Mutation S543N in the thumb subdomain of the *Taq* DNA polymerase large fragment suppresses pausing associated with the template structure

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Abstract Substitution of Asn for the conserved Ser^{543} in the thumb subdomain of the *Taq* DNA polymerase large fragment (*Klentaq* DNA polymerase) prevents pausing during DNA synthesis and allows the enzyme to circumvent template regions with a complex structure. The mutant enzyme (*KlentaqN* DNA polymerase) provides specific PCR amplification and sequencing of difficult templates, e.g. those with a high GC% content or strong secondary structure.

Key words: Polymerase chain reaction; DNA sequencing; *Taq* DNA polymerase; *Klentaq* DNA polymerase

1. Introduction

DNA-dependent DNA polymerases from thermophilic bacteria are widely used for DNA sequencing by Sanger's method, PCR amplification, etc. Studies on the factors affecting DNA synthesis by DNA polymerases may contribute much to understanding the mechanism of function of these enzymes and selecting optimal conditions for their in vitro application.

The efficiency of DNA polymerases is limited by various factors that cause pausing during elongation of the DNA strand and synthesis termination. The two most principle factors are (1) the presence in the template of fragments with complex secondary or primary structures [1,2], especially those with a high GC% content [2–4], and (2) the incorporation of mismatching nucleotide in the synthesized DNA strand [5]. The latter factor is of particular importance in the case of DNA polymerases with no $3' \rightarrow 5'$ endonuclease activity [6]. The negative effects of these factors on the function of DNA polymerases may be decreased by adding to the reaction mixture an auxiliary DNA polymerase with the $3' \rightarrow 5'$ endonuclease activity [6,7] and compounds facilitating the melting of DNA double-stranded regions [1–4]. Another approach to increasing the efficiency of DNA synthesis is modification of DNA polymerases themselves in order to decrease their sensitivity to the limiting factors.

Earlier [8], we described the S543N mutation in the thumb subdomain of the *Taq* DNA polymerase large fragment (*Klentaq* DNA polymerase [5]) that increased the efficiency of DNA synthesis in PCR amplification by the mutant enzyme (*KlentaqN* DNA polymerase [8]). The thumb subdomain is a fragment of the polymerase domain and plays an important role in the polymerase interaction with the template-primer duplex [9–13]. Mutations in this subdomain result in considerable changes in the polymerase processivity and efficiency of synthesis of long DNA molecules [14,15].

The goal of this work was to study the effect of the S543N mutation on the enzyme capacity for DNA strand elongation in the case of difficult templates and after incorporation of mismatching nucleotides.

2. Materials and methods

2.1. PCR amplification of λDNA fragments

PCR was performed in a DNA Thermal Cycler 480 (Perkin-Elmer-Cetus). The reaction mixture (50 μl) contained 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 0.2 mM of each dNTPs, 3.5 mM $MgCl_2$, 17 pmol of each primer, 15 ng of λDNA as a template, and 2–12 U of either *Klentaq* or *KlentaqN* DNA polymerases. The reaction proceeded in 25 cycles: 94°C, 30 s; 58°C, 30 s; 72°C, 100 s.

λDNA fragments were amplified with the primers P1 (5′-GATGAGTTCGTGTCCGTACAACTGG) and P3 (5′-TTCCCAGCCACACGCTGCATGACAT) (a 1270-bp fragment); P1 and P4 (5′-TAGCTGTCGTCATAGGACTCAGCG) (a 2000-bp fragment); and P1 and P5 (5′-TGTTGACCTTGCCTGCAGCAACGC) (a 2500-bp fragment).

2.2. Determination of the efficiency of DNA synthesis in the presence of 3′-mismatching nucleotide

The efficiency of DNA synthesis by *Klentaq* and *KlentaqN* DNA polymerases in the presence of 3′-mismatching nucleotide was estimated by comparing the amounts of DNA synthesized in PCR with the primers either containing or not the 3′-mismatching nucleotide. PCR amplification of the 500-bp λDNA fragment was performed with the primer pairs P2/P1 (5′-GGTTATCGAAATCAGCCACAGCGCC), P2/P12 (5′-GATGAGTTCGTGTCCGTACAACTGC), P2/P13 (5′-GATGAGTTCGTGTCCGTACAACTGA), and P2/P14 (5′-GATGAGTTCGTGTCCGTACAACTGT). The primers P1 and P2 were complementary to the corresponding fragments of λDNA; the primers P12, P13 and P14 were identical to P1, except the 3′-terminal nucleotide. λDNA was used as a template. The reaction mixture (50 μl) contained 12 U of either *Klentaq* or *KlentaqN* DNA polymerases. The reaction proceeded in 25 cycles: 94°C, 45 s; 59°C, 30 s; 72°C, 30 s.

