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Improvement of single nucleotide polymorphism genotyping by allele-specific PCR using primers modified with an ENA residue

Makoto Koizumi [a,*], Koji Morita [a], Miho Takagi [a], Hiroaki Yasumo [b], Atsushi Kasuya [c]

[a] *Lead Discovery Research Laboratories, Sankyo Company Ltd., 2-58, Hiromachi 1-Chome, Shinagawa-ku, Tokyo 140-8710, Japan*
[b] *Biomedical Research Laboratories, Sankyo Company Ltd., 2-58, Hiromachi 1-Chome, Shinagawa-ku, Tokyo 140-8710, Japan*
[c] *Drug Metabolism and Pharmacokinetics Research Laboratories, Sankyo Company Ltd., 2-58, Hiromachi 1-Chome, Shinagawa-ku, Tokyo 140-8710, Japan*

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Abstract

When we placed an ENA residue into primers at the 3′ end, or the *n*-1, *n*-2, or *n*-3 position, which included a single nucleotide polymorphism (SNP) site at the 3′ end, only primers containing the ENA residue at the *n*-2 position were read by *Taq* DNA polymerase for amplification. The use of the ENA primers avoided the generation of undesired short products, which are thought to be derived from primer–dimers. A greater discrimination of the SNP site by these primers containing the ENA residue was observed compared with that of the corresponding unmodified DNA primers that are often used for allele-specific polymerase chain reaction (AS–PCR). This improvement is probably due to the difficulty of incorporating a nucleotide into the mismatched ENA primer by *Taq* DNA polymerase in the modified primer–template duplex. These results demonstrate that ENA primer-based AS–PCR would enable a rapid and reliable technique for SNP genotyping.

Keywords: Allele-specific polymerase chain reaction; BNA; ENA; LNA; SNP; Modified nucleoside; AS–PCR

Single nucleotide polymorphisms (SNPs)[1] represent substitutions with other nucleotides in the genome by point mutations. They occur at a frequency of 1 per 1000, and their number would be 3–10 million in human genomes [1]. The genotyping of a large number of SNPs will help to understand not only common human diseases but also complex human diseases. To genotype individuals, a rapid, reliable, and inexpensive methodology is required. Several innovative SNP genotyping techniques have been developed, as described in a recent review [2].

Allele-specific polymerase chain reaction (AS–PCR) is a conventional method for SNP genotyping that is based on DNA amplification by using an allele-specific primer at the 3′ position, which binds to the SNP allele [3]. After binding, the primers are extended by *Taq* DNA polymerase and PCR products are synthesized (Fig. 1A). On the other hand, the primers containing a mismatched nucleoside at the 3′ end are neither efficiently nor completely extended in a PCR (Fig. 1B). One benefit of AS–PCR is that probes such as TaqMan are not necessary. However, it has been reported that *Taq* DNA polymerase extends mismatched allele-specific DNA primers, thereby generating false-positive results [4]. To solve the false-positive problem, a 2′,4′-BNA/LNA (bridged nucleic acid/locked nucleic acid) residue, which is a

* Corresponding author. Fax: +81 3 5436 8570.
E-mail address: koizum@sankyo.co.jp (M. Koizumi).

[1] *Abbreviations used:* SNP, single nucleotide polymorphism; AS–PCR, allele-specific polymerase chain reaction; BNA, bridged nucleic acid; LNA, locked nucleic acid; ENA, 2′-*O*,4′-*C*-ethylene nucleic acid; ESI–MS, electrospray ionization–mass spectrometry; ESI–HRMS, electrospray ionization–high-resolution mass spectrometry; FAB–MS, fast atom bombardment–mass spectrometry.

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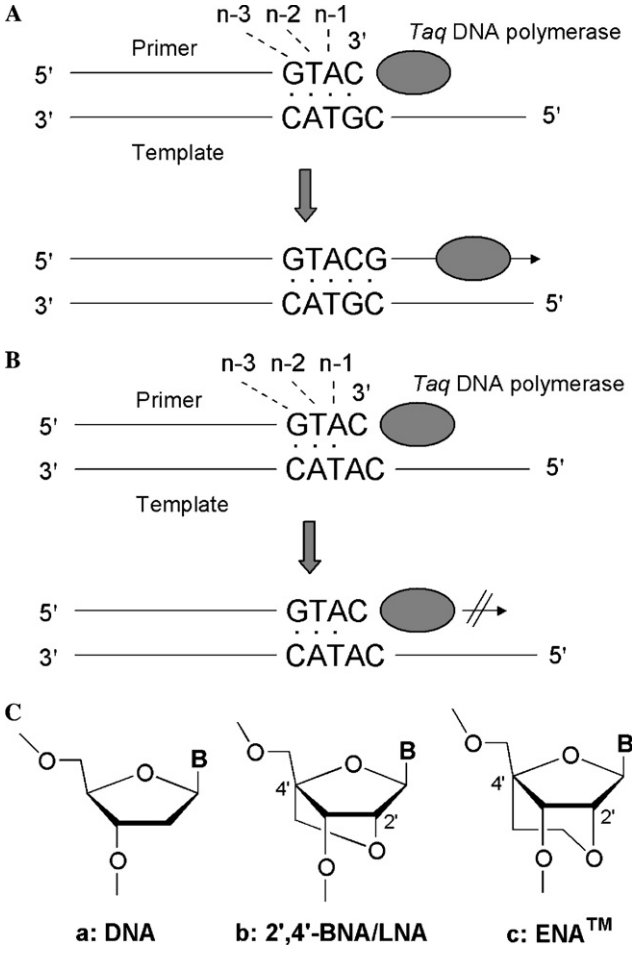


