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Cooperative DNA Probing Using a β-Cyclodextrin−DNA Conjugate and a Nucleobase-Specific Fluorescent Ligand

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In most gene analysis techniques, DNA probes that carry reporter groups such as fluorophores and electrochemically active groups are used to discriminate single nucleotide polymorphisms (SNPs).[1] The detection specificity solely relies on the difference in thermal stabilities of the duplexes originating from the one-base mismatch. This places a limit on the length of the probes, operation temperature, and the kinds of SNPs themselves. In addition, in some techniques, free or nonspecifically bound probes must be thoroughly washed from the plate to obtain a signal with a good contrast, due to signal interference from unbound probes.

We have developed a convenient technique for SNP genotyping in a homogeneous solution. Probes to be used in solution are expected to emit a signal only when they bind to the targets. We achieved this using cooperative action between two probes.[2] This system, in which several weak interactions work cooperatively, takes advantage of the programmability of DNA as a scaffold. Here we synthesized the DNA conjugates, β-cyclodextrin (β-CyD)-modified oligodeoxyribonucleotide (**CyD-ODN**), and the nucleobase-specific fluorescent DNA ligand (**MNDS**) and simultaneously used them for SNP analysis in aqueous solution. **MNDS** consists of two parts, 2-acetoamide-7-methyl-1,8-naphthyridine (AcMND) and 2,6-dansyl as a recognition and a signaling site, respectively. AcMND is known to bind with a specific base (guanine, G) by complementary hydrogen bonding.[3] AcMND-based ligands that recognize G in a bulge or G-G mismatch have been reported.[4]

In this assay, SNP bases (N) on the targets are displayed at a gap in ternary duplexes (N-gap duplexes) consisting of the targets, **CyD-ODN**, and **mask** as shown in Figure 1. That is, the sequences of **CyD-ODN** and **mask** are designed to be complementary to both adjacent sequences to the SNP base of the target. Therefore, both ODNs form a stable tandem duplex regardless of the type of SNP base. **MNDS** is then added to these ternary duplexes with a displayed SNP base. The dansyl group is expected to form a luminous inclusion complex with nearby β-CyD, only when the AcMND moiety recognizes G displayed in the gap. Base discrimination is not based on the hybridization of a long DNA probe as in conventional methods but on the complementarity of a small ligand only with a displayed base. This would be expected to increase the contrast of the signal.

All **ODN**s were synthesized by an automated DNA synthesizer (Expedite 8900) using conventional methods.[5] **CyD-ODN** conjugates were prepared as described in the Supporting Information (SI). β-CyD was selectively modified on one of the C6 positions (upper-rim) with a thiol group. Separately, an amine group tethered to the 3′- or 5′-end of an ODN was activated with a heterobifunctional cross-linking agent, SPDP (*N*-succinimidyl 3-(2-pyridyldithio)propionate) to form a thiol-reactive ODN. Both of them were coupled in a buffered solution, purified with HPLC, and identified with MALDI-TOF mass spectrometry. **MNDS** was prepared as shown in the SI. Other chemicals were purchased as special grade and used without further purification.

