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Sensing Domain and Extension Rate of a Family B-Type DNA Polymerase Determine the Stalling at a Deaminated Base

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The uracil-sensing domain in archaeal family B-type DNA polymerases recognizes pro-mutagenic uracils in the DNA template, leading to stalling of DNA polymerases. Here, we describe our new findings regarding the molecular mechanism underpinning the stalling of polymerases. We observed that two successive deaminated bases were required to stall TNA1 and KOD1 DNA polymerases, whereas a single deaminated base was enough for stalling Pfu DNA polymerase, in spite of the virtually identical uracil-sensing domains. TNA1 and KOD1 DNA polymerases have a much higher extension rate than Pfu DNA polymerase; decreasing the extension rate resulted in stalling by TNA1 and KOD1 DNA polymerases at a single deaminated base. These results strongly suggest that these polymerases require two factors to stop DNA polymerization at a single deaminated base: the presence of the uracil-sensing domain and a relatively slow extension rate.

Keywords: Archaeal family B-type DNA polymerase, uracil sensing, TNA1 DNA polymerase, extension rate, hypoxanthine

DNA is at risk of damage by reactive oxygen species [7, 17], which can deaminate bases (adenine to hypoxanthine, guanine to xanthine, and cytosine to uracil [17, 21, 26]), eventually resulting in an increased transition mutation during replication *in vivo* [12, 17, 22]. It has been also reported that the deamination frequency at DNA bases increases at high temperature [14, 20], and hyperthermophilic archaea inhabiting high temperature environments are exposed to an increased risk of DNA damage by deamination [10]. To circumvent the highly detrimental effect of deamination, hyperthermophilic archaea have apparently evolved efficient enzymes that hydrolyze unusual bases in solution or remove deaminated bases from DNA templates, such as UDGase [24, 25, 29], dUTPase [4, 13], and hypoxanthine/xanthine dNTP pyrophosphatase [5]. In addition, the unique domain at the N-terminus of family B-type DNA polymerases, called the uracil-sensing domain, recognizes the pro-mutagenic uracil that is present four bases ahead of the primer-template junction and stalls replication [6, 9, 27]. Together with hydrolysis enzymes toward unusual bases, the uracil-induced stalling of Pfu DNA polymerase appears to be a molecular tool to insure the replication accuracy and efficiency by avoiding the incorporation of pro-mutagenic uracil into DNA. The remaining questions are whether the uracil-induced stalling is common to all archaeal B-type DNA polymerases that encounter uracil and how they handle other deaminated bases like hypoxanthine and xanthine, which are also generated *in vivo*.

Recently, we isolated a new hyperthermophilic strain from a deep-sea hydrothermal vent area in the PACMANUS field. It was identified as a species of *Thermococcus* based on its 16S rDNA sequence [1], and various thermostable enzymes have been studied by combination of conventional molecular engineering and genomic approachs [16, 19]. In this study, we report that various archaeal B-type DNA polymerases are inhibited by dITP and their common uracil-sensing domain recognizes both hypoxanthine and uracil, consistent with the recent reports [8, 11]. We also observed that the stalling pattern was not uniform in various family B-type DNA polymerases, but was related to the extension rate of the specific DNA polymerase.

Materials and Methods

Construction of Mutant DNA Polymerase Genes

Site-specific mutagenesis of TNA1 and Pfu DNA polymerase genes was carried out using a QuikChange site-directed mutagenesis kit

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Table 1. The position of amino acid substitution and the primer sequences for mutation.

Name of mutants	Position of amino acid substitution	Mutation primer sequence (5'-3')
TNA1		
T_Y7F	Tyr→Phe	CTCGACGTCGATTTCATCACAGAGGACGGAAAGC
T_V93Q	Val→Gln	CACCCGCAGGACCAACCCGCAATCCGCGACAAGATAAGG
T_F116E	Phe→Glu	CGACATACCCGAAGCCAAGCGCTACCTC
Pfu		
P_Y7F	Tyr→Phe	TTAGATGTGGATTTCATAACTGAAGAAGGAAAAC
P_V93Q	Val→Gln	CATCCCCAAGATCAACCCACTATTAGAGAAAAAGTT
P_F116E	Phe→Glu	TACGATATTCCAGAAGCAAAGAGATACCTCATCGAC

(Stratagene, La Jolla, CA, U.S.A.). The primers for the mutation in this study are summarized in Table 1.

Expression and Purification of Mutant DNA Polymerases

Plasmids were transformed into *Escherichia coli* BL21 Rosetta(DE3)pLysS. Overexpression of genes was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at the mid-exponential growth phase, followed by additional 3 h incubation at 37°C. Cells were then harvested by centrifugation (6,000 ×*g* at 4°C for 20 min) and resuspended in 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 M KCl and 10% glycerol, followed by sonication. The cell lysate was centrifuged (20,000 ×*g* at 4°C for 30 min), and crude samples were prepared by heat treatment at 80°C for 20 min. The resulting supernatant was applied to a column of TALON metal affinity resin (BD Biosciences, Clontech, Palo Alto, CA, U.S.A.) and washed with 10 mM imidazole (Sigma, St. Louis, MO, U.S.A.) in 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 M KCl and 10% glycerol, and the protein was eluted with 300 mM imidazole in the same buffer. The pooled fractions were dialyzed in storage buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, and 10% glycerol.

