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Fidelity of *Thermococcus litoralis* DNA polymerase (Vent™) in PCR determined by denaturing gradient gel electrophoresis

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ABSTRACT

DNA synthesis fidelities of two thermostable DNA polymerases, Thermus aquaticus (Taq) and Thermococcus litoralis (Tli, also known as Vent™), and a non-thermostable enzyme, a modified T7 DNA polymerase (Sequenase®), were determined by analyzing polymerase chain reaction (PCR) products using denaturing gradient gel electrophoresis (DGGE). The error rates were 4.4, 8.9, and 2.4×10^{-5} errors/bp for modified T7, Taq, and Tli polymerase, respectively. Reducing the nucleotide triphosphate concentration for Tli polymerase during PCR did not alter the fidelity. The ability of DGGE to detect a mutant present at several percent in a wild type population is related to the polymerase fidelity. To examine the sensitivity of mutant detection, human genomic DNA containing a 1% fraction of a known base pair substitution mutant was PCR-amplified with the three enzymes using primers that flank the mutant sequence. The PCR products were analyzed by DGGE. The signal from the mutant present at 1% was visible in the samples amplified with modified T7 and Tli polymerase, but the higher error rate of Taq polymerase did not permit visualization of the signal in DNA amplified with Taq polymerase.

INTRODUCTION

Polymerase chain reaction (PCR) has found numerous applications in molecular biology (1,2). The first thermostable DNA polymerase used in PCR was isolated from Thermus aquaticus (Taq). Taq polymerase permitted the automation of PCR since the polymerase is not inactivated by the temperature used in the denaturation step of PCR. Taq polymerase has a reported error rate of 2×10^{-4}/bp (3,4), therefore, depending on the length of the amplified DNA and the degree of amplification, the fraction of molecules containing an incorrect base can approach 100% due to polymerase error (5). While the majority of the molecules may contain a polymerase-induced error, the fraction of molecules with an error at a given base is small, and the DNA can generally be directly sequenced with no ambiguity. However, when the number of starting template molecules approaches one, or when one wishes to analyze a complex population of mutants, the polymerase-induced errors can limit the sensitivity of the system (6,7).

Several other thermostable DNA polymerases have recently become commercially available, including Thermococcus litoralis DNA polymerase (Tli), also called Vent™. According to New England Biolabs product literature, Tli polymerase contains a 3′ to 5′ proofreading exonuclease activity. Taq polymerase does not contain a 3′ to 5′ exonuclease activity (4), and the proofreading function may increase the fidelity of Tli polymerase compared to Taq polymerase.

To examine fidelity of the polymerases we used the method of Keohavong and Thilly (8). Briefly, (i) the target DNA was amplified with radiolabelled oligonucleotide primers, (ii) the DNA was loaded onto a polyacrylamide gel and the desired fragment was recovered and electroeluted, (iii) the DNA was denatured and allowed to hybridize in solution, (iv) molecules containing a PCR-induced mutation were separated from wt molecules by denaturing gradient gel electrophoresis (DGGE). Under our conditions, essentially all the DNA molecules containing a PCR-induced mutation will be in a mismatched heteroduplex after solution hybridization. DGGE can resolve nearly all heteroduplexes containing a single base mismatch from the wild type homoduplex. The fidelity of the polymerases was calculated after determining the fraction of non-wild type and wild type molecules in the denaturing gradient gel. We examined the fidelities of Tli polymerase, Taq polymerase, and a non-thermostable modified T7 DNA polymerase (Sequenase®).

MATERIALS AND METHODS

Plasmids and DNA

Plasmid pλ3001, containing exon 3 and flanking regions of the human hypoxanthine guanine phosphoribosyltransferase gene (HPRT), was a gift from C.T.Caskey, Baylor College of

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Medicine, Houston TX. Wild type human genomic DNA was from the cell line TK6. Human genomic DNA containing a single base pair substitution in HPRT exon 3 DNA was from the cell line HPRT Munich (9).

