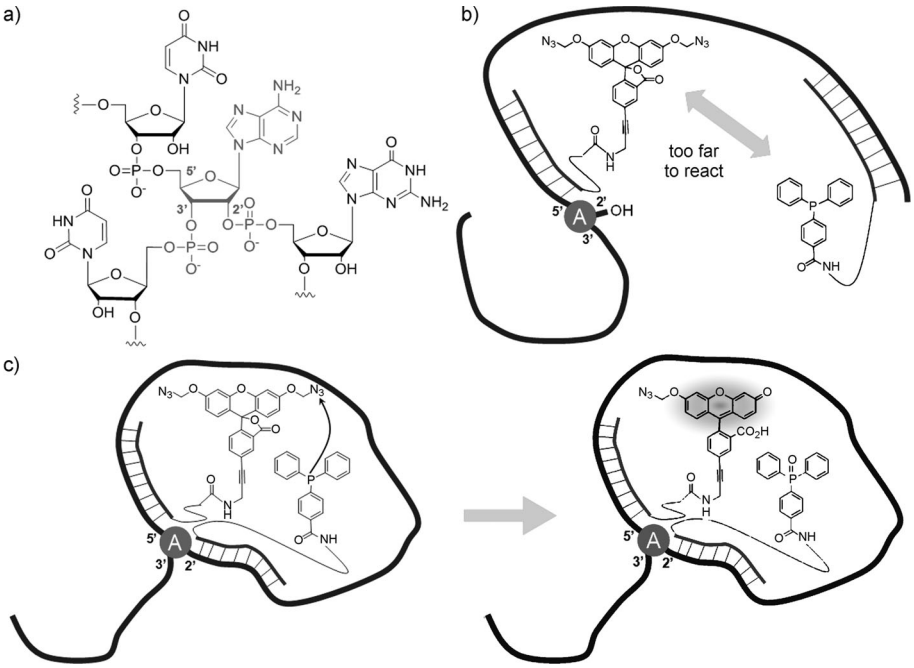

Figure 1. a) Chemical structure of the branched point of lariat RNA. Detection of intron b) prelariat RNA (pLaRNA) and c) lariat RNA (LaRNA) by using a reduction-triggered fluorescent (RETF) probe.

Despite the biological importance of LaRNA, a rapid method for its detection remains to be established. Some methods for the detection of LaRNA have been reported, such as the reverse transcription/polymerase chain reaction (RT-PCR), RNase R treatment, two-dimensional gel electrophoresis, and debranching enzyme assays.[5] In these assays, LaRNA is incubated with enzymes, and after terminating the reaction, the cleaved products and the fragment of LaRNA that includes the branch point are separated by gel electrophoresis and visualized by fluorescence or autoradiography.

[*] Dr. K. Furukawa,[+] Prof. Dr. H. Abe, Y. Tamura, Prof. Dr. Y. Ito
Nano Medical Engineering Laboratory
RIKEN Advanced Science Institute
2-1, Hirosawa, Wako-Shi, Saitama, 351-0198 (Japan)
E-mail: h-abe@riken.jp
y-ito@riken.jp
Prof. Dr. H. Abe
PRESTO, Japan Science and Technology Agency
4-1-8, Honcho, Kawaguchi, Saitama 332-0012, (Japan)
Dr. K. Furukawa,[+] Y. Tamura, Prof. Dr. S. Tsuneda
Department of Life Science and Medical Bio-Science
Waseda University
2-2, Wakamatsu-cho, Shinjuku-ku, Tokyo 162-8480 (Japan)
Dr. R. Yoshimoto, Prof. Dr. M. Yoshida
Chemical Genetics Laboratory
RIKEN Advanced Science Institute
2-1, Hirosawa, Wako-Shi, Saitama, 351-0198 (Japan)
[+] Department of Molecular, Cellular, and Developmental Biology
Yale University.