The protein concentration was determined by Bradford assay [2], and protein purity was examined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using standard procedures [18].

dITP Incorporation Assay

Incorporation assay was carried out as described by Chung *et al.* [5] with slight modification. In brief, a 5'-^{32}P-labeled 23-mer primer was annealed to the underlined portion of the 44-mer template (Table 2) and the primer-template complex (200 fmol) was incubated at 75°C for 15 min with 1.25 units of TNA1 DNA polymerase in 120 mM Tris-HCl (pH 8.8), 60 mM KCl, 30 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, and 0.1 mM dITP. The reaction mixtures for Pfu (Promega) and KOD1 (Novagen) DNA polymerases were modified using each manufacturer's buffer. After incubation, each sample was analyzed by 15% polyacrylamide gel electrophoresis, followed by autoradiography.

Primer Extension Assay

Primer extension assay was performed as described by Fogg *et al.* [6] with slight modification. In brief, templates (Table 2) containing uracil, hypoxanthine, or xanthine were used. The primer-template complex (400 fmol) was incubated at 75°C for 15 min with 1.25 units of TNA1 DNA polymerase in 120 mM Tris-HCl (pH 8.8), 60 mM KCl, 30 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, and 0.25 mM of each dNTP. The reaction mixtures for *rTaq* (Takara), Pfu (Promega), Deep-vent (New England Biolabs, Inc., Beverly, MA, U.S.A.), Vent (New England Biolabs, Inc.), and KOD1 (Novagen) DNA polymerases were slightly modified using each manufacturer's buffer. After incubation, each sample was analyzed by 15% polyacrylamide gel electrophoresis, followed by autoradiography.

PCR Amplification

PCR amplification of a 2-kb target from λ DNA was carried out by TNA1 and Pfu DNA polymerases using the manufacturer's buffer. TNA1 DNA polymerase buffer consisted of 120 mM Tris-HCl (pH 8.8), 30 mM $(NH_4)_2SO_4$, 60 mM KCl, and 1 mM $MgCl_2$. After a single 1 min denaturation step at 95°C, 30 cycles of denaturation (20 sec at 94°C), annealing, and extension (2 kb, 2 min; 5 kb, 3.5 min; 8 kb, 5 min; 10 kb, 6 min; 12 kb, 7 min at 72°C) were performed, followed

Table 2. Oligonucleotides used as substrates in this study.

Name	Oligonucleotide sequence (5'-3')
5'-^{32}P-labeled 23-oligonucleotide primer	
Labeled primer	GGGGAGCCGCTAGAGTCGACCTC
44-Oligonucleotide templates	
C_tem	GGAGACAAGCTTGATTGCCTCGAGGTCGACTCTAGCGGCTCCCC
1U_tem	GGAGACAAGCTTGAUTGCCTCGAGGTCGACTCTAGCGGCTCCCC
2U_tem	GGAGACAAGCTTGUUTGCCTCGAGGTCGACTCTAGCGGCTCCCC
1H_tem	GGAGACAAGCTTGAHTGCCTCGAGGTCGACTCTAGCGGCTCCCC
2H_tem	GGAGACAAGCTTGHHTGCCTCGAGGTCGACTCTAGCGGCTCCCC
1X_tem	GGAGACAAGCTTGAXTGCCTCGAGGTCGACTCTAGCGGCTCCCC
2X_tem	GGAGACAAGCTTGXXTGCCTCGAGGTCGACTCTAGCGGCTCCCC

U, uracil; H, hypoxanthine; X, xanthine.

by a final 7 min extension at 72°C. PCR products were analyzed using 0.8% agarose gel electrophoresis. The primers as previously reported [16] were used to amplify the 2-kb fragment from λ DNA.

Structural Analysis of Uracil-Sensing Domain of Pfu and KOD1 DNA Polymerases

For the structural analysis of the uracil-sensing domain, PDB ID, 2JGU, and 1WNS were used. A hypoxanthine was snugly docked into the binding pocket in the uracil-sensing domain using the "solid docking" module in QUANTA (Molecular Simulation, Inc.). The program considers an electrostatic and geometric complementarity in docking a guest molecule into a host molecule.

