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Allele Specific C-Bulge Probes with One Unique Fluorescent Molecule Discriminate the Single Nucleotide Polymorphism in DNA

Fumie Takei,[a] Hitoshi Suda,[b] Masaki Hagihara,[a] Jinhua Zhang,[a] Akio Kobori,[b] and Kazuhiko Nakatani*[a, b]

Abstract: A combination of an allele specific C-bulge probe and the fluorescent molecule *N*,*N*′-bis(3-aminopropyl)-2,7-diamino-1,8-naphthyridine (DANP) that binds specifically to the C-bulge provides a method for single nucleotide polymorphism (SNP) typing with only one fluorescent molecule without covalent modification of the DNA probe. The allele specific C-bulge probe contains one additional cytosine and produces a C-bulge directly flanking the SNP site upon hybridization to the target DNA. The C-bulge is a scaffold to recruit and retain DANP directly neighboring the SNP site. The DANP fluorescent probe was selectively modulated by the flanking matched and mismatched base pairs. The mutation type could be discriminated by the modulated fluorescent intensity with respect to the allele specific C-bulge probes used for the assay.

Keywords: cytosine bulge · DNA recognition · fluorescent probes · mutation detection

Introduction

Typing of the single nucleotide polymorphisms (SNPs) in an individual genome against sets of predetermined disease-related SNPs is essential to achieve personalized medicine.[1–6] Besides methods which exploit the sequence-specific reactions of enzymes,[1–4] the challenge toward novel SNP typing methods based on organic chemistry draws attention to molecular systems that emit fluorescence in an allele specific manner.[7–19] Base discrimination by fluorescent molecules largely relies on the change in the fluorescent intensity upon formation of the matched or mismatched base pair. The chemical basis of the fluorescence change is a result of the modulation of the dielectric and hydrogen-bonding environments of the fluorescent chromophore. To exert the maximum influence of the base pairing on the fluorescence intensity, the fluorescent molecules have thus been developed near the SNP site by covalently linking the probe DNA and/or directly bind to the base at the SNP site.[7,15–19] In the latter strategy, the use of four fluorescent molecules selectively responding to each one of the four nucleotide bases has been investigated.[15–19]

In terms of convenience and simplicity of the assay, typing methods using fluorescent molecules would be particularly effective if the covalent modification of the probe DNA were not required, and if all four nucleotide bases could be analyzed by one unique fluorescent molecule. To address these issues, we have investigated a method exploiting allele specific C-bulge probe DNA and a fluorescent molecule selectively binding to the C-bulge. The C-bulge probe DNA is allele specific by alternating the nucleotide base (^{Y}N) opposite the SNP site (^{X}N) and contains one additional cytosine directly neighboring ^{Y}N. The probe DNA provides a C-bulge as a scaffold to recruit and keep the fluorescent molecule directly neighboring the SNP site (Figure 1). We have reported that *N*,*N*′-bis(3-aminopropyl)-2,7-diamino-1,8-naphthyridine (DANP) selectively and strongly bound to the C-bulge with an exclusive 1:1 stoichiometry.[20] DANP binds to the C-bulge as the protonated form DANPH$^+$ at a neutral pH (Figure 2). Therefore, DANP would be recruited to the cytosine bulge that is located directly adjacent to the matched or mismatched base pair ^{X}N–^{Y}N at the SNP site. DANPH$^+$ emitted characteristic fluorescence upon binding to the cytosine bulge. Upon binding, both absorption and emission maximum of DANPH$^+$ were shifted by 30 nm to a longer wavelength from those of the unbound DANP. Therefore, it is possible to selectively monitor the DANPH$^+$

[a] Dr. F. Takei, Dr. M. Hagihara, Dr. J. Zhang, Prof. K. Nakatani
Department of Regulatory Bioorganic Chemistry
The Institute of Scientific and Industrial Research
Osaka University, 8-1 Mihogaoka, Ibaraki 567-0047 (Japan)
Fax: (+81) 6-6879-8459
E-mail: nakatani@sanken.osaka-u.ac.jp

[b] H. Suda, Dr. A. Kobori, Prof. K. Nakatani
Department Synthetic Chemistry and Biological Chemistry
Kyoto University, Nishigyou-ku, Kyoto 615-8510 (Japan)

