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'Cyclicons' as Hybridization-Based Fluorescent Primer-Probes: Synthesis, Properties and Application in Real-Time PCR

Ekambar R. Kandimalla and Sudhir Agrawal*

Hybridon, Inc., 155 Fortune Boulevard, Milford, MA 01757, USA

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Abstract—We have studied the use of 'pseudocyclic oligonucleotides' (PCOs) (Jiang et al. *Bioorg. Med. Chem.* **1999**, *7*, 2727) as hybridization-based fluorescent probes. The resulting fluorescent tag-attached PCOs are called 'cyclicons'. Cyclicons consist of two oligonucleotides linked to each other through 3′-3′ or 5′-5′ ends. One of the oligos is the probe or primer-probe sequence that is complementary to a target nucleic acid (mRNA/DNA), and the other is a modifier oligo that is complementary to one of the ends of the probe oligo. A fluorescence molecule and a quancher molecule are attached at an appropriate position in the cyclicons. In the absence of the target nucleic acid, the fluorophore and the quencher are brought in close proximity to each other because of the formation of an intramolecular cyclic structure, resulting in fluorescence quenching. When the cyclicon hybridizes to the complementary target nucleic acid strand, the intramolecular cyclic structure of the cyclicon is destabilized and opened up, separating the fluorophore and quencher groups, resulting in spontaneous fluorescence emission. Fluorescent studies in the presence and absence of a target nucleic acid suggest that cyclicons exist in intramolecular cyclic structure form in the absence of the target and form the duplex with the target sequence when present. Both the cyclicons are useful for nucleic acid detection. The studies with DNA polymerase on 5′-5′-attached cyclicons suggest that the presence of quencher moiety in the probe sequence does not inhibit chain elongation by polymerase. The experiments with a 5′-5′-attached cyclicon suggest the new design serves as an efficient unimolecular primer-probe in real-time PCR experiments.

Introduction

In recent years, techniques based on the complementary hybridization between oligonucleotides and nucleic acid targets have been widely applied in molecular diagnostics, therapeutics development, and mechanistic and molecular biological studies. As a result of human genome analysis, these techniques have become routine and there is an ever-increasing demand for more rapid, accurate, and effective nucleic acid detection and measurement methods. Fluorescence-based methods are more rapid and sensitive for hybridization detection and measurement than are the methods based on absorbance spectroscopy, calorimetry, and magnetic resonance spectroscopy. The advantage of the fluorescence-based techniques for monitoring complementary hybridization is that they can be used in both solution and solid-phase applications.

The polymerase-chain reaction (PCR) is extensively used in molecular biological and genetics-based research and is increasingly becoming an essential tool for molecular diagnostics. Several homogenous fluorescence assay methods for probing amplification products in PCR reactions have been developed in recent years. These include TaqMan,[1,2] molecular beacon,[3] hairpin-primer,[4] and scorpion.[5]

We have designed oligodeoxynucleotides containing two segments attached through their 3′-3′ or 5′-5′ ends.[6] One of the segments of these oligonucleotides is 20 nucleotides long and is complementary to a target nucleic acid, and is referred to here as the 'probe' or 'primer-probe' oligo. The other segment is six to eight nucleotides long and is complementary to the 3′- or 5′-end of the primer-probe segment. This short segment is referred to here as the 'modifier' oligo, because it modifies the structure and fluorescence of the primer-probe depending on the absence or presence of the target nucleic acid strand. As a result of complementarity between the primer-probe and modifier oligos, they form an intramolecular cyclic structures in the absence of the target. When the single-stranded region of cyclicon hybridizes to the complementary sequence on a target nucleic acid, the cyclic structure opens up and a linear duplex is formed between the cyclicon and the target nucleic acid. We have attached a fluorophore and a quencher at the

*Corresponding author. Tel.: +1-508-482-7500; fax: +1-508-482-7791; e-mail: sagrawal@hybridon.com

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appropriate positions on this oligonucleotide such that when the oligonucleotide is in the cyclic form (in the absence of the target nucleic acid), the fluorophore and quencher are brought close together and the fluorophore does not fluoresce because of fluorescence resonance energy transfer (FRET). When the primer-probe sequence binds to the complementary sequence on a target nucleic acid, the cyclic structure is opened up and the fluorophore and quencher are separated far enough to disrupting FRET between the donor and acceptor molecules, resulting in spontaneous fluorescence. We report here the synthesis, properties and use of cyclicons in PCR-related applications.