PCR

Oligonucleotide primers complementary to the intron sequence immediately flanking exon 3 were used to amplify a 224bp fragment containing exon 3 from (i) 3×10^4 copies of plasmid DNA, and (ii) 500ng of wt human genomic DNA containing 1% genomic DNA from the HPRT exon 3 mutant HPRT Munich.

The primer sequences were:

5′ CATATATTAA ATATACTCAC (downstream primer)
5′ TCCTGATTTT ATTTCTGTAG (upstream primer).

For Taq polymerase amplification, the reaction mixture contained 16.6mM $(NH_4)_2SO_4$, 67mM Tris HCl (pH 8.7 at 25°C), 6.7mM $MgCl_2$, 0.1mM EDTA, 10mM 2-mercapto-ethanol, 500nM primers, 100μM dNTPs (Pharmacia, Piscataway, NJ), 2U Taq polymerase (AmpliTaq, Perkin Elmer Cetus, Norwalk, CT). One cycle consisted of 1 min. at 94°C, 1 min. at 53°C, and 2 min. at 72°C.

The buffer for modified T7 DNA polymerase amplification contained 10mM Tris HCl (pH 8.0), 5mM $MgCl_2$, 1μM primers, and 2.7mM dNTPs. A cycle consisted of boiling for 1.25 min., cooling at room temperature for 45 sec., addition of 2U of modified T7 DNA polymerase (Sequenase^R, Version 2.0, United States Biochemical Corp., Cleveland, OH), and incubating for 2 min. at 37°C.

The Tli polymerase buffer was provided by the supplier (New England Biolabs, Beverly, MA) and consisted of 10mM KCl, 10mM $(NH_4)_2SO_4$, 20mM Tris HCl (pH 8.8 at 25°C), 2mM $MgSO_4$, and 0.1% Triton X-100. For plasmid DNA, the PCR solution contained Tli buffer, plasmid template, 1μM primers, 200μM dNTPs, and 1U of Tli (Lot numbers 2 and 5); the reaction mixture was overlaid with 60μl of mineral oil. One cycle consisted of 1 min. at 94°C, 1 min. at 30°C, a 3 min. temperature ramp to 72°C, and 30s at 72°C. The PCR reaction mixture with human genomic DNA contained Tli buffer, genomic DNA, 9mM total $MgSO_4$, 500nM primers, 200μM dNTPs, and 1U of Tli polymerase (no oil was used). One cycle consisted of 1 min at 94°C, 1 min. at 50°C, and 1 min. at 72°C. The volume for all PCR reactions was 100μl.

The PCR amplifications were done in two steps, the first amplification used nonradiolabelled primers, then an aliquot was used as a template for a second amplification with radiolabelled primers. The initial amplification from plasmid DNA started with 3×10^4 copies and yielded roughly 3×10^{11} copies, an amplification of about 10^7-fold. 10^9-10^{10} copies of amplified DNA served as a template for the second PCR with end-labelled primers. The second PCR amplification produced $10^{11}-10^{12}$ copies, yielding an overall amplification of 10^8-10^{10}-fold.

Both primers were 5′ end-labelled with ^{32}P-α-ATP (6000 Ci/mmol, New England Nuclear, Boston, MA) and the reagents in the BRL 5′ terminus labelling kit (BRL, Gaithersburg, MD). The specific activity of the DNA produced with radiolabelled primers was 3.3×10^5 dpm/pmol.

After PCR, the DNA was loaded onto a polyacrylamide gel, the 224bp HPRT exon 3 fragment was cut from the gel with no exposure to ethidium bromide or UV light; these procedures produce damage that can be detected by DGGE (10). The DNA was electroeluted from the gel slice, ethanol precipitated, and resuspended in water.