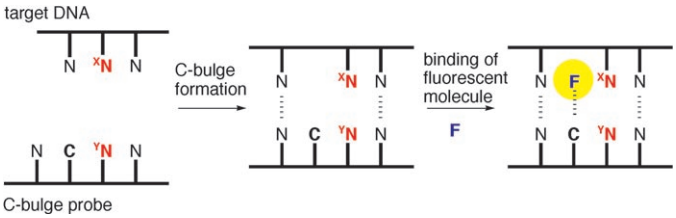

Figure 1. Illustration of the SNP typing exploiting the C-bulge binding molecule DANP and C-bulge probe as a scaffold to recruit DANP to the region directly neighbouring the SNP site. ^{X}N: nucleotide to be determined at the SNP site; ^{Y}N: nucleotide opposite ^{X}N in the probe; C: the bulged cytosine; F: fluorescent molecule binding to C-bulge (DANP).

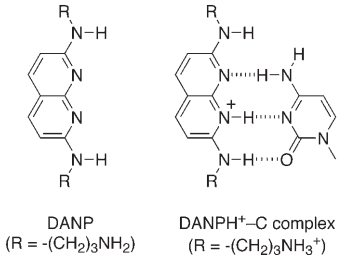

Figure 2. Structures of DANP and DANPH^{+}–C complex.

bound to the C-bulge even in the presence of unbound DANP.

The feasibility of the proposed method depends entirely on 1) the DANPH^{+} is bound to the C-bulge flanking the mismatched base pair and 2) the DANPH^{+} fluorescence could be modulated by the neighboring base pair. We have investigated the binding of DANP to the C-bulge flanking the mismatched base pairs and the fluorescence behavior upon binding. We report here that the fluorescence of the DANPH^{+} bound to the C-bulge was, in fact, selectively modulated by the neighboring matched or mismatched base-pair formation. The profile in the fluorescence change of DANPH^{+} regarding the allele specific C-bulge probes may be used for the determination of the allele type at the predetermined SNP site.

Results and Discussion

First we looked at the DANPH^{+} binding to the C-bulge flanking the mismatched base pairs. The formation of the DANPH^{+}-bound complex was investigated by measuring the melting temperature (T_m) of the 16-mer target DNA 5′-d(ACATCCAA^{X}NCAACCAC)-3′ and 17-mer C-bulge probes 5′-d(GTGGTTG^{Y}N*C*TTGGATGT)-3′ in the presence of DANP. The C-bulge probes contained one additional cytosine (italic) flanking ^{Y}N (N = A, T, C, G, and I) opposite ^{X}N. We have used inosine (I) in addition to A, T, C, and G to avoid the fluorescence quenching by G in the C-bulge probe. Upon hybridization, a single C-bulge was produced directly neighboring the ^{X}N–^{Y}N base pair. By alternating ^{Y}N with A, T, G, C, and I with respect to four ^{X}N in the target DNA, C-bulge duplexes flanking 20 different matched and mismatched base pairs were produced. The T_m of the duplex contained ^{X}T, ^{X}A, and ^{X}C increased in the presence of DANP regardless to the base at ^{Y}N (Table 1). Based on the

Table 1. Melting temperatures of C-bulge duplexes with DANP.[a]

	5′-d(ACATCCAA_^{X}NCAACCAC)-3′ 3′-d(TGTAGGTTC^{Y}NGTTGGTG)-5′			
^{Y}N	^{X}T	^{X}A	^{X}G	^{X}C
A	52.2 (4.8)[b]	46.7 (2.7)	52.2 (0.6)	46.3 (3.9)
T	47.3 (5.3)	50.4 (3.2)	53.5 (1.0)	46.6 (5.0)
C	48.1 (5.5)	46.7 (4.0)	56.0 (2.3)	48.1 (6.7)
G	50.3 (6.0)	51.8 (2.9)	54.3 (1.1)	54.6 (3.6)
I	48.2 (5.5)	52.2 (4.2)	52.5 (0.5)	49.4 (3.1)

[a] T_m of duplexes 5′-d(ACATCCAA^{X}NCAACCAC)-3′/5′-(GTGGTTG^{Y}NCTTGGATGT)-3′ (2 μM) were measured in 10 mM Na cacodylate (pH 7.0) with 100 mM NaCl in the presence of DANP (50 μM). [b] The values in a parenthesis refer to the increase of T_m compared with the values measured without DANP. The reported values are the average of three independent measurements.