Results and Discussion

Cyclicon design and synthesis

The cyclicons are designed to contain a long primer-probe and a short modifier oligo attached through a 3′-3′ or 5′-5′ linkage. The primer-probe studied herein was a 20 nucleotide-long sequence that is complementary to a portion of the human MDM2 mRNA. The modifier oligo is complementary to six to eight nucleotides at the 3′ or 5′ end of the primer-probe sequence. If the modifier oligo is complementary to the 5′ end of the primer-probe, the two oligos are attached through a 3′-3′ linkage. If the modifier oligo is complementary to the 3′ end of the primer-probe, the two oligos are attached through a 5′-5′ linkage.

Fluorescent cyclicon probe design

To detect the hybridization between the probe sequence of cyclicon and the target nucleic acid, a fluorophore (fluorescein) or fluorescence resonance energy donor (FRED) was attached to the free end (3′ or 5′) of the modifier oligo. A fluorescence quencher (DABCYL) or fluorescence resonance energy acceptor (FREA) on a thymine base (at the 5-position) was incorporated in the primer-probe sequence (Fig. 1). We attached the quencher at the 5-position of thymine in the present study; these molecules can also be attached on at other positions, including the 2′-position of a sugar moiety.[10] In the absence of a target nucleic acid, cyclicons form intramolecular cyclic structures; in this state, the fluorophore and the quencher are brought in close proximity to each other (Fig. 1A), resulting in the loss of fluorescence because of FRET. In the presence of a target nucleic acid sequence in solution, the probe sequence hybridizes to the complementary sequence on the target, destabilizing the intramolecular cyclic structure and causing it to open up, resulting in spontaneous fluorescence emission. The presence of fluorescence indicates hybridization between the target nucleic acid and the probe sequence of the cyclicon (Fig. 1).

Cyclicon structure

We examined if cyclicons form an intramolecular cyclic structure (Fig. 1A) and/or intermolecular cyclic or linear (Fig. 1B) structures by incubating a small amount (~20 nM) of fluorescent cyclicon with increasing concentrations (up to 1:1000 ratio) of the same cyclicon synthesized without fluorescent and quencher tags, and measuring the fluorescence. If cyclicons do not form intermolecular linear or cyclic structures, there should not be any fluorescence signal in solution as a result of excess non-fluorescent cyclicon in solution. The inset in Figure 1 shows that the increase in fluorescence as a result with increasing concentration of non-fluorescent cyclicon is minimal. When a large excess of target DNA over non-fluorescent cyclicon was added to the same solution, however, full fluorescence was detected (Fig. 1 inset). These results suggest that cyclicons exist in intramolecular cyclic structure form in the absence of the target sequence. In addition, concentration-dependent UV thermal melting studies of cyclicons alone showed T_{ms} within ±1.0 °C, suggesting intramolecular cyclic structure formation in the absence of the target sequence (data not shown).

Detection of target nucleic acid with cyclicons

We monitored hybridization of the cyclicon with the target nucleic acid and measured the fluorescence increase as a function of target nucleic acid concentration. Figure 2A

