Gene, 108 (1991) 1–6

GENE 06172

High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*

(Polymerase chain reaction; mutation frequency; *lacI*; proofreading; 3′-to-5′ exonuclease; recombinant DNA; archaebacteria)

Kelly S. Lundberg[a], Dan D. Shoemaker[a], Michael W.W. Adams[b], Jay M. Short[a], Joseph A. Sorge[a] and Eric J. Mathur[a]

[a] Division of Research and Development, Stratagene, Inc., La Jolla, CA 92037 (U.S.A.), and [b] Center for Metalloenzyme Studies, University of Georgia, Athens, GA 30602 (U.S.A.)
Tel. (404)542-2060

Received by M. Salas: 16 May 1991
Revised/Accepted: 11 August/13 August 1991
Received at publishers: 17 September 1991

SUMMARY

A thermostable DNA polymerase which possesses an associated 3′-to-5′ exonuclease (proofreading) activity has been isolated from the hyperthermophilic archaebacterium, *Pyrococcus furiosus* (*Pfu*). To test its fidelity, we have utilized a genetic assay that directly measures DNA polymerase fidelity in vitro during the polymerase chain reaction (PCR). Our results indicate that PCR performed with the DNA polymerase purified from *P. furiosus* yields amplification products containing less than 10% of the number of mutations obtained from similar amplifications performed with *Taq* DNA polymerase. The PCR fidelity assay is based on the amplification and cloning of *lacI*, *lacO* and *lacZα* gene sequences (*lacIOZα*) using either *Pfu* or *Taq* DNA polymerase. Certain mutations within the *lacI* gene inactivate the Lac repressor protein and permit the expression of βGal. When plated on a chromogenic substrate, these LacI$^-$ mutants exhibit a blue-plaque phenotype. These studies demonstrate that the error rate per nucleotide induced in the 182 known detectable sites of the *lacI* gene was 1.6×10^{-6} for *Pfu* DNA polymerase, a greater than tenfold improvement over the 2.0×10^{-5} error rate for *Taq* DNA polymerase, after approx. 10^5-fold amplification.

INTRODUCTION

The polymerase chain reaction (PCR) has become an important tool in molecular biology. The automated in vitro amplification process utilizes a thermostable DNA polymerase; at present, the enzyme of choice is *Taq* DNA polymerase (Mullis et al., 1986; Saiki et al., 1988). Purified *Taq* DNA polymerase is devoid of 3′-to-5′ exonuclease (proofreading) activity and thus cannot excise misincorporated nt (Tindall and Kunkel, 1988). The estimated error rate (mutations per nt per cycle) of *Taq* polymerase varies from 2×10^{-4} during PCR (Saiki et al., 1988; Keohavaong and Thilly, 1989) to 2×10^{-5} for nt substitution errors produced during a single round of DNA synthesis of the *lacZα* gene (Eckert and Kunkel, 1990). Such polymerase-induced mutations have hindered applications requiring high fidelity (Reiss et al., 1990; Ennis et al., 1990).

P. furiosus ('rushing fireball'; DSM3638) was first iso-

Correspondence to: Dr. E.J. Mathur, Stratagene, Inc., 11099 N. Torrey Pines Rd., La Jolla, CA 92037 (U.S.A.)
Tel. (619)535-5400, ext. 5481; Fax (619)535-0071.

Abbreviations: βGal, β-galactosidase (encoded by *lacZ*); bp, base pair(s); BSA, bovine serum albumin; Δ, deletion; dNTP, deoxyribonucleoside triphosphate; ER, error rate; EtdBr, ethidium bromide; IPTG, isopropyl-β-D-thiogalactopyranoside; kb, kilobase(s) or 1000 bp; *lacIOZα*, gene sequence of *lacI*, *lacO* and *lacZα* from *E. coli*; LB, Luria–Bertani (medium); mf, mutant frequency; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; *Pfu*, *Pyrococcus furiosus*; PCR, polymerase chain reaction; PNK, T4 polynucleotide kinase; *Taq*, *Thermus aquaticus*; u, unit(s); wt, wild type; XGal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

lated from geothermally heated marine sediments in Vulcano, Italy (Fiala and Setter, 1986). This hyperthermophilic archaebacterium grows optimally at 100°C by an unusual fermentative-type metabolism in which H_2 and CO_2 are the only detectable end products (Adams, 1990). In this study, we demonstrate that a DNA polymerase isolated from *P. furiosus* contains 3′-to-5′ exonuclease (proofreading) activity and works effectively in PCR. Moreover, during PCR, *Pfu* DNA polymerase exhibits eleven- to twelvefold greater replication fidelity than *Taq* DNA polymerase.

