

PCR fidelity of *Pfu* DNA polymerase and other thermostable DNA polymerases

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ABSTRACT

The replication fidelities of *Pfu*, *Taq*, *Vent*, *Deep Vent* and *UITma* DNA polymerases were compared using a PCR-based forward mutation assay. Average error rates (mutation frequency/bp/duplication) increased as follows: *Pfu* (1.3×10^{-6}) < *Deep Vent* (2.7×10^{-6}) < *Vent* (2.8×10^{-6}) < *Taq* (8.0×10^{-6}) \ll *exo⁻ Pfu* and *UITma* ($\sim 5 \times 10^{-5}$). Buffer optimization experiments indicated that *Pfu* fidelity was highest in the presence of 2–3 mM MgSO₄ and 100–300 μ M each dNTP and at pH 8.5–9.1. Under these conditions, the error rate of *exo⁻ Pfu* was ~40-fold higher (5×10^{-5}) than the error rate of *Pfu*. As the reaction pH was raised from pH 8 to 9, the error rate of *Pfu* decreased ~2-fold, while the error rate of *exo⁻ Pfu* increased ~9-fold. An increase in error rate with pH has also been noted for the exonuclease-deficient DNA polymerases *Taq* and *exo⁻ Klenow*, suggesting that the parameters which influence replication error rates may be similar in pol I- and α -like polymerases. Finally, the fidelity of 'long PCR' DNA polymerase mixtures was examined. The error rates of a *Taq/Pfu* DNA polymerase mixture and a *Klentaq/Pfu* DNA polymerase mixture were found to be less than the error rate of *Taq* DNA polymerase, but ~3–4-fold higher than the error rate of *Pfu* DNA polymerase.

INTRODUCTION

The use of high fidelity DNA polymerases in the polymerase chain reaction (PCR) is essential for reducing the introduction of amplification errors in PCR products that will be cloned, sequenced and expressed. Several thermostable DNA polymerases with 3'→5' exonuclease-dependent proofreading activity (*Pfu*, *Vent*, *Deep Vent* and *UITma*) have been introduced for high fidelity PCR amplification (1–3). Flaman *et al.* have reported that the error rate of *Pfu* was 5- and 30-fold lower than the error rates of the proofreading enzymes *Deep Vent* and *UITma*, respectively (4). Using several different fidelity assays, the error rate of *Pfu* has been found to be ~10-fold lower than that of the non-proofreading enzyme *Taq* (1,4,5).

The parameters which contribute to the replication fidelity of DNA polymerases need to be investigated, as very little is known about the molecular features of these enzymes which give rise to

variations in replication fidelity and mutational spectra. A number of factors are thought to contribute to the overall fidelity of a DNA polymerase (reviewed in 6–8). These parameters include the tendency of a polymerase to incorporate incorrect nucleotides and the presence of an integral 3'→5' exonuclease activity which can remove mispaired bases (proofreading activity).

The importance of proofreading activity to replication fidelity has been demonstrated for both the Klenow fragment (9) and for *Vent* polymerase (10), which exhibit 10- and 5-fold increases in error rates, respectively, when the associated 3'→5' exonuclease activity is inactivated. The contribution of proofreading activity to DNA polymerase fidelity is also evident when the error rates of proofreading and non-proofreading enzymes are compared. Kunkel has noted that the average base substitution error rates exhibited by non-proofreading DNA polymerases range from 10⁻² to $\geq 10^{-6}$, while the error rates of proofreading enzymes range from 10⁻⁶ to 10⁻⁷ (7). The parameters which contribute to error rate variations among proofreading enzymes may reflect inherent differences in 3'→5' exonuclease activity, the tendency to discriminate mispaired versus correctly paired bases and/or the efficiency of shuttling between polymerization and proofreading modes.

Recently, mixtures of non-proofreading and proofreading DNA polymerases have been reported to synthesize higher yields of PCR product and to allow amplification of longer templates than is possible with single enzyme formulations ('long PCR') (5). The addition of a low level of a proofreading enzyme (e.g. *Pfu* DNA polymerase) to PCR reaction mixtures has been proposed to improve the performance of non-proofreading polymerases (e.g. *Taq* DNA polymerase) by correcting mismatches introduced during PCR which prevent the efficient synthesis of full-length products (5). The PCR fidelity of DNA polymerase mixtures has not yet been determined, but error rates are likely to reflect the fidelity of the component polymerases and the ratio of non-proofreading to proofreading enzyme activities.