K_d value of 10 mM for the DANPH^{+} binding to the C-bulge,[20] it was estimated that about 80% of the C-bulge duplex was present as the DANPH^{+}-bound form under these conditions. Duplexes containing ^{X}G showed much smaller increases of T_m than other duplexes especially when ^{Y}N was A, T, G, and I. This is probably due to the unavoidable formation of ^{X}G–C base pair between ^{X}G and the extra cytosine, producing a single ^{Y}N bulge instead of the C-bulge.

Having confirmed the binding of DANP to the C-bulge flanking the ^{X}T–^{Y}N, ^{X}A–^{Y}N, and ^{X}C–^{Y}N base pairs, we focused our attention on the fluorescence of the DANPH^{+} bound to the C-bulge. The fluorescence measurements were performed in the presence of an excess amount of C-bulge duplex to make sure that DANP was completely bound to the C-bulge. The DANPH^{+} fluorescence was selectively modulated by the base pairs flanking the C-bulge (Figure 3). Strong fluorescence was observed when the base pair flanking the C-bulge was ^{X}A–^{Y}T, whereas only weak fluorescence was observed for ^{X}C–^{Y}G. It is often observed that the guanine base quenches the fluorescence of the neighboring fluorophore.[17,21,22] We found that the substitution of G in the ^{X}C–^{Y}G base pair to I in the ^{X}C–^{Y}I base pair resulted in a large increase in the fluorescence intensity. The use of I in the C-bulge probe would be effective for suppressing the quenching of the DANPH^{+} fluorescence by the neighboring G in the C-bulge probe. The effect of the flanking mismatched base pair on the DANPH^{+} fluorescence was quite marked. While the DANPH^{+} fluorescence was quite weak when the base pair flanking the C-bulge was ^{X}C–^{Y}T, quite strong fluorescent was observed for the ^{X}A–^{Y}G base pair. Based on the T_m increase, DANPH^{+} bound to the C-bulge flanking both ^{X}C–^{Y}T and ^{X}A–^{Y}G. Therefore, the DANPH^{+} fluorescence was modulated by the flanking mismatched base pairs. It is particularly noteworthy that the guanine in the ^{X}A–^{Y}G did not quench the fluorescence of the neighboring DANPH^{+} bound to the C-bulge. While we do not have

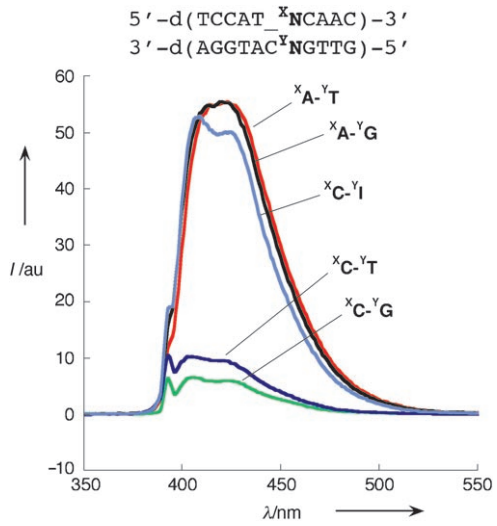

Figure 3. Fluorescence spectra of DANP (10 μM) was measured in the presence of C-bulge duplexes (50 μM) in a phosphate buffer (pH 7.0) and NaCl (100 mM). Excitation wavelength was 394 nm. ^{X}N–^{Y}N = A—T (red), A—G (black), C—T (blue), C–G (green), C—I (sky blue).

a rational explanation for these observations at this moment, this fluorescent property of DANPH$^+$ is worthy of future investigations.