Solution hybridization and DGGE

$2-3\times10^4$ cpm of end-labelled DNA (0.06−0.1 pmol) was boiled 5 min. in 30ml of 300mM NaCl, 10mM Tris HCl (pH 8.0), 1mM EDTA, then placed at 65°C for 4 hr. The DNA was recovered by ethanol precipitation and resuspended in water.

The DNA was loaded onto a 1mm thick, 12.5% polyacrylamide (37.5/1, polyacrylamide:bisacrylamide), 20−35% linear denaturing gradient gel (100% = 7M urea, 40% formamide). The gel was formed using a gravity flow gradient maker. The gel was run submerged at 60°C for 18 hr. at 150V in TAE buffer, dried, and exposed to X-ray film.

The X-ray film was used as a template to cut both the wild type band (homoduplex) and the region above the wild type band (heteroduplex) from the denaturing gradient gel. The gel slices were rehydrated, placed in ScintiVerse™ II fluor (Fisher Scientific, Pittsburgh, PA), and the dpms were determined by liquid scintillation counting.

RESULTS

Figure 1 shows the results of plasmid amplification with Tli polymerase using 2−10mM $MgSO_4$. Only the 2mM and 3mM $MgSO_4$ concentrations produced a band of the expected size, 224bp, as well as a less-intense, higher molecular-weight band (lanes 2 and 3). A large quantity of lower molecular weight product, probably primer dimers, was observed at $MgSO_4$ concentrations greater than 2mM. The fidelity experiments were performed at 2mM $MgSO_4$. The efficiency of plasmid amplification with 2mM $MgSO_4$ was 55% (see Table 1). Taq and modified T7 polymerase both produced a single band of the expected size on an ethidium-bromide stained gel.

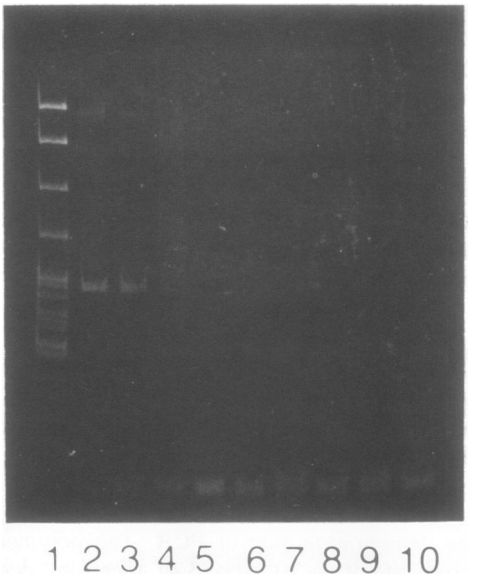

Fig. 1. Ethidium bromide-stained polyacrylamide gel showing the effect of $MgSO_4$ concentration on efficiency of amplification with Tli polymerase. 3×10^4 copies of plasmid template were amplified 38 cycles with Tli polymerase. One-tenth of the reaction volume was loaded onto an 8% polyacrylamide gel. Lane 1: 100ng pBR322/MspI marker; lane 2: 2mM $MgSO_4$; lane 3: 3mM $MgSO_4$; lane 4: 4mM $MgSO_4$: lane 5: 5mM $MgSO_4$; lane 6: 6mM $MgSO_4$; lane 7: 7mM $MgSO_4$; lane 8: 8mM $MgSO_4$; lane 9: 9mM $MgSO_4$; lane 10: 10mM $MgSO_4$.

The amplification factor was determined by running an aliquot of the PCR mixture on a gel with known amounts of DNA as a standard. The quantity of DNA in the PCR aliquot was determined by comparison with the standards and the total copies of DNA produced during PCR was determined. The efficiency of PCR amplification is given by:

$$\text{amplification factor} = X^n$$

where efficiency $= X-1$, and n = the number of cycles. The efficiencies of modified T7, Tli, and Taq polymerase in amplifying plasmid DNA are given in Table 1. Conditions for amplification with modified T7 and Taq polymerase have been examined previously (7,8). For the initial fidelity studies, we selected conditions that produced maximum amplification efficiency for all three polymerases.