[**] K.F., H.A., and Y.I. were financially supported by JSPS, MEXT, and NEDO and MEXT, respectively. We thank the BSI Research Resources Center for the mass spectrum analysis and Dr. A. Roth for helpful comments on the manuscript.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201104425.

However, the gel-based assays are laborious and time-consuming, and cannot be utilized for detection of LaRNA structures in living cells.

Recently, we have reported a reduction-triggered fluorescent (RETF) probe[6] that has an azidomethyl fluorescein derivative (Flu-az) as the fluorogenic molecule and could detect nucleic acids based on a fluorescence signal in vitro and endogenous mRNA in living human cells.[6a] The chemistry of the RETF probe is based on a nucleic acid templated chemical reaction[7] and involves the reaction between the azidomethyl group of a fluorescein derivative and triphenylphosphine (TPP) as reducing reagent, both of which are attached to oligonucleotide templates (see Figure 1b,c and Figure S4 in the Supporting Information). Hybridization of these oligonucleotides to the complementary template brings both reactive groups close enough to react with each other. Therefore, they cannot react as a template on the pre-lariat RNA (pLaRNA, Figure 1b), but they can react as a template on the lariat structure and subsequently emit strong fluorescence (Figure 1c). In this study, we report the development of a simple, rapid, and nonenzymatic fluorescence detection method for LaRNAs with RETF probes.

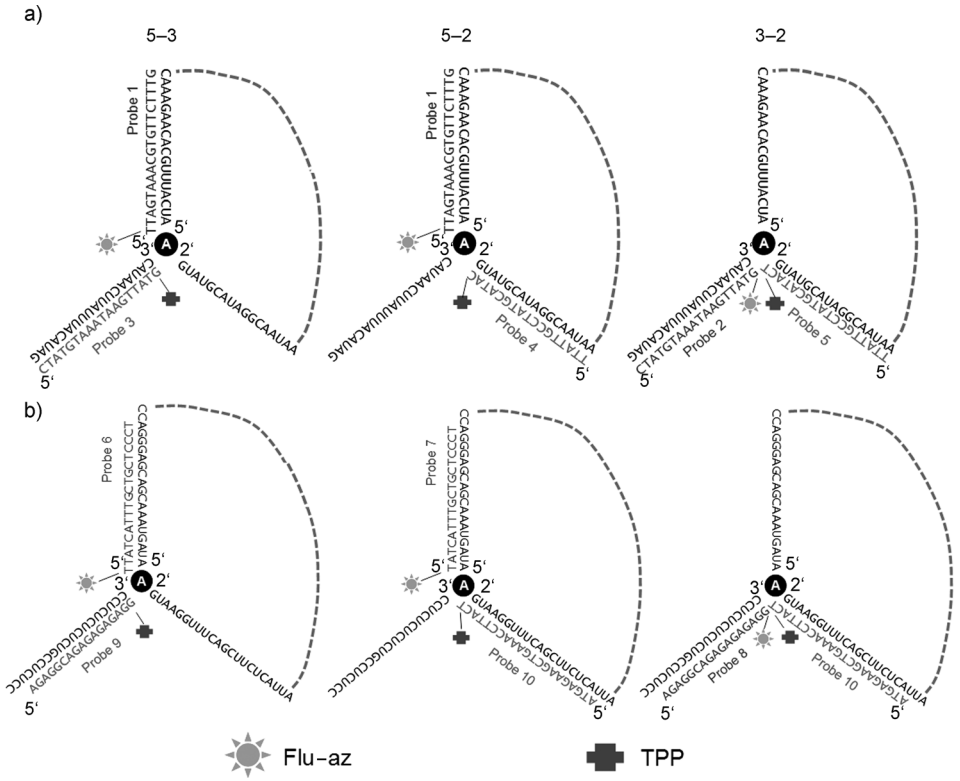

Figure 2. Probe design and sequences for the detection of a) YBL059W (51 nt loop) or b) CDC (230 nt loop) intron lariat RNAs. Only core-branched structures from each lariat RNA are shown. Flu-az = azidomethyl fluorescein, TPP = triphenylphosphine.