Pfu DNA polymerase has been found to be useful in high fidelity amplifications (1,4) of DNA targets up to 25 kb (K. Nielson, personal communication). In this report we use the previously described *lacI* PCR mutation assay (1) to compare the error rate of *Pfu* with an expanded number of PCR polymerases, including *exo⁻ Pfu*, *Deep Vent*, *Vent*, *UITma* and *Taq*, as well as 'long PCR' DNA polymerase mixtures. Polymerase error rates have been found to vary with buffer composition, including pH, Mg²⁺ concentration and nucleotide concentration (11–13). PCR reaction conditions have been optimized with respect to fidelity for

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both *Vent* and *Taq* DNA polymerases (11). Buffer optimization studies with *Pfu* DNA polymerase were performed here to assess whether the fidelity of *Pfu* DNA polymerase could be further enhanced. Error rate comparisons between *Pfu* and *exo⁻Pfu* are expected to illuminate the contribution of proofreading activity to the fidelity of *Pfu* DNA polymerase. Finally, PCR fidelity comparisons between *Pfu* DNA polymerase and *Pfu*-containing DNA polymerase mixtures will allow evaluation of the contribution of the predominant non-proofreading enzyme to the error rate of 'long PCR' mixtures.

MATERIALS AND METHODS

DNA polymerases

Cloned *Pfu*, *exo⁻Pfu* and *Taq* DNA polymerases were prepared at Stratagene. *Deep Vent* and *Vent* polymerases were purchased from New England BioLabs, *UITma* was obtained from Perkin-Elmer and KlenTaqLA (KTLA) was provided by Wayne Barnes (Washington University School of Medicine, St Louis, MO). Except where indicated, PCR amplifications were performed in the presence of buffers supplied by the manufacturers. The KTLA PCR buffer used was buffer PC2 of Barnes (5).

PCR fidelity assay

The fidelity of DNA replication during PCR was measured using a previously described assay (1,14). Briefly, a 1.9 kb sequence encoding *lacIOZα* was PCR amplified as described below with oligonucleotide primers containing 5' *EcoRI* restriction sites (1). The amplified fragments were digested with *EcoRI*, purified by gel electrophoresis and ligated into λ gt10 arms. The ligation reactions were packaged and the λ phage used to infect an α -complementing *Escherichia coli* host strain. Aliquots of infected cells were plated on LB plates with top agar containing either X-gal (1 mg/ml) or X-gal plus IPTG (1.5 mM). Error rates were calculated as described in the legend to Table 1.

PCR amplifications

Except where indicated, PCR amplifications were performed in 100 μ l reaction volumes in the presence of the appropriate Tris-based buffer, using 5 U polymerase, 200 μ M each dNTP, 250 ng each primer and 24 ng *lacIOZα* target (50 ng *lacIOZα* plasmid template). The PCR mixtures were denatured by heating at 95°C for 30 s. Thirty cycles of amplification were performed using the following conditions: 5 s at 95°C; 1 min at 55°C; 2.5 min at 72°C.

RESULTS

PCR fidelity of thermostable DNA polymerases

Replication fidelities of thermostable DNA polymerases were compared using a previously described assay (1) which measures the frequency of mutations introduced into the *lacI* target gene during PCR amplification. PCR amplification was performed in the presence of each enzyme's optimal PCR buffer. All other PCR parameters remained constant, including the dNTP, primer and template concentrations, the PCR cycling parameters and the number of PCR cycles performed.

Pfu DNA polymerase exhibited the greatest PCR fidelity, with an average error rate of 1.3×10^{-6} mutation frequency/bp/duplication (Table 1). The *lacI* target size used in these calculations was estimated to be 349 bp, based upon the most recent analysis of *lacI⁻* mutant DNA sequences (15). Previous error rate calculations assumed a *lacI* target size of 182 bp (1). After recalculating error rates based on a *lacI* target size of 349 bp, the mean error rate of *Pfu* DNA polymerase obtained in this study (1.3×10^{-6} mutation frequency/bp/duplication) was found to be similar to previous estimates obtained using an identical assay (0.8×10^{-6} ; 1) or an alternative PCR-based assay employing a p53 target gene ($\leq 1.0 \times 10^{-6}$; 4).