RESULTS AND DISCUSSION

(a) *Pyrococcus* DNA polymerase possesses 3′-to-5′ exonuclease activity

A thermostable DNA polymerase isolated from *P. furiosus* was purified to greater than 99% homogeneity as visualized by polyacrylamide-gel electrophoresis. The purification and characterization of *Pfu* DNA polymerase will be described elsewhere (E.J.M., in preparation). *Taq* DNA polymerase was purchased from Perkin-Elmer/Cetus. Table I lists the polymerase and 3′-to-5′ exonuclease-specific activities of *Pfu* and *Taq* DNA polymerases utilized in this study.

TABLE I

Specific activities of purified *Pfu* and *Taq* DNA polymerases

Source of DNA polymerase	Protein concentration[a] (mg/ml)	Polymerase activity[b] (u/mg)	3′ to 5′ exonuclease activity[c] (u/mg)
P. furiosus	0.13 (± 0.004)	31713 (± 1015)	9200 (± 294)
T. aquaticus	0.30 (± 0.015)	16667 (± 850)	negligible

[a] Protein determinations were performed by the method described by Bradford (1974).

[b] Polymerase activity assays were performed essentially as described by Grippo and Richardson (1971) except that the reaction mixture was incubated at 72°C instead of 37°C and that incorporated labeled nt were counted on DEAE filter disks (DE-81, Whatman), not by trichloroacetic acid precipitation. One unit of DNA polymerase activity catalyzed the incorporation of 10 nmol total nt into a DEAE-bound form in 30 min at 72°C.

[c] The exonuclease assay was performed essentially as described by Chase and Richardson (1974) except that the reaction was incubated at 72°C instead of 37°C and the substrate was 3′ labeled phage λ DNA. Briefly, the substrate was prepared by digesting 100 μg phage λ DNA with 500 u *Taq*I restriction endonuclease in 100 mM K · acetate/25 mM Tris · acetate pH 7.6/10 mM Mg · acetate/0.5 mM β-mercaptoethanol/ and 10 μg BSA per ml for 2 h at 65°C. This leaves dG and dC 5′ overhangs at 121 sites per phage λ DNA molecule. These sites were filled-in with 50 u modified T7 DNA polymerase and excess [^{3}H]dGTP and [^{3}H]dCTP in 200 mM Tris · HCl pH 7.5/100 mM $MgCl_2$/250 mM NaCl at 37°C for 30 min. One unit of exonuclease activity catalyzed the acid solubilization of 10 nmol of total nt in 30 min at 72°C.

(b) Proofreading ability of *Pfu* DNA polymerase

To analyze the 3′-to-5′ exonuclease activity at the molecular level, we designed an assay to elucidate how a DNA polymerase responds to a mismatched 3′ terminus. Three events can occur when a DNA polymerase interacts with a mismatched 3′ primer terminus: no extension of the mismatched primer, extension with the incorporation of the mismatched nt, or extension following excision of the 3′ mismatched nt. To address this question, a 35-nt synthetic DNA template was constructed along with four 15-mer oligo primers complementary to the template but containing 0, 1, 2, and 3 mismatched nt at the 3′ termini (Fig. 1). The template was designed with an internal *Eco*RI site which coincides with the mismatched nt at the 3′ end of the 15-mer primers. The 5′ end-labeled primers were annealed to the 35-mer template prior to incubation with either *Pfu* or *Taq* DNA polymerase. Following extension, half of the reaction mixture was digested with *Eco*RI. *Pfu* DNA polymerase extended all the primers tested, as demonstrated by the presence of 35-bp product before restriction digestion (Fig. 1). In addition, *Pfu* DNA polymerase excised the mismatched nt of the primers and restored the *Eco*RI site, as demonstrated by the digestion of 35-bp products. The presence of degradation products (13- and 14-mers) seen with some of the mismatched primers can be attributed to the active 3′-to-5′ exonuclease activity associated with *Pfu* DNA polymerase. These reaction intermediates would not be detected if the extension reactions were to go to completion. *Taq* DNA polymerase was capable of extending the wt control 15-mer primer to form a 35-bp product susceptible to digestion with *Eco*RI, but the absence of labeled 35-bp product in the reactions with the mismatched primers suggested that *Taq* DNA polymerase was unable to extend off any of these 3′ terminal mismatched primers (we chose a G : G mismatch which is not efficient substrate for *Taq* DNA polymerase). To fully characterize the proofreading activity of a DNA polymerase, the composition and position of all possible mismatches must be considered. The assay described above was simply an initial screen for the existence of proofreading activity in *Pfu* DNA polymerase.