We first synthesized two LaRNA sequences to test the utility of the RETF system for the fluorescence-based detection of LaRNA. These sequences are the 69 nucleotide (nt) LaRNA derived from yeast YBL059W intron with a 51 nt lariat loop, and the 258 nt LaRNA derived from chicken δ-crystallin (CDC) intron[5b] with a 230 nt lariat loop. The LaRNA sequences were synthesized by using in vitro transcribed RNA and deoxyribozymes according to the method by Silverman and co-workers (see the Supporting Information for details).[5c,8] The core-branched structures of each LaRNA and probe sequences are shown in Figure 2. We designed three different probe pairs, which hybridize 1) 5′ upstream and 3′ downstream from the branch-site adenosine (5–3 in Figure 2), 2) 5′ upstream and 2′ downstream from the branch-site adenosine (5–2 in Figure 2), and 3) 3′ downstream and 2′ downstream from the branch-site adenosine (3–2 in Figure 2). The 5–3 probe pair can react on both pLaRNA and LaRNAs, therefore, we used the 5–2 or 3–2 probe pairs for the specific detection of lariat structures.

The fluorescence intensities of YBL059W and CDC intron LaRNA were measured after incubation with RETF probes for 30 min at 37 °C (Figure 3). For the specific detection of lariat RNA, the fluorescence signal from LaRNA should be significantly higher than that from pLaRNA. Firstly, we tested whether the RETF probe can detect YBL059W LaRNA (Figure 3a), and, as expected, the 5–3 probe pair can detect both YBL059W LaRNA and pLaRNA. The fluorescence signal was 1.8 ± 0.6 times higher with pLaRNA than with LaRNA. This result might arise from the higher steric accessibility of pLaRNA compared with

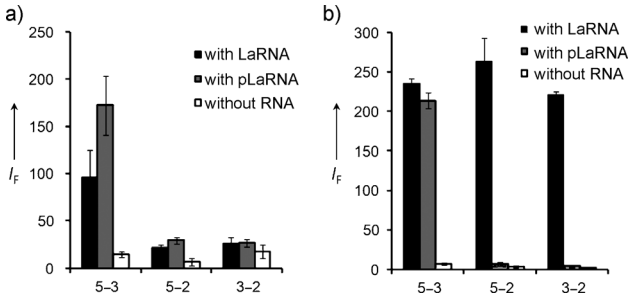

Figure 3. Comparison of the fluorescence detection of a) YBL059W or b) CDC LaRNA and pLaRNA. Reactions were performed in phosphate buffered saline (100 mL, pH 7.4) containing PEG6000 (0.025 %) and calf thymus DNA (1 mg mL^{-1}) with two probes (100 nM) in the presence of LaRNA or pLaRNA (100 nM) at 37 °C for 30 min. Excitation and emission wavelength were 490 nm and 520 nm, respectively. Error bars represent the standard deviation of three independent analyses.

LaRNA. However, no significant differences between the fluorescence signals of LaRNA and pLaRNA were observed when either the 5–2 or 3–2 probe pairs were used. There are two possible explanations for these observations: 1) although both oligonucleotides can hybridize the YBL059W LaRNA, the Flu-az and TPP molecules might not be able to get close enough to react with each other, 2) the Flu-az and/or TPP probes might not be able to hybridize with the branched site of YBL059W LaRNA. To assess scenario (1), an alkyl (C_{12}) or poly(ethylene glycol) linker were inserted between Flu-az and the oligonucleotide (Figure S4), because the insertion of a flexible linker could increase the chemical reactivity. However, the YBL059W LaRNA could not be detected when this modification was used (data not shown). This result indicated that the reason we were unable to detect YBL059W LaRNA is not the low reactivity between Flu-az and TPP (scenario 1), but a low accessibility of the oligonucleotides near the branched sites of LaRNA (scenario 2). To confirm this hypothesis, we synthesized YBL059W branched RNA (bRNA), which does not have a loop region and should therefore be more accessible than LaRNA. Both 5–2 and 3–2 probe pairs could detect the bRNA with significant fluorescence signals, although these signals were lower than that from the 5–3 probe pair (Figure S5). These data suggest that the lower accessibility of the small loop structure with 51 nt is caused by the considerable strain that is induced when probes bind and form double strands with the targets. Therefore, we designed LaRNA with a bigger loop to increase the accessibility of the RETF probes.