RESULTS AND DISCUSSION

The Mechanistic Explanation of Sensing Hypoxanthine

Now that family B-type DNA polymerases of hyperthermophilic archaea have been reported to sense uracil, it was postulated that the presence of hypoxanthine in the template could be also sensed by family B-type DNA polymerases, making the polymerases stalled at encountering a hypoxanthine, eventually leading to a decreased efficiency of polymerization. To address the issue, incorporation assay using dITP as a sole dNTP was tested to see whether dITP could affect DNA polymerase activity itself. Consequently, Pfu, KOD1, and TNA1 polymerases could replicate the template, indicating that the polymerase activities of Pfu, KOD1, and TNA1 polymerases were not inhibited by dITP (Fig. 1). On the other hand, it is worthy to note that there was intriguing difference between Pfu and the other DNA polymerases in the incorporation assay. As shown in Fig. 1, Pfu DNA polymerase stopped right after adding a hypoxanthine to pair with cytosine. It is known that the pairing with cytosine is the most stable even though hypoxanthine can pair with all four natural bases. In contrast, KOD1 and TNA1 DNA polymerases could replicate the template up to the end (44-mer) using only dITP as a sole dNTP. It was thought that KOD1 and TNA1 DNA polymerases were relatively insensitive to the wobble base pairing between hypoxanthine and other bases, whereas Pfu DNA polymerase extremely prefers hypoxanthine and cytosine base-paring. Regardless of the disparity in the incorporation pattern, it was obvious that DNA polymerase activity itself was not affected by the presence of dITP.

Then, primer extension assay was performed to test whether the presence of hypoxanthine in the DNA template

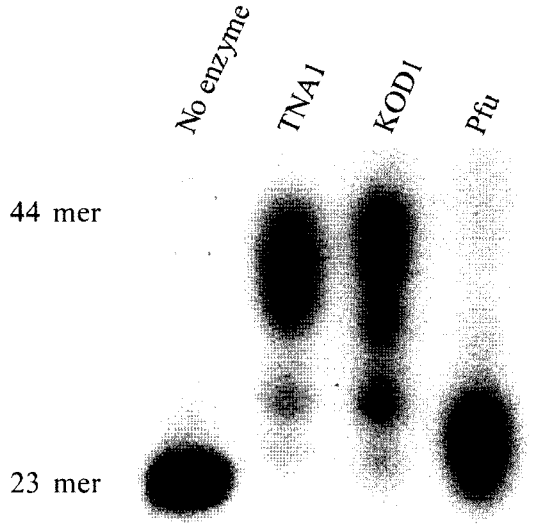

Fig. 1. dITP incorporation assay using Pfu, KOD1, and TNA1 family B-type DNA polymerases.
The reaction contained primer-template complees (200 fmol), 1.25 units of TNA1, 50 mM Tris-HCl, pH 8.5, 60 mM KCl, 30 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, and only 0.1 mM dITP.

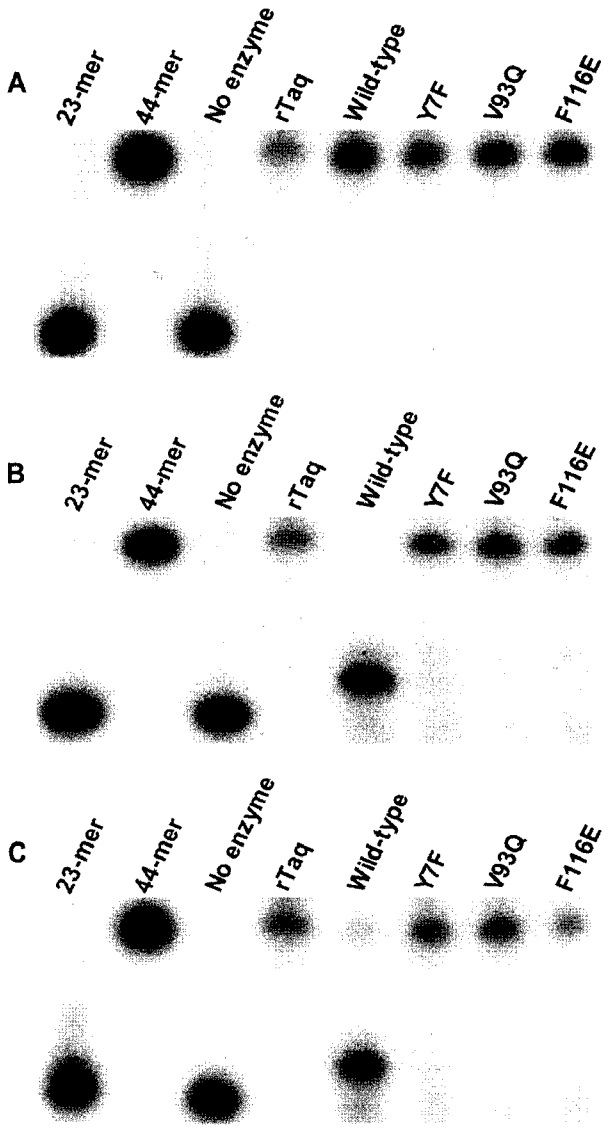

Fig. 2. Primer extension assays using Pfu DNA polymerase.
Templates containing no deaminated base (**A**), a single uracil (**B**), or a single hypoxanthine (**C**) were used. The first and second lanes in each panel exhibit 23-mer primer and 44-mer template, respectively.

