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Triple-Stem DNA Probe: A New Conformationally Constrained Probe for SNP Typing

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The specificity of nucleic acid hybridization has been a subject of intensive investigation due largely to its significance in single-nucleotide polymorphism (SNP) analysis. SNPs represent the most abundant form of genetic variations, accounting for 80 to 90% of the differences between two human genomes. SNP analysis is important in population-based genetic risk assessment, molecular diagnostics, pharmaceutical drug development, linkage analysis, and identity testing in forensic applications.[1] Allele-specific hybridization is a technique used in SNP typing among others.[2] In this approach, an allele-specific probe should hybridize to the perfectly matched target sequence while remaining unbound to a target containing a single base mispairing under the same hybridization conditions (Figure 1A, left). The simplicity of the probe and the assay design is an important advantage of this method when compared with pyrosequencing, invasive cleavage, primer extension, or ligation techniques, which use extra protein-mediated steps for SNP discrimination. However, the low specificity of the probe–analyte hybridization is a major challenge in the application of hybridization approaches to SNP typing. Indeed, an oligonucleotide probe of a practical, useful length hybridizes with similar affinities to the perfectly matched nucleic acid and to a target containing a single noncomplementary base.[3]

The formation of at least 15–20 nucleotide hybrids between the probe and the analyte is required to uniquely define a specific fragment in a genome-sized nucleic acid. Hybrids of such length are too stable to be sensitive to a base mispairing, since a single mismatched unit results in an energetic penalty equivalent to only a small fraction of the total energy gain upon duplex formation (Figure 1A, right).[3,4] It is possible to discriminate between two alleles if the energy of the probe–analyte dissociated state (DS) has a value between the energies of associated states (AS) for matched and mismatched duplexes. In this energy disposition, which is optimal for SNP typing, the fully matched complex is formed, while the mismatched hybrid is dissociated. This difference can be easily detected, thus constituting a means for SNP discrimination by hybridization techniques. In order to achieve these conditions, conventional techniques use destabilization of the probe–analyte AS (Figure 1A, right, dashed bars) by employing buffers of low ionic strength, denaturing agents,

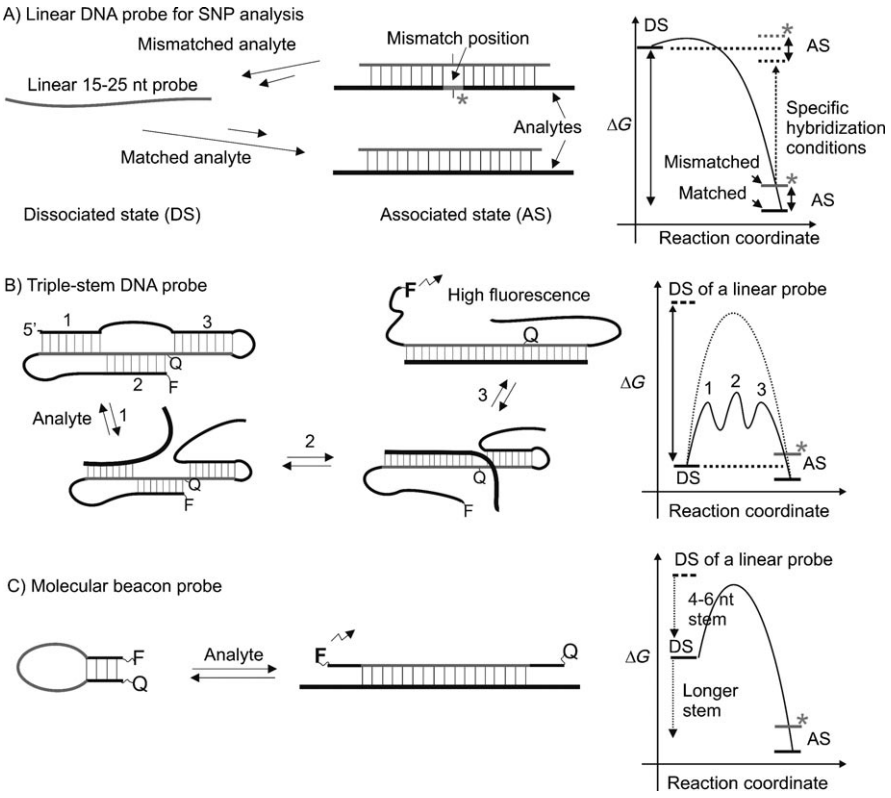

Figure 1. Schemes and energy diagrams for the hybridization of oligonucleotide probes to a nucleic acid analyte. "DS" and "AS" represent probe–analyte dissociated and associated (hybrid) states, respectively. A) Hybridization of a linear oligonucleotide probe. The difference in the energy between matched and mismatched duplexes is much smaller than the energy gap between DS and AS. At high temperature or in denaturing buffers, the DS energy is higher than the energy of the fully matched hybrid, but lower than the energy of the mismatched hybrid. B) A triple-stem DNA probe hybridizes to the analyte by consecutive unwinding of the three stems. Local minima in the energy curve correspond to the partially hybridized analyte. "F" and "Q" indicate a fluorophore and a quencher dyes, respectively. C) Hybridization of a molecular beacon probe. The energy of DS is reduced due to the formation of a 4–6 nt stem. All energy graphs represent theoretical predictions rather than experimental curves.