Encouraged by the fluorescent measurements on selected matched and mismatched base pairs flanking the C-bulge, we have looked at the comprehensive survey of the effect of the flanking base pairs on the DANPH$^+$ fluorescence. Because the assay conditions with an excess amount of DNA are not feasible in practical SNP typing, the fluorescence measurements of DANPH$^+$ were investigated in the presence of a limited amount of C-bulge duplex. Since the absorption and emission spectra of the DANPH$^+$–C-bulge complex was shifted by 30 nm to a longer wavelength compared with those of unbound DANP, it was anticipated that we could selectively monitor the DANPH$^+$ bound to the C-bulge in the presence of unbound DANP. The DANPH$^+$ fluorescence was measured with 410 nm excitation and 460 nm emission filters to selectively monitor the DANPH$^+$-bound complex in the presence of unbound DANP (λ_{max} 365 nm). The observed fluorescence intensity (I_{obs}) in the presence of the C-bulge duplex is reported as a value (I_{rel}) relative to that of the background (I_{back}) measured without the C-bulge duplex.

There were two sequence motifs for the C-bulge probe in terms of the relative position of the C-bulge to the ^{X}N–^{Y}N base pair. The sequences 5′-A_^{X}N-3′/3′-T*C*^{Y}N-5′, 5′-T_^{X}N-3′/3′-A*C*^{Y}N-5′, and 5′-C_^{X}N-3′/3′-I*C*^{Y}N-5′ contained the bulged cytosine at the 3′ end of ^{Y}N, whereas other three sequences 5′-^{X}N_A-3′/3′-^{Y}N*C*T-5′, 5′-^{X}N_T-3′/3′-^{Y}N*C*A-5′, and 5′-^{X}N_C-3′/3′-^{Y}N*C*I-5′ contained the bulged cytosine at the 5′ end of ^{Y}N. The 5′-G_^{X}N-3′/3′-*C*C^{Y}N-5′ and 5′-^{X}N_G-3′/3′-^{Y}N*C*C-5′ sequence contained G at the 5′ or 3′ end of ^{X}N produced the I_{rel} to be 1 regardless to the ^{X}N–^{Y}N base pair and are not discussed here. The G flanking C-bulge in the probe was replaced with inosine to avoid fluorescence quenching by G.

The C-bulge in the first sequence series showed a common tendency of I_{rel} as representatively described for 5′-A_^{X}N-3′/3′-TC^{Y}N-5′(Table 2). Among 20 C-bulges in the sequence series of 5′-A_^{X}N-3′/3′-TC^{Y}N-5′, six C-bulges flanking ^{X}T–^{Y}A, ^{X}A–^{Y}A, ^{X}A–^{Y}T, ^{X}A–^{Y}G, ^{X}A–^{Y}I, and ^{X}C–^{Y}I showed the I_{rel} larger than 2. The largest I_{rel} was 4.2 for the

Table 2. I_{rel} of DANPH$^+$ bound to the C-bulge in the A_^{X}N/TC^{Y}N sequence.[a]

	5′-d(ACATCCAA_^{X}NCAACCAC)-3′ 3′-d(TGTAGGTTC^{Y}NGTTGGTG)-5′			
^{Y}N	^{X}N			
	T	A	G	C
^{Y}A	2.8	2.5	1.0	1.5
^{Y}T	1.4	3.5	0.9	1.2
^{Y}C	1.1	1.4	1.0	1.0
^{Y}G	1.4	3.1[b]	1.0	1.3
^{Y}I	1.9	4.2	1.1	2.8

[a] Fluorescence measurements were carried out for the solution containing 2 μM each of two C-bulge duplex and 50 μM DANP in a phosphate buffer (pH 7.0) and 100 mM NaCl. $I_{rel} = I_{obs}/I_{back}$. The error (s.e.m.) was 0.1 for three independent measurements unless otherwise noted. [b] The error was 0.2.

C-bulge flanking ^{X}A–^{Y}I. When the ^{X}N was guanine, the I_{rel} was nearly 1 regardless to the ^{Y}N. It was also characteristic that the I_{rel} was below 1.5 for the C-bulge containing C at either ^{X}N or ^{Y}N position. The only exception was the C-bulge flanking to ^{X}C–^{Y}I. The ^{X}A–^{Y}G mismatch produced a large I_{rel}. The data clearly show that the flanking sequence to the C-bulge had considerable effects on the DANPH$^+$ fluorescence bound to the C-bulge. Other two sequences 5′-T_^{X}N-3′/3′-AC^{Y}N-5′ (Table 3), and 5′-C_^{X}N-3′/3′-IC^{Y}N-5′ (Table 4) showed the similar I_{rel} values for a given ^{X}N–^{Y}N base pair.