Fig. 1. Schematic illustrations of the interactions between the template and primer with an SNP site at the 3′ end. (A) The primer specific for the allele is amplified. (B) The primer with a mismatch at the SNP site is not amplified. (C) Structures of DNA and modified nucleosides for allele-specific primers: (a) DNA; (b) 2′,4′-BNA/LNA (bridged nucleic acid/locked nucleic acid); (c) ENA (2′-*O*,4′-*C* ethylene-bridged nucleic acid). B, nucleobases.

nucleic acid analog with a 2′-*O*,4′-*C*-methylene linkage (structure b in Fig. 1C) [5–7] (i.e., LNA primers), was placed at the 3′ end or the *n*-1 position of the allele-specific primer [8–12]. *Taq* DNA polymerase could extend LNA primers only when the template–primer formed perfectly matched base pairing at the 3′ end. The *Taq* DNA polymerase could not bind to a template–primer complex containing a mismatched base pair at the 3′ end [8]. This might be due to the steric hindrance of the methylene linkage of the 2′,4′-BNA/LNA residue around the 3′ hydroxy group that is observed with mismatched pairs.

Recently, we reported the synthesis of novel 2′-*O*,4′-*C*-ethylene nucleosides (ENA)[2] (structure c in Fig. 1C) that have a less strained six-member ring than the five-member ring of 2′-*O*,4′-*C*-methylene nucleosides [13,14]. The oligonucleotides with the ENA residues exhibit a binding affinity to ssDNA and ssRNA as high as 2′,4′-BNA/LNA and show excellent triplex formation with dsDNA [13–16]. Their ENA oligonucleotides also exhibit much higher nuclease resistance than do oligonucleotides containing 2′,4′-BNA/LNA residues [13,14] (Scheme 1).

In this article, we investigated the specificity and sensitivity of PCR with primers containing the ENA residue at the 3′ end or the *n*-1, *n*-2, or *n*-3 position. The ENA primers modified at the *n*-2 position were allele specific and were efficiently amplified in mouse and human genomic targets.

Materials and methods

General methods

^{1}H NMR was recorded on a Varian Mercury 400 (400 MHz). All reactions were monitored by thin-layer chromatography performed on Merck silica gel plates (Art 5715, 60 F_{254} plates) with UV detection. For column chromatography, Kanto Chemicals silica gel 60, spherical particles (40–100 μm), was used.

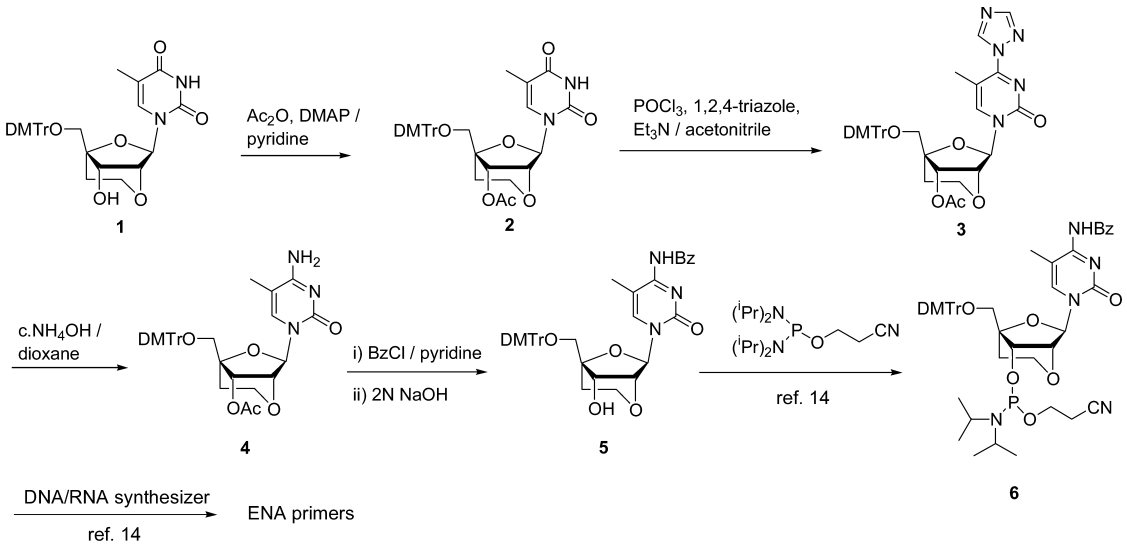
5′-O-(4,4′-Dimethoxytrityl)-3′-O-acetyl-2′-O,4′-C-ethylene-5- methyluridine (2)