An aliquot of the amplified fragment was used as template for a second round of PCR with end-labelled primers. After gel purification, the radiolabelled fragment was denatured and annealed in solution, then loaded onto a denaturing gradient gel. Under these conditions, mutations produced by the polymerases are found above the wild type position in the gel. By inspection, it is apparent that Taq polymerase produced more errors during PCR than either Tli or modified T7 polymerase (see Figure 2).

The proportion of molecules containing a mutation is termed the mutant fraction, *MF*. The mutant fraction is directly proportional to the error rate of the polymerase. The *MF* was determined by separately excising the wt homoduplex and the area above the wt band (heteroduplex) and determining the dpms by liquid scintilation counting.

Simply counting the dpms in the heteroduplex region of the gel will overestimate the fraction of mutant molecules since the heteroduplex region contains mutant:wt heteroduplexes. For example, if we have a DNA population containing a 1% *MF*, after solution hybridization, essentially all of the mutant DNA will be in a mutant:wt homoduplex. If this DNA is loaded onto a denaturing gradient gel, we will find 2% of the DNA in the heteroduplex fraction, thus counting the dpms in the heteroduplex region would produce and error of 100%.

If a DNA population containing a fraction w of wt molecules and a fraction m of a particular mutant is boiled and annealed, the distribution of the molecules will be: $w^2 + 2wm + m^2$, where w^2 is the fraction of wt homoduplex, $2wm$ is the fraction of wt:mutant heteroduplex molecules, and m^2 is the fraction of mutant homoduplex molecules. It is assumed that mismatches do not significantly reduce the rate of formation of wt:mutant heteroduplexes compared to wt:wt or mutant:mutant homoduplexes. In our gel system, we determine the fraction of wt homoduplex molecules after solution hybridization, thus the fraction of wt molecules in the population is the square root of the fraction of molecules in the wt homoduplex band. The fraction of molecules in the population with a mutation, *MF*, is 1−(wt fraction in the population).

The presence of a diffuse smear in the heteroduplex region of the denaturing gradient gel indicates that many different mutants are present, each at a low frequency. In this case, after solution hybridization, essentially all of the mutant molecules will be in a wt:mutant or mutant:mutant heteroduplex; the original mutant homoduplex be present at the square of its frequency in the population. Mutant homoduplexes can be found above, below, or at the wt position in a denaturing gradient gel, and mutant homoduplexes could introduce an artifact into the fidelity calculations. However, given that essentially all the mutant molecules are in a heteroduplex, and that all heteroduplexes will

Table 1. The efficiencies and fidelities for modified T7 (Sequenase^R), Taq, and Tli (Vent^{TM}) polymerase.

Polymerase	Amplification factor	Efficiency	Het dpms / wt dpms	Error rate (x 10^{-5})	Average error rate (x 10^{-5})
Sequenase	3.4 x 10^8	56%	3411 / 7007	5.3	4.4
			1274 / 4469	3.5	
Taq	6.4 x 10^8	58%	6993 / 5234	9.9	8.9
			3919 / 4319	7.9	
Vent	6.7 x 10^9	55%	2768 / 9105	3.2	2.4
			1080 / 6007	2.0	
	1.3 x 10^9		1275 / 6853	2.1	

Our best estimates for the fidelity of the polymerases is the average of three experiments for Tli polymerase, and the average of two experiments for both modified T7 and Taq polymerase. Het dpms and wt dpms represent the radioactivity in the heteroduplex and wt homoduplex region of the denaturing gradient gel, respectively.