(c) PCR amplification with *Pfu* DNA polymerase

Pfu DNA polymerase was also evaluated for use in PCR. Amplification reactions were performed with several different primer : template combinations using either *Pfu* or *Taq* DNA polymerase. In all cases tested, PCR performed with *Pfu* DNA polymerase produced reaction products comparable to those obtained with *Taq* DNA polymerase. Four examples of amplification reaction products are shown in Fig. 2. In most cases tested the specificity of amplification was increased with *Pfu* DNA polymerase. We do not yet know the reason for this, although *Pfu* DNA polymerase exhibits only half the activity of *Taq* polymerase at room

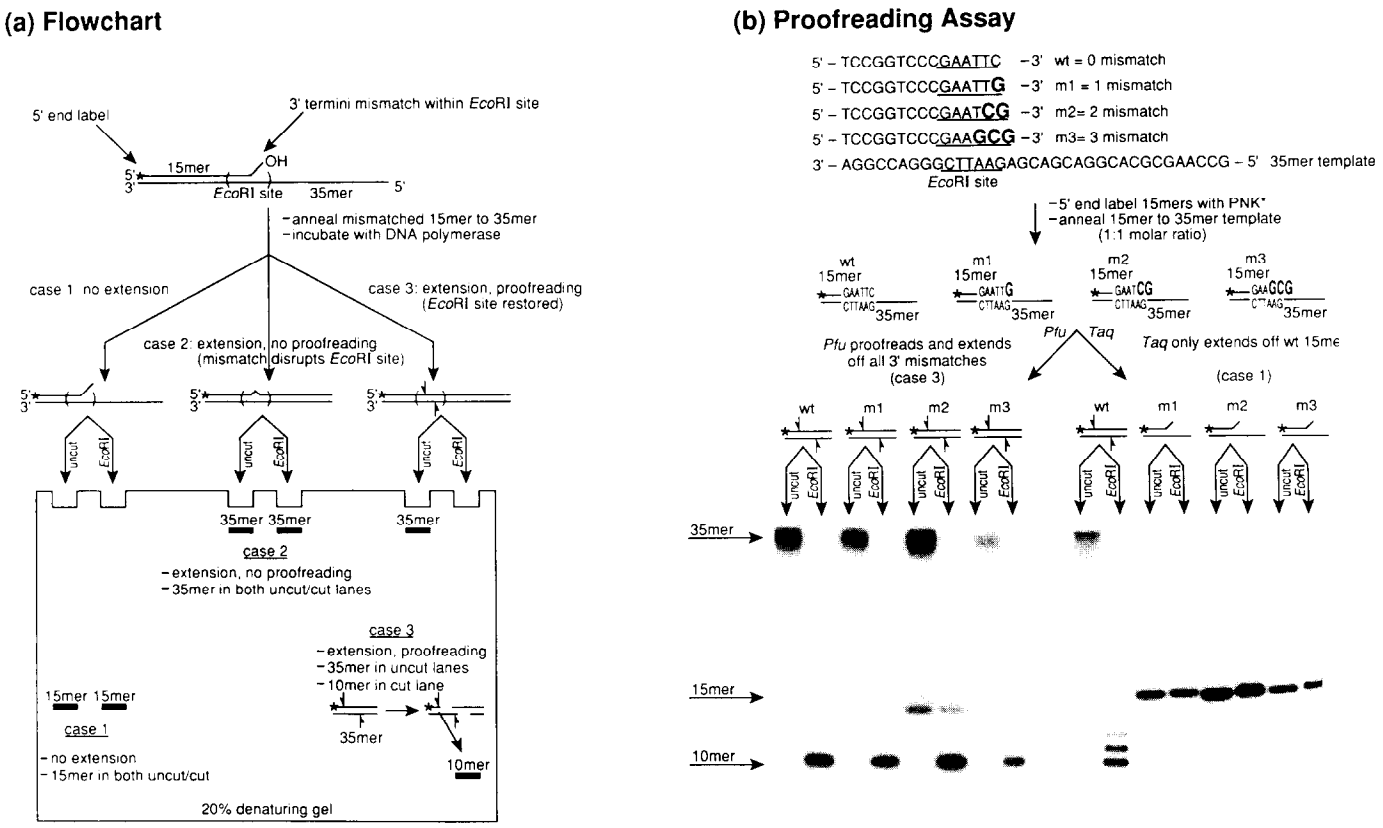

Fig. 1. Proofreading activity of *Pfu* and *Taq* DNA polymerases. The proofreading activities of *Pfu* and *Taq* DNA polymerases were characterized by annealing a 35-mer template containing an internal *Eco*RI site to 15-mer primers with 0, 1, 2 and 3 mismatches nt at the 3′ terminus (wt, m1, m2 and m3, respectively) at the *Eco*RI site. The 15-mers were 5′ end-labeled with [^{32}P]dATP and PNK (Maniatis et al., 1989). Of each labeled 15-mer (wt, m1, m2 and m3) 10 ng was annealed to 40 ng of the 35-mer synthetic template in 20 μl of buffer containing 20 mM Tris · HCl pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.1% gelatin/200 μM each dNTP. One unit of either *Pfu* or *Taq* DNA polymerase was added and the mixture incubated at 72°C for 15 min. Of each reaction 10 μl was removed and lyophilized. The pellet was resuspended in 5 μl of a stop solution (95% formamide/20 mM EDTA/0.05% bromophenol blue/0.05% xylene cyanol FF). The remaining 10 μl of each reaction was digested with 5 u of *Eco*RI in 100 mM K · acetate/25 mM Tris · acetate pH 7.6/10 mM Mg · acetate/0.5 mM β-mercaptoethanol/10 μg BSA per ml at 37°C for 30 min. The reaction was terminated by lyophilizing and resuspending the pellet in 5 μl stop solution. The uncut and cut extension products from each 15-mer primer (wt, m1, m2 and m3) were run side by side on a 20% polyacrylamide/7 M urea denaturing gel and visualized by autoradiography. The presence of the 35-mer band in the uncut