Table 1. Average error rates of thermostable DNA polymerases during PCR^a

DNA polymerase	No. of PCRs	Target (ng)	Template doublings ^b	<i>lacI⁻</i> plaques ^c (% \pm SD)	Error rate ^d ($\times 10^{-6} \pm$ SD)
<i>Pfu</i>	10	24	9.7	0.42 \pm 0.08	1.3 \pm 0.2
	2	2	12.7	0.30 \pm 0.06 ^{e,f}	0.7 \pm 0.1 ^e
	2	0.2	16.0	0.47 \pm 0.03 ^{e,f}	0.8 \pm 0.02 ^e
	2	0.02	19.4	0.66 \pm 0.03 ^{e,f}	1.0 \pm 0.04 ^e
<i>Deep Vent</i>	4	24	9.7–10	0.9 \pm 0.1	2.7 \pm 0.2
<i>Vent</i>	6	24	8.7–10	0.9 \pm 0.3	2.8 \pm 0.9
<i>Taq</i>	11	24	8.7–11	2.7 \pm 1.1	8.0 \pm 3.9
<i>UITma</i>	2	24	9.7	18.8 \pm 0.8 ^e	55 \pm 2 ^e

^aPCRs were performed in each manufacturers' recommended buffer (all pH 8.8) in the presence of 200 μ M each dNTP and 2 mM MgSO₄ (*Pfu*, *Deep Vent* and *Vent*), 2 mM MgCl₂ (*UITma*) or 1.5 mM MgCl₂ (*Taq*).

^bTemplate doublings (*d*) were determined using the equation $2^d = (\text{amount of PCR product}) / (\text{amount of starting target})$. 24 ng *lacI* target corresponds to 50 ng *lacIOZα* plasmid template. The range of *d* obtained is indicated.

^cMutant frequencies (*mf*) were determined by dividing the total number of blue plaques (*lacI⁻* mutants) on X-gal plates by the total number of plaques containing a functional *lacZα* sequence (blue plaques on X-gal plus IPTG plates).

^dError rates were calculated using the equation $ER = mf / (bp \times d)$, where *mf* is the mutation frequency, *bp* is the number of detectable sites in *lacI* (=349; 15) and *d* is the number of template doublings.

^e \pm indicates range of duplicate measurements.

^fMutation frequencies for *Pfu* amplification of 0.02–2 ng target were normalized such that the mean mutation frequency for *Pfu* amplification of 24 ng target (assay internal control) was 0.42%.

Average error rates of thermostable DNA polymerases were found to increase in the following order: *Pfu* (1.3×10^{-6}) < *Deep Vent* (2.7×10^{-6}) < *Vent* (2.8×10^{-6}) < *Taq* (8.0×10^{-6}) << *UITma* (5.5×10^{-5}). These results are in excellent agreement with the relative error rates measured by Flaman *et al.* (4), who reported that *Pfu* exhibits the greatest PCR fidelity, followed by *Deep Vent*, *Taq* and *UITma* DNA polymerases. The relative error rates obtained here are also consistent with DGGE analyses showing that *Pfu* exhibits a lower error rate than *Vent* and *Taq* DNA polymerases (16). We found that relative error rates observed using the *lacI* screening assay were consistent from PCR reaction to PCR reaction.

The influence of template doublings (*d*) on error rate estimates of *Pfu* DNA polymerase was also examined (Table 1). Amplification reactions described above and resulting in the *Pfu* error rate of 1.3×10^{-6} employed 24 ng *lacI* target (10^{10} copies). Approximately 10 doublings were observed in 30 PCR cycles. When the input *lacI* target DNA was decreased from 10^{10} copies (24 ng) to 10^7 copies (0.02 ng), the number of template doublings increased from 9.7 (~900-fold amplification) to 19.4 (~700 000-fold amplification) after 30 cycles of PCR. The error rate of *Pfu* DNA polymerase varied from 0.7 to 1.3×10^{-6} over the 1000-fold range of DNA target concentrations tested. Flaman *et al.* have also reported that polymerase error rates do not appear to be significantly influenced by the number of template doublings (4).