Acetic anhydride (0.87 ml, 9.22 mmol) and 4-(dimethylamino)pyridine (5 mg, 0.04 mmol) were added to 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*,4′-*C*-ethylene-5-methyluridine (**1**, 1.08 g, 1.84 mmol [14]) in anhydrous pyridine (10 ml). After stirring for 30 min, the reaction was quenched with EtOH (2 ml) and CH_2Cl_2 (30 ml) was added. The organic layer was washed with saturated $NaHCO_3$ solution, dried over $MgSO_4$, and then evaporated in vacuo. The residue was purified by chromatography on silica gel (CH_2Cl_2:MeOH = 100:2 v/v) to give compound **2** as a white solid (1.14 g, 98%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.29 (3H, s), 1.44 (1H, dd), 2.02–2.13 (1H, m), 2.13 (3H, s), 3.19 (1H, d), 3.44 (1H, d), 3.80 (6H, s), 3.93–4.02 (2H, m), 4.54 (1H, d), 5.24 (1H, d), 6.09 (1H, s), 6.82–6.87 (4H, m), 7.13–7.42 (9H, m), 7.88 (1H, s), 8.11 (1H, brS). Electrospray ionization–mass spectrometry (ESI–MS) (positive): 651$(M + Na)^+$. Electrospray ionization–high-resolution mass spectrometry (ESI–HRMS) (positive): calcd. for $C_{35}H_{36}N_2O_9Na_1$ $[M + Na]^+$ 651.23185, found 651.23240.

5′-O-(4,4′-Dimethoxytrityl)-2′-O, 4′-C-ethylene-4-N-benzoyl-5- methylcytidine (5)

$POCl_3$ (1.5 ml, 16.1 mmol) was added slowly to a stirred suspension of 1,2,4-triazole (4.53 g, 65.6 mmol) in acetonitrile (100 ml), followed by triethylamine (10 ml, 71.7 mmol), at 0 °C. After stirring for 40 min at 0 °C, a

[2] ENA is a registered trademark of Sankyo Lifetech Company Ltd.



Scheme 1. Synthesis of ENA primers containing the 2′-*O*,4′-*C*-ethylene-5-methylcytidine phosphoramidite unit.

solution of compound **2** (1.27 g, 2.02 mmol) in acetonitrile (20 ml) was added slowly. After stirring for another 3 h at 0 °C, the reaction mixture was warmed to room temperature and stirred for another 3 h. The reaction was quenched by the addition of saturated $NaHCO_3$ solution (30 ml), and then the mixture was extracted with CH_2Cl_2 (60 ml) twice. The organic layer was washed with saturated $NaHCO_3$ solution and brine, dried over $MgSO_4$, and then evaporated in vacuo to afford compound **3** (1.52 g, quant.). Aqueous ammonium solution (28%, 10 ml) was added to compound **3** (1.52 g, 2.02 mmol) in 1,4-dioxane (20 ml). After stirring for 30 min, the mixture was evaporated in vacuo to give compound **4** (1.27 g, quant.). Benzoyl chloride (1.0 ml, 8.61 mmol) was added to compound **4** (1.27 g, 2.02 mmol) in anhydrous pyridine (10 ml) on ice. The reaction mixture was warmed to room temperature and stirred for 45 min. The reaction was quenched by the addition of H_2O (1 ml) and cooled on ice, and then MeOH (5 ml) and 2 N NaOH (10 ml) were added to the mixture. After stirring for 30 min, CH_2Cl_2 (30 ml) was added and the mixture was partitioned between CH_2Cl_2 and H_2O. The organic layer was washed with brine, dried over $MgSO_4$, and then concentrated in vacuo. The residue was purified by chromatography on silica gel (CH_2Cl_2:MeOH = 100:1 v/v) to give compound **5** as a white solid (1.02 g, 73% in three steps). The data of ^1H NMR, fast atom bombardment–mass spectrometry (FAB–MS), and FAB–HRMS are identical to those of compound **5** as described in [14].

Synthesis of primers for AS–PCR

PCR primers containing 2′-*O*,4′-*C*-ethylene nucleosides were prepared using compound **6** and 2′-*O*,4′-*C*-ethylene nucleoside-3′-*O*-phosphoramidite units according to reported methods [13,14]. DNA primers and some ENA primers were purchased from Sigma–Aldrich Japan. LNA primers were synthesized according to reported methods [5,7].

PCR conditions for SNP detection in the human prothrombin gene and the promoter region of the mouse angiopoietin-like 3 gene

Forward PCR primers for the human prothrombin gene (coagulation factor II, GenBank Accession No. M17262, with the SNP site being in nucleotide 26784 of this accession number) [17] are listed in Table 1. Human genomic DNA from the TrueSNP kit (Proligo) was used. PCR was conducted using human genomic DNA (Proligo), 0.25 μM forward primer, 0.25 μM reverse primer 1 (5′-GGGTGAAGGCTGTGACCG-3′), and 1.25 U premix *Taq* (Takara Bio, Japan). Thermal cycling was carried out using a PCR Thermal Cycler PERSONAL (Takara Bio) programmed as follows: 10 min at 94 °C, followed by 30 cycles at 94 °C for 60 s, 63 °C for 60 s, 72 °C for 60 s, and final elongation at 72 °C for 10 min. Electrophoresis was conducted using a 10% polyacrylamide gel in 1× TBE buffer. The gel was stained with SYBR Green I (Cambrex). The bands on the gel (216 bp) were visualized with a Molecular Imager FX Fluorescent Imager system (Bio-Rad) and quantified with Quantity One software (Bio-Rad). The image of the gel was optimized using Paint Shop Pro (Jasc Software). This study was approved by the Ethical Committee of Sankyo Company.