lanes demonstrated that *Pfu* DNA polymerase extended all the mismatched primers while *Taq* polymerase was only able to extend the annealed wt primer (note that residual activity of *Taq* polymerase at lower temperatures resulted in partial fill-in products of the 10-mer cleavage product). Moreover, the complete digestion (35-mer to 10-mer) of all four *Pfu* DNA polymerase extension products demonstrated that the *Eco*RI site was restored by the excision of the 3′ mismatches prior to extension.

temperature (Fig. 1), which would reduce primer extension by *Pfu* DNA polymerase under non-stringent conditions.

To demonstrate that the lower mutation frequency exhibited by *Pfu* polymerase was not due to less efficient amplification after 30 cycles of PCR, the relative amplification efficiencies of *Pfu* and *Taq* DNA polymerases were compared. PCR products from the *lacIOZ*α template used in these studies were analyzed by agarose-gel electrophoresis following 15, 20, and 30 amplification cycles. As can be seen in Fig. 3, amplification of the target sequence proceeded in a comparable manner and yielded similar amounts of product after 30 cycles. In experiments where the *lacIOZ*α product was used for fidelity studies, final amplification was estimated to be 10^5 after 30 cycles for both *Pfu* and *Taq* polymerases (see legend to Fig. 3).

(d) Fidelity of DNA amplification

Numerous independent studies suggest that 3′-to-5′ exonuclease-dependent proofreading enhances the fidelity of DNA synthesis (Muzyczka et al., 1972; Fersht and Knill-Jones, 1983; Sinha, 1987; Reyland et al., 1988; Kunkel et al., 1986; Bernad et al., 1989). Therefore, we examined the relative accuracies of *Pfu* and *Taq* DNA polymerases in PCR by modifying in vivo mutagenesis assay (Kohler et al., 1991). Transgenic mouse genomic DNA containing a *lacIOZ*α transgene in 33 copies per cell, was isolated from brain tissue. The *lacIOZ*α nt sequence was amplified from a transgenic mammalian template rather than from an *E. coli* genomic template to mimic the complex genomic templates frequently used in PCR. The *Pfu* and *Taq* DNA polymerase amplification products were cleaved at unique

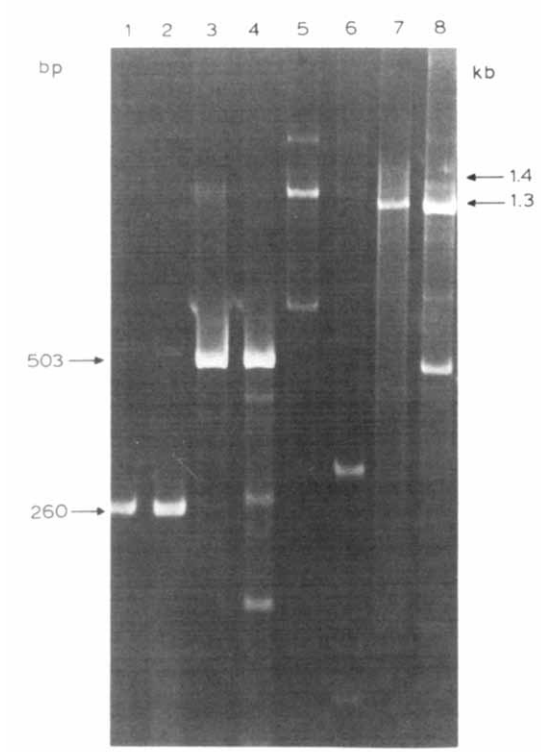

Fig. 2. Amplification reaction products from four primer : template combinations using either *Pfu* or *Taq* DNA polymerase. Similar PCR conditions were employed except that the buffer used in *Pfu* DNA polymerase reactions contained 20 mM Tris · HCl pH 8.8/10 mM KCl/6 mM $(NH_4)_2SO_4$/2 mM $MgCl_2$ (1.5 mM for the reaction in lane **1**)/0.1% Triton X-100. The buffer used in *Taq* polymerase reactions (*Taq* buffer) contained 20 mM Tris · HCl pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.1% gelatin. In addition, all amplification reactions included 200 μM each dNTP, 250 ng each primer and 100 ng genomic DNA template. PCR was performed using the GeneAmp kit (Perkin-Elmer/Cetus) according to the manufacturer's instructions. Briefly, 100 μl reactions without DNA polymerase were incubated at 95 °C for 5 min to denature the template DNA, then at 54 °C for 5 min to allow primer annealing. Next, 2.5 u of either *Pfu* or *Taq* DNA polymerase was added and the samples were overlayed with 50 μl mineral oil. The reactions were then amplified for 30 cycles in a DNA thermocycler (Perkin-Elmer/Cetus) using the following parameters: 72 °C for 3 min, 95 °C for 1 min, and 54 °C for 3 min. Following the final cycle, PCR was completed with a 10-min incubation at 72 °C. Of each reaction 20 μl was combined with 2 μl XC-dye (0.04% bromophenol blue/0.04% xylene cyanol/50% glycerol), subjected to nondenaturing electrophoresis on a 6% polyacrylamide gel in 1 × TBE (89 mM Tris · borate/89 mM boric acid/2 mM EDTA) and stained with EtdBr. Lanes **1**, **3**, **5** and **7** represent amplification products from PCR performed with *Pfu* DNA polymerase; lanes **2**, **4**, **6** and **8** represent reaction products from PCR performed with *Taq* DNA polymerase. In lanes **1** and **2**, the expected 260-bp amplification product is from Epstein-Barr virus template using the following primers: 5′-GGCATCAGCATCCGGGTCTC-3′ and 5′-TTCGGGGGGGAACCTCCGGG-3′. Lanes **3** and **4** show the expected 503-bp reaction product from a transgenic mouse template using the primers 5′-GGTGGCGACGACTCCTGGAGCCC-3′ and 5′-GACAGTCACTCCGGCCGGTGCCG-3′. Lanes **5** and **6** depict the amplification of a 1.4-kb product from a human genomic template using the primers 5′-TCTGGCTCCAGCCAAAGCCACCCTAG-3′ and 5′-GGCTGAGCCCAGTGCCTCCTTCAGTA-3′. The expected 1.3-kb product from first-strand synthesis of a mouse mRNA template, shown in lanes **7** and **8**, was amplified with 5′-GCTGTTGGGCTGTTCTGCCTGG-3′ and 5′-GGCATCCACACAGGGCCTTGGA-3′.

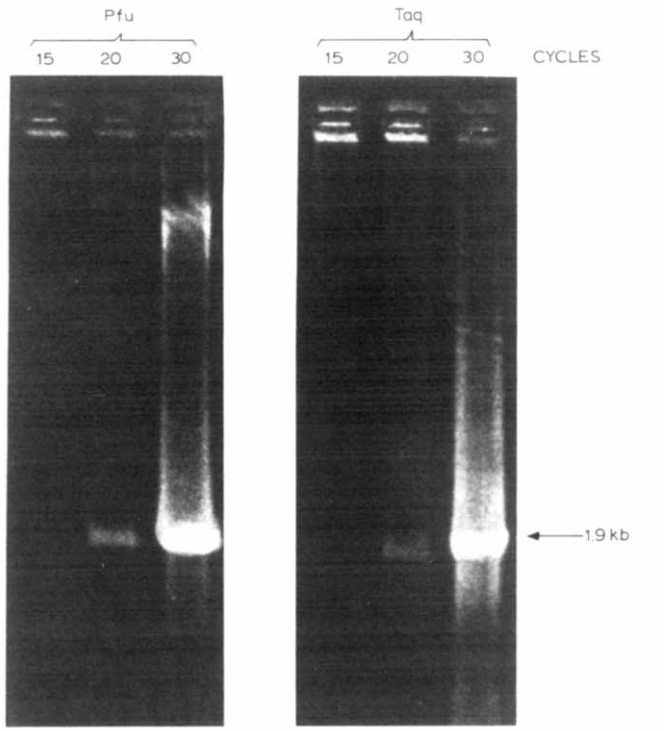

Fig. 3. PCR products of the *lacIOZα* gene from mouse genomic DNA evaluated at 15, 20, and 30 cycles of amplification. The primer sequences were as follows: 5′-CATAGCGAATTCGCAAAACCTTTCGCGGTATGG-3′; 5′-ACTACGGAATTCCACGGAAAATGCCGCTCATCC-3′. The conditions for PCR with *Pfu* and *Taq* DNA polymerases were identical. Amplification reactions contained *Taq* buffer (see legend to Fig. 2)/200 μM each dNTP/250 ng each primer, and approx. 250 ng of transgenic mouse genomic DNA which contained 0.1 ng of the *lacIOZα* gene (33 copies per cell). The 100-μl reactions were incubated at 95 °C for 5 min to denature the template DNA and cooled to 40 °C for 5 min to allow primers annealing. Next, 2.5 units of either *Pfu* or *Taq* DNA polymerase was added and the samples were overlayed with 50 μl mineral oil. Amplification was performed for 30 cycles in a DNA thermocycler (Perkin-Elmer/Cetus) using the following parameters: 72 °C for 3 min, 95 °C for 1 min, and 54 °C for 3 min. From each reaction 20-μl aliquots were removed after 15, 20 and 30 cycles, electrophoresed on a 1% agarose (Seakem, FMC) gel and stained with EtdBr. In experiments requiring the cloning of the *lacIOZα*-amplified target, 20-μl of the amplified product was also electrophoresed on a 1% agarose gel and stained with EtdBr. Amplification was estimated to be approx. 10^5-fold (0.1–10-μg).

*Eco*RI sites introduced at the ends of the fragments by the PCR primers, cloned into the phage λgt10 vector, packaged and plated with a *lacZΔ*M15 *E. coli* host strain containing the α-complementing portion of the *lacZ* gene. Certain mutations incurred within the *lacI* gene during the amplification process result in a nonfunctional repressor protein. In the absence of an active repressor protein, the α portion of βGal within the λgt10 phage is expressed and can complement the ω portion within the cell, generating a blue plaque when plated with top agar containing the chromogenic substrate, XGal. Therefore, the observed mutation frequency (frequency of phenotypic mutants) can be calculated by dividing the number of blue plaques ($LacI^-$

mutants) by the total number of plaques scored (Table II). The phage were also scored on plates with XGal and IPTG to demonstrate that 99% of the *Pfu* DNA polymerase amplified phage clones and 89% of the *Taq* DNA polymerase amplified phage clones were blue and therefore contained functional *lacZα* genes.

TABLE II

Fidelity of *Pfu* and *Taq* DNA polymerases in PCR fidelity assay[a]

DNA polymerase	Plaques scored		Mutant frequency[d] (%)	Error rate[e]
	Total[b]	Mutant[c]		
Pfu				
PCR				
1	9044	62	0.60	2.0×10^{-6}
2	17972	84	0.47	1.6×10^{-6}
3	15903	56	0.35	1.2×10^{-6}
4	19171	103	0.54	1.8×10^{-6}
Taq				
PCR				
1	9376	700	7.38	2.5×10^{-5}
2	10190	538	5.20	1.7×10^{-5}
3	13002	570	4.30	1.4×10^{-5}
4	14640	916	6.25	2.1×10^{-5}

