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Monitoring triplex DNA formation with fluorescence resonance energy transfer between a fluorophore-labeled probe and intercalating dyes

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ABSTRACT

Triplex-forming oligonucleotides (TFOs) are sequence-dependent DNA binders that may be useful for DNA targeting and detection. A sensitive and convenient method to monitor triplex formation by a TFO and its target DNA duplex is required for the application of TFO probes. Here we describe a novel design by which triplex formation can be monitored homogeneously without prelabeling the target duplex. The design uses a TFO probe tagged with a fluorophore that undergoes fluorescence resonance energy transfer with fluorescent dyes that intercalate into the target duplex. Through color compensation analysis, the specific emission of the TFO probe reveals the status of the triple helices. We used this method to show that triple helix formation with TFOs is magnesium dependent. We also demonstrated that the TFO probe can be used for detection of sequence variation in melting analysis and for DNA quantitation in real-time polymerase chain reaction.

Triplex-forming oligonucleotides (TFOs)[1] are oligonucleotides that can associate with duplex DNA in the major groove through Hoogsteen hydrogen bonding between nucleobases. Currently, TFOs with natural nucleobases can bind only to duplex DNA with oligopurine•oligopyrimidine sequences, generating three types of triple helices: (i) a TC triplex, in which the third strand is parallel to the polypurine strand of the DNA duplex in the Hoogsteen conformation, forming TA*T and CG*C+ triplets; (ii) a GA triplex, in which the third strand is antiparallel to the polypurine strand in reverse Hoogsteen conformation, forming CG*G and TA*A triplets; and (iii) a GT triplex, in which the third strand is either antiparallel to the polypurine strand in reverse Hoogsteen conformation, forming CG*G and TA*T triplets, or parallel to the polypurine strand in Hoogsteen conformation, forming CG*G and TA*T triplets (see Ref. [1] for a review).

The association between TFOs and duplex DNA is sequence specific, making TFOs good ligands for DNA targeting without the need for denaturing the double-stranded DNA. Recently, TFOs have been applied in several biomedical research studies such as transcription inhibition [2,3], sequence-specific cleavage [4] and crosslinking [5–8], and chromosome mapping [9]. TFOs have also been explored for their therapeutic use [10–13].

In addition to gene targeting, TFOs have the potential to serve as sequence-specific probes for detecting DNA in vitro. However, two bottlenecks limit the application of TFO probes. First, the TFO target is limited to oligopurine•oligopyrimidine sequences; it is difficult to design a TFO that binds efficiently to arbitrary sequences. Several attempts have been made to solve this limitation. For example, introducing synthesized nucleoside analogues into the TFO has allowed recognition of a pyrimidine that interrupts the oligopurine stretch [14–17]. Modification of the backbones or the sugars [18–21], modification of the 3′ or 5′ termini [22], and insertion of intercalating nucleic acids [23,24] in TFOs improve the affinity to their target duplex and may benefit the association with unfavorable sequences. In vitro selection with SELEX (systematic evolution of ligands by exponential enrichment) has been used to identify the most stable TFO from a library of oligonucleotides [25], but this method has yet to be applied to DNA duplexes of arbitrary sequences.

The second bottleneck is the lack of a convenient method to measure the formation of triple helices. Conventional methods to measure triplex formation include gel mobility shift assays, DNase I protection assays, and dimethyl sulfate (DMS) footprinting. These methods are labor intensive and not real time. Ultraviolet (UV) spectrometry is convenient and real time, but it suffers from low sensitivity, low resolution, and low throughput [26,27]. Several researchers have used fluorescence resonance energy transfer (FRET) and melting analysis to monitor the formation of triple

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[1] *Abbreviations used:* TFO, triplex-forming oligonucleotides; SELEX, systematic evolution of ligands by exponential enrichment; DMS, dimethyl sulfate; UV, ultraviolet; FRET, fluorescence resonance energy transfer; PCR, polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid; T_m, melting temperature; Ts1/Ts2, first/second transmission.

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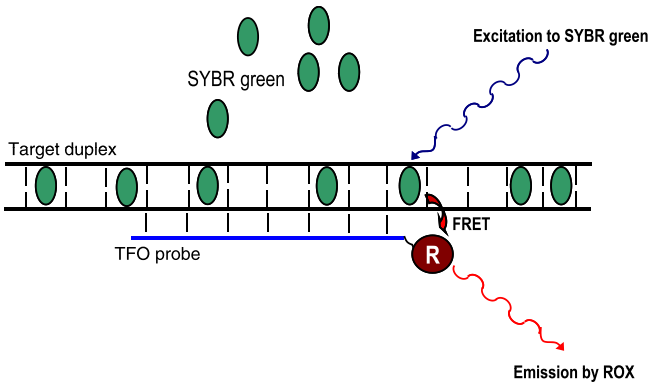

Fig.1. Schematic diagram of the experimental design. The TFO probe (represented by a blue line) is labeled with ROX (represented by an R), which undergoes FRET with SYBR green I (represented by green circles) intercalating into the target DNA duplex (represented by black lines), resulting in specific fluorescence emission. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

helices [28,29]. This approach uses a pair of fluorophores, a donor and an acceptor, to label the target duplex and its TFO, respectively. The formation of the triple helix brings the two fluorophores close to each other, allowing FRET to occur. Darby and coworkers reported a modified design that labels a fluorophore and a quencher on the oligonucleotide that can fold into duplex, triplex, and quadruplex structures [26]. Different structures result in different fluorescence melting profiles that can be measured by a real-time polymerase chain reaction (PCR) instrument such as a Roche LightCycler 480.