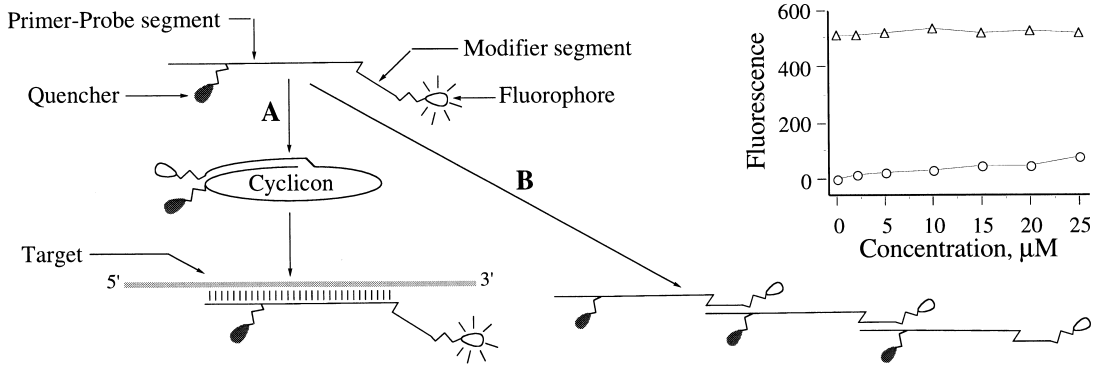

Figure 1. Schematic representation of cyclicon structure and its hybridization with a complementary target nucleic acid strand. (A). Cyclic structure formation brings fluorophore and quencher molecules in close proximity, resulting in FRET. When the cyclicon binds to the target strand, the cyclic structure is destabilized to open resulting in fluorescence emission. (B). Possible intermolecular linear structure of cyclicons. Inset: Fluorescence of cyclicon **3** with increasing concentrations of the same sequence synthesized without fluorophore and quencher tags (o), and the same solution in the presence of a complementary oligodeoxynucleotide strand (Δ).

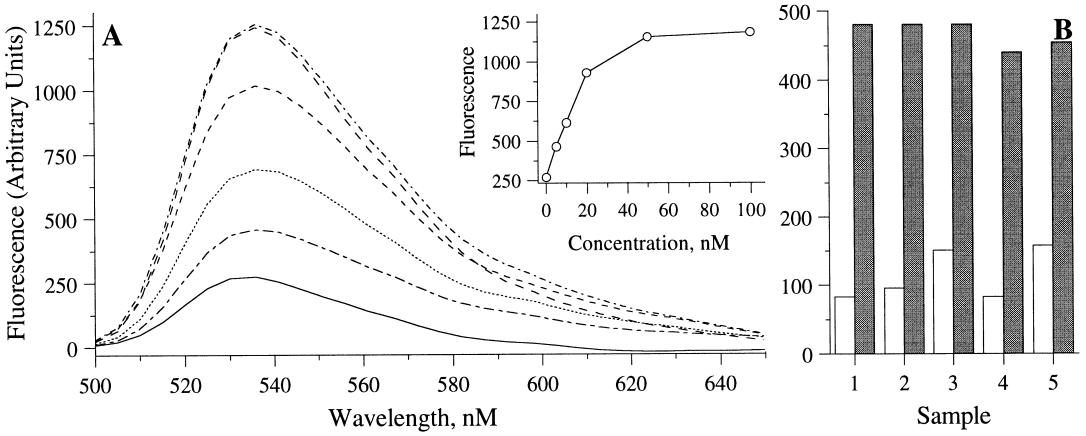

Figure 2. (A). Fluorescence spectra of cyclicon **1** alone (—) and in the presence of increasing concentrations of complementary oligodeoxynucleotide as determined by ABI Prism 7700 Sequence Detection System in plate-read mode. Inset: A plot showing linear increase in fluorescence as a function of increasing target concentration in solution. (B). Demonstration of sequence-specificity of cyclicons. Cyclicon **2** (20 nM) in the absence (white bars) and presence (shaded bars) of DNA target (50 nM). In 1, no other non-specific oligonucleotides were added. In 2, a 20-mer random oligonucleotide sequence (1 μM) was added. In 3, two 20-mer oligonucleotides (1 μM) that did not have any complementarity to the cyclicon were added. In 4, four 20-mer oligonucleotides (2 μM) that did not have any complementarity to the cyclicon were added. In 5, eight 20-mer oligonucleotides (2 μM) that did not have any complementarity to the cyclicon were added.