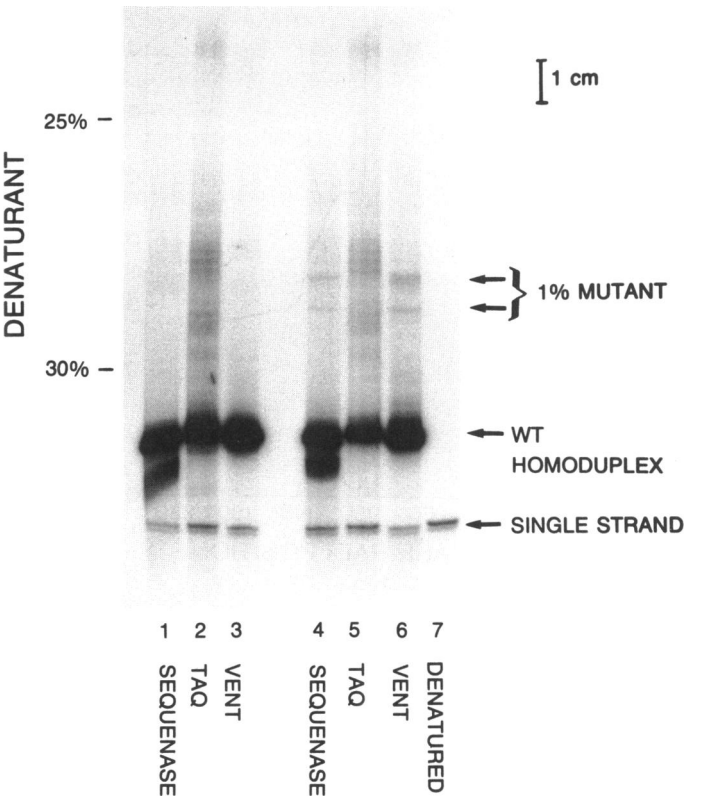

Fig. 2. Autoradiogram of denaturing gradient gel showing HPRT exon 3 amplified 10^8-10^{10}-fold from plasmid template and from human genomic DNA containing a 1% fraction of a known base pair substitution mutant in a wild type background. Lanes 1−3 show DNA amplified from plasmid template with modified T7 (Sequenase^R), Taq, and Tli (Vent^{TM}) polymerase. PCR-induced mutations are found above the wt homoduplex. Lanes 4−6 show human genomic DNA with a 1% fraction of a known base pair substitution mutant (HPRT Munich) amplified with the three polymerases. The arrows indicate the position of the two mutant heteroduplex strands formed when wt DNA hybridizes to the two mutant HPRT Munich strands. The unhybridized single strand DNA is found below the wt homoduplex, lane 7 shows denatured DNA.

be found above the wt position, the bias introduced by mutant homoduplexes is negligible.

The error rate, f, of the polymerases was determined by $f = MF/(b \times d)$, where MF is the fraction of molecules with a mutation, b is the region of the molecule (in bp) where mutations can be detected and d is the number of DNA duplications (e.g., 29.9 duplications yields a 10^9-fold amplification). The region of the DNA molecule where mutations can be detected by DGGE is given is Figure 3; mutations at 119 of the 224 bp locations are predicted to be resolved from the wild type. The polymerase error rates were calculated using plasmid DNA as template, this data is given in Table 1. The average error rates for modified T7, Taq, and Tli polymerase were 4.4, 8.9, and 2.4×10^{-5} errors/bp, respectively.

An application of PCR/DGGE is in the analysis of complex mutant populations (7). Typically, one seeks to detect a mutant present at a frequency of several percent in a wild type background. We amplified human genomic DNA containing a 1% fraction of a known HPRT exon 3 mutant, HPRT Munich (9). Taq and modified T7 polymerase produced a single band of the expected size on an ethidium bromide-stained gel. The major product produced by Tli polymerase was of the expected size, however, a fainter band of about 575 was also observed; this band was also present when Tli was used to amplify plasmid DNA (Figure 1). Figure 2 shows the results of the denaturing gradient gel. The 1% HPRT Munich band was visible in the DNA amplified with modified T7 and Tli polymerase, however, the band could not be identified in DNA amplified with Taq polymerase.