The CDC intron LaRNA was successfully detected with all probe pairs (Figure 3b). Both 5–2 and 3–2 probe pairs generate a significantly higher fluorescence signal with the CDC LaRNA than with the pLaRNA, thus indicating that RETF probes specifically detect the lariat structure. The signal-to-background (S/B) ratios of the fluorescence signals were calculated by dividing the fluorescence signal intensities with LaRNA by those with pLaRNA, and were 1.1 ± 0.1, 38.7 ± 16.2, and 52.9 ± 12.5 for the 5–3, 5–2, and 3–2 probe pairs, respectively. Therefore, the sites for both 5–2 and 3–2 probe pairs on CDC LaRNA can be used to detect the CDC intron LaRNA with RETF probes.

These results indicated that it is difficult to detect relatively small LaRNA (< 100 nt) with RETF probes, which is likely due to steric effects. However, the majority of human introns are longer than 1000 nt and the group II intron is 400–1000 nt in size;[1a,9] therefore, most biologically related LaRNAs are expected to have sufficient accessibility to be detectable with RETF probes.

We next conducted an assay under conditions identical to those summarized in Figure 3 except that four LaRNA samples (50 pM–50 nM) were used (Figure 4). The results indicated that the detection limit for LaRNA with the RETF probe is at an LaRNA concentration of around 500 pM. Interestingly, the fluorescence signals with 500 pM and 5 nM LaRNA were higher than one-hundredth and one-tenth of the signal with 50 nM LaRNA, respectively. This result is probably caused by multiple turnover reactions that use target LaRNA as a catalytic template. Given that the volume of a typical mammalian cell is 10^{-12} liter, an RNA concentration of

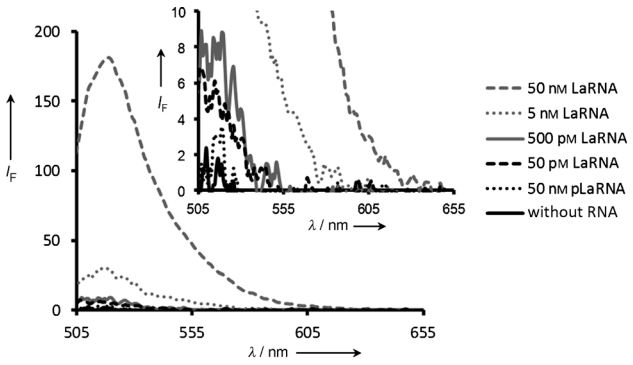

Figure 4. Detection limit of LaRNA with RETF probe. Conditions were identical to those stated in Figure 3 except that the concentration of LaRNA was varied. The 3–2 probe pair for CDC LaRNA was used.

500 pM corresponds to approximately 300 copies per cell. Since the amount of LaRNA in cells should theoretically be equal to that of pre-mRNA, the LaRNAs derived from moderate and abundant mRNAs, of which more than 300 copies per cell exist, could be detected. Generally, the splicing and debranching reactions occur in the nucleus, therefore, the RETF probes should be delivered into the nucleus when they are applied for the visualization of intracellular LaRNA. It has been reported that the bulk of short oligonucleotides are rapidly transported from the cytoplasm into the nucleus when they are introduced into cells with the help of pore-forming agents, such as streptolysin O.[10] We have also reported that RETF probes can be delivered into the nucleus with streptolysin O and that the probes can be used to visualize 28S rRNA in the nucleolus.[6a] The RETF probes that target LaRNA will most likely be delivered into the nucleus in a similar manner. Furthermore, the RETF probes might interfere with the binding of spliceosomes to pre-mRNAs and inhibit the splicing reaction. To validate this hypothesis, we carried out in vitro splicing reactions with nuclear extracts in the presence or absence of probes. As shown in Figure S6, similar amounts of LaRNA were observed in each lane (6.7% and 8.9% for lane 1 and 2, respectively), although possible RNase H cleavage products were observed only in the presence of probes (lane 1). This result indicates that RETF probes have only little influence on the function of the spliceosome and the production of LaRNA in cells.