Table 3. I_{rel} of DANPH$^+$ bound to the C-bulge in the T_^{X}N/AC^{Y}N sequence.[a]

	5′-d(ACATCCAT_^{X}NCAACCAC)-3′ 3′-d(TGTAGGTAC^{Y}NGTTGGTG)-5′			
^{Y}N	^{X}N			
	T	A	G	C
^{Y}A	2.6	2.6	0.9	1.3
^{Y}T	1.6	4.0	0.9	1.0
^{Y}C	1.2	1.5	0.9	1.0
^{Y}G	1.1	2.7	0.9	1.0
^{Y}I	2.1	3.7	1.0	2.3

[a] See Table 2.

In the second sequence series, where C-bulge was located at the 5′ end of the ^{Y}N, the I_{rel} was also sensitive to the flanking base pairs (Tables 5–7). When ^{X}N was G, the I_{rel} was almost 1 regardless to ^{Y}N as observed in the first sequence context. In contrast, the I_{rel} for the C-bulges containing ^{X}C was about two times higher than that observed in the first sequence context. This flanking base-pair dependency of the I_{rel} was common for the second sequence context. On

Table 4. I_{rel} of DANPH$^+$ bound to the C-bulge in the C_^{X}N/IC^{Y}N sequence.[a]

	5′-d(ACATCCAC_^{X}NCAACCAC)-3′ 3′-d(TGTAGGTIC^{Y}NGTTGGTG)-5′			
^{Y}N	^{X}N			
	T	A	G	C
^{Y}A	2.3	2.2	0.9	1.2
^{Y}T	1.5	3.3	0.9	1.2
^{Y}C	1.1	1.3	0.9	1.0
^{Y}G	1.2	3.0	0.9	1.0
^{Y}I	2.1	4.2	1.0	2.4

[a] See Table 2.

Table 5. I_{rel} of DANPH$^+$ bound to the C-bulge in the ^{X}N_A/^{Y}NCT sequence.[a]

	5′-d(ACATCCA^{X}N_ACAACCAC)-3′ 3′-d(TGTAGGT^{Y}NCTGTTGGTG)-5′			
^{Y}N	^{X}N			
	T	A	G	C
^{Y}A	4.0	3.0	1.0	1.9
^{Y}T	2.6	3.5	0.9	1.8
^{Y}C	2.0	2.5	1.0	1.8
^{Y}G	1.9	2.7	0.9	1.1
^{Y}I	2.4	4.8	0.9	3.3

[a] See Table 2.

the basis of these fluorescence measurements, the fluorescence of DANPH$^+$ bound to the C-bulge was selectively modulated by the ^{X}N–^{Y}N base pair directly neighboring 5′ and 3′ sides. The G directly flanking SNP site quenched the DANPH$^+$ fluorescence and thus did not allow fluorescence measurements. There were three sequence series regarding the position of G relative to ^{X}N; ^{X}N was flanked by 1) one G at the 5′ end (5′-G^{X}NN-3′), 2) one G at the 3′ end (5′-N^{X}NG-3′), and 3) two Gs at both 5′ and 3′ end (5′-G^{X}NG-3′). In the first two sequences, fluorescence quenching by G could be circumvented by the design of C-bulge probe. Thus, the position of the C-bulge was chosen such that the C-bulge was produced at the opposite side of the G as shown in Figure 4a and b. In the third sequence, a probe