Optimization of the PCR fidelity of *Pfu*

We attempted to further improve the fidelity of *Pfu* by optimizing PCR reaction conditions. PCR error rates were measured at varying concentrations of MgSO_4 (Fig. 1) and dNTPs (Fig. 2) and at varying pHs (Fig. 3). The indicated pH values are those measured at room temperature. Where noted, the pH of Tris buffers at elevated temperatures was estimated using the formula: $\text{pH}_T = \text{pH}_{25^\circ\text{C}} + [(T^\circ\text{C} - 25^\circ\text{C}) \times (-0.03 \text{ pH U}^\circ\text{C})]$ (where *T* is the reaction temperature; 17). The lowest error rates for *Pfu* were observed when PCR amplifications were performed in the presence of 2–3 mM MgSO_4 , 100–300 μM each dNTP and in a pH range between 8.5 and 9.1 (pH ~7.1–7.7 at 72°C). These conditions have been found to give optimal yield of PCR product as well (18).

In the presence of 1 mM MgSO_4 and 800 μM total dNTPs, the error rate of *Pfu* was ~3-fold higher than when PCR amplifications were performed in 2 mM MgSO_4 at the same dNTP concentration (Fig. 1). The error rate did not vary significantly as the MgSO_4 concentration was increased from 2 to 10 mM (~1.2–9.2 mM free Mg^{2+}). The error rate of *Vent* polymerase has also been shown to decrease significantly between 0.5 and 2 mM MgSO_4 in the presence of 2 mM total dNTPs and thereafter remains constant with increasing concentrations of free Mg^{2+} (11). These results are in contrast to those reported for *Taq*, in which error rates are lowest at equimolar concentrations of MgCl_2 and dNTPs (1 mM) and increase with increasing concentration of free Mg^{2+} (12). Error rate variations of *Pfu* and *Vent* likely reflect the Mg^{2+} dependency of both proofreading and polymerase activities.

In Figure 2, the error rate of *Pfu* was found to increase 2.4-fold as the total dNTP concentration was raised from 0.4 to 4 mM in the presence of a constant amount of free Mg^{2+} (~1.2 mM). These results are consistent with the observations of Clayton *et al.* (19), who report that high concentrations of dNTPs diminish the

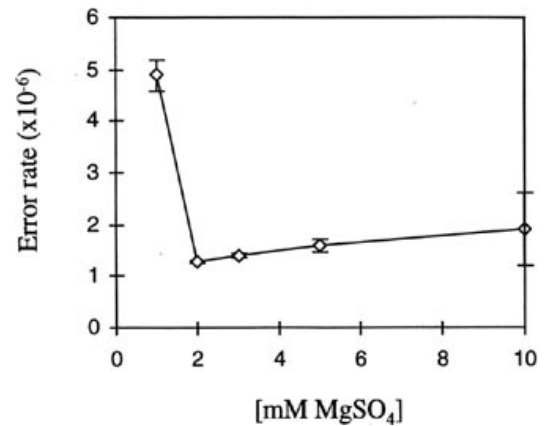


Figure 1. Variation of the PCR error rates of *Pfu* DNA polymerase with MgSO_4 concentration. PCR amplification was performed in buffer containing 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 0.1% Triton X-100, 100 $\mu\text{g}/\text{ml}$ BSA, 200 μM each dNTP and varying concentrations of MgSO_4 (1–10 mM). Error rates shown are the average (\pm range) values obtained from two independent PCR amplifications.