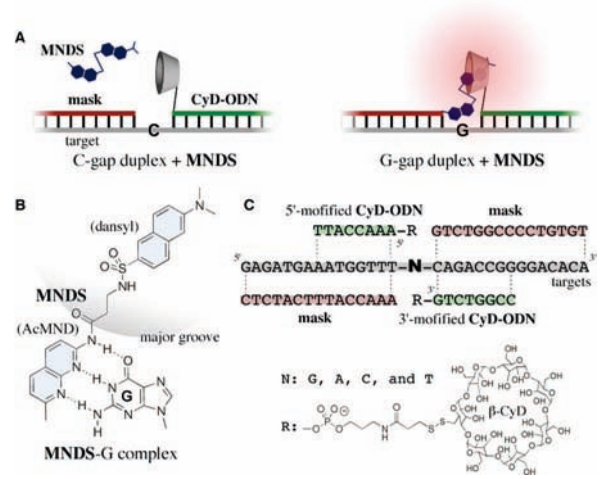

Figure 1. (A) Schematic illustration of nucleobase recognition by **MNDS** in N-gap duplexes. (B) Complementary hydrogen bonding between **MNDS** and guanine base (G). Dansyl moiety is protruded to the major groove of the duplex. (C) The structures and sequences of **CyD-ODN** and other ODNs used in this study. The target is a part of the TMPT (thiopurine *S*-methyltransferase) gene containing one of the hot spots.

Fluorescent SNP analyses were performed using N-gap duplexes containing 3′- and 5′-modified **CyD-ODN**s. Figure 2A shows the normalized fluorescence intensities at 443 nm measured at 0 °C. The signal was enhanced significantly, when G was displayed in the gap of the tandem duplex (G-gap duplex) containing 3′-modified **CyD-ODN**. As we expected, the AcMND moiety in **MNDS** seemed to recognize G by complementary hydrogen bonding in the gap. The contrasts of the fluorescence signal of G were 13.3, 25.6, and 23.8 in regard to A, C, and T, respectively. Interestingly, the recognition of the nucleobases in N-gap duplexes of 5′-modified **CyD-ODN** failed. The signal contrast for the nucleobases was not high.

The interactions of **MNDS** with the G-gap duplexes were studied further by fluorescence titration at 0 °C. The spectral changes on

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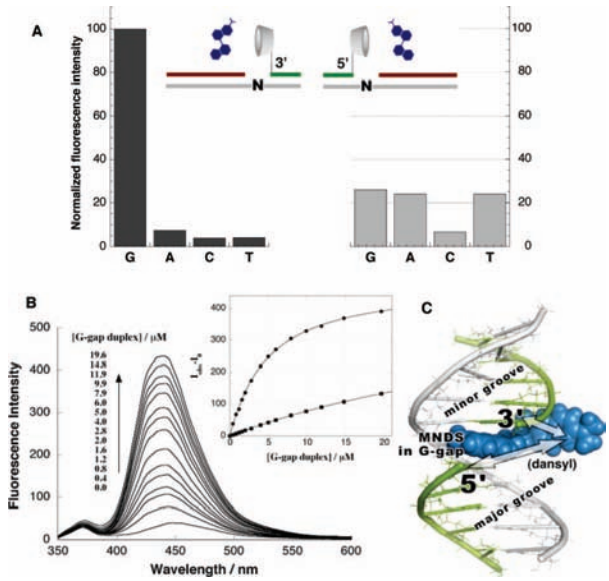

Figure 2. (A) Normalized fluorescence intensities of **MNDS**/N-gap duplexes. Left and right bar charts indicate the results of N-gap duplexes carrying 3′- and 5′-modified **CyD-ODN** conjugates, respectively. Relative intensities at 443 nm were obtained after subtraction of the signal of **MNDS** alone from each signal. 1.0 μM N-gap duplexes, 5.0 μM **MNDS**, 1 M NaCl, 10 mM phosphate-Na buffer (pH7.0), 0.83% DMSO, λ_{ex} = 328 nm, 0 °C. (B) Fluorescence titration. G-gap duplex carrying 3′-modified **CyD-ODN** was titrated (0−19.6 μM) into the solution containing **MNDS** (1.0 μM). Other conditions are the same as those for part A. The inset shows the change in fluorescence intensities for G-gap duplexes carrying 3′- (●) and 5′-modified (■) **CyD-ODN**. The two curves are the theoretical ones optimized by nonlinear least-squares method. (C) One of the possible 3D structures of **MNDS**/G-gap duplex. The model was geometry-optimized by AMBER* force field with GB/SA (generalized Born/surface area) solvent model using MacroModel version 9.1. The distances from the dansyl moiety of bound **MNDS** to both (3′- and 5′-) ends of ODNs are quite different. β-CyD was omitted from the structure for clarity (see TOC for entire structure).