To estimate the amount of the synthesized DNA, [α-^{32}P]dATP was added to the reaction mixture (2 μCi/50 μl reaction mixture), and radioactivity of the acid-insoluble fraction was then determined. For this purpose, the reaction was performed, and 20 μl of the resulting mixture was applied on a GF/B filter (Whatman). The filter was washed with 10% trichloroacetic acid and dried. The radioactivity was determined with a Beckman LS 9800 scintillation counter using Ready-Solv HP scintillation liquid (Beckman).

2.3. PCR amplification of the GC-rich template

As a GC-rich template, we used a 368-bp fragment of the *Thermus thermophilus* DNA polymerase gene (GC pair content of 67%) flanked by the TN1 (5′-CTTCCGCTTGGCGGGCCACCCCTT) and TN2 (5′-CCCGGCGGATCCTCTGGCCCAAG) primers. DNA from *T. thermophilus* HB8 was used as a template. The reaction mixture (50 μl) contained 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 0.2 mM of each dNTPs, 3.5 mM $MgCl_2$, 17 pmol of each primer, 100 ng of *T. thermophilus* HB DNA, and 12 U of either

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Abbreviations: S543N, mutation Ser^{543} to Asn; PCR, polymerase chain reaction

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Klentaq or *KlentaqN* DNA polymerases. PCR was performed in the following regimes: (I) 94°C, 30 s; 56°C, 30 s; 72°C, 30 s; or (II) 94°C, 30 s; 61°C, 30 s; 72°C, 30 s. The reaction included 25 cycles. When the regime (I) was used, the reaction mixture either contained or not 1.2 M betaine (Sigma).

2.4. DNA sequencing

Dideoxy sequencing reactions were carried out using *Klentaq* or *KlentaqN* DNA polymerases according to the protocol recommended for Δ*Taq* Ver. 2.0 DNA polymerase (USB). A 3-kb *Bss*HII-*Eco*RI fragment of the human *RIL* gene [16] cloned in the pBluescript SKII-vector (Stratagene) was used as a double-stranded template. Each sequencing reaction was performed using 1 unit of one of the two enzymes and 6 μg of the template.

3. Results

λDNA fragments of 1270, 2000, and 2500 bp were amplified by PCR using *Klentaq* or *KlentaqN* DNA polymerases. Fig. 1 shows that, in order to achieve similar efficiencies of the DNA amplification, the amount of *Klentaq* DNA polymerase should be six times greater than that of *KlentaqN* DNA polymerase. This difference in the efficiency of the two enzymes became more evident with the increase in the amplified fragment length.

The sensitivities of *Klentaq* and *KlentaqN* DNA polymerases to the presence of mismatching nucleotide at the 3′-terminus of the elongated DNA strand were compared as described in [17]. The 500-bp λDNA fragment was amplified using *Klentaq* and *KlentaqN* DNA polymerases and pairs of primers P1/P2, P12/P2, P13/P2, and P14/P2. The P1 and P2 primers were complementary to the corresponding fragments of λDNA; the primers P12, P13, and P14 were identical to the P1 primer except the 3′-terminal oligonucleotides. When estimating the amplification efficiency from incorporation of radioactivity-labeled nucleotide in the synthesized product, we found that the efficiency of PCR in the presence of 3′-mismatching nucleotide decreased by more than an order of magnitude for both enzymes (Table 1). Therefore, the non-modified (*Klentaq*) and mutant (*KlentaqN*) enzymes exhibited similar sensitivity for the 3′-mismatching nucleotide.

To estimate the effect of template structure on the efficiency of DNA synthesis by *Klentaq* and *KlentaqN* DNA polymerases, we performed DNA sequencing by Sanger's method and PCR amplification of the GC-rich template using these enzymes. The efficiencies of *Klentaq* and *KlentaqN* DNA polymerases in PCR amplification of the GC-rich DNA sequence were determined by amplifying a 368-bp fragment of the *Tth* DNA polymerase gene (GC pair content of 67%). PCR was performed at different primer annealing temperatures in the presence or absence of betaine in the reaction mixture. Betaine is known to promote the melting of GC-rich DNA sequences [18]; it eliminates pausing during DNA strand elongation [2] and considerably facilitates PCR amplification of GC-rich templates [4]. Fig. 2 shows that *KlentaqN* DNA polymerase efficiently amplified the GC-rich DNA sequence at different primer annealing temperatures both in the presence or absence of betaine, whereas *Klentaq* DNA polymerase was capable of DNA amplification only at high primer annealing temperature or in the presence of betaine.