[a] The *lacIOZα* gene sequence was amplified in four separate experiments with either *Pfu* or *Taq* DNA polymerase with oligo primers which contained *Eco*RI recognition sites at the 5′-terminal regions (see legend to Fig. 3). Following amplification, polymerase activity was removed from PCR products with StrataClean resin (Stratagene, La Jolla, CA) following the manufacturer's instructions. The amplification products were digested with 5 u of *Eco*RI in 150 mM K · acetate/35 mM Tris · acetate pH 7.6/15 mM Mg · acetate/0.7 mM B-mercaptoethanol/15 μg BSA per ml at 37°C for 1 h, and purified by agarose gel (1%) elution with the GeneClean kit (Bio101) according to directions supplied by the manufacturer. The isolated fragments were then ligated into *Eco*RI λgt10 in 5 μl with 2.9 u of T4 DNA ligase and 1 mM rATP in 50 mM Tris · HCl pH 7.5/7 mM $MgCl_2$/1 mM dithiothreitol. Following an overnight incubation at 4°C. Of the ligation mix 4 μl was packaged with high efficiency λ packaging extract (Gigapack, Stratagene, La Jolla, CA) according to the manufacturer's instructions. The packaged phage were incubated with an α-complementing *E. coli* strain {F⁻ *endA*1 *hsdR*17 (r_k^-, m_k^+) *supE*44 λ⁻ *thi*-1 *recA*1 *gyrA*96 *relA*1 *deoRΔ* (*lacZYA-argF*)U169 (*φ*80*lacZΔ*M15)} for 30 min at 37°C and plated on LB plates with top agar containing 1 mg XGal/ml.

[b] The total number of plaques scored included clear and blue plaques.

[c] LacI⁻ mutants were identified as blue plaques on the bacterial lawn.

[d] The mutation frequency of observed phenotypic mutants is expressed as the % of blue plaques in relation to the total number of plaques.

[e] The ER was calculated using the equation $ER = \frac{mf}{bp \times d}$, where *mf* is the observed mutation frequency (%) minus the background frequency of 0.0017%, *bp* is the number of detectable sites in the *lacI* gene sequence (182), and *d* is the number of duplications (16.6 for 10^5-fold amplification).

Errors introduced during 10^5-fold amplification yielded an observed *mf* of 0.5% for *Pfu* DNA polymerase and 5.8% for *Taq* DNA polymerase. The background *mf* in *lacI* in mouse somatic tissues was determined to be $1.7 \times 10^{-5} \pm 0.45$ (Kohler et al., 1991). Using these values, the ER (mutations per nt per cycle) was calculated for each DNA polymerase as ER = *bp* × *d*, where *bp* is the number of sites within the *lacI* gene known to yield a mutant phenotype, and *d* is the number of effective duplications during PCR (16.6 for 10^5-fold amplification). The *bp* value of 182 was used because Gordon et al. (1988) have shown that 102 different sites within the *lacI* gene harbored various missense mutations after sequencing over 6000 spontaneous and induced lacI⁻ mutants, and there are 80 nt sites at which nonsense mutations can be produced (Schaaper et al., 1986). It is important to note that a subset of mutations may be suppressed in our assay due to the presence of the *supE* tRNA in the *E. coli* plating host. Also, any errors incurred during the final cycle will yield heteroduplexes which may be subject to correction in vivo. However, our calculated error rate for *Taq* DNA polymerase (Table II) is in close agreement with that reported by Eckert and Kunkel (1990).

The average error rate was determined to be 1.6×10^{-6} for *Pfu* DNA polymerase and 2.0×10^{-5} for *Taq* DNA polymerase. Thus, if a 1-kb sequence is amplified for 20 effective cycles with *Taq* DNA polymerase, 40% of the amplification products will contain mutations (both silent and phenotypically detectable). In contrast, if PCR under identical conditions is performed with *Pfu* DNA polymerase, only 3.2% of the reaction products will harbor mutations.

(e) Conclusions

(*1*) A thermostable DNA polymerase isolated from *P. furiosus* can serve as a effective substitute for *Taq* DNA polymerase for most applications in PCR. Unlike *Taq* polymerase, *Pfu* DNA polymerase possesses a 3′-to-5′ exonuclease activity which enables the polymerase to proofread errors. This appears to contribute to the observed higher fidelity during DNA synthesis.

(*2*) Results from the PCR-based fidelity assay demonstrate that *Pfu* DNA polymerase yields PCR products containing less than 10% of the mutations that are obtained from similar amplifications performed with *Taq* DNA polymerase. These findings are significant for PCR techniques which require high-fidelity DNA synthesis, including the direct cloning of PCR amplification products, PCR-based procedures for high-efficiency double-stranded mutagenesis and amplification techniques designed to detect specific point mutations.