Amplification from human genomic DNA with Tli polymerase required a modification of the conditions used with plasmid DNA. When conditions used for amplification of plasmid DNA were applied to human genomic DNA, no product band was visible on an ethidium bromide-stained gel after 43 PCR cycles. Altering the PCR cycle (1 min. 94°C, 1 min. 42°C, 2 min. ramp to 72°C, 30s 72°C; 60μl oil) and varying the primer concentration from 250−1500nM also did not produce a band. Increasing the annealing temperature to 50°C and the $MgSO_4$ concentration to

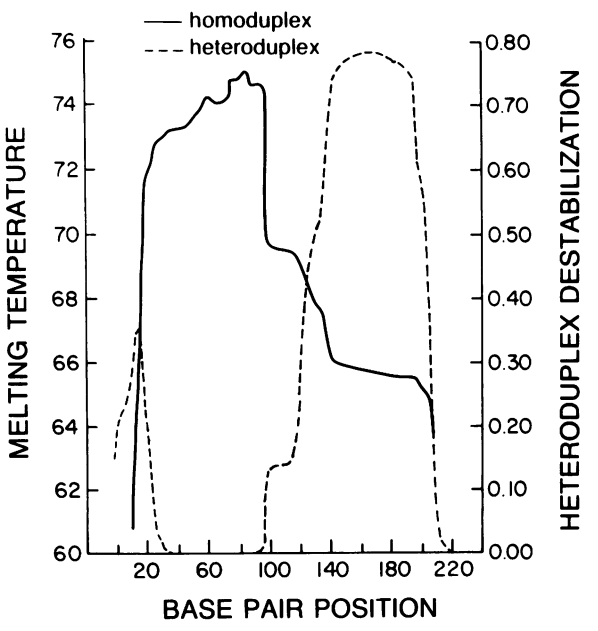

Fig. 3. Calculated destabilization produced by mismatches at each base pair location of human HPRT exon 3. The wild type homoduplex melting map is shown as a solid line. This represents the Centigrade temperature at which each base pair is in a 50:50 equilibrium between the helical and melted state (Y1 axis). The dotted line shows the predicted reduction in melting temperature (°C) caused by a single mismatch at each base pair location (Y2 axis). A reduction in melting temperature of 0.025°C caused by a single base pair mismatch should produce about a 1mm shift the denaturing gradient gel (15% change in denaturant, 17cm gel, 1% denaturant = 0.313°C). A one mm shift or greater is predicted if a mismatch occurs at base pair locations 1−28 and 95−215. However, mutations occurring in a primer sequence will be converted to the wild type sequence during subsequent PCR cycles, thus mutations at the 5′ and 3′ 20 base pairs should not be detected and are not considered in the calculations of fidelity. Excluding the primer sequences, mismatches at base pairs 21−28 and 95−205 should produce a 1mm shift of greater in a denaturing gradient gel. 119 base pairs are predicted to be sensitive to single base substitutions and this value was used in the fidelity calculations. The 5′ nucleotide of the coding region is at bp 21. The program SQHTX was used to calculate the melting maps (16). The program was kindly provided by L. Lerman (Massachusetts Institute of Technology, Cambridge. MA) and was run with the following values; destabilization = 50°C, retardation length = 75, velocity zero = 2.0, 5hr electrophoretic run.