Forward PCR primers for mouse angiopoietin-like 3 gene (GenBank Accession No. AL935325, with the SNP sites being nucleotide numbers 60556 and 60522 of this accession number) [18] are listed in Tables 2 and 3. Mouse genomic DNA was isolated according to reported methods [18]. The SNP sites were determined by the direct sequencing of the genomic DNA from each strain

Table 1
PCR primer sequences for human prothrombin gene

Primer name	Sequence (5′–3′)	Modified position	Mutant or wild type
Forward DNA primer 1	CACTGGGAGCATTGAGCTC	None	Wild type
Forward DNA primer 2	CACTGGGAGCATTGAGCTT	None	Mutant
Forward ENA primer 1	CACTGGGAGCATTGAGCTC	3′	Wild type
Forward ENA primer 2	CACTGGGAGCATTGAGCTT	3′	Mutant
Forward ENA primer 3	CACTGGGAGCATTGAGCTC	*n*-1	Wild type
Forward ENA primer 4	CACTGGGAGCATTGAGCTT	*n*-1	Mutant
Forward ENA primer 5	CACTGGGAGCATTGAGCTC	*n*-2	Wild type
Forward ENA primer 6	CACTGGGAGCATTGAGCTT	*n*-2	Mutant
Forward ENA primer 7	CACTGGGAGCATTGAGCTC	*n*-3	Wild type
Forward ENA primer 8	CACTGGGAGCATTGAGCTT	*n*-3	Mutant
Forward LNA primer 1	CACTGGGAGCATTGAGcTC	*n*-2	Wild type
Forward LNA primer 2	CACTGGGAGCATTGAGcTT	*n*-2	Mutant

Note. ENA residues are underlined, and LNA residues are shown in lowercase. C indicates 2′-*O*,4′-*C*-ethylene-5-methylcytosine. In the case of the ENA primer 1, 2′-*O*,4′-*C*-ethylenecytosine was used.

Table 2
PCR primer sequences for mouse angiopoietin-like 3 gene

Primer name	Sequence (5′–3′)	Modified position	Mouse strain
Forward DNA primer 3	ATCTGTCTACATATATATACACACACAT	None	AKR
Forward DNA primer 4	ATCTGTCTACATATATATACACACACAC	None	KK/Nga, KK/Snk
Forward ENA primer 9	ATCTGTCTACATATATATACACACACAT	3′	AKR
Forward ENA primer 10	ATCTGTCTACATATATATACACACACAC	3′	KK/Nga, KK/Snk
Forward ENA primer 11	ATCTGTCTACATATATATACACACACAT	*n*-1	AKR
Forward ENA primer 12	ATCTGTCTACATATATATACACACACAC	*n*-1	KK/Nga, KK/Snk
Forward ENA primer 13	ATCTGTCTACATATATATACACACACAT	*n*-2	AKR
Forward ENA primer 14	ATCTGTCTACATATATATACACACACAC	*n*-2	KK/Nga, KK/Snk
Forward ENA primer 15	ATCTGTCTACATATATATACACACACAT	*n*-3	AKR
Forward ENA primer 16	ATCTGTCTACATATATATACACACACAC	*n*-3	KK/Nga, KK/Snk

Note. The SNP site sequences of mouse angiopoietin-like 3 gene were analyzed. A colon (:) represents a deletion site. ENA residues are underlined.
KK/Nga, KK/Snk (wild type) —ATCTGTCTACATATATATACACACACAT—
AKR (mutant) —ATCTGTCTACATATATATACACACACA::T—

Table 3
PCR primer sequences for mouse angiopoietin-like 3 gene

Primer name	Sequence (5′–3′)	Modified position	Mouse strain
Forward DNA primer 5	CATGTCTACTGCTACTTCACATGTG	None	AKR
Forward DNA primer 6	CATGTCTACTGCTACTTCACATGTA	None	KK/Nga, KK/Snk
Forward DNA primer 7	CATGTCTACTGCTACTTCACATGGG	None	AKR
Forward DNA primer 8	CATGTCTACTGCTACTTCACATGGA	None	KK/Nga, KK/Snk
Forward ENA primer 17	CATGTCTACTGCTACTTCACATGTG	*n*-2	AKR
Forward ENA primer 18	CATGTCTACTGCTACTTCACATGTA	*n*-2	KK/Nga, KK/Snk
Forward ENA primer 19	CATGTCTACTGCTACTTCACATGGG	*n*-2	AKR
Forward ENA primer 20	CATGTCTACTGCTACTTCACATGGA	*n*-2	KK/Nga, KK/Snk