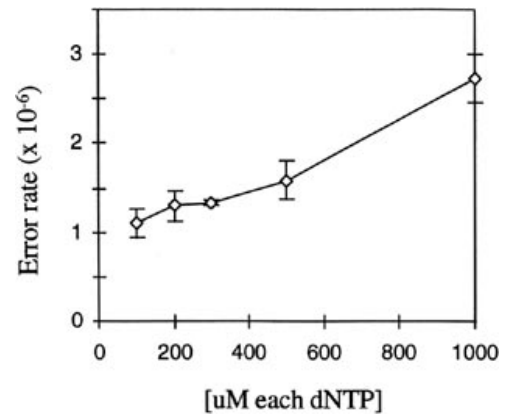


Figure 2. Variation of the PCR error rates of *Pfu* DNA polymerase with dNTP concentration. PCR amplification was performed in buffer containing 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 0.1% Triton X-100, 100 $\mu\text{g}/\text{ml}$ BSA and varying concentrations of dNTPs (100–1000 μM each). The MgSO_4 concentration of the PCR mixtures was adjusted to give a constant free Mg^{2+} concentration (1.2 mM) at each dNTP concentration: 1.6 mM MgSO_4 /0.4 mM total dNTPs; 2 mM MgSO_4 /0.8 mM total dNTPs; 2.4 mM MgSO_4 /1.2 mM total dNTPs; 3.2 mM MgSO_4 /2 mM total dNTPs; 5.2 mM MgSO_4 /4 mM total dNTPs. Error rates shown were normalized such that the mean mutation frequency for *Pfu* amplifications with 0.8 mM total dNTPs (assay internal control) was 1.3×10^{-6} mutation frequency/bp/duplication. The average error rates (\pm range) from two independent PCR amplifications are shown.

proofreading ability of exonuclease-proficient polymerases by increasing the efficiency of mispair extension. It is likely that the fidelity of *Pfu* DNA polymerase could be further increased by reducing the total dNTP concentration below 0.4 mM total dNTPs. However, using lower dNTP concentrations to increase the fidelity of PCR amplification reactions is not practical, as PCR product yields decrease significantly below 0.4 mM total dNTPs.

In Figure 3 (inset), the error rate of *Pfu* was measured as a function of pH. The error rate of *Pfu* was found to decrease 4-fold between pH 7.5 and 8.5 in the presence of 2 mM MgSO_4 and 0.8 mM total dNTPs. *Vent* polymerase has also been reported to

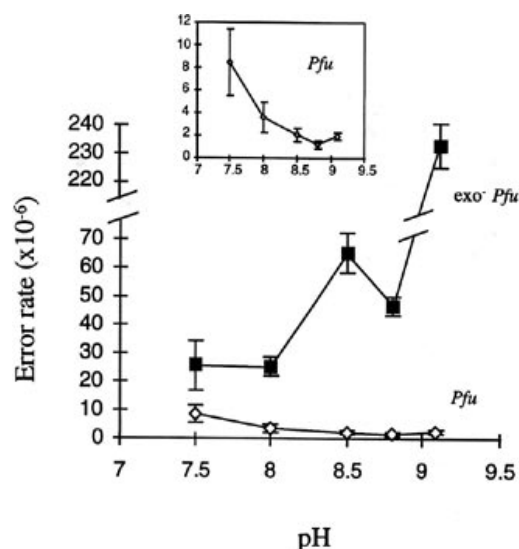


Figure 3. Variation of the PCR error rates of *Pfu* and *exo⁻ Pfu* DNA polymerases with pH. PCR amplification was performed in 20 mM Tris-HCl buffers whose pH values ranged from 7.5 to 9.1 (pH at 25°C). In addition, the buffer contained 2 mM MgSO₄, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100, 100 µg/ml BSA and 200 µM each dNTP. The average error rate of *Pfu* (open diamonds) is shown in comparison with *exo⁻ Pfu* (filled diamonds) and in the accompanying inset. Error rates shown are the average (± range) values obtained from two independent PCR amplifications.

exhibit a significant decrease in error rate as the pH is increased from 7 to 8 in the presence of 2 mM MgSO₄ (11). For *Taq* DNA polymerase, a 2-fold increase in error rate was observed when the reaction pH was raised from 8 to 9 (11). Eckert and Kunkel have also reported that the base substitution and frameshift error rates of *Taq* (12) and *exo⁻ Klenow* (13) increase >10-fold as the reaction pH is raised from ~6.5 to 9.5 (25°C estimates of pH from 12,13).

pH dependency of the fidelity of *Pfu* and *exo⁻ Pfu*

The error rates of *Pfu* and *exo⁻ Pfu* were compared to assess the contribution of 3'→5' exonuclease activity to fidelity. In the presence of *Pfu* PCR buffer (2 mM MgSO₄, 200 µM each dNTP,

pH 8.8), *exo⁻ Pfu* exhibited an error rate of 4.7×10^{-5} mutation frequency/bp/duplication, which is ~40-fold higher than that determined for exonuclease-proficient *Pfu*.

Figure 3 shows the error rate variation of *exo⁻ Pfu* and *Pfu* as a function of pH. *Exo⁻ Pfu* shows a dramatic increase in error rate (~9-fold) as the reaction pH is raised from pH 8 to 9.1 (or from 6.6 to 7.7 at 72°C). In contrast to *exo⁻ Pfu*, the error rate of *Pfu* decreased ~2-fold in this pH range. Presumably, the fidelity of *Pfu* is maintained at high pH (pH 9) by enhanced proofreading activity, which accompanies the dramatic increase in nucleotide misincorporation occurring between pH 8 and 9.1 (identified using *exo⁻ Pfu*). These results and those reported by others for *Taq* and *exo⁻ Klenow* (11–13) indicate that the error rates of exonuclease-deficient enzymes, *Taq*, *exo⁻ Klenow* and *exo⁻ Pfu*, are similarly increased by pH. The significance of the apparent biphasic relationship between error rate and pH is currently under investigation.

PCR fidelity of 'long PCR' DNA polymerase mixtures

The fidelities of *Pfu* and *Taq* DNA polymerases were compared with the fidelities of two *Pfu*-containing DNA polymerase mixtures (Table 2). A *Taq/Pfu* (16 U:1 U) mixture was prepared and shown to amplify DNA targets >30 kb (data not shown). The *Taq/Pfu* mixture exhibited an average error rate of 5.6×10^{-6} mutation frequency/bp/duplication when amplifications were performed in *Taq* PCR buffer. The mean error rate of the *Taq/Pfu* mixture was 30% lower than the mean error rate of *Taq* DNA polymerase when amplifications were conducted in *Taq* PCR buffer. When compared with the error rate of *Pfu* DNA polymerase in the same buffer system, the error rate of the *Taq/Pfu* mixture was found to be 6-fold higher.

Similar observations were made for a second 'long PCR' mixture, KTLA, which consists of *Klentag* (N-terminally truncated *Taq*) and *Pfu* DNA polymerases (5). When PCR amplifications were conducted as described in this report, KTLA exhibited a mean error rate of 3.9×10^{-6} mutation frequency/bp/duplication, which was 3-fold higher than the error rate of *Pfu* DNA polymerase (Table 2). When PCR conditions from Barnes (5) were used (Table 2, condition 2), KTLA exhibited a mean error rate (9.4×10^{-6}) which was 4-fold higher than the error rate of *Pfu* DNA polymerase assayed under identical conditions.

Table 2. Error rate comparisons of DNA polymerases and 'long PCR' DNA polymerase mixtures

PCR condition ^a	DNA polymerase	No. of PCRs	Template doublings ^b	Error rate ($\times 10^{-6} \pm SD$) ^b
1	<i>Pfu</i>	10	8.7–9.7	1.3 ± 0.2
	<i>Taq</i>	11	8.7–11	8.0 ± 3.9
	<i>Taq/Pfu</i> (16:1) ^c			
	<i>Taq</i> buffer	8	9.7–10	5.6 ± 1.6
	<i>Pfu</i> buffer	11	9.7–11	7.6 ± 1.2
	KTLA	2	9.7	3.9 ± 0.1^d
2	<i>Pfu</i>	2	8.1	2.3 ± 0.2^d
	<i>Taq</i>	2	8.1	9.4 ± 0.9^d
	KTLA	2	8.1	9.4 ± 0.9^d

^aPCR condition 1 is described in Materials and Methods, PCR amplification. PCR condition 2 is from Barnes (5) and differs in the following respects. PCR amplifications were performed on a Robocycler 40 (Stratagene) using thin-walled PCR tubes and 7.2 ng target (15 ng *lacIOZα* plasmid). Sixteen cycles of amplification were performed using the following conditions: 30 s at 99°C, 30 s at 67°C, 3 min at 68°C.

^bDefined in the legend to Table 1.

^cThe *Taq/Pfu* mixture consists of *Taq* (5 U/µl) and *Pfu* (0.31 U/µl) DNA polymerases.

^d± indicates range of duplicate measurements.