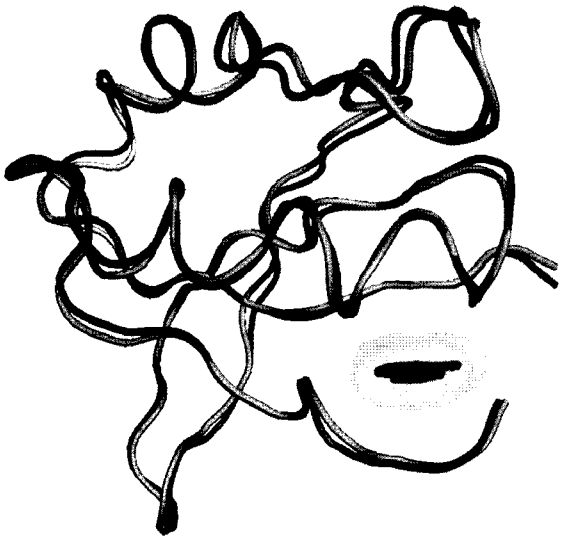

Fig. 3. Superimposed uracil-sensing domains and molecular docking of hypoxanthine.
Cα-Tracing of superimposed uracil-sensing domains with RMSD of 0.313 Å from KOD1 DNA polymerase (dark gray; PDB code, 1WNS) and Pfu DNA polymerase (light gray; PDB code, 2JGU). Hypoxanthine is represented in stick surrounded by transparent surface.

was a cause for the inhibitory effect of dITP. Obviously, Pfu DNA polymerase was stalled at a DNA template with hypoxanthine (Fig. 2), similar to stalling at uracil, raising the possibility that Pfu DNA polymerase can sense hypoxanthine in the DNA template. To elucidate the mechanism of how the DNA polymerase can sense hypoxanthine in the DNA template, structural and biochemical analysis were performed. Consequently, we found that the uracil-sensing domain of the DNA polymerase was responsible for recognizing hypoxanthine, in that mutations at critical residues (Y7, V93, and F116) in the uracil-sensing domain could get over the stalling in the presence of hypoxanthine (Fig. 2), consistent with the recent report [6]. The molecular docking model supports the finding that hypoxanthine was well fitted to the uracil-sensing domain (Fig. 3). The docking with uracil may be the most favorable than other bases with less free energy; however, the hole could hold hypoxanthine as well.

dITP Inhibits PCR Amplification of Family B-Type DNA Polymerase

The ability of family B-type DNA polymerase to synthesize DNA in a PCR reaction was significantly inhibited by the addition of dITP in the reaction, whereas *Taq*, a family A-type DNA polymerase, was unaffected by dITP (Fig. 4). Biochemical and structural investigations of Pfu DNA polymerase to unveil the molecular basis for the inhibitory effect of dITP demonstrated that dITP was incorporated into newly synthesized strands in the first round of the PCR, and subsequently the uracil-sensing domain would sense the incorporated hypoxanthine in the template,

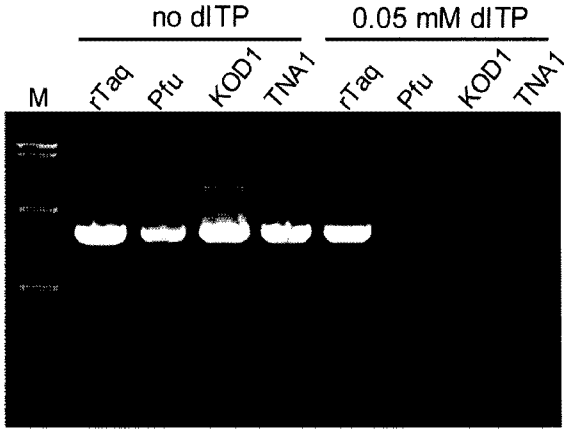

Fig. 4. Effect of dITP on PCR amplification by family A and B type DNA polymerases.
A 2-kb target from λ DNA was amplified using 2.5 units of *rTaq* (Takara) or 1.5 units of various family B-type DNA polymerases. The reaction mixture contained 5 ng of λ DNA, 10 pmol of primers, 0.25 mM dNTPs, 0.05 mM dITP, and PCR reaction buffer.

eventually stalling replication, consistent with the recent reports [8] (Fig. 1, Fig. 2, and Fig. 3).