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or elevated temperatures (usually 50–60 °C). However, the optimal hybridization conditions are sequence-dependent, and the temperature and buffer that enable analysis of one nucleotide substitution often fail in discriminating others. Therefore, optimization of the multiplex hybridization reactions, for example, in microarrays, requires a substantial investment of capital and time. Alternatively, the optimal energy disposition can be created by reducing the free energy of DS. One approach to achieve this situation was originally suggested by Roberts and Crothers for DNA triplexes[4] and later demonstrated for DNA duplexes by introducing conformationally constrained probes, the probes that form competing secondary structures in DS.[5,6] Molecular beacon (MB)[5] and displacement hybridization probes[6] are two well-studied representatives of conformationally constrained probes. In addition to an improved specificity, both oligonucleotide constructs are designed to detect specific nucleic acids in real time, that is, they fluoresce instantly after hybridization to the cognate targets.

Xiao et al. have designed and characterized a new, representative, conformationally constrained probe named a "triple-stem DNA probe" that is capable of detecting nucleic acid analyte fluorescence immediately after hybridization.[7] The probe is a single-stranded oligodeoxyribonucleotide that is folded in a compact secondary structure with three separate relatively short (7-nucleotide) stems (stems 1, 2, and 3 in Figure 1B, top left). In the absence of a complementary target, the fluorescence of the 3′-end fluorophore group is quenched by a closely located quencher. Hybridization of the complementary oligonucleotide unwinds all three stems and separates the fluorophore from the quencher (Figure 1B top right). As a result, the fluorescence is increased by up to 30 times. At the same time, only a negligible fluorescence increase is observed in the presence of the oligonucleotides containing a single noncomplementary base. Remarkably, the high probe specificity is maintained over the wide temperature range, that is from 20 to 60 °C.

What are the factors that make the new probe highly specific even at room and physiological temperatures? What distinguishes the triple-stem DNA probe from widely used MB probes? MB probes are oligonucleotide hairpins with a fluorophore and a quencher conjugated to the opposite ends of the oligomer (Figure 1C). Binding to complementary nucleic acids causes MBs to switch to their elongated conformation, thereby increasing their fluorescence. In this case, the reduction in the free energy of DS is achieved by Watson–Crick base-pair formation in the stem of the hairpin (Figure 1C, left). MBs distinguish mismatches over a wider temperature range than linear probes do;[8] however, thermal destabilization of the hybrid is still required for accurate SNP discrimination[9,10] because short 4–6 nt MB stems cannot bring the energy of DS to the optimal dislocation. Elongation of the MB stem improves the mismatch discrimination ability, but at the same time slows down the hybridization due to the larger activation energy required for duplex formation.[10]

The secret behind the excellent specificity of the triple-stem DNA probe designed by Xiao et al. lies in the greater level of conformational constraint provided by the three stems, which in total form 21 Watson–Crick base pars in DS. It is reasonable to suggest that the energy of DS for this probe is brought to the optimal dislocation, that is, between the energy of AS for the fully matched and mismatched duplexes (Figure 1B, right). One can argue that the same situation can be achieved by the introduction of a single long (~20 nt) nucleotide stem. That is true; however, the hybridization of such a probe would be kinetically hindered by the high energy barrier (dotted curve in Figure 1B) required for the unwinding of a 20 nt stem. At the same time, the three 7 nt stems of the triple-stem DNA probe can be unwound consecutively during hybridization with the analyte (Figure 1B). In this stepwise process, the high activation-energy barrier is divided into three lower barriers (solid curve). Hence, the triple-stem design should provide a high level of conformational constraint without greatly reducing the hybridization rate, thus representing a balance between thermodynamic stability and kinetic flexibility. In addition, the three-stem architecture should enable fine tuning of the probe specificity simply by adjusting the stability of each stem. Therefore, the approach suggested by Xiao et al. demonstrates that the probe design itself rather than the hybridization conditions can predetermine high specificity of analyte recognition.

Nevertheless, the hybridization kinetics for the triple-stem DNA probe were found to be relatively slow: the fluorescent signal only approaches a plateau about three hours after mixing the probe with the target. In comparison, hybridization of a five-stem MB with the corresponding analyte is substantially completed in one minute.[10] The slow binding kinetics might hinder the application of the new probe in real-time PCR, for which a quick fluorescent response during each amplification cycle is desirable. Furthermore, the probe responds to the presence of ~60 nM fully complementary analyte by generating a fluorescent signal with a signal-to-noise ratio of ~3, whereas MBs generate signals of comparable intensity in the presence of only ~5–10 nM target. Therefore, the highly stable secondary structure of the triple-stem probe predetermines its high specificity, while slowing down the response and reducing the limit of detection.

The displacement hybridization probe is another type of conformationally constrained probe that can recognize mismatches at room temperature in real time.[6] The design of this probe takes advantage of intermolecular DNA duplexes to bring a fluorophore-conjugated oligonucleotide to the partially complementary competitive strand conjugated with a quencher. When the probe strand hybridizes to the target, the quencher-conjugated competitor is displaced into solution, thus generating high fluorescence. The hybridization of these probes is essentially complete in a few minutes, thus enabling their application in real-time PCR.[6b] It would be interesting to compare the performance of displacement hybridization probes with that of the triple-stem DNA probe in a systematic study.

In conclusion, Xiao et al. have suggested an original design for a probe that

forms a competing secondary structure when unbound to nucleic acid analytes and generates a fluorescent signal in the analyte-bound state. The introduction of a new structurally constrained probe broadens the spectrum of highly specific tools for nucleic acid analysis and undoubtedly represents a step toward new multiplex SNP-typing techniques that are free from the meticulous optimizations of hybridization conditions.

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