Measuring the specific emission from FRET between the fluorophore pair on the TFO and the target duplex allows sensitive, real-time, and high-throughput detection of triplex formation. However, this format is not suitable for in vitro detection of DNA because in most situations the target DNA is unlabeled. Here we demonstrate a novel FRET design to detect the formation of triple helices without prelabeling the target duplex. This design uses a fluorophore-labeled TFO probe that undergoes FRET with a double-stranded DNA intercalating dye (the concept is depicted in Fig. 1). Thus, the association and dissociation of the TFO probe can be monitored.

Materials and methods

TFO probes, primers, and target DNAs

The ROX-labeled TFO probes with GA-rich (GA–TFO) or TC-rich (TC–TFO) sequences were synthesized by MWG Biotech (Ebersberg, Germany). The target duplex DNAs and PCR primers were synthesized by Purigo Biotech (Taipei, Taiwan). DNA duplex 1 is a pair of complementary oligonucleotides with a stretch of oligopurine•oligopyrimidine pairs in the center. Duplex 2 has the same sequence as duplex 1 except for a G-to-T change that interrupts the oligopurine•oligopyrimidine stretch. Duplex 3 has the same sequence as duplex 1 but lacks the 5′ region outside of the oligopurine•oligopyrimidine stretch. Duplex 4 has the same sequence as duplex 1 but lacks both the 5′ and 3′ regions. GA–TFO probe has a GA-rich sequence and a ROX fluorescent dye labeled at its 3′ end. TC–TFO probe has a TC-rich sequence and a ROX at its 3′ end. The deoxycytidines in the TC–TFO were substituted with 5-methyldeoxycytidines. The sequences of these oligonucleotides are shown in Fig. 2A. For preparation of the target duplex, equal amounts of the paired oligonucleotides were dissolved in an annealing buffer (10 mM Tris–HCl [pH 8.0], 1 mM ethylenediaminetetraacetic acid [EDTA], and 50 mM NaCl), heated to 95 °C for 5 min, and slowly cooled to room temperature (1 °C/min) to allow formation of duplex DNA. The DNA duplexes were stored at −30 °C until use. To make the PCR templates, the DNA duplexes were ligated into a vector (T&A vector, RBC Bioscience, Taipei, Taiwan) (Fig. 2B) and transformed into DH5α *Escherichia coli* cells. The plasmids extracted from the *E. coli* were used as PCR templates. A pair of PCR primers was designed to amplify a 68-bp product that contains the target region (Fig. 2B).

Triple helix formation and melting analysis

Unless otherwise stated, triplex formation was carried out in 10 mM Tris–HCl (pH 7.2) containing 10 mM $MgCl_2$ and 0.5× SYBR green I (Invitrogen, Carlsbad, CA, USA) for GA–TFO or in 10 mM sodium acetate buffer (pH 5.0) containing 10 mM $MgCl_2$ and 0.5× SYBR green I for TC–TFO. The concentrations of the target duplexes and the TFO probes were 0.95 and 1 μM, respectively. The reaction mixture was incubated at 37 °C for 1 h before melting analysis and mobility shift assays. Melting analysis was performed on a Roche LightCycler 480. The reaction mixture was slowly heated from 37 to 95 °C at a rate of 0.1 °C/s. The change in fluorescence emission was measured at two acquisitions/°C in two channels: (i) channel 533, excitation at 483 nm and detection at 533 nm, for SYBR green I emission; and (ii) channel 610, excitation at 483 nm and detection at 610 nm, for ROX emission. ROX emission was generated through FRET between the intercalated SYBR green I (donor) and the ROX dye (acceptor).

Color compensation

Because the SYBR green I emission spectrum overlaps with the ROX emission spectrum at 610 nm, fluorescence due to SYBR green I emission can be detected in channel 610. This SYBR green I signal in channel 610 can be corrected by color compensation analysis. The color compensation algorithm is built in the LightCycler 480 software. The color compensation experiment measures the main emission of SYBR green I in channel 533 and its bleeding fluorescence in channel 610, and it generates a relationship (or color compensation object) of signal intensity between both channels. When applying the color compensation object to filter the signals in channel 610, the bleeding fluorescence from SYBR green I can be subtracted. To conduct the color compensation experiment, 10 μg/ml linearized plasmid DNA (~3000 bp) plus 0.5× SYBR green I was used for channel 533. The ROX-labeled probe (0.5 μM) was used for channel 610. The buffer without plasmid DNA, probes, or SYBR green I was used as a blank. Five replicates of each of the above mixtures were prepared for the compensation experiment. A temperature gradient program was performed, increasing the temperature from 40 to 95 °C and measuring the fluorescence at two acquisitions/°C. Color compensation was selected for this temperature gradient program in the analysis mode. The results were saved as a color compensation object, which was applied in the following experiments. In this study, we generated two color compensation objects for two different buffer systems. One performed in 10 mM Tris–HCl (pH 7.2) buffer containing 10 mM $MgCl_2$ as described above, and the other performed in the PCR buffer (see below).

Mobility shift assay

The TFO probe and the target duplex were mixed to allow triplex formation as described above. The mixture was then loaded onto a 12% polyacrylamide gel (19:1) in a buffer containing 17.8 mM Tris–borate (pH 7.2) and 10 mM $MgCl_2$ for GA–TFO or in a buffer containing 40 mM sodium acetate (pH 5.0) and 10 mM $MgCl_2$ for TC–TFO. Electrophoresis was carried out at 4 °C and 70 V for 2.5 h. The gel was then removed from the electrophoresis apparatus and soaked in its electrophoresis buffer and 1×