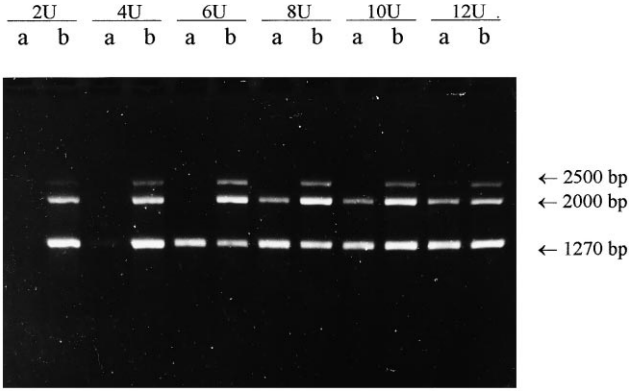

Fig. 1. Electrophoretic analysis of PCR amplification products of λDNA (1270, 2000, 2500 bp) in 1.5% agarose gel. The fragments were amplified with 2, 4, 6, 8, 10, and 12 U of (lanes a) *Klentaq* or (lanes b) *KlentaqN* DNA polymerases.

The results of DNA sequencing by Sanger's method using *Klentaq* and *KlentaqN* DNA polymerase are shown in Fig. 3. The application of *KlentaqN* DNA polymerase allowed us to decrease considerably (compared to *Klentaq* DNA polymerase) the number of pause sites associated with the DNA template structure (Fig. 3). The data on DNA sequencing and PCR indicate that *KlentaqN* DNA polymerase is less sensitive to the template structure than *Klentaq* DNA polymerase.

4. Discussion

The efficiency of in vitro DNA amplification is limited by sensitivity of DNA polymerases to the following factors: complex template structure and attachment of mismatching nucleotide resulting in the DNA synthesis termination. To increase the efficiency of in vitro DNA synthesis, two principal approaches may be used. The first approach is addition to the reaction mixture of components that may decrease the nega-

Table 1
Radioactive label incorporation into the 500-bp λ phage DNA fragment synthesized by PCR with primers containing or not containing 3′-mismatching nucleotides

DNA polymerase	Primers	Radioactivity, 10^5 cpm	Student's error[a] ($P = 0.05$), 10^3 cpm
Klentaq	Prλ 1/Prλ 2	2.55	7.3
	Prλ 12/Prλ 2	0.15	1.7
	Prλ 13/Prλ 2	0.13	1.2
	Prλ 14/Prλ 2	0.16	1.5
KlentaqN	Prλ 1/Prλ 2	2.70	7.8
	Prλ 12/Prλ 2	0.17	1.2
	Prλ 13/Prλ 2	0.16	1.4
	Prλ 14/Prλ 2	0.17	1.6
Background (without polymerase)		0.012	0.2

[a]Computed using statistics program Sigma Plot (Jandel Scientific).

tive effects of these factors. The other approach is modification of DNA polymerases in order to decrease the sensitivity of these enzymes to the negative factors. An example of such modification is substitution of Asn for Ser^{543} in the large fragment of *Taq* DNA polymerase that was found to increase significantly the efficiency of DNA amplification (Fig. 1).

Ser^{543} of *Taq* DNA polymerase is a highly conserved residue [9] that directly participates in the enzyme interaction with the template [13]. The significance of this residue for the DNA polymerase interaction with DNA was demonstrated in the study [14], in which the conserved fragment of the thumb subdomain of DNA polymerase from the Ø29 phage was studied. Thus, substitution of Gly for Ser^{338} (which is homologous to Ser^{543} of *Taq* DNA polymerase [9]) decreased the efficiency of the Ø29 phage DNA replication [14]. Apparently, substitution of Ser at this position affects the interactions between DNA polymerase with the primer-template duplex and, therefore, changes the efficiency of synthesis of long DNA sequences [8,14]. These effects may be associated with either changes in the sensitivity of DNA polymerase to incorporation of mismatching nucleotides or alterations in the enzyme sensitivity to the template structure.

