High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase

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ABSTRACT

We demonstrate that despite lacking a 3′→5′ proofreading exonuclease, the *Thermus aquaticus (Taq)* DNA polymerase can catalyze highly accurate DNA synthesis *in vitro*. Under defined reaction conditions, the error rate per nucleotide polymerized at 70°C can be as low as 10^{-5} for base substitution errors and 10^{-6} for frameshift errors. The frequency of mutations produced during a single round of DNA synthesis of the *lac* Zα gene by *Taq* polymerase responds to changes in dNTP concentration, pH, and the concentration of $MgCl_2$ relative to the total concentration of deoxynucleotide triphosphates present in the reaction. Both base substitution and frameshift error rates of <1/100,000 were observed at pH 5–6 (70°C) or when $MgCl_2$ and deoxynucleotide triphosphates were present at equimolar concentrations. These high fidelity reaction conditions for DNA synthesis by the *Taq* polymerase may be useful for specialized uses of DNA amplified by the polymerase chain reaction.

INTRODUCTION

Our laboratory has previously analyzed the fidelity of DNA synthesis *in vitro* by the thermostable, exonuclease-deficient DNA polymerase isolated from *Thermus aquaticus* (*Taq*)[1] (1). Using reaction conditions similar to those employed with a variety of other polymerases, *Taq* polymerase generates base substitution and one-base frameshift errors at a rate of 1/9,000 and 1/41,000, respectively, during a single round of DNA replication (1). We are interested in examining the effects of reaction conditions on the fidelity of DNA polymerization by the *Taq* polymerase for several reasons. First, the *Taq* polymerase has no detectable 3′→5′ proofreading exonuclease activity (1), allowing the accuracy inherent to DNA synthesis by the polymerase to be examined. Second, this enzyme is capable of DNA synthesis using a wide range of *in vitro* reaction conditions (2). Variations in the type and concentration of activating divalent metal ion, the pH, and the dNTP concentration are known to influence the rate and processivity of polymerization with other DNA polymerases (3,4), and several observations suggest that these parameters may influence fidelity as well (5,6).

The *Taq* polymerase is the enzyme of choice in amplification of genetic information by the polymerase chain reaction (PCR). DNA amplification by PCR is being performed using a variety of *in vitro* reaction conditions, each determined by the DNA target sequence, the set of primers and the preference of the investigator (7). The observed error rate (mutations per nucleotide per cycle) of *Taq* polymerase during PCR can differ at least 10-fold, from 2×10^{-4} to $<1.2\times10^{-5}$, using different target sequences and reaction conditions (8,9), suggesting that these variations may influence the fidelity of *Taq* polymerase. In this report, we demonstrate that in a defined polymerase reaction, the thermostable *Taq* polymerase can perform highly accurate DNA synthesis. These data may be especially useful for those investigations in which highly accurate DNA synthesis throughout the PCR process is required to maintain the integrity of the original information.

METHODS

Enzymes

The recombinant form of *Taq* DNA polymerase was a gift from David Gelfand (Cetus corporation, Emeryville, CA). All other materials were from previously described sources (10).

DNA polymerase reactions

M13mp2 gapped DNA substrates were constructed as previously described (10), using wild-type M13mp2 (forward assay), M13mp2A89 (opal codon reversion assay) or M13mp2+T70 (frameshift reversion assay) DNA.

Unless otherwise noted, polymerase reactions (100 μl) contained approximately 500 ng (reversion assays) or 600 ng (forward assay) gapped DNA substrate, 20 mM TrisHCl (pH 7.3 at 70°C), 50 mM KCl, 10 mM $MgCl_2$, and 1 mM each of dATP, dGTP, dCTP and dTTP. The reaction mixtures were prewarmed to 70°C for five minutes, followed by the addition of 0.1 pmol of *Taq* DNA polymerase (a 1:1 molar equivalent of enzyme to DNA) in 1/10th volume of enzyme diluent (10 mM TrisHCl, pH 8.0, 50 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 0.5% Tween 20, and 500 μg/ml gelatin). After incubation at 70°C for five minutes, the reactions were stopped by the addition of EDTA to a final concentration of 15 mM. Variations from these conditions are indicated in the legends to

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individual Figures and Tables. Samples of each reaction were analyzed for the extent of gap-filling synthesis by agarose gel electrophoresis as previously described (11). In all cases reported here, polymerization yielded reaction products that migrated coincident with a double-stranded nicked (RFII) DNA standard (data not shown).

M13mp2 fidelity assays

All classes of DNA polymerase errors are measured in the M13mp2 forward mutation assay as a result of a single round of *in vitro* DNA synthesis to fill a 390 base gap located opposite the wild-type M13mp2 *lac* Zα target sequence (11). Mutations are scored as light blue or colorless plaques after transfection of competent *Escherichia coli* strain MC1061 with the synthesis products, as described previously (10). Included in the types of errors detected in this assay are single base substitution errors at 114 template positions (12, and unpublished observations) and single-base frameshifts at 150 template positions (13).

The DNA templates for the reversion assays are made using defined *lac* Zα mutant sequences which are completely defective for α-complementation and result in colorless plaque phenotