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Genetic Analysis: Biomolecular Engineering
14 (1999) 151–156

GENETIC ANALYSIS
Biomolecular Engineering

Multiplex detection of single-nucleotide variations using molecular beacons

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Abstract

We demonstrate that single-nucleotide differences in a DNA sequence can be detected in homogeneous assays using molecular beacons. In this method, the region surrounding the site of a sequence variation is amplified in a polymerase chain reaction and the identity of the variant nucleotide is determined by observing which of four differently colored molecular beacons binds to the amplification product. Each of the molecular beacons is perfectly complementary to one variant of the target sequence and each is labeled with a different fluorophore. To demonstrate the specificity of these assays, we prepared four template DNAs that only differed from one another by the identity of the nucleotide at one position. Four amplification reactions were prepared, each containing all four molecular beacons, but each initiated with only one of the four template DNAs. The results show that in each reaction a fluorogenic response was elicited from the molecular beacon that was perfectly complementary to the amplified DNA, but not from the three molecular beacons whose probe sequence mismatched the target sequence. The color of the fluorescence that appeared in each tube during the course of the amplification indicated which nucleotide was present at the site of variation. These results demonstrate the extraordinary specificity of molecular beacons. Furthermore, the results illustrate how the ability to label molecular beacons with differently colored fluorophores enables simple multiplex assays to be carried out for genetic analysis.

Keywords: Hybridization probes; Single-nucleotide polymorphisms; Mismatch discrimination; Genetic analysis; Multiplex assays

1. Introduction

Single-nucleotide substitutions represent the largest source of diversity in the human genome. Some of these variations have been directly linked to human disease, though the vast majority are neutral. Even neutral variations are important because they provide guideposts in the preparation of detailed maps of the human genome, serving as essential elements in linkage analyses that identify genes responsible for complex disorders [1]. Although sequencing is adequate for the initial discovery of single-nucleotide variations, simpler, faster, and more automated genotyping methods are needed for routine clinical diagnostics and population studies. High-throughput methods are essential for understanding the distribution of genetic variations in populations, as well as for identifying the genes responsible for genetic disorders. Current alternatives to sequence analysis [2] either miss some single-nucleotide substitutions or are too complex to enable high-throughput assays.

Here we describe a simple, one-tube assay that can determine the identity of a nucleotide at a particular position in an otherwise invariant sequence. In this method, a polymerase chain reaction is carried out to amplify the DNA region of interest and a set of molecular beacons is used to identify the amplified sequence in real time in a sealed reaction tube. Molecular beacons are single-stranded oligonucleotide probes that become fluorescent when they bind to perfectly complementary nucleic acids [3]. Because they are nonfluorescent when they are not bound to their target, they can be used in hybridization reactions without having to separate the probe-target hybrids from the nonhybridized probes. Molecular beacons possess a stem-and-

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1050-3862/99/$ - see front matter
PII: S1050-3862(98)00018-7

loop structure. A fluorophore is covalently linked to one end of the molecule and a quencher is covalently linked to the other end. When not bound to target, the hairpin stem keeps the fluorophore so close to the quencher that fluorescence does not occur. The energy absorbed by the fluorophore is transferred to the quencher and released as heat. However, when the probe sequence in the loop anneals to its target sequence, the rigidity of the probe-target hybrid forces the hairpin stem to unwind, separating the fluorophore from the quencher, and restoring fluorescence (Fig. 1). Because molecular beacons can possess a wide variety of differently colored fluorophores [4], multiple targets can be distinguished in the same solution, using several different molecular beacons, each designed to detect a different target, and each labeled with a different fluorophore.

Molecular beacons are uniquely suited for the detection of single-nucleotide variations because they recognize their targets with significantly higher specificity than conventional oligonucleotide probes [4,5]. Their high specificity is a consequence of their hairpin structure [5]. When a molecular beacon binds to its target sequence, the formation of the probe-target hybrid occurs at the expense of the stem hybrid. Molecular beacons can be designed so that over a wide range of temperatures only perfectly complementary probe-target hybrids are sufficiently stable to force open the stem hybrid. Mismatched probe-target hybrids do not form except at substantially lower temperatures [5]. Therefore, a relatively wide range of temperatures exists in which perfectly complementary probe-target hybrids elicit a fluorogenic response, while mismatched molecular beacons remain dark. Consequently, assays using molecular beacons robustly discriminate targets that differ from one another by as little as a single nucleotide.

In this report we demonstrate that all four variants of a particular nucleotide in a target sequence can be distinguished from one another in a single polymerase chain reaction. Multiplex detection was achieved with four molecular beacons, each designed to bind to only one of the variants, and each labeled with a different fluorophore. The color of the fluorescence that appeared during amplification identified the variant nucleotide.

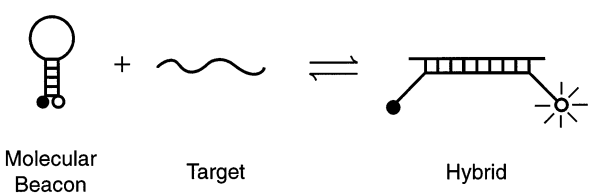

Fig. 1. Principle of operation of molecular beacons. Free molecular beacons are nonfluorescent because the stem hybrid keeps the fluorophore close to the quencher. When the probe sequence in the loop hybridizes to its target, forming a rigid double helix, a conformational reorganization occurs that removes the quencher from the vicinity of the fluorophore, thereby restoring fluorescence.

2. Materials and methods

2.1. Synthesis of molecular beacons

Four molecular beacons were synthesized, each containing 5-nucleotide-long arms and a 16-nucleotide-long probe sequence, the only difference between their sequences being the identity of the 11th nucleotide: fluorescein-5′-CCACGCTTGTGGGTCAACCCCCG-TGG-3′-DABCYL (FAM-G), tetrachloro-6-carboxy-fluorescein-5′-CCACGCTTGTTGGTCAACCCCCGT-GG-3′-DABSYL (TET-T), DABMI-5′-CCACGCTTG-TAGGTCAACCCCCGTGG - 3′- 5-carboxyrhodamine (RHD-A), and tetramethylrhodamine-5′-CCACGCTT-GTCGGTCAACCCCCGTGG-3′-DABCYL(TMR-C), where the underlined nucleotides identify the site of variation. Molecular beacons FAM-G, RHD-A, and TMR-C were synthesized from oligodeoxyribonucleotides that contained a sulfhydryl group at their 5′ end and a primary amino group at their 3′ end, using our previously described protocol that is available at http://www.phri.nyu.edu/molecular_beacons [4]. The succinimidyl ester of the quencher 4′-(4-dimethylaminophenylazo)benzoic acid (DABCYL), was used for the construction of molecular beacons FAM-G and TMR-C, and 4-dimethylaminophenylazophenyl-4′-maleimide (DABMI) was used for the construction of molecular beacon RHD-A. The basic protocol was modified for the synthesis of molecular beacon RHD-A, so that the succinimidyl ester of 5-carboxyrhodamine-6G could be coupled to the 3′-amino group and DABMI could be coupled to the 5′-sulfhydryl group. All of the fluorophores and quenchers were obtained from Molecular Probes. Molecular beacon TET-T was synthesized completely on a DNA synthesizer, utilizing a controlled-pore glass column (Glen Research) to introduce a 4-dimethylaminoazobenzene-4′-sulfonyl group (DABSYL) at the 3′ end of the oligodeoxyribonucleotide and a tetrachloro-6-carboxyfluorescein phosphoramidite (Glen Research) to introduce the fluorophore at the 5′ end of the molecule. Each molecular beacon was purified by high-pressure liquid chromatography.

2.2. Generation of DNA templates that differ at one position

We prepared a set of four DNA templates, each possessing nucleotides 1521–1602 of the RNA polymerase gene of *Mycobacterium tuberculosis* [6], but

differing from one another by the identity of the nucleoside at position 1578. They contained either a thymidine, a cytidine, an adenosine, or a guanosine at that position. These templates were produced in four separate polymerase chain reactions that were initiated with wild-type genomic DNA from *M. tuberculosis*. Each reaction utilized oligodeoxyribonucleotide 5′-GGCCGGTGGTCGCCGCG-3′ as the forward primer and 5′-ACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTNGGTCA-3′ as the reverse primer, where the underlined nucleotide was either A, C, G, or T, depending on which template was being synthesized. Each amplified DNA was purified by gel electrophoresis and its nucleotide sequence was determined to confirm that the desired nucleotide was present.

2.3. Thermal denaturation profiles

For each molecular beacon, five tubes were prepared, each containing 50 μl of 250 nM molecular beacon, 4 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris–HCl (pH 8.0). Four of the tubes contained 1 μM of target oligonucleotide, 5′-GGGGTTGACCNACAAG-3′, where the underlined nucleotide was either A, C, G, or T, and the fifth tube did not contain target. The fluorescence of each solution was monitored, in parallel, as a function of temperature on a spectrofluorometric thermal cycler (Applied Biosystems Prism 7700). The temperature was decreased from 80 to 25°C in 1°C steps of 60 s each. Fluorescence was monitored during each step.

2.4. Polymerase chain reactions

Five polymerase chain reactions were prepared: four were initiated with one of the four DNA templates, and the fifth was initiated without any template. Each of these reactions contained all four molecular beacons along with the forward primer, 5′-GGCCGGTGGTCGCCGCG-3′, and the reverse primer, 5′-ACGTGACAGACCGCCGGGC-3′. Each 50-μl reaction contained 50 nM FAM-C, 50 nM TET-A, 500 nM RHD-T, 500 nM TMR-G, 500 nM of each primer, 2 units of Amplitaq Gold DNA polymerase (Perkin-Elmer), 250 μM dATP, 250 μM dCTP, 250 μM dGTP, 500 μM dUTP, 4 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris–HCl (pH 8.0). 100 000 template molecules were used to initiate each reaction. The thermal cycling program consisted of 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 45 s at 56°C, and 30 s at 72°C. Fluorescence was monitored during the 56°C annealing steps. Prior to carrying out the polymerase chain reactions, the emission spectrum of each molecular beacon hybridized to an excess of perfectly complementary target at 56°C in the presence of the reaction buffer was 'memorized' by the spectrofluorometric thermal cycler. This enabled the complex fluorescent emission spectra generated during the polymerase chain reactions to be decomposed into the contributions from each of the four differently colored molecular beacons that were present in each reaction.

3. Results

3.1. Design of molecular beacons for multiplex detection

To demonstrate that molecular beacons readily distinguish single-nucleotide substitutions, we prepared four molecular beacons that were identical to one another except that each possessed a different nucleotide at one position in their probe sequence and each was labeled with a differently colored fluorophore. These molecular beacons were used together in polymerase chain reactions that were initiated with one of four DNA templates that differed from one another by the identity of a single nucleotide in the region to which the molecular beacons bind. The variant nucleoside in the target, either adenosine, cytidine, guanosine, or thymidine, was located at a position complementary to the site of variation in the molecular beacons. If these molecular beacons only hybridize to perfectly complementary targets, then the color of the fluorescence that results from the amplification of one of the four templates would identify the variant nucleotide.

3.2. Selection of fluorophores

To distinguish the fluorescence of one molecular beacon from another, fluorophores were selected so that their emission maxima were well spaced from each other across the visible spectrum. Any fluorophore can be used as the reporter moiety in molecular beacons, because the fluorophore and the quencher are in such close proximity at the end of the hairpin stem that the energy received by the fluorophore is directly transferred to the quencher and released as heat. This mode of energy transfer does not impose any restriction on the kind of fluorophore that can be quenched by a particular quencher. Thus, the nonfluorescent chromophore DABCYL is able to serve as a universal quencher for all fluorophores [4]. Therefore, in a multiplex detection experiment, the fluorophores can be chosen in accordance with the requirements of the instrument that is used. The spectrofluorometric thermal cycler that we used to monitor the polymerase chain reactions employs a 488 nm laser to excite the fluorophores and it monitors the entire emission spectrum from each reaction in real time. It is therefore desirable that the chosen fluorophores can be excited

reasonably well by 488 nm light and that their emission spectra can be resolved from each other. We chose fluorescein, tetrachlorofluorescein, 5-carboxyrhodamine, and tetramethylrhodamine, which have emission maxima at 515, 538, 553 and 575 nm, respectively. Although, the fluorescence yield of 5-carboxyrhodamine and tetramethylrhodamine was rather low compared to that of fluorescein and tetrachlorofluorescein when excited at 488 nm, the signals from each fluorophore could easily be distinguished from each other and from the background.

3.3. Determination of the window of discrimination

To ensure that each molecular beacon can discriminate single-nucleotide variations, the following criteria should be met at the detection temperature: in the absence of targets, the hairpin stem must be closed; in the presence of perfectly complementary targets, the molecular beacons must form a stable probe-target hybrid; and in the presence of mismatched targets, the molecular beacons must remain closed. Using a DNA folding program to estimate the stability of the hairpin-stem (available at http://www.ibc.wustl.edu/~zuker/), we chose hairpin stems that dissociate at a temperature 7–8°C higher than the 56°C detection temperature. Using the 'percent-G:C rule' [7], we chose a probe sequence length that results in perfectly complementary probe-target hybrids that dissociate at a temperature 7–8°C higher than the detection temperature.

We then synthesized the four molecular beacons and tested them to see whether they met all three criteria. Utilizing the fluorescence of the molecular beacons as an indicator of their state, we measured the fluorescence of each molecular beacon as a function of temperature in the absence of targets, in the presence of perfectly complementary target oligonucleotides, and in the presence of each of the three mismatched target oligonucleotides. The resulting temperature-fluorescence profiles for one of the four molecular beacons is shown in Fig. 2. Significantly, the three mismatched probe-target hybrids melted apart approximately 13°C below the melting temperature of the perfectly complementary probe-target hybrids, and the window of discrimination was centered on 56°C. At this temperature, perfectly complementary probe-target hybrids are stable, mismatched probe-target hybrids are dissociated, and in the absence of targets the hairpin stems of the molecular beacons remain closed. Consequently, at 56°C only perfectly complementary targets elicit fluorescence. Similar results were obtained for the hybrids formed by the other three molecular beacons. Taken together, these results indicate that in a mixture of all four molecular beacons at 56°C, the only molecular beacon that should fluoresce is the one whose probe sequence is perfectly complementary to the target.

3.4. Detection of single-nucleotide variants in real time polymerase chain reactions

We prepared four DNA templates that differed from one another by a single nucleotide at one position using a site-directed mutagenesis procedure. We then performed five polymerase chain reactions, four initiated with one of the four DNA templates, and a control reaction initiated without any template. Each reaction contained all four molecular beacons, along with the other components of the reaction, from the outset of amplification. The emission spectra of each reaction was monitored during the annealing stage of every thermal cycle (56°C). The spectrofluorometric thermal cycler recorded the emission spectrum at the end of each annealing stage. Utilizing stored spectra obtained from pure solutions of each molecular beacon hybridized to a perfectly complementary target, the emission spectra were decomposed into the contributions of each of the four differently colored molecular beacons. Fig. 3 shows the results of these amplification reactions. For each amplification reaction, the fluorescence of each molecular beacon is plotted as a function of the number of cycles of amplification. The results show that the amplification of each target resulted in an increase in the fluorescence of only one of the four molecular beacons, and that molecular beacon was the one that was perfectly complementary to the template DNA. The color of the fluorescent signal identified the variant nucleotide. Thus, molecular beacons are so specific that they can distinguish sequence differences as small as a single-nucleotide substitution.

4. Discussion

The results demonstrate that all four nucleotide substitutions at a particular position in a sequence can be distinguished from one another in a polymerase chain reaction that utilizes differently colored molecular beacons. There are several distinguishing aspects of molecular beacons that enabled their use in these multiplex assays [4]: enhanced specificity due to the presence of the hairpin stem, efficient quenching of fluorophores due to the formation of the stem hybrid, and the use of a universal nonfluorescent quenching moiety. The signals generated from the molecular beacons that were hybridized to perfectly complementary targets were distinguishable from the background fluorescence because the mismatched molecular beacons did not form hybrids and the excess nonhybridized molecular beacons remained highly quenched. Furthermore, the fluorescence spectra of the four molecular beacons were readily distinguished from one another because the quencher was nonfluorescent and did not crowd the emission spectrum.

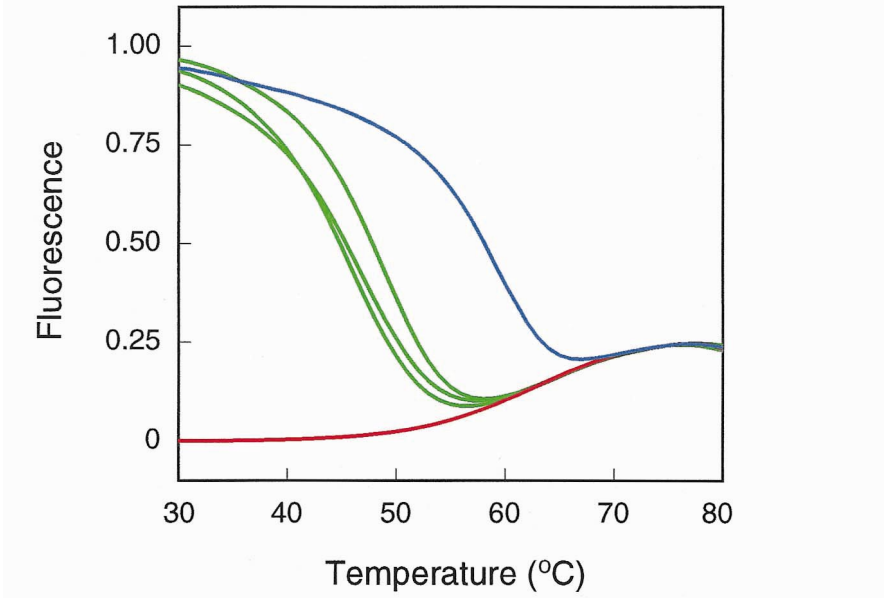

Fig. 2

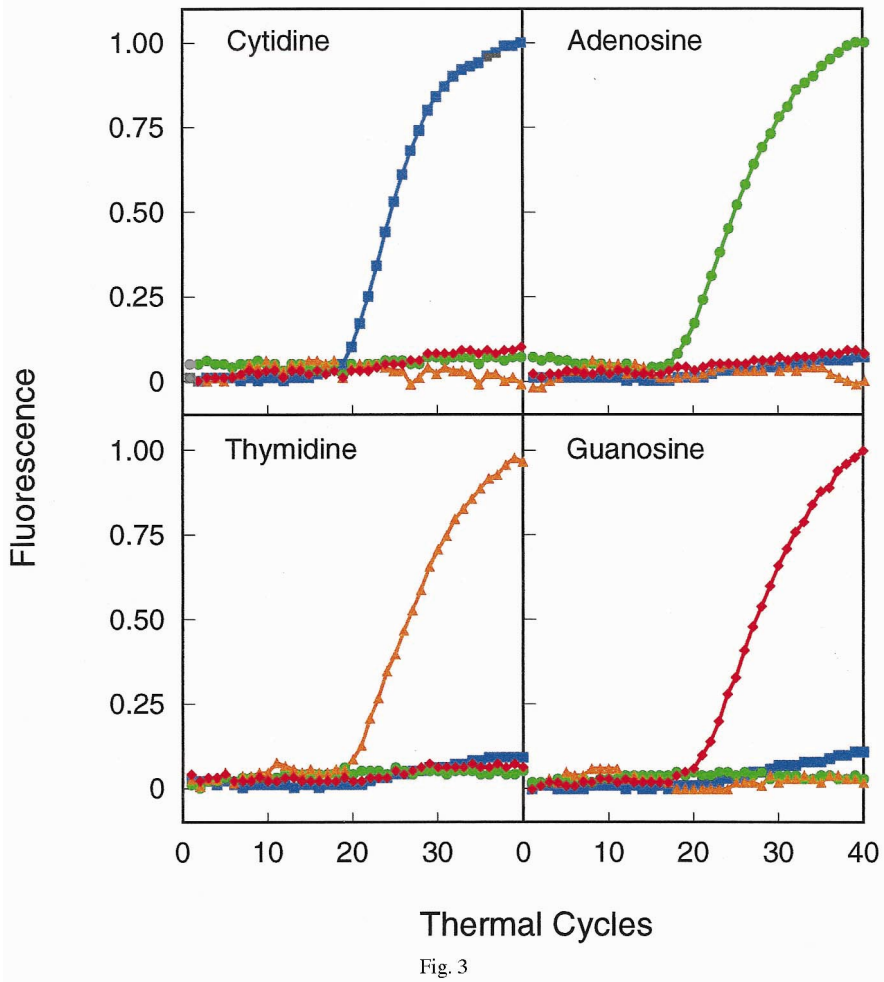

Fig. 3

Fig. 2. Determination of the range of temperatures in which molecular beacon FAM-G can discriminate between perfectly complementary targets and targets possessing a single-nucleotide mismatch. The fluorescence of solutions of molecular beacon FAM-G was plotted as a function of temperature: in the absence of targets (red curve), in the presence of perfectly complementary targets (blue curve), and in the presence of each of the three mismatched targets (green curves).

Fig. 3. Multiplex detection of single-nucleotide variations in real time polymerase chain reactions. Four differently colored molecular beacons were present in each reaction: the fluorescence of molecular beacon FAM-G is plotted in blue, the fluorescence of TET-T is plotted in green, the fluorescence of RHD-A is plotted in orange, and the fluorescence of TMR-C is plotted in red. The nucleoside at the site of variation in the template DNA used to initiate each amplification reaction is indicated at the top of each panel. The color of the fluorescence that developed in each reaction identified the variant nucleotide. No fluorescence developed in a control reaction that did not contain template DNA.

Other detection schemes that monitor the progress of polymerase chain reactions in real time lack some of these attributes [8–11]. For example, assays based on the 5'-endonucleolytic activity of *Taq* DNA polymerase [8,9] utilize conventional linear probes ('TaqMan probes') that have difficulty discriminating single-nucleotide variants. Furthermore, TaqMan probes utilize resonance energy transfer to quench fluorescence, and cannot employ a universal quencher without sacrificing quenching efficiency. For efficient resonance energy transfer, the emission spectrum of the fluorophore must substantially overlap the absorption spectrum of the quencher [12]. Consequently, a different quencher is required to quench each fluorophore, and since these quenchers are themselves fluorescent moieties, there is little room in the spectrum to accommodate all the probes required for a multiplex assay. Furthermore, it is necessary to space the fluorophore and the quencher far apart from each other to maximize the probability of endonucleolytic cleavage occurring between them, further compromising quenching efficiency. Thus, there would be a relatively large background from mismatched TaqMan probes and it would be difficult to perform a multiplex assay that could distinguish all four single-nucleotide variations in a single polymerase chain reaction.

Irrespective of the type of fluorescent probes used in a multiplex assay, the number of different probes that can be distinguished from one another is determined by the design of the instrument. In our experiments, the use of an instrument possessing a monochromatic laser light source for the stimulation of fluorescence limited the number of fluorophores to those that could be excited reasonably well at that wavelength. The use of a variable-wavelength light source would enable additional fluorophores to be used in multiplex detection assays.

We have demonstrated multiplex discrimination among four single-nucleotide variants in reactions in which amplification and detection are carried out at the same time. Since current instruments perform 96 reactions in parallel, these assays can be used to characterize a large population with relative ease. Molecular beacons have already been used to determine the genotype at a human chemokine receptor 5 allele [13], a human chemokine receptor 2 allele [14], a human methylenetetrahydrofolate allele [15], and several alleles of the RNA polymerase gene of *M. tuberculosis* [6]. In addition to carrying out the same genotyping assay on different individuals, a large number of different genotyping assays can be carried out on genetic material from the same individual. Thus, these assays will be particularly useful for linkage analysis. Molecular beacon assays are so simple that the characterization of single-nucleotide polymorphisms should become routine, speeding the exploration of the genetic basis of pathological processes.

Acknowledgements

We thank Amy S. Piatek, Jacqueline A.M. Vet, Diana P. Bratu, Leondios G. Kostrikis, and Musa M. Mhlanga for their advice and assistance. This work was supported by National Institutes of Health Grant HL-43521.

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