DISCUSSION

The intrinsic properties of thermostable DNA polymerases which contribute to variation in PCR fidelity are not fully understood. In general, enzymes which possess an associated 3'→5' exonuclease-dependent proofreading activity are thought to exhibit higher replication fidelity than non-proofreading DNA polymerases (7). Variation in fidelity among proofreading enzymes, such as *Pfu*, *Vent* and *Deep Vent*, may reflect differences in the rate of mispair excision, the level of discrimination between mispaired and correctly paired bases, the rate of mispair extension and/or the efficiency of shuttling the 3' primer terminus between the polymerase and exonuclease active sites.

The contribution of 3'→5' exonuclease activity to the PCR fidelity of *Pfu* was demonstrated directly by comparing the error rates of *Pfu* and *exo⁻Pfu*. The error rate of *exo⁻Pfu* was found to be 7-fold higher than the error rate of *exo⁺Pfu* at pH 8.0 and 40-fold higher at pH 8.8 (*Pfu* PCR buffer).

Despite the importance of proofreading activity to the fidelity of *Pfu* and *Vent* (10), the presence of 3'→5' exonuclease activity does not necessarily guarantee high fidelity DNA synthesis, as illustrated by *UITma* DNA polymerase. The poor fidelity of *UITma* DNA polymerase may be related to the relatively low level of 3'→5' exonuclease activity exhibited by this enzyme. In a preliminary analysis of exonuclease activity, *UITma* was found to exhibit significantly lower levels of 3'→5' exonuclease activity than *Pfu*, *Deep Vent* and *Vent* DNA polymerases (A. Lovejoy, personal communication). However, other parameters are likely to contribute to low fidelity, since *UITma*, an N-terminally deleted version of *Thermatoga maritima* DNA polymerase (20), exhibits an ~7-fold higher error rate than *Taq*, which is completely devoid of proofreading activity.

In the absence of proofreading activity, a DNA polymerase like *Taq* is thought to accomplish high fidelity DNA synthesis by inefficient incorporation of non-complementary dNTPs and a reduced tendency to extend from mismatched 3' primer termini. Huang *et al.* (21) have shown that, with the exception of C–T mispairs, *Taq* polymerase exhibits ~100–1000-fold greater discrimination against mispair extension, as compared with avian myeloblastosis and HIV-1 reverse transcriptases, which extend most mispairs permissively. The rate at which DNA polymerases extend from mispaired 3' primer termini, however, does not contribute to the actual fidelity of non-proofreading enzymes. The mismatch extension rate only contributes to fidelity in the sense that if the mismatch is extended inefficiently, the DNA will not be replicated to completion and the mutation will not be scored. Therefore, the mispair extension rate influences the number of detected mutants, rather than reflecting the inherent fidelity of a non-proofreading DNA polymerase.

The observed 6-fold difference in error rate between *Taq* (8×10^{-6}) and *exo⁻Pfu* (4.7×10^{-5}) suggests that the misincorporation and/or misextension rates of *Pfu* (as measured with *exo⁻Pfu*) are significantly higher than those of *Taq*. Apparently, a lower degree of discrimination against misinsertion or mispair extension errors can be tolerated when an associated proofreading activity is present, as is the case with exonuclease-proficient *Pfu*.

Further fidelity measurements with *exo⁻Pfu* revealed that the fidelity of dNTP incorporation was significantly influenced by the pH of the PCR buffer. The error rate increased by ~9-fold as the pH was raised from pH 6.6 to 7.7 (pH at 72°C). The error rates

of both *Taq* (12) and *exo⁻Klenow* (13) increase similarly at higher pH. Eckert and Kunkel have attributed the lower fidelity of *exo⁻Klenow* at high pH to an increase in both nucleotide misinsertion and mispair extension (13).

It is tempting to speculate that the lower fidelity of *exo⁻Pfu* at high pH may also reflect increased misinsertion and mispair extension, analogous to the observations made for *exo⁻Klenow* (13). If so, it would suggest that the parameters which contribute to fidelity are similar, despite the structural differences which are thought to exist between the α -like (*exo⁻Pfu*; 22) and pol I-like (*exo⁻Klenow* and *Taq*) DNA polymerases. For example, the observed variation in error rates with pH suggests that an active site histidine residue may play a role in fidelity, possibly in the discrimination of mismatched 3' primer termini. Alternatively, protonation of the primer, template or substrate dNTP may enhance error discrimination (13). Finally, pol I- and α -like polymerases may undergo a similar conformational change at low pH which may alter template binding properties, thereby improving error discrimination. Such a mechanism was proposed for *exo⁻Klenow* by Eckert and Kunkel (13) and was supported by additional data showing that lower error rates at low pH were accompanied by an increase in polymerase processivity.