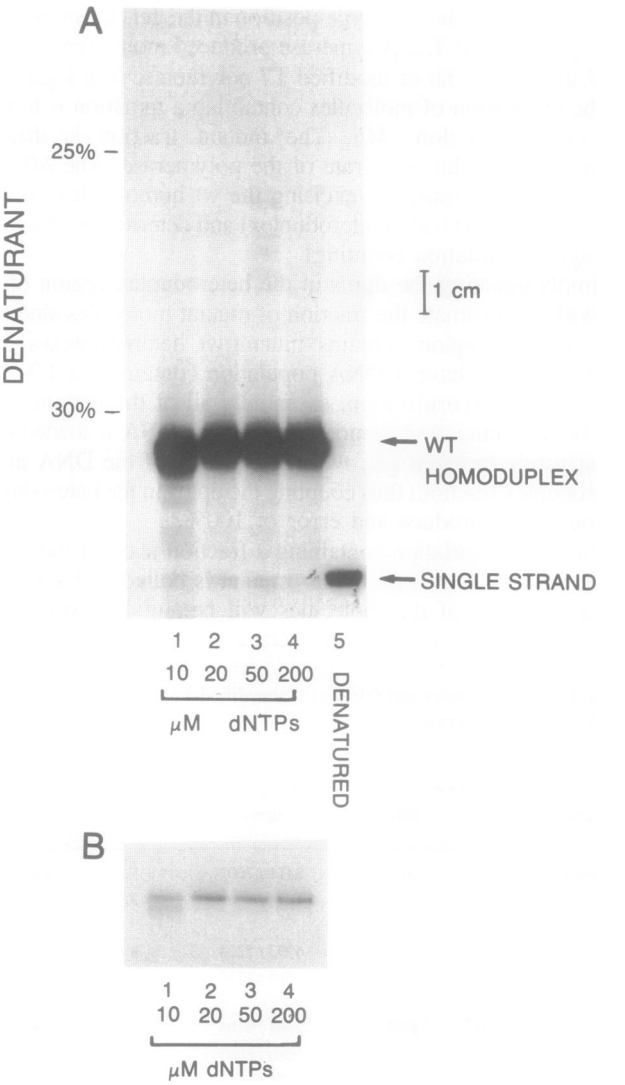

Fig. 4. Autoradiogram of denaturing gradient gel showing plasmid DNA amplified with Tli polymerase using different concentrations of dNTPs. Lanes 1−4 of panel A shows amplification with 10, 20, 50, and 200μM dNTPs. Lane 5 of panel A shows the position of single-stranded DNA (denatured). Panel B is a lighter exposure of the wt homoduplex bands.

9mM as described in Materials and Methods did produce a product, however, the efficiency of amplification was reduced to about 30%.

The effect of dNTP concentration on the fidelity of Tli polymerase was also examined. Plasmid template was amplified with Tli polymerase using 200, 50, 20, and 10μM dNTPS. As the dNTP concentration was reduced, the efficiency of amplification decreased. The efficiency of amplification was 55%, 51%, 43%, and 30% using 200, 50, 20, and 10μM dNTPs, respectively. The DNA was loaded onto a denaturing gradient gel as shown in Figure 4 and the fidelity was determined as described above. At 200, 50, 20, and 10μM dNTPs the error rates were 2.1, 3.2, 2.2, and 1.8×10^{-5} errors/bp, respectively.

DISCUSSION

The PCR error rates of modified T7, Taq, and Tli polymerase were 4.4, 8.9, and 2.4×10^{-5} errors/bp, respectively. The fidelity was determined by amplifying an identical plasmid template 10^8-10^{10}-fold and determining the fraction of molecules with a mutation. Molecules containing a mutation were forced into a mismatched heteroduplex by denaturing and annealing the amplified DNA in solution, then the mutant molecules were separated from wt molecules by denaturing gradient gel electrophoresis. The ratio of mutant to wt molecules was used to calculate the error rate of the polymerase.

Both modified T7 (11) and Taq (4) polymerase contain no $3'\rightarrow5'$ exonuclease activity, while Tli possesses an exonuclease activity, according to product literature. The exonuclease activity may contribute to the higher fidelity observed with Tli polymerase.