The Stalling of Family B-Type Polymerases Depends on Intrinsic Extension Rate

Even though it was anticipated that family B-type DNA polymerases would share the sensing mechanism toward hypoxanthine based on the common inhibitory effect of dITP (Fig. 4) and the sequence similarities among their uracil-sensing domains (Fig. 5), primer extension analysis with five family B-type DNA polymerases revealed that

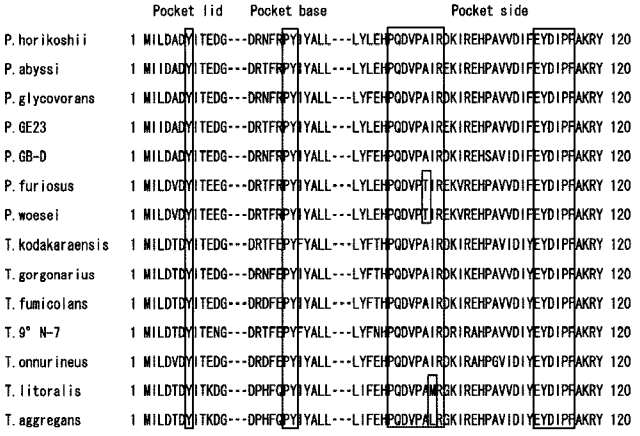

Fig. 5. Multi-alignment of the uracil-sensing domain of archaeal family B-type DNA polymerases from *Pyrococcus* and *Thermococcus* genera.
The amino acid sequence accession numbers are *Pyrococcus horikoshii* (O59610), *P. abyssi* (P77916), *P. glycovorans* (CAC12849), *Pyrococcus* sp. GE23 (CAA90887), *Pyrococcus* sp. GB-D (Q51334), *P. furiosus* (P61875), *P. woesei* (P61876), *Thermococcus kodakaraensis* (P77933), *T. gorgonarius* (P56689), *T. fumicolans* (P74918), *T.* sp. 9°N-7 (Q56366), *T. onnurineus* NA1 (ABC11972), *T. litoralis* (P30317), and *T. aggregans* (O33845).

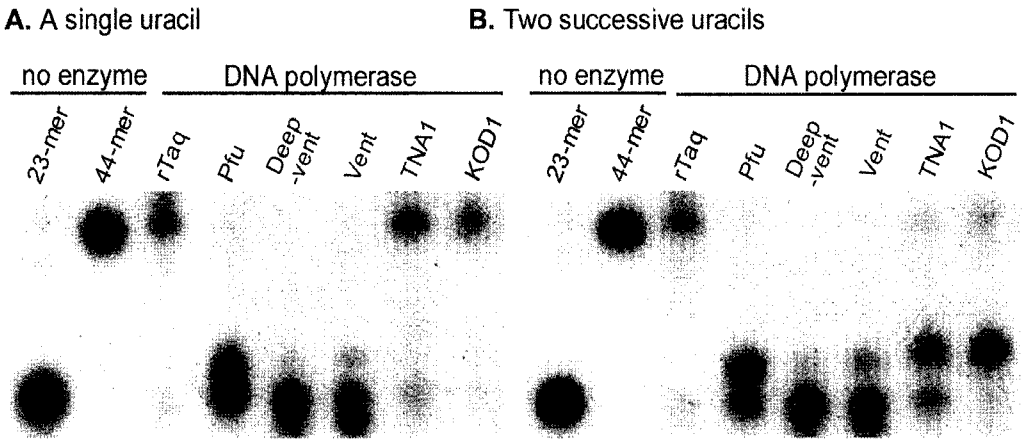

Fig. 6. Primer extension assays using various archaeal family B-type DNA polymerases.
Templates containing a single uracil (**A**), or two successive uracils (**B**) were used. The first and second lanes in each panel exhibit 23-mer primer and 44-mer template, respectively.

they could be divided into two groups. The first group, including Pfu, Vent, and Deep-vent DNA polymerases, stall when they encounter a single uracil in the template (Fig. 6A). The second group, KOD1 and TNA1 DNA polymerases, did not stall at a single uracil, but were stalled by two successive uracils (Fig. 6B). Similarly, two successive hypoxanthines were required to stall TNA1 DNA polymerase (Fig. 7C), whereas a single hypoxanthine