The relative error rates for *Pfu*, *Vent* and *Taq* were found to parallel the terminal transferase activities of DNA polymerases. Hu (23) has compared the tendency of DNA polymerases to catalyze the addition of non-template-directed bases to the 3'-end of a DNA fragment (terminal transferase activity). Terminal transferase activity is high in *Taq* but low (*Klenow* and *Vent*) or absent (*Pfu*, *T4* and *T7*) in proofreading enzymes, which presumably edit the misextended base. The absence of terminal transferase activity appears to correlate with high fidelity. Fidelity measurements compiled by Cha and Thilly show that the error rates of *Pfu*, *T4* and *T7* DNA polymerases are lower than the error rates of *Vent* and *Klenow* (16). Thus, the parameters which give rise to terminal transferase activity may be similar to those which contribute to lower fidelity. The lower error rate and lack of terminal transferase activity for *Pfu* (as compared with *Vent*) may be the result of a reduced tendency of *Pfu* to incorporate a mismatch or a base opposite an abasic site. Alternatively, *Pfu* may excise misincorporated bases more readily or shuttle between the exonuclease and polymerase active sites more efficiently.

Finally, fidelity comparisons with *Pfu*-containing 'long PCR' DNA polymerase mixtures have shown that the error rate of mixtures appears to be intermediate between the error rate of *Pfu* and the non-proofreading DNA polymerase. The lower error rate of a *Taq/Pfu* mixture, as compared with *Taq* alone, suggests that *Pfu* is editing a certain percentage of mismatches that have been introduced by *Taq* during the PCR process. Editing may occur at the 3'-terminus after *Taq* has introduced a mismatch and dissociated from the incomplete PCR product (5). In the absence of *Pfu*, *Taq* presumably extends some of these putative stalling mismatches during the course of the PCR process; otherwise the mutations would not be scored in the *lacI* screening assay and there would be no apparent difference in error rate between *Taq* and the *Taq/Pfu* mixture. *Pfu* may also reduce the overall error rate of *Taq* DNA polymerase by degrading *Taq*-generated duplex DNA containing mismatches and resynthesizing the correct sequence.

Although the error rate of the *Taq/Pfu* mixture is somewhat lower than the error rate of *Taq* alone, it is still 4–6-fold higher than the error rate of *Pfu* alone (Table 2). These results indicate that the majority of PCR products are synthesized by *Taq*. This

result is not surprising, since *Taq* is present in this particular mixture at a 16-fold higher polymerase unit concentration than *Pfu* DNA polymerase. Hence, the misincorporation rate of *Taq* DNA polymerase contributes significantly to the error rate of *Taq/Pfu* DNA polymerase mixtures.

KTLA, a 'long PCR' mixture of *Klentaq* and *Pfu* DNA polymerases, was also found to exhibit an error rate significantly higher than the error rate of *Pfu*. Our results are inconsistent with the results of Barnes (5), who has compared the error rates of *Pfu*, *Klentaq* and KTLA-64 (~640 U *Klentaq*:1 U *Pfu*) using a similar PCR forward mutation assay based on the mutational target gene *lacZ*. Barnes reported that the error rate of the KTLA mixture was 2-fold lower than the error rate of *Pfu* DNA polymerase (5). There are several differences between the Barnes assay and the assay performed here, including PCR amplification conditions (see Table 2 legend), number of clones screened [500–4200 clones/1 PCR in Barnes (5) versus 10 000–50 000 clones/PCR/4 PCRs in this study] and the mutational target gene used (*lacZ* versus *lacI*), as well as possible unknown variations in the KTLA mixtures. The results in Table 2 demonstrate that differences in the PCR amplification conditions employed are not likely to contribute to the differences in relative error rates observed in the two studies. Fidelity analyses of additional DNA polymerase mixtures are currently under way to help elucidate the role of component enzymes and buffer composition in the fidelity of 'long PCR' amplifications.

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