Eckert and Kunkel have shown that the fidelity of Taq polymerase can be increased by decreasing the pH, dNTP, and $MgCl_2$ concentrations (15). We wished to determine if reducing the dNTP concentration during PCR would likewise increase the fidelity of Tli polymerase. While the efficiency of PCR declined with decreasing dNTP concentration, we found that the fidelity of Tli polymerase was essentially unaffected by varying the dNTP concentration from 10−200μM. At 10μM dNTPs, a smear was seen below the wt homoduplex (Figure 4, panel B, lane 1); this smear was not seen at higher dNTP concentrations. It is possible that at 10μM dNTPs, Tli polymerase was not fully extending the template and the smear represents molecules truncated by one or more base pairs. A smear below the wt position in the samples amplified with modified T7 DNA polymerase was also observed (Figure 2, lanes 1 and 4), the nature of the smear is not known; it is not generally seen in samples amplified with modified T7 polymerase.

The fidelity of Taq polymerase in our experiments, 8.9×10^{-5} errors/bp, agrees fairly well with other reports. Keohavong and Thilly used DGGE/PCR to examine the fidelity of Taq and modified T7 polymerase, they found Taq polymerase to have an error rate of $1.9-2.1\times10^{-4}$ error/bp (8); it should be noted that we used a slightly different method for calculating fidelity. In other test systems, the fidelity of Taq polymerase was found to be 2×10^{-4} (cloning and sequencing of PCR products), 7.0×10^{-4} (cloning and sequencing cDNA PCR products), and 1.1×10^{-4} (strand synthesis in an M13mp2 construct) (3,4,12).

Our value for the fidelity of modified T7 DNA polymerase in PCR, 4.4×10^{-5} errors/bp, agrees well with the values found by Keohavong and Thilly (8), $3.4-3.6\times10^{-5}$ errors/bp, using the same test system. The choice of DNA template was the same, human HPRT exon 3, and the PCR conditions (concentration of dNTPs, $MgCl_2$, buffer, etc.) were almost identical in the experiments in both laboratories. However, the efficiency of PCR with modified T7 polymerase in our laboratory was 56%, while Keohavong and Thilly reported an efficiency of 90%. Perhaps lot-to-lot variations from the supplier, or subtle differences in PCR conditions may affect the PCR efficiency.

DGGE can resolve base pair substitutions, frameshifts, and small deletions from the wild type, however, deletions larger than about 10 bp are not expected to be detected by DGGE. Although no smaller molecular-weight bands were visible after PCR on an ethidium bromide-stained polyacrylamide gel, it is possible that these species exist and that they were not detected by our denaturing gradient gel technique.

One application of PCR/DGGE is examining DNA from complex mutant populations (7). In this approach, a population of mutants is examined simultaneously, and identical mutants that comprise roughly 1% or more of the population will be visible as a faint, non-wild type band. Previously, modified T7 polymerase was employed in this type of analysis because DNA amplified with this enzyme had a lower DGGE background noise than DNA amplified with Taq polymerase (9). Since modified T7 polymerase is not thermostable and enzyme must be added every PCR cycle, we wished to determine if the thermostable Tli polymerase could be used for automated PCR. Based on the observations that the fidelity of Tli polymerase is greater than that of modified T7 polymerase, and that a 1% mutant fraction can be detected with both enzymes, it seems likely that Tli polymerase can be used in place of modified T7 polymerase in the analysis of complex populations, thus permitting automated PCR to be applied to the study of complex populations.

Heat-induced abasic sites, particularly apurinic sites, will be produced during PCR and abasic sites are mutagenic both in vitro and in vivo (13,14 for review). If mutation at heat-induced apurinic sites were causing a significant fraction of the PCR errors, we would expect both Taq and Tli polymerase to show a lower fidelity than modified T7 polymerase, since the polymerization temperature of the thermostable enzymes is 72°C while modified T7 polymerase functions at 37°C. However, the thermostable enzyme Tli polymerase showed the highest fidelity, so depurination at the elevated temperatures at which the thermostable enzymes function does not make a significant contribution to the total PCR errors.

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