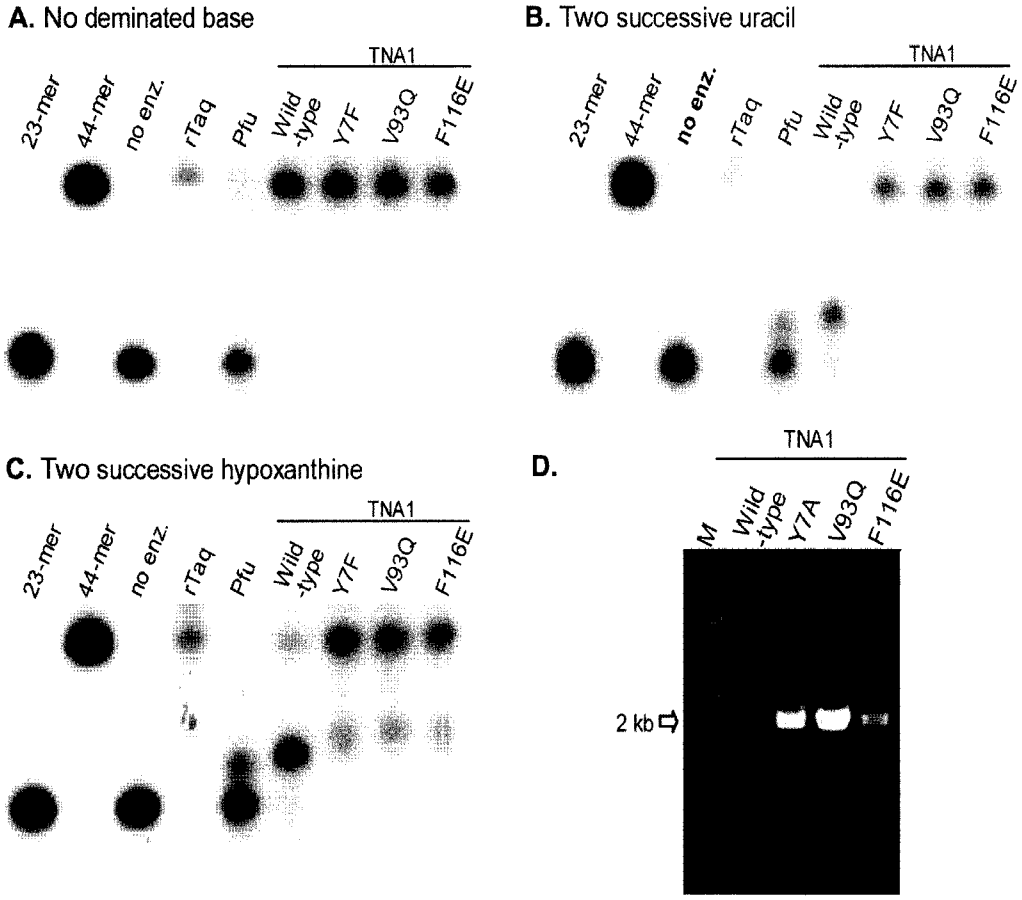

Fig. 7. Primer extension assays using TNA1 DNA polymerase in the presence of hypoxanthine.
Templates containing no deaminated base (**A**), two successive uracils (**B**), or two successive hypoxanthines (**C**) were used. The first and second lanes exhibit 23-mer primer and 44-mer template, respectively. **D**. PCR amplification using wild-type and mutant of TNA1 DNA polymerases in the presence of dITP. The reaction contained 5 ng of λ DNA, 10 pmol of primers, 0.25 mM dNTPs, 0.025 mM dITP, and PCR reaction buffer.

was bypassed (data not shown). These observations differ from previous reports, which suggested that all family B-type polymerases stall on encountering a single deaminated base [6, 8, 9, 11, 27]. Site-specific mutations at residues Y7 (lid of uracil-sensing pocket), V93 (side of the pocket), or F116 (side of the pocket) (Fig. 5), consisting of the uracil-sensing domain of TNA1 DNA polymerase, alleviated the stalling at two successive hypoxanthines or uracils in the primer extension assay (Figs. 7B and 7C), implicating that the uracil-sensing domain is still a key determinant in the stalling at two successive deaminated bases. It seemed likely that by bypassing stalling, PCR amplification by TNA1 DNA polymerase in the presence of dITP was significantly increased (Fig. 7D).

Given these results, we were interested in determining why some family B-type polymerases are stalled only by two successive deaminated bases, whereas others are stalled by a single deaminated base, in spite of their common ability to recognize deaminated bases. To elucidate the molecular mechanism underpinning the difference in the stalling pattern, we thoroughly compared residues forming the uracil-sensing domain between the two groups and found that most residues in the lid, base, and side of the uracil-sensing pocket were identical, with a few exceptions (Fig. 5). In addition, the structures of the uracil-sensing domains from KOD1 and Pfu DNA polymerases are virtually identical to each other (Fig. 3). It is well known that sometimes a single amino acid difference can generate considerable functional differences in homologous proteins. To investigate the effect of the subtle differences in the amino acid composition of the uracil-sensing domains, we selected Glu35, Phe87, and Ala95 of the uracil-sensing domain as mutation targets and replaced each residue in TNA1 DNA polymerase with the corresponding residues from Pfu DNA polymerase. The purified mutant proteins (E35R, F87L, and A95T) still required two successive deaminated bases to be stalled, similar to the wild-type TNA1 DNA polymerase (data not shown). Chimeric proteins generated by whole domain swapping of uracil-sensing domains or exonuclease domains between Pfu and TNA1 DNA polymerases, as denoted in Fig. 8, were tested; the stalling pattern was that of the parent polymerase domain rather than the uracil-sensing domain. For example, a fusion protein with the uracil-sensing domain from Pfu DNA polymerase and the exonuclease/polymerase domain from TNA1 DNA polymerase followed the pattern of TNA1 DNA polymerase and *vice versa* (Fig. 8). Consequently, it is reasonable to conclude that the discrepancy in the stalling pattern was neither brought about by the uracil-sensing domain nor the exonuclease domain.