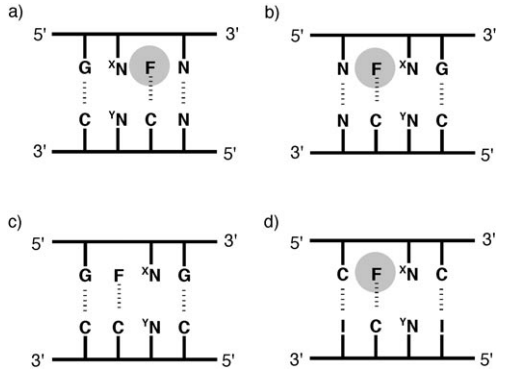

Figure 4. Discrimination of allelic types in six mutations with two C-bulge probes and DANP. Key: a) A to T; b) C to T; c) A to C; d) C to G; e) A to G; f) G to T mutations.

design was not effective to circumvent fluorescence quenching because C-bulge was always flanked by the G (Figure 4c). In this case, the fluorescence measurements should be done on the complementary strand in the 5′-C^{X}NC-3′ with the C-bulge probe having two inosine (I) opposite C (Figure 4d).

In the proposed SNP typing, the single-stranded DNA containing the SNP site was obtained by PCR followed by asymmetric PCR, then hybridized with the C-bulge probes. The DANP was added to the duplex and the fluorescence was measured. The fluorescence profiles of the given DNA sample regarding the C-bulge probes determined the SNP

Table 6. I_{rel} of DANPH$^+$ bound to the C-bulge in the ^{X}N_T/^{Y}NCA sequence.[a]

	5′-d(ACATCCA^{X}N_ACAACCAC)-3′ 3′-d(TGTAGGT^{Y}NCTGTTGGTG)-5′			
^{Y}N	^{X}N			
	T	A	G	C
^{Y}A	2.6	2.5	1.0	1.8
^{Y}T	2.3	2.8	1.0	1.9
^{Y}C	2.1	2.5	0.9	2.0
^{Y}G	1.3	1.7	0.9	1.0
^{Y}I	1.9	3.1	0.9	2.3

[a] See Table 2.

Table 7. I_{rel} of DANPH$^+$ bound to the C-bulge in the ^{X}N_C/^{X}NCI sequence.[a]

	5′-d(ACATCCA^{X}N_ACAACCAC)-3′ 3′-d(TGTAGGT^{Y}NCTGTTGGTG)-5′			
^{Y}N	^{X}N			
	T	A	G	C
^{Y}A	2.3	2.5	1.0	2.2
^{Y}T	1.9	2.8	0.9	2.3
^{Y}C	1.8	2.3	1.0	2.5
^{Y}G	1.3	1.7	0.9	1.0
^{Y}I	1.8	3.6	0.9	2.4

[a] See Table 2.

type. The SNP typing needs to discriminate three allelic types of wild type and mutant homozygotes and heterozygote. In homozygotes, the nucleotide base at the SNP site (^{X}N) is identical for two alleles, whereas a different base is found in each one of two alleles of heterozygotes. For example, in the A to C mutation, the homozygote of wild type has ^{X}A at the SNP site in two alleles (^{X}A/^{X}A), whereas the homozygote of mutant has ^{X}C in two alleles (^{X}C/^{X}C). The heterozygote has ^{X}A and ^{X}C at the SNP site in each allele (^{X}A/^{X}C). Six combinations of two bases (^{X}A/^{X}C, ^{X}A/^{X}G, ^{X}A/^{X}T, ^{X}C/^{X}G, ^{X}C/^{X}T, and ^{X}G/^{X}T) were correlated to the six types of single nucleotide mutations. The I_{rel} data shown in Tables 2–7 were obtained for the C-bulge flanked by one ^{X}N and indicated that the data were corresponding to those of homozygote samples.

The I_{rel} of heterozygote samples were measured for the DNA obtained by mixing two heterozygote samples. In Table 8, the I_{rel} data for the heterozygote samples in the 5′-A_^{X}N-3′/3′-TC^{Y}N-5′ sequence, where ^{X}N consisted of two

different nucleotide bases $^{X}N_1$ and $^{X}N_2$, is shown. The I_{rel} value was found to be the average of I_{rel} obtained for each one of two ^{X}N. This indicates that the binding of DANPH$^+$ to two different C-bulge duplexes is independent from each other under the conditions. With the I_{rel} profile of the homo- and heterozygote DNA against each one of C-bulge probes (Tables 2 and 8), the allelic type could be clearly discriminated by comparing the I_{rel} in terms of the ratio and the magnitude obtained for two C-bulge probes (Figure 5).