Note. The SNP site sequences of the mouse angiopoietin-like 3 gene were analyzed. ENA residues are underlined.
KK/Nga, KK/Snk (wild type) —CATGTCTACTGCTACTTCACATGCA—
AKR (mutant) —CATGTCTACTGCTACTTCACATGCG—

(Tables 2 and 3). PCR was carried out using 0.5 ng/μl mouse genomic DNA, 0.25 μM forward primer, 0.25 μM reverse primer 2 (5′-GTCACTAGACTACTGCTTACT GTCC-3′), and 1.25 U premix *Taq*. Thermal cycling was performed using a PCR Thermal Cycler PERSONAL programmed as described above. Electrophoresis was performed, and the band on the gel was visualized as described above. The PCR analysis were repeated two or three times to observe the reproducibility.

Results

AS–PCR SNP analysis for human prothrombin gene in human genomes

To investigate the effect of incorporating an ENA residue into primers and their extension by *Taq* DNA polymerase, forward ENA primers 1 to 8 were designed with an SNP site (C to T transition) at the 3′ end to distin-

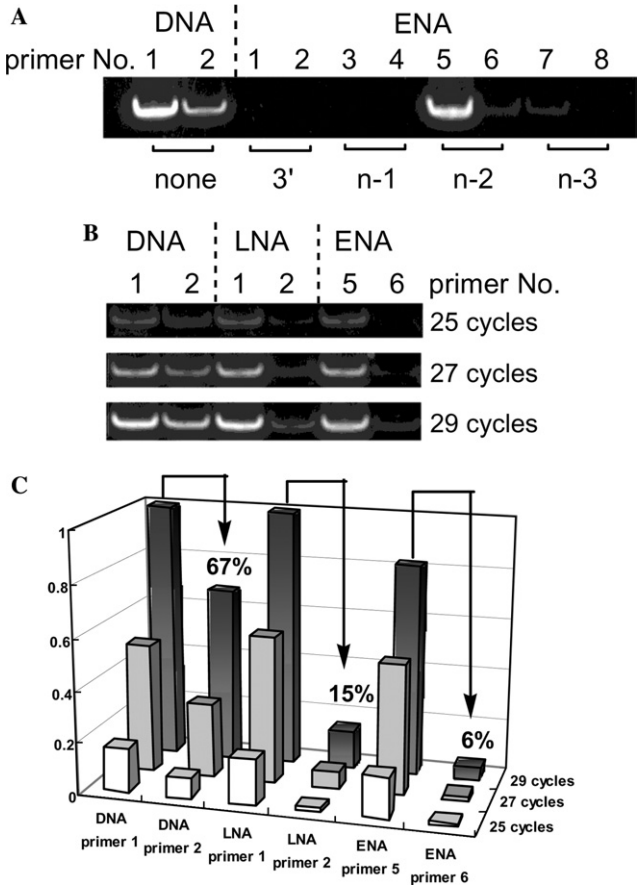


Fig. 2. (A) Comparison of DNA primers with ENA primers modified at various positions. (B) Discrimination of ENA primers with DNA and LNA primers modified at the *n*-2 position. Amplicons (216 bp) show products of the human prothrombin gene from human genomic DNA templates. (C) Histogram comparing the results of ENA primers with those of DNA and LNA primers. The ratios of intensities of amplicons using DNA primer 1 to the other matched or mismatched DNA, LNA, and ENA primers are plotted. The percentage values after 29 PCR cycles were obtained from the ratio of each mismatched primer to perfectly matched primer multiplied by 100.

guish between the wild type and mutant sequences of the human prothrombin gene (Table 1). Fig. 2A illustrates that only the primer modified with the ENA residue at the *n*-2 position, forward ENA primer 5 for the wild type, gave levels of amplification comparable to those of the forward DNA primer 1. In addition, *n*-2 position-modified ENA primer 6 for the mutant had lower levels of mismatch extension than did DNA primer 2 for the mutant. To investigate the effect of the ethylene bridge of the ENA residue on the PCR amplification, we compared AS–PCR using ENA primers with that using LNA primers modified with the less hindered methylene bridge rather than the ethylene bridge. Forward ENA primer 6 yielding a faint mismatched amplicon band showed a band of low intensity compared with *n*-2 position-modified LNA primer 2 after 25, 27, and 29 cycles of PCR, as shown in Fig. 2B. Incorporation of the ENA residue into the primers reduced mismatched extension down to approximately 6% relative to the case of DNA primers (67%) and LNA primers (15%), as shown in Fig. 2C. This discrimination ratio between matched and mismatched products using ENA primers was quite comparable to that using primers with 2′,4′-BNA/LNA at the 3′ end [8].