ACKNOWLEDGEMENTS

We thank Sandra Mathur and Kirk Nielson for their expert technical assistance, Jae-Bum Park for sharing his expertise in metabolism and fermentation of archaebacteria, and Steve Kohler and Scott Provost for the transgenic mouse genomic DNA used in these studies. MWWA acknowledges the support of the Department of Energy (FG09-88-ER-13901) and the National Science Foundation (BCS-9011583 and DIR 9014281).

REFERENCES

Adams, M.W.W.: The metabolism of hydrogen by extremely thermophilic, sulfur-dependent bacteria. FEMS Microbiol. Rev. 75 (1990) 219–238.

Bernad, A., Blanco, L., Lazaro, J.M., Martin, G. and Salas, M.: A conserved 3′-5′ exonuclease active site in prokaryotic and eukaryotic DNA polymerases. Cell. 59 (1989) 219–228.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72 (1976) 248–254.

Chase, J.W. and Richardson, C.C.: Exonuclease VII of *Escherichia coli*. J. Biol. Chem. (1974) 4545–4552.

Eckert, K.A. and Kunkel, T.A.: High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase. Nucleic Acids Res. 18 (1990) 3739–3744.

Ennis, P.D., Zemmour, J., Salter, R.D. and Parham, P.: Rapid cloning of HLA-A,B cDNA by using the polymerase chain reaction: frequency and nature of errors produced in amplification. Proc. Natl. Acad. Sci. USA. 87 (1990) 2833–2837.

Fersht, A.R. and Knill-Jones, J.W.: Contribution of 3′ to 5′ exonuclease activity of DNA polymerase III holoenenzyme from *Escherichia coli* to specificity. J. Mol. Biol. 165 (1983) 669–682.

Fiala, G. and Stetter, K.O.: *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°C. Arch. Microbiol. 145 (1986) 56–61.

Gordon, A.J.E., Burns, P.A., Fix, D.F., Yatagai, F., Allen, F.L., Horsfall, M.J., Halliday, J.A., Gray, J., Bernelot-Moens, C. and Glickman, B.W.: Missense mutation in the *lacI* gene of *Escherichia coli*: inferences on the structure of the repressor protein. J. Mol. Biol. 200 (1988) 239–251.

Grippo, P. and Richardson, C.C.: Deoxyribonucleic acid polymerase of bacteriophage T7. J. Biol. Chem. 246 (1971) 6867–6872.

Keohavong, P. and Thilly, W.G.: Fidelity of DNA polymerases in DNA amplification. Proc. Natl. Acad. Sci. USA. 86 (1989) 9253–9257.

Kohler, S.W., Provost, G.S., Fieck, A., Kretz, P.L., Bullock, W.O., Sorge, J.A., Putman, D.L. and Short, J.M.: Spectra of spontaneous and mutagen-induced mutations in the *lacI* gene in transgenic mice. Proc. Natl. Acad. Sci. USA. 88 (1991) 7958–7962.

Kunkel, T.A., Beckman, R.A. and Leob, L.A.: On the fidelity of DNA synthesis. J. Biol. Chem. 261 (1869) 13610–13616.

Mullis, K., Faloona, F., Saiki, R., Horn, G. and Erlich, H.: Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harbor Symp. Quant. Biol. 51 (1986) 263–273.

Muzyczka, N., Poland, R.L. and Bessman, M.J.: Studies on the biochemical basis of spontaneous mutation. J. Biol. Chem. 247 (1972) 7116–7122.

Reiss, J., Krawczak, M., Schloesser, M., Wagner, M. and Copper, D.N.: The effect of replication errors on the mismatch analysis of PCR-amplified DNA. Nucleic Acids Res. (1990) 973–978.

Reyland, M.E., Lehman, I.R. and Loeb, L.A.: Specificity of proofreading by the 3′ to 5′ exonuclease of the DNA polymerase-primase of *Drosophila melanogaster*. J. Biol. Chem. 263 (1988) 6518–6524.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A.: Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239 (1988) 487–491.

Sambrook, J., Fritsch, E.F., and Maniatis, T.: Molecular Cloning. A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

Schaaper, R.M., Danforth, B.N. and Glickman, B.W.: Mechanisms of spontaneous mutagenesis: an analysis of the spectrum of spontaneous mutation in the *Escherichia coli lacI* gene. J. Mol. Biol. 189 (1986) 273–284.

Sinha, N.K.: Specificity and efficiency of editing of mismatches involved in the formation of base-substitution mutations by the 3′ to 5′ exonuclease activity of phage T4 DNA polymerase. Proc. Natl. Acad. Sci. USA. 84 (1987) 915–919.

Tindall, K.R. and Kunkel, T.A.: Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. Biochemistry 27 (1988) 6008–6013.