Table 8. I_{rel} of DANPH$^+$ bound to the C-bulge in heterozygote duplexes.[a]

	5′-d(ACATCCAA_$^{X}N_1$CAACCAC)-3′ 5′-d(ACATCCAA_$^{X}N_2$CAACCAC)-3′ 3′-d(TGTAGGTTC^{Y}NGTTGGTG)-5′					
^{Y}N	$^{X}N_1/^{X}N_2$					
	$^{X}A/^{X}C$	$^{X}A/^{X}G$	$^{X}A/^{X}T$	$^{X}C/^{X}G$	$^{X}C/^{X}T$	$^{X}G/^{X}T$
^{Y}A	2.0	1.7	2.6	1.2	2.1	1.9
^{Y}T	2.4	2.2	2.5	1.1	1.3	1.1
^{Y}C	1.2	1.2	1.2	1.0	1.1	1.0
^{Y}G	2.2	2.0[b]	2.3	1.1	1.3	1.2
^{Y}I	3.5[b]	2.7	3.2	2.0	2.4	1.5

[a] Fluorescence measurements were carried out for the solution containing 1 μM each of two 16 mer 5′-d(ACATCCAA$^{X}N_1$CAACCAC)-3′, 5′-d(ACATCCAA$^{X}N_2$CAACCAC)-3′ and 2 μM each of 17 mer 5′-(GTGGTTG^{Y}NCTTGGATGT)-3′ and 50 μM DANP in a phosphate buffer (pH 7.0) and 100 mM NaCl. $I_{rel}=I_{obs}/I_{back}$. The error (s.e.m.) was 0.1 for three independent measurements unless otherwise noted. [b] The error was 0.2.

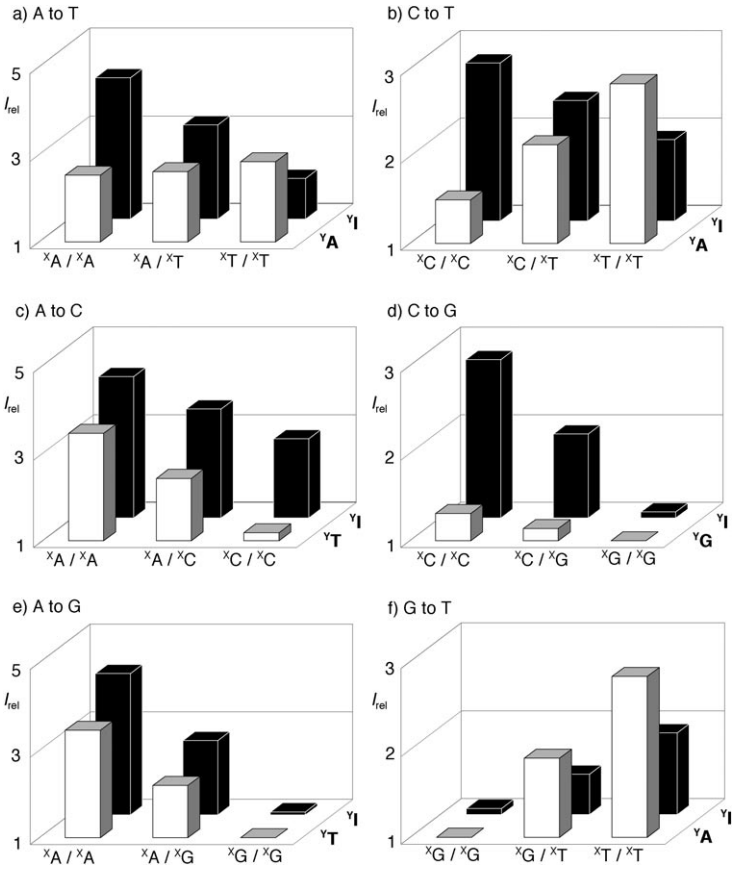

Figure 5. Selection of C-bulge probe with respect to the target sequence. a) C-bulge probe with the extra cytosine at the 5′ end of ^{Y}N; target: 5′-G^{X}NN-3′, probe: 5′-NC^{Y}NC-3′. b) C-Bulge probe with the extra cytosine at the 3′ side to ^{Y}N; target: 5′-N^{X}NG-3′, probe: 5′-C^{Y}NCN-3′. c) ^{X}N flanked by two G; target: 5′-G^{X}NG-3′, probe: 5′-C^{Y}NCC-3′. d) SNP typing should be done on the complementary strand having 5′-C^{X}NC-3′ sequence with the C-bulge contained two inosines; target: 5′-C^{X}NC-3′, probe: 5′-I^{Y}NCI-3′.