AS–PCR SNP analysis for mouse angiopoietin-like 3 gene in mouse genomes

To further investigate the effect of incorporating ENA residues into primers and their extension by *Taq* DNA polymerase, forward ENA primers 9 to 16 targeting a sequence of mouse angiopoietin-like 3 gene were designed with a deletion site (CA deletion, Table 2). The 2-bp deletion was recognized by primers designed with a single 3′ mismatch compared with the wild type. In the case where the mouse AKR strain was used as a deletion genome, only the primer modified with the ENA residue at the *n*-2 position, forward ENA primer 13, gave a higher amplification yield than did forward DNA primer 3 (Fig. 3A). In addition, *n*-2 position-modified ENA primer 14 had no mismatch extension in comparison with forward DNA primer 4, which was accompanied by an undesired band. In the case of using the mouse KK/Nga strain, the ENA primer modified at the *n*-2 position, forward ENA primer 13, yielded no amplicon, and corresponding ENA primer 14 gave the predicted result, as shown in Fig. 3B. The genomic DNA of the KK/Snk strain was analyzed in the same manner as the genomic DNA of the KK/Nga strain (Fig. 3C). A heterozygous DNA template (Mix) was prepared by mixing both the AKR and KK/Nga strain genomic DNAs in equivalent amounts, and their AS–PCR using DNA and ENA primers was conducted. Although nearly an equal amount of PCR products was detected in the case of using forward ENA primers 13 and 14, it was difficult to distinguish the allele in the case of using DNA primers 3 and 4, as shown in Fig. 3C. These results indicated that AS–PCR using ENA primers could highly discriminate against not only homozygous alleles but also heterozygous alleles.

AS–PCR SNP analysis using ENA primers having an unpaired nucleoside at the n-1 position

As another SNP detection method by AS–PCR, a method using primers having an unpaired nucleoside at the *n*-1 position has been reported [19,20]. This has improved recognition of the nucleoside at the SNP site. We investigated the influence of the *n*-2 position-modified ENA primer on the introduction of an unpaired nucleoside at the *n*-1 position of an ENA primer that was recognized as containing an SNP site (G to A transition) of the mouse angiopoietin-like 3 gene, as shown in Table 3. As a preliminary result, primers

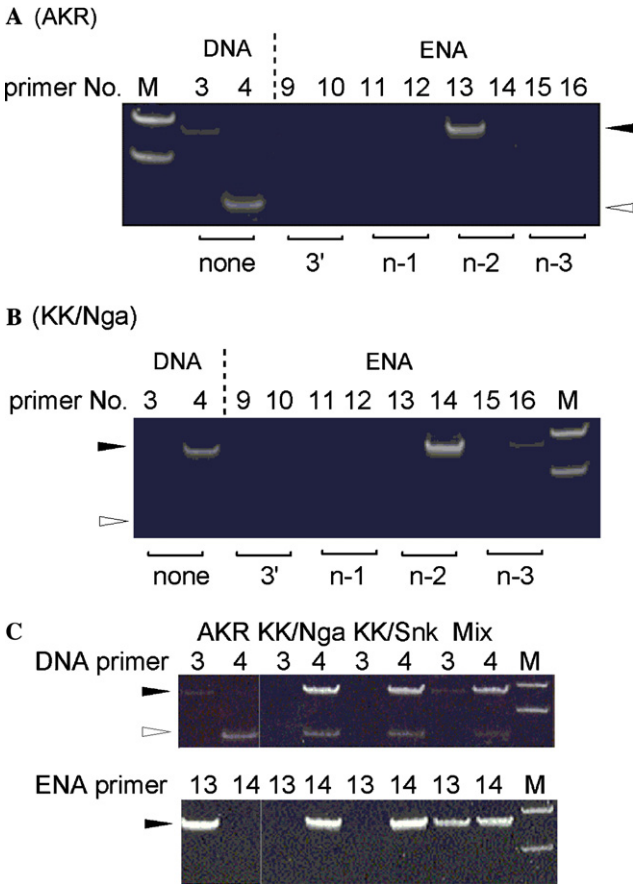


Fig. 3. Comparison of DNA primers with ENA primers modified at various positions. Amplicons show products of mouse angiopoietin-like 3 gene from (A) mouse AKR strain and (B) mouse KK/Nga strain genomic DNA templates. (C) Comparison of DNA primers 3 and 4 with ENA primers 13 and 14 amplified from mouse AKR, KK/Nga, and KK/Snk strain genomic DNA templates and heterozygous DNA templates (Mix) of both AKR and KK/Nga strain genomic DNAs. Closed arrowheads and open arrowheads indicate desired products (152 bp for AKR strain, 154 bp for KK/Nga strain) and undesired products, respectively. Two bands in the marker lane (M) indicate lengths of 200 and 100 bp.

having a perfectly matched base pair at the *n*-1 position did not dramatically improve the SNP discrimination (data not shown). The newly designed primers had an unpaired nucleoside (T or G) at the *n*-1 position. In the case of DNA primers 5 and 6, which have an unpaired T nucleoside at the *n*-1 position, there was perfect discrimination of the G and A nucleosides at the SNP site of the genomic DNA from the AKR strain. However, a minor band was observed in the analysis of the genomic DNA from KK/Nga mice using DNA primer 5 (Fig. 4A). On the other hand, ENA primers 17 and 18 having an unpaired T nucleoside discriminated between the G and A nucleosides at the SNP site without producing any undesired bands in comparison with corresponding DNA primers 5 and 6. In the case of the DNA and ENA primers having an unpaired G nucleoside at the *n*-1 position, ENA primers 19 and 20 gave much better results