According to previous studies on DNA polymerases of this family [16, 28], TNA1 and KOD1 DNA polymerases have faster extension rates than other polymerases in the family. TNA1 is three times faster than Pfu, and KOD1 is six times faster than deep-vent DNA polymerase. If the reason why TNA1 and KOD1 bypass single uracil residues is their faster extension rate, we predicted that slowing the extension rate by lowering the temperature might lead to

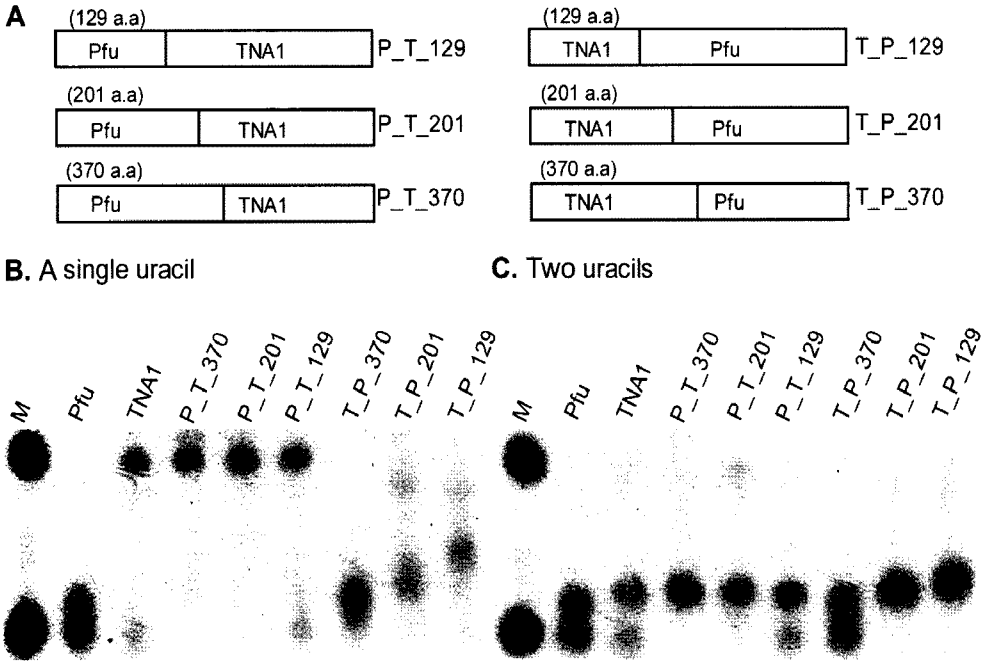

Fig. 8. Whole domain swapping experiments.
A. Schematic representation of Pfu and TNA1 fusion proteins. The numbers indicate the residues of NA1. In (**B**) and (**C**), primer extension assays using Pfu and TNA1 fusion proteins show the recognition of uracil. Templates containing a single uracil (**B**) or two successive uracils (**C**) were used. The first lane shows 23-mer primer and 44-mer template, respectively.

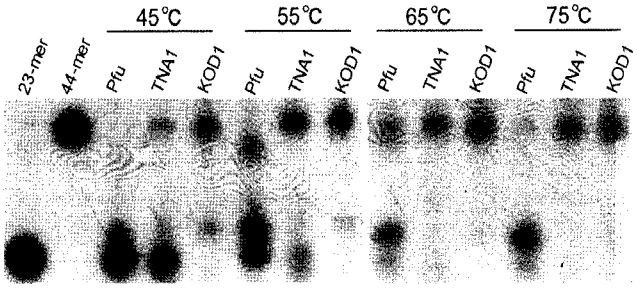

Fig. 9. Temperature effects of TNA1 DNA polymerases in primer extension assays.
Templates containing a single uracil were used. The first and second lanes exhibit 23-mer primer and 44-mer template, respectively. The primer-template complex (400 fmol) was incubated at various temperatures (45°C, 55°C, 65°C, and 75°C) for 15 min with 1.25 units of TNA1 DNA polymerase in 120 mM Tris-HCl (pH 8.8), 60 mM KCl, 30 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, and 0.25 mM of each dNTP.

stalling at single uridine residues. We carried out primer extension assays at various temperatures to test this prediction. Interestingly, TNA1 and KOD1 DNA polymerases were stalled at as low as 45°C by a single deaminated base, but the stalling disappeared at higher temperatures (Fig. 9). Therefore, these results support a model in which the stalling pattern is dictated by the extension rate. Binding of the first deaminated base to the uracil-sensing domain may not produce enough binding energy to stall the fast-moving TNA1 and KOD1 polymerases. However, the interaction between the first deaminated base and the uracil-sensing domain may act as a brake to retard the enzyme, and subsequently, the DNA polymerase with a decreased mobility is then stalled by the second deaminated base. We tested whether two single deaminated bases separated by a few nucleotides would stall the DNA polymerase, and it was found that three nucleotide spaces between the two deaminated bases were not enough to stall the DNA polymerase. In summary, the binding of uracil (hypoxanthine) to a uracil-sensing domain, which commonly occurs in family B-type DNA polymerases, does not necessarily lead to the stalling of enzymes. The stalling requires the recognition of deaminated bases, but is also likely to be determined by the extension rate of the DNA polymerase.