shows fluorescence spectra of cyclicon **1** in the absence and presence of a DNA target nucleic acid strand as determined by ABI Prism 7700 Sequence Detector in the plate-read mode. The background fluorescence in the absence of a target was the result of the greater distance between the fluorophore and quencher in 3′-3′ linked cyclicons. The addition of a DNA target strand to the solution increased fluorescence as a result of hybridization of the cyclicon probe sequence to the target nucleic acid strand and opening up of the intramolecular cyclic structure (Fig. 2A). The linear increase in fluorescence with the increase in target concentration (Fig. 2A inset) suggests that the method can be used for quantitative detection of the target nucleic acid sequence in solution.

To verify if full fluorescence was emitted upon hybridization of cyclicon to the target nucleic acid, cyclicons alone and in the presence of the target DNA were treated with DNase I and the fluorescence was measured (data not shown). The fluorescence readings were within ±10% of those observed without DNase I treatment in the presence of target nucleic acid, suggesting that full fluorescence was emitted upon hybridization of the cyclicon to the target nucleic acid under study.

Effect of nature of linkage in cyclicons

The quencher is incorporated in the probe sequence in such a way that when the cyclic structure is formed, the fluorophore comes close to the FREA and the fluorescence is quenched completely. Examination of fluorescence of cyclicons **1**–**4** alone showed that 3′-3′ -attached cyclicons **1** and **2** have had higher background fluorescence than 5′-5′ -attached cyclicons **3** and **4**. This difference could reflect the greater distance between the fluorophore and quencher in 3′-3′ -attached cyclicons as a result of 3′-skewing of the two strands of the duplex DNA. It would be appropriate not to skip a base between the fluorophore and quencher in 3′-3′ -attached cyclicons to reduce background fluorescence. The optimum distance between the FRED and FREA molecules would be about 70 to 100 Å to disrupt FRET and attain full fluorescence.[11,12] We maintained a distance of about 19 internucleotide phosphate bridges between the donor and acceptor molecules. When the cyclicon hybridizes to the target nucleic acid sequence, the FRED and FREA molecules are pulled apart about 70 Å, effectively diminishing FRET between the molecules and resulting in spontaneous fluorescence emission. The linkage between the probe and the short oligos can be either 3′-3′ or 5′-5′ as required for the application of the cyclicon. For example, for use in cellular studies, a 3′-3′ linkage would be more appropriate because of higher stability of 3′-3′-attached oligonucleotides against nucleases in in vitro and in vivo studies,[6] whereas for a primer in PCR reactions, a free 3′ end containing 5′-5′-attached cyclicon is more appropriate.

Specificity of hybridization of cyclicons to target nucleic acid

To determine the specificity of cyclicons, a synthetic mixture containing one to eight different oligonucleotide sequences (20- to 24-mers) was made up and mixed with cyclicons in the absence and presence of perfectly complementary target DNA. In the absence of a matched target DNA, cyclicons showed no or little fluorescence (Fig. 2B). When the perfectly matched target DNA strand was added to the same solution, fluorescence was detected, suggesting that the intramolecular cyclic structure of the cyclicon was opened up in the presence of a target nucleic acid but not in the presence of non-targeted nucleic acid sequences.

Effect of the presence and position of Dabcyl on primer extension

To determine if the presence of quencher on a thymine of the primer would interfere with Taq polymerase extension, we have synthesized four 20-mer oligodeoxynucleotides with Dabcyl-thymine at nucleotide positions 2, 3, 5, and 7

from the 3′ end and compared the primer chain extension with that of a primer without Dabcyl. The results showed extension of each primer by Taq-polymerase (Fig. 3), suggesting that the presence of Dabcyl in the primer did not interfere with the chain extension activity of polymerase. This allows the flexibility to incorporate the quencher in the sequence at appropriate sites.

Cyclicons as unimolecular primer-probes in real-time PCR amplification

The 5′-5′-attached cyclicon can be used as an unimolecular primer-probe to monitor real-time RT-PCR amplification as shown schematically in Figure 4A. The 5′-5′-attached cyclicon has two 3′ ends. Only the primer-probe oligo segment is complementary to the MDM2 RNA and this 3′ end is extended when it recognizes and hybridizes to the RNA in the first round of RT-PCR. The 3′ end of the modifier oligo is not extendible, because the modifier oligo does not bind to the RNA, and its 3′ end is blocked by fluorescein moiety.

To validate the use of cyclicon as primer-probe we performed RT-PCR of MDM2 RNA extracted from JAR cells as described in Materials and Methods. We have compared the amplification results obtained with TaqMan primers and probe with those of the cyclicon primer-probe (Fig. 4B). In these experiments, the forward primer was the same in both the reactions and the bimolecular TaqMan reverse primer and probe were replaced with a unimolecular 5′-5′-attached cyclicon primer-probe. The plots in Fig. 4B suggest that the 5′-5′-attached cyclicon served as an efficient unimolecular primer-probe. In order to examine the amplification product, we labeled the 5′ end of each product from the PCR reactions with ^{32}P and analyzed them by denaturing PAGE. The results showed the presence of the expected length amplification product in which cyclicon was used as the primer-probe, as was the case with the TaqMan primer (data not shown).

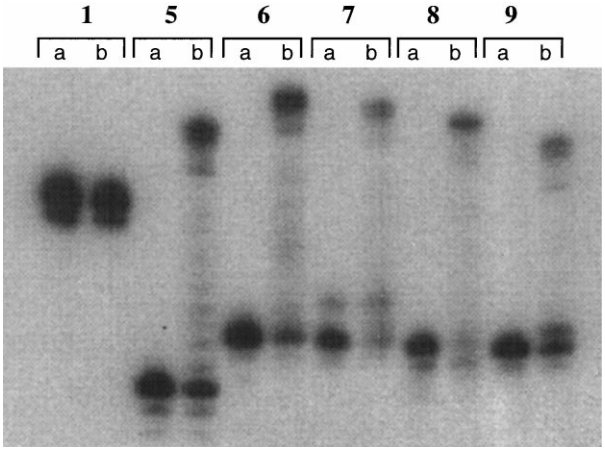

Figure 3. An autoradiogram showing DNA polymerase chain extension on the 40-mer template using different primers containing Dabcyl at different positions on the primers. The lane numbers shown on the top of the gel correspond to primer number given in Experimental. The lanes marked 1 contained cyclicon **1**, shows no extension. The labels a and b represent reaction mixture before and after extension, respectively.

Quantitaion of MDM2 RNA using cyclicons as the primer-probe in real-time RT-PCR

To examine further the applicability of a cyclicon primer-probe for the determination of mRNA levels in unknown samples by real-time quantitative RT-PCR, we have constructed a standard curve with known amounts of MDM2 mRNA extracted from JAR cells (Fig. 5A) using cyclicon **3** as a reverse primer-probe. The standard curve shown in Fig. 5B suggests that the method could be applicable for accurate measurement of MDM2 mRNA in unknown samples.

Mechanism of signaling

To examine the mechanisms of fluorescence signaling by cyclicon during PCR cycles, we plotted the fluorescence signal for PCR cycles 14–40 for both the TaqMan probe and cyclicon (Fig. 6). As expected, the signaling mechanisms were different for the two probes and they showed different characteristics. As seen in Figure 6A, the TaqMan probe registered an increase in fluorescence signal with amplification and never showed a decrease in fluorescence signal as expected. In contrast, the cyclicon gave an increase in the fluorescence signal with amplification and a lower fluorescence signal during the 60 °C hold (Fig. 6B).

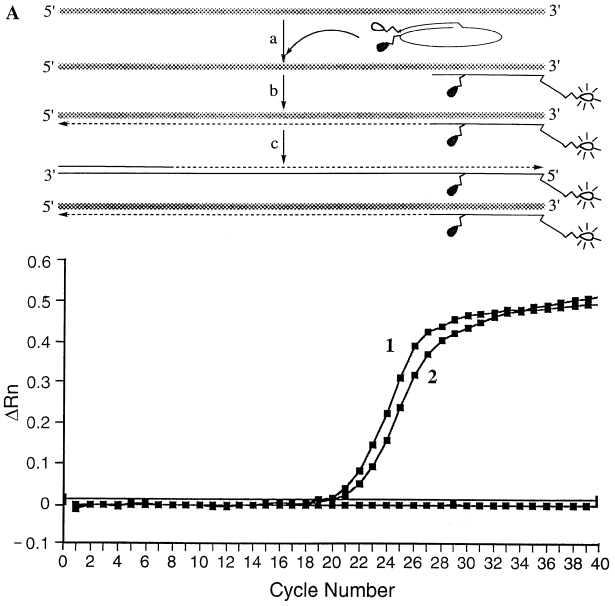

Figure 4. (A). Representation of polymerase chain extension with a 5′-5′-attached cyclicon as a primer-probe in RT-PCR. Thick lines represent RNA template strand that requires to be amplified. Thin lines represent forward and reverse primers. Dotted lines represent chain elongation and its direction. Step a: Hybridization of cyclicon primer-probe to the RNA strand. Step b: Extension of reverse primer in the first cycle of reverse transcription cycle. Step c: Extension of forward primer and PCR amplification of cDNA. (B). Real-time amplification plots, ΔRn versus cycle number, for MDM2 mRNA extracted form JAR cells using cyclicon **3** as a unimoleuclar reverse primer-probe (curve 1), and TaqMan primers and probe (curve 2). Buffer and no-template control plots are at the base line.

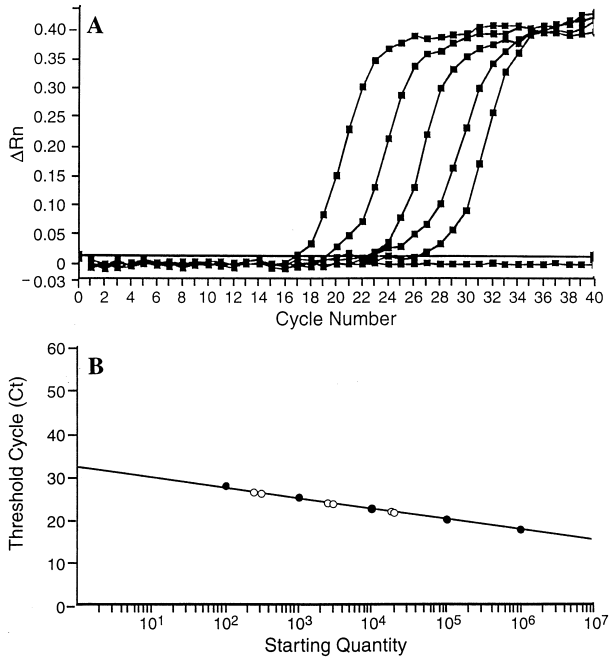

Figure 5. (A). Real-time amplification plot, ΔRn versus cycle number, for various concentrations of MDM2 mRNA extracted form JAR cells using cyclicon 3 as reverse primer-probe. (B). Standard curve obtained from the data shown in (A) and the concentrations measured for unknown samples (open circles).

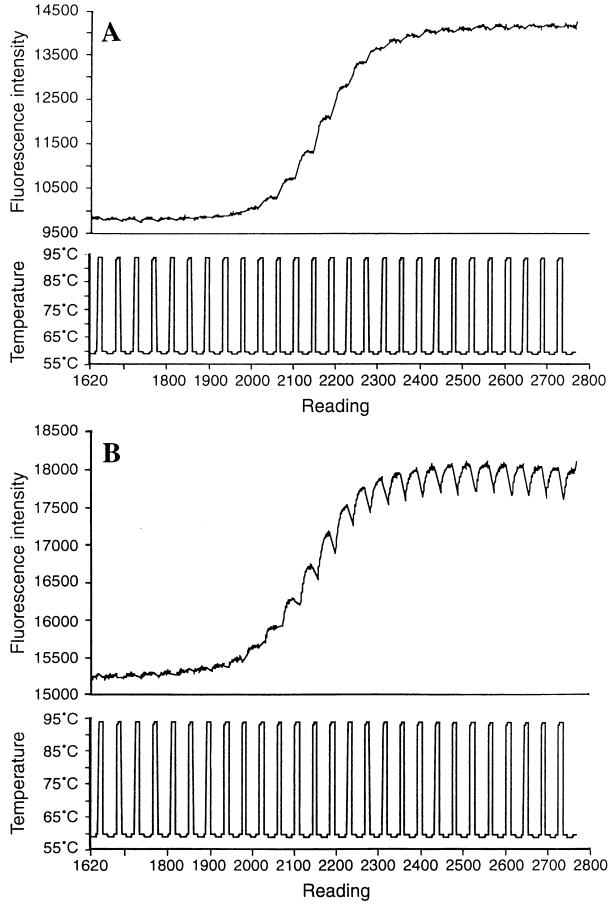

Figure 6. Plots showing change in fluorescence pattern during amplification in each cycle with (A). TaqMan probe, and (B). cyclicon 3.

Conclusions

We have designed and developed novel, efficient probes for the detection of complementary nucleic acids in solution. The 3′-3′-attached cyclicon is useful as a probe in both end-point and real-time PCR detection, as is the case for the molecular beacon. The 5′-5′-attached cyclicon is useful as an integrated primer-probe in real-time PCR detection. The new design is better than the TaqMan probe, in which a probe is labeled with a quencher and fluorophore on either end. The TaqMan probe is cleaved by the 5′-exonuclease activity of DNA polymerase during PCR, releasing free fluorophore and thereby increasing the fluorescence signal.[1,2] The placement of fluorophore and quencher at the two ends of the probe causes increased fluorescence background, because of decreased FRET, with the inverse sixth power of the distance between the fluorophore and quencher.[12] Cyclicons can be used with polymerases devoid of nuclease activity. Cyclicons function like recently introduced molecular beacons[3] and scorpions.[5] The molecular beacon works as a probe but not as a primer unless an extension is attached, as in the case of hairpin primer-probes,[4] and the scorpion serves as a primer-probe but not as a probe alone. In that sense, cyclicons can be used for either function. The integrated primer-probe function of cyclicons is an important step forward in PCR detection and diagnostics without compromising the total length of the oligonucleotide (compared with the length and modifications incorporated in the scorpion) and related costs. In addition, the use of a unified primer-probe in PCR detection simplifies the reaction set up and avoids unnecessary carry-over contaminations. Cyclicons can also be directly attached to solid supports to use on chips for high-throughput screening, solid-phase PCR, and other applications. Studies on these applications are in progress.

Experimental

Synthesis of oligonucleotides

The 3′-3′- and 5′-5′-attached cyclicons were synthesized on a 1 to 2 μmol scale on a Biosearch 8900 DNA synthesizer as described earlier,[6–8] using 3′- and 5′-phosphoramidites as required. The 5′-phosphoramidites, 6-FAM phosphoramidite, 6-FAM-CPG, Dabcyl-T phosphoramidite, and Dabcyl-CPG were obtained from Glen Research Corporation. Deoxynucleoside-3′-phosphoramidites were obtained from Perkin–Elmer. After the synthesis, oligonucleotides were deprotected with concentrated ammonium hydroxide and purified on non-denaturing polyacrylamide gels. After excision and extraction of the appropriate full-length oligonucleotide band from the gels, they were desalted using Waters C_{18} Sep-Pack cartridges. The oligonucleotides were dried in a Speed-Vac under vacuum and the concentrations were determined by absorbance measurement at 260 nm. All the oligonucleotides were characterized by reverse phase HPLC, capillary gel electrophoresis (CGE), polyacrylamide gel electrophoresis (PAGE) and MALDI-TOF mass spectrometry.