titration of the G-gap duplex carrying 3′-modified **CyD-ODN** was shown in Figure 2B. The spectral changes observed here indicated the formation of an inclusion complex between dansyl and β-CyD, because the **MNDS** spectra scarcely changed by the addition of the control G-gap duplex lacking β-CyD. The change in fluorescence intensities at 443 nm is shown in the inset. The plot was fitted to the theoretical curve, which is derived assuming 1:1 interaction. The binding constant of **MNDS** with the G-gap duplex carrying 3′-modified **CyD-ODN** was calculated to be 2.4×10^5 M^{-1}.[6] In the same way, the binding constant of **MNDS** with β-CyD was estimated to be 5.2×10^2 M^{-1} under the same conditions (SI). The enhancement in binding constant (*ca.* 500 times) observed for the G-gap duplex with 3′-modified **CyD-ODN** would be due to the cooperative interaction of AcMND and dansyl moieties in **MNDS**, as expected. That is, AcMND inserts into the G-gap to form the complementary hydrogen bonding with G and, simultaneously, the dansyl group is accommodated in the nearby β-CyD modified at the 3′-end of ODN. Signal changes observed in the interaction with A-, C-, and T-gap duplexes carrying 3′-modified **CyD-ODN** were too small to be treated quantitatively.

The interaction of **MNDS** with the G-gap duplex carrying the 5′-modified **CyD-ODN** was also studied. The binding constant was roughly estimated to be 2×10^4 M^{-1} (Figure 2B inset). Therefore, the difference in fluorescence signals observed for 3′- and 5′-modified **CyD-ODN** shown in Figure 2A would be attributed to that in the binding constant for both duplexes. How can we account for this disparity between 3′- and 5′-modified **CyD-ODN**? **MNDS** protrudes its dansyl moiety into the major groove of the duplex, when AcMND forms complementary hydrogen bonds with G in the gap as shown in Figure 1B.[7] The distances from both ODNs' ends that face into the gap to the dansyl group of bound **MNDS** would be quite different (Figure 2C). Molecular modeling studies showed that the distances from 3′- and 5′-ends of both ODNs to the center of dansyl were *ca.* 10.4 and 17.8 Å, respectively. The conformational freedom would be limited for the linker chain that connects the 5′-end of ODN with β-CyD, when the dansyl group of **MNDS** is included in the 5′-modified β-CyD. That would make the binding of **MNDS** with G-gap duplex of 5′-modified **CyD-ODN** weaker because of the entropic disadvantage.

Another measurement using 3′-modified longer (15 mer) **CyD-ODN** conjugates, which form the duplexes with higher thermal stability, gave similar results under the same conditions. One of the merits of the present method is that all measurements using different DNA probes could be performed under the same conditions, because the recognition does not rely on the subtle difference in thermal stabilities of the probes.

We can design other small ligands that recognize any specific nucleobases in consideration of their complementarity for hydrogen bonding.[8] Some of them would protrude their reporter moiety into the minor groove, where it is close to β-CyD modified on the 5′-end of ODN. The combined use of 3′- or 5′-modified **CyD-ODN**s with dansyl-tethered ligands would be a general and flexible method for fluorescent SNP analysis in a homogeneous solution.

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Supporting Information Available: Synthesis details of **CyD-ODN** and **MNDS**, UV melting data of N-gap duplexes, fluorescence titration between **MNDS** and β-CyD, and raw spectra of SNP detection. This material is available free of charge via the Internet at http://pubs.acs.org.

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(6) All **MNDS**s cannot bind to the G-gap under experimental conditions. The quality of the signal would be improved by modification of the ligand so as to increase its affinity.

(7) **MNDS** could bind with A, C, and T through two hydrogen bonds (see SI). In this case, the dansyl moiety protrudes to the minor groove of N-gap duplexes (both sides possible for T).

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