The experiments described here, in which DNA polymerases with high extension rates require two successive deaminated bases to be stalled, may not reflect the situation *in vivo*. DNA polymerase works in concert with various auxiliary factors such as PCNA, replication factor, helicase, and so on [3, 15, 23]. It is possible that the factors or the condition *in vivo* could change the microenvironment of

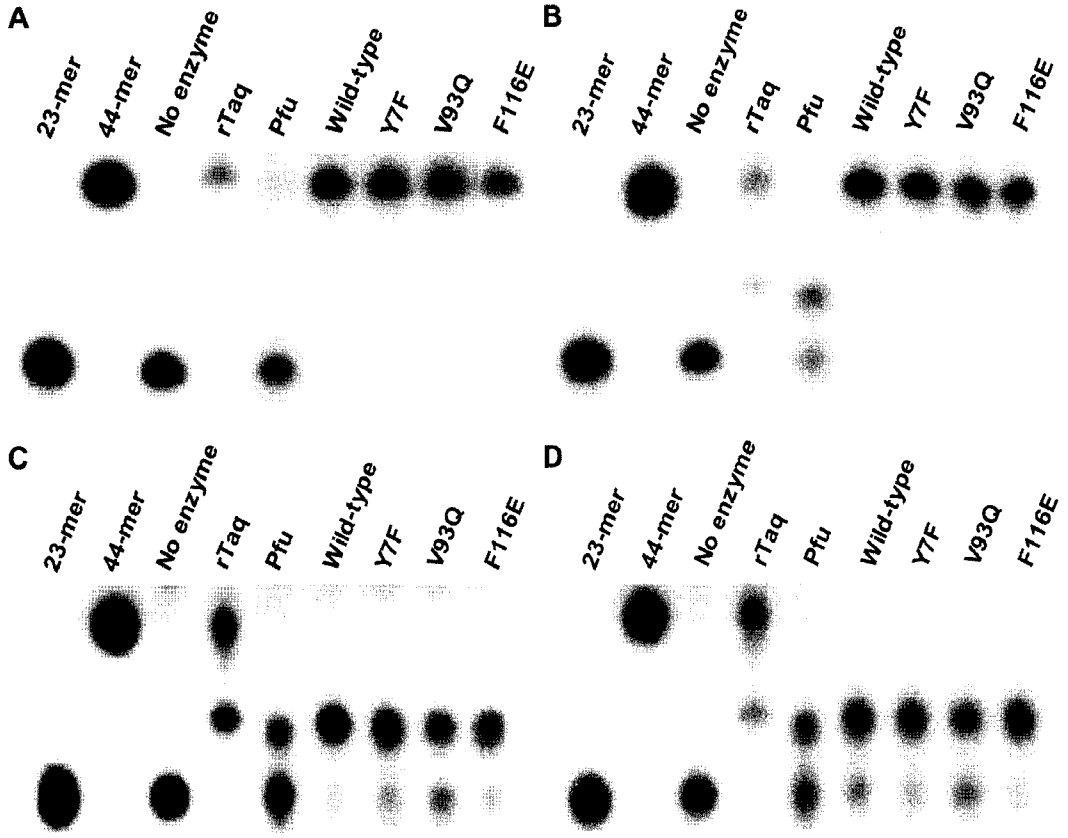

Fig. 10. Primer extension assays using various archaeal family B-type DNA polymerases.
Templates containing no deaminated base (**A**), a single xanthine (**B**), two successive xanthines (**C**), and four successive xanthines (**D**) were used. The first and second lanes in each panel exhibit 23-mer primer and 44-mer template, respectively.

the enzyme, and enable these fast polymerases to sense even one base.

On the other hand, the synthesis of a DNA oligomer containing the xanthine base enabled us to show that the polymerization of 44 mer template was incomplete in the presence of xanthine (Fig. 10), although the underlying mechanism behind the stalling seems different from that of hypoxanthine and uracil. The stalling position was not the same as that of hypoxanthine or uracil and the mutations in the uracil-sensing domain could not reverse the stalling. Furthermore, *Taq* DNA polymerase, a family A-type DNA polymerase, was also stalled in the presence of xanthine base. As discussed in Gill *et al.* [8], the active site may not easily incorporate a xanthine base at the site, giving truncated products but longer than those seen for uracil-sensing mediated stalling.

In conclusion, the recognition (sensing) of deaminated bases is an intrinsic property of family B-type DNA polymerases of hyperthermophilic archaea living at temperatures above 80°C, preventing transition mutation from deaminated bases. However, the stalling of DNA polymerase seems to rely on the molecular mobility during DNA replication.

Acknowledgments

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