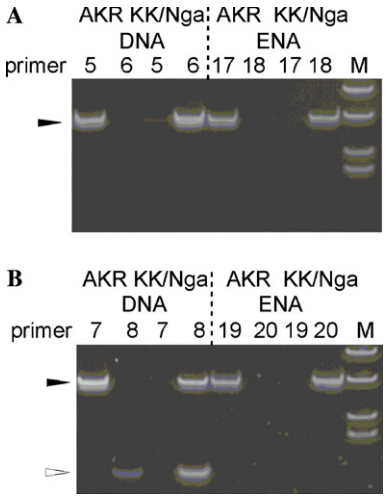


Fig. 4. (A) Comparison of DNA primers 5 and 6 with ENA primers 17 and 18 having an unpaired T nucleoside at the *n*-1 position. (B) Comparison of DNA primers 7 and 8 with ENA primers 19 and 20 having an unpaired G nucleoside at the *n*-1 position. Amplicons show products of mouse angiopoietin-like 3 gene from mouse AKR or KK/Nga strain genomic DNA templates. Closed arrowheads and open arrowheads indicate desired products (182 bp for AKR strain, 184 bp for KK/Nga strain) and undesired products, respectively. The four bands in the marker lane (M) indicate lengths of 300, 200, 100, and 80 bp (Sigma–Aldrich, Japan).

than did DNA primers 7 and 8, which gave undesired products (Fig. 4B).

Discussion

Relationship between ENA-modified residue position of the primers and their efficiency of amplification in PCR

We showed three examples of ENA primers in Figs. 2–4. In the PCR with *Taq* DNA polymerase, only the *n*-2 position-modified ENA primers could be compared with the DNA primers. The other primers, which were modified with ENA residues at the 3′ end, or the *n*-1 or *n*-3 position, were not amplified effectively, although it has been reported that LNA primers modified at the 3′ end or the *n*-1 position were amplified effectively [8,10,21]. For other sugar-modified primers, it has been reported that with primers containing a C4′-methylated thymidine at the 3′ end, *Taq* DNA polymerase can discriminate against a mismatched nucleotide in the template [22]. Our results seem to show that *Taq* DNA polymerase can tolerate the ethylene bridge of an ENA residue located only at the *n*-2 position of the primers because at this site the *Taq* DNA polymerase and template–primer ternary complex can be formed with the least steric hindrance. To investigate whether the ethylene bridge did not inhibit the ternary complex formation, we superimposed the structure of 2′-*O*,4′-*C*-ethylene adenosine [14] on the structure of the complex reported previously [23].

When the 2′-*O*,4′-*C*-ethylene adenosine was placed at the *n*-1 position, the ethylene bridge clashed with the main chain of the *Taq* DNA polymerase at Gln582 and Asn583 (data not shown). Placing 2′-*O*,4′-*C*-ethylene adenosine at the *n*-2 position of the primer in the ternary complex was tolerated with some minor rotation of the side chain of Tyr545, as shown in Fig. 5. Although the sugar conformations of three nucleosides from the 3′ end in the primer of the ternary complex all were in the N conformation, the fourth nucleoside from the 3′ end, which corresponds to the nucleoside at the *n*-3 position, was in the S conformation [23]. This conformation of the nucleoside at the *n*-3 position was not compatible with the N conformation of 2′-*O*,4′-*C*-ethylene adenosine. On the basis of these observations, modification at the *n*-2 position of primers was found to be reasonable in view of the ternary complex formation.

In the case of SNP detection in the mouse angiopoietin-like 3 gene, although a low level of desired product and/or undesired side product (which were thought to be derived from a primer–dimer) was observed among the PCR amplicons when using DNA primers, only desired amplicons were observed when ENA primers modified at the *n*-2 position were used. It is thought that the primer–dimer formation, which leads to mismatched extension, might occur in a tail-to-tail interaction mechanism with *Taq* DNA polymerase, meaning an interaction between the 3′ regions of the two primers [24]. These results suggest that the primers modified with ENA residues at the *n*-2 position could inhibit the primer–dimer extension by *Taq* DNA polymerase even though the ENA primers may interact in a tail-to-tail fashion. Furthermore, we showed that the efficiency of amplification using ENA primers was nearly the same as that using DNA primers (Figs. 2 and 3). These results indicate that the use of the *n*-2 position-modified ENA primer improved mismatch discrimination without affecting PCR efficiency.