To prove the efficacy of RETF probes, we next used fluorescence resonance energy transfer (FRET) probes for the detection of CDC intron LaRNA, and compared the potential utility of FRET probes with that of RETF probes. The probe sequences of the FRET probes were identical to those of the 5–2 probe pair for CDC LaRNA. The probe pair was labeled with fluorescein and tetramethylrhodamine (TAMRA) to generate FRET signals (Figure 5a). FRET probes and CDC LaRNA were incubated under the same conditions as RETF probes, and fluorescence spectroscopic analysis was carried out. As shown in Figure 5b, a FRET signal was observed only in the presence of LaRNA. We optimized the distance between the two fluorophores by changing the nucleotide attachment points for the fluoro-

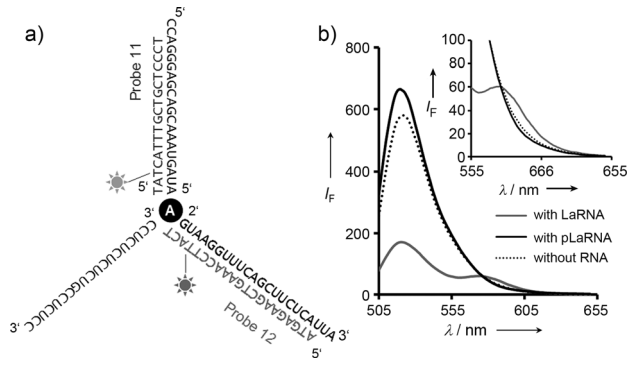

Figure 5. FRET detection of CDC intron LaRNA and pLaRNA. a) FRET probe pair: probe 11 labeled with fluorescein, probe 12 labeled with TAMRA. b) Fluorescence spectrum analysis. Reactions were performed in PBS buffer (100 mL, pH 7.4) containing PEG6000 (0.025%) and calf thymus DNA (1 mg mL^{-1}) with two probes (100 nM) in the presence of LaRNA or pLaRNA (100 nM) at 37°C for 30 min. Excitation and emission wavelengths were 490 nm.

phores, and estimated their FRET values (ratio of FRET signal with LaRNA to FRET signal with pLaRNA; Figure S7).[11] The FRET value 7.3 is obtained when the distance is six nucleotides (Figure 5b and Figure S7). It must be noted that the FRET signal might be improved by optimizing acceptor and/or hybridization sites. However, we successfully detected the CDC LaRNA without any optimization, thus indicating that the RETF system is more straightforward for the detection of LaRNAs than the FRET system.

In summary, we succeeded in the fluorescence detection of intron LaRNA by using RETF probes. Moreover, our RETF probes are superior to FRET probes in terms of the ability to discriminate LaRNAs from pLaRNAs. To the best of our knowledge, this is the first report on the fluorescence detection of intron LaRNA, although there are a few reports on the visualization of splicing through the detection of the exon junction sites by using fluorescent oligonucleotide probes both in vitro[12] and in vivo.[13] Furthermore, this is the first example for the detection of higher-order-structured RNA by using a nucleic acid templated chemical reaction, although the DNA-templated detection of G-quadruplex or hairpin DNA was recently reported.[14] Studies on the real-time detection of LaRNA with RETF probes both in vitro and in vivo are currently under way.

Received: June 27, 2011
Revised: September 22, 2011
Published online: October 21, 2011

Keywords: biosensors · fluorescent probes · lariat RNA · oligonucleotides · RNA structures

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