The allelic types in the A to T (or T to A) mutation were determined by two C-bulge probes containing ^{Y}A and ^{Y}I (Figure 5a). The C-bulge probe contained ^{Y}I was chosen instead of that containing ^{Y}T because the I_{rel} difference between ^{X}A and ^{X}T was larger for ^{Y}I than for ^{Y}T. Three allelic types of $^{X}A/^{X}A$, $^{X}A/^{X}T$, and $^{X}T/^{X}T$ produced a similar I_{rel} for the ^{Y}A-containing probe, but gave markedly different I_{rel} values for the ^{Y}I-containing probe. The ratio of I_{rel} obtained for the two C-bulge probes determined the allelic type. In the C to T (or T to C) and A to C (or C to A) mutations (Figure 5b and c), the allelic type could be also determined by the ratio metric analysis. The other three mutations of G to N (N = A, T, and C) could be analyzed by the difference in the intensity of I_{rel} for each of the C-bulge probes used for the analysis. Because the $^{X}G/^{X}G$ homozygote showed an I_{rel} of 1.0, the I_{rel} of the heterozygote ($^{X}G/^{X}N$) would be one half of that obtained for $^{X}N/^{X}N$ homozygote DNA. For a practical applicability, the predetermined profile of I_{rel} for the allelic type could be used as a standard for the diagnosis of testing samples.

Conclusion

The SNP-typing method described here exploits the C-bulge as a scaffold to recruit and keep DANP directly neighboring the SNP site. The method does not require covalent modification of the probe DNA and uses only one fluorescent molecule, that is, DANP, for the assay. The high flexibility in the probe design is another characteristic of the proposed SNP typing and makes the method applicable in principle to any target sequence. Especially, the fluorescence quenching by G could be circumvent by probe design and the effective use of inosine in the probe. However, bulge binding molecules with improved fluorescence properties, stronger fluorescence intensity, a large absorption and emission shift upon binding to C-bulge, and a large fluorescence difference with neighboring base pairs are necessary for this proposed method to be applicable for SNP typing. It is also important to understand the chemical basis that the DANPH$^+$–C bulge complex neighboring ^{X}A–^{Y}G, ^{X}C–^{Y}I and ^{X}A–^{Y}I base pairs emitted strong fluorescence. These studies may provide a way to avoid the fluorescence quenching by ^{X}G.

Experimental Section

Measurements of melting temperature of bulge-containing duplexes: DANP (50 μM) was dissolved in a sodium cacodylate (10 mM, pH 7.0) containing bulge duplex (2 μM) and NaCl (100 mM). The thermal denaturation profile was recorded on a Shimadzu UV2550 spectrometer equipped with a Shimadzu TMSPC-8 temperature controller. The absorbance of the sample was monitored at 260 nm from 4 °C to 80 °C with a sample heating rate of 1 °C min^{-1}.

UV and fluorescent spectra measurements: UV spectra were recorded on a Shimadzu UV2550 spectrometer. Fluorescent spectra were recorded on a Shimadzu RF-5300PC. DNA samples were prepared in 10 mM sodium phosphate buffer at the designated pH 7.0 in the presence of 100 mM sodium chloride. Excitation wavelength for the fluorescent measurements was the wavelength at the absorption maximum unless otherwise noted.

The fluorescence intensity was recorded on a Berthold Mithras LB 940 with 410 nm excitation and 460 nm emission filters. The measurements were carried out for the solution containing 2 μM each of two C-bulge duplex and 50 μM of DANP on the phosphate buffer (pH 7.0) and 100 mM NaCl.

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