Discrimination against mismatched template ENA primer by Taq DNA polymerase

DNA primers fail to discriminate against point mutations in the template, producing a false-positive result [3,4]. However, ENA primers could be used to specifically amplify the target gene in a wild type or mutant template (Figs. 2–4). The proposed mechanism of specific template discrimination by the *n*-2 position-modified ENA primer can be described as follows. The X-ray analysis showed that the three nucleosides from the 3′ end of the primer in the *Taq* DNA polymerase–template–primer ternary complex preferred the N conformation because this produced a wider minor groove than that of the B-form structure where the sugar puckering of each nucleoside was all in the S conformation [23]. On the other hand, it was also observed that a DNA–ENA duplex formed an A-like structure similar

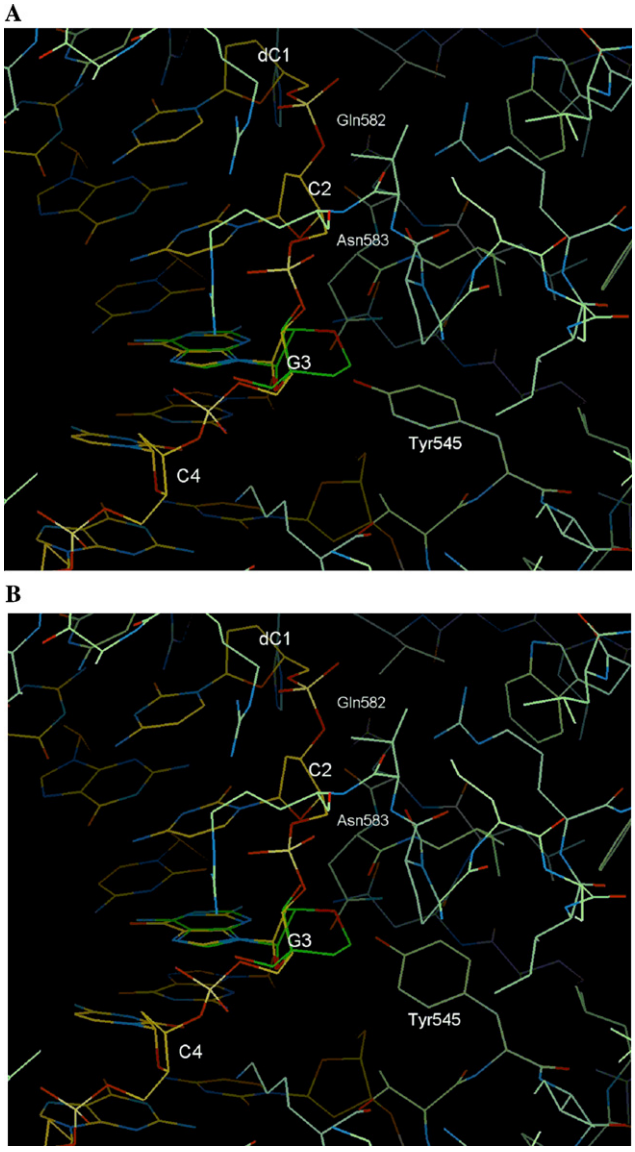


Fig. 5. Two superimposed structures of *Taq* DNA polymerase–template–primer ternary complex and 2′-*O*,4′-*C*-ethylene adenosine. (A) The data of *Taq* DNA polymerase–template–primer ternary complex and 2′-*O*,4′-*C*-ethylene adenosine were obtained based on the data of Li et al. [23] and Morita et al. [14], respectively, and were superimposed using QUANTA software (Accelrys, San Diego, USA). The carbon atoms of the primer in the ternary complex and 2′-*O*,4′-*C*-ethylene adenosine are shown in yellow and yellow-green, respectively. dC1, C2, G3, and C4 represent four nucleotides of the primer from the 3′ end [23]. The structure of 2′-*O*,4′-*C*-ethylene adenosine was superimposed on that of G3 at the *n*-2 position of the primer of the ternary complex. The ethylene bridge of the 2′-*O*,4′-*C*-ethylene adenosine clashed with the side chain of Thr545. (B) The side chain of Tyr545 of the ternary complex was fixed in a position that did not hinder the ethylene bridge of the 2′-*O*,4′-*C*-ethylene adenosine with some minor rotation of the side chain of Thr545 (see text).

to that of a DNA–LNA duplex [14]. In the DNA duplex containing the LNA residues, the sugar conformation of the LNA residue and that of the two nucleotides at both sides of the LNA residue changed from S conformation to N conformation [25]. Such conformational changes

seen in the LNA residues may also occur with conformationally restricted ENA residues. *Taq* DNA polymerase did extend the ENA primer–template with some minor structural exchange, as shown in Fig. 5. Nevertheless, the sugar conformations of the ENA residues and those of the nucleosides on either side would be different from natural DNA. However, *Taq* DNA polymerase did not extend the ENA primer containing a mismatched SNP at the 3′ end such as G:T or A:C. This is probably because the *Taq* DNA polymerase cannot simultaneously recognize the conformational change in nucleosides derived from the incorporation of the ENA residues and the terminal SNP site.

Conclusion

We have shown that modification of primers with ENA residues placed at the *n*-2 position contributed to the effective production of amplicons by *Taq* DNA polymerase. Furthermore, we demonstrated an improved method for SNP detection using ENA-modified primers and conventional AS–PCR. By using this method, an SNP in genomes could be detected accurately. This ENA-based AS–PCR could be combined with various other methods for the detection of PCR products such as real-time PCR and mass spectroscopy detection. Such improved AS–PCR methods could then be automated to detect a variety of SNP sites in genomes.

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