To determine the effect of the S543N mutation in the *Taq* DNA polymerase large fragment on the enzyme sensitivity to incorporation of mismatching nucleotide, we compared the capacities of the non-modified (*Klentaq* DNA polymerase) and modified (*KlentaqN* DNA polymerase) enzymes for elongation of the DNA strand in the presence of 3′-mismatching nucleotide. We found (Table 1) that the presence of mismatching nucleotide at the 3′-end of the elongated DNA strand decreased to the same extent the PCR amplification efficiency by both enzymes.

The effects of the template structure on the efficiency of DNA synthesis by *Klentaq* and *KlentaqN* DNA polymerases were compared by amplifying a DNA fragment with the high content of GC pairs (67%) (Fig. 2). *Klentaq* DNA polymerase amplified this fragment only at a high primer annealing temperature or in the presence of betaine, which is known to facilitate amplification of GC-rich templates [4]. These facts

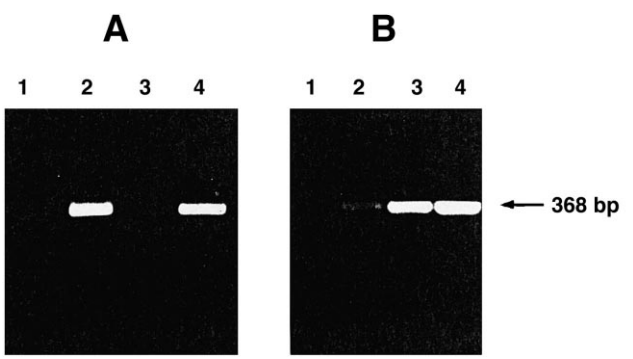

Fig. 2. Electrophoretic analysis of PCR amplification products of the GC-rich (67%) DNA template (368 bp) in 1.5% agarose gel. A: PCR was performed with 12 U of (lanes 1, 3) *Klentaq* or (lanes 2, 4) *KlentaqN* DNA polymerases in the following regimes: (lanes 1, 2) 94°C, 30 s; 56°C, 30 s; 72°C, 30 s; or (lanes 3, 4) 94°C, 30 s; 61°C, 30 s; 72°C, 30 s. The reaction included 25 cycles. B: Amplification was performed in the absence (lanes 1, 3) or presence (lanes 2, 4) of 1.2 M betaine with 12 U of (lanes 1, 2) *Klentaq* or (lanes 3, 4) *KlentaqN* DNA polymerases in the following regime: 94°C, 30 s; 56°C, 30 s; 72°C, 30 s.

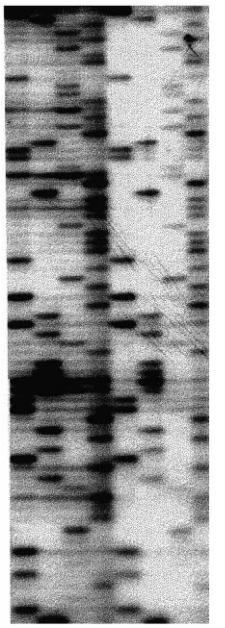

Fig. 3. Effect of the S543N mutation on (A) *Klentaq* and (B) *KlentaqN* DNA polymerase pausing in DNA sequencing. Lanes contain reactions terminated by ddTTP, ddCTP, ddGTP, and ddATP from left to right, respectively. The arrows indicate pauses.

indicate that difficulties in amplification of this template are related to the complexity of its secondary structure stabilized by the high amount of GC pairs. *KlentaqN* DNA polymerase efficiently amplified this DNA fragment in the absence of betaine and at a low primer annealing temperature, which suggests the low sensitivity of the mutant enzyme to the structure of the template DNA.

Site-specific pausing associated with the template structure is clearly manifested in DNA sequencing by Sanger's method. The application of *KlentaqN* DNA polymerase for DNA sequencing allowed us to decrease significantly the number of pause sites compared to *Klentaq* DNA polymerase (Fig. 3).

The results on PCR amplification of the GC-rich DNA fragment (Fig. 2) and DNA sequencing (Fig. 3) with *Klentaq* and *KlentaqN* DNA polymerases show that the S543N mutation suppresses pausing and facilitates synthesis of template fragments with complex structures. Apparently, the substitution of Asn for Ser^{543} in the *Taq* DNA polymerase large fragment enhances the polymerase interaction with the primer-template duplex and prevents the termination of DNA synthesis, thereby increasing the enzyme efficiency.

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