The oligonucleotide sequences used in the study are

1. 5′-TGACACCT(Q)GTTCTCACTCAC-3′-3′-ACTGTG-F (+364 to +383);
2. 5′-TGACACCTGT(Q)TCTCACTCAC-3′-3′-ACTGTGGA-F;
3. 3′-CACTCACT(Q)CTTGTCCACAGT-5′-5′-GTGAGT-F;
4. 3′-CACTCACTCT(Q)TGTCCACAGT-5′-5′-GTGAGTGA-F (Q and F stand for quencher DABCYL and fluorophore fluorescein);
   DNA target: 5′-ATCTGTGAGTGAGAACAGGTGTCAC CTT-3′.

Fluorescence measurements were carried out in Perkin–Elmer's ABI Prism 7700 machine in plate-read mode in a 96-well plate. Each sample was 50 μL. All the reactions were carried out in 50 mM Tris, pH 8.0, containing 1 mM $MgCl_2$.

DNA polymerase chain extension

The 40-mer template (3′-TTCCACTGTGGACAA-GAGTGAGTGTCTACATGGACCCAGG-5′) (0.15 A_{260} units) was mixed with 5′-^{32}P-end labeled primers (0.08 A_{260} units) **5**. 5′-TGACACCTGTTCTCACTCAC-3′, **6**. 5′-GGTGACACCTGTTCTCACT*C-3′, **7**. 5′-GTGACACCTGTTCTCACT*CA-3′, **8**. 5′-GACACCTGTTCTCACT*CACA-3′ or **9**. 5′-CACCTGTTCTCACT*CACAGA-3′ (*represents thymine containing Dabcyl at 5-position) in 16 μL of 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 9.0. The samples were heated to 95 °C for 5 min and cooled to room temperature for 15 min before lyophylization. In a final volume of 30 μL, the annealed template/primers were then incubated for 3 h at 37 °C with 5 units of Taq DNA polymerase (Amersham Pharmacia), 200 μM dNTPs (Perkin–Elmer), 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 9.0. The extension products were fractionated by 15% denaturing PAGE and visualized by autoradiography.

RT-PCR

MDM2 mRNA was extracted from JAR cells as described[9] and used in the PCR reactions. All the PCR reagents were purchased from PE-BioSystems, CA. The TaqMan primers and probes used are 3′-GACTAACT-GATGATGGTTCAAGGACA-5′ (forward primer; +311 to +366), 3′-CACTCTTGTCCACAGTGGAACT-5′ (reverse primer; +368 to +389), 3′-TAMRA-TCCTTAGTAGCCTGAGTCCATGTAGACAC-6-FAM-5′ (probe; +338 to +389) and 3′-CATGTTCTC-GAAGTCCTTCTC-5′ (reverse primer-2; +415 to +435). Typically each reaction was carried out in a final volume of 200 μL containing 1×TaqMan EZ buffer, 3 mM manganese, 300 μM each of dATP, dCTP, and dGTP, 600 μM dUTP, 200 nM each of forward and reverse primers, 100 nM TaqMan probe, 0.1 U/μL rTth DNA polymerase, 0.01 U/μL AmpErase UNG and 1 to 50 ng of template RNA. In reactions where the TaqMan probe and reverse primer were replaced with 5′-5′-attached cyclicon, the final concentration of cyclicon was 300 nM. All the RT-PCR reactions were carried out in triplicate (50 μL each). Amplification was performed in Perkin–Elmer's ABI Prism 7700 Sequence Detection System. The thermal cycling parameters were- an initial 2-min hold at 50 °C and 30-min hold at 60 °C, a deactivation hold of 5 min at 95 °C, followed by 38 to 40 cycles of a 20-s denaturation step at 94 °C and a 1-min anneal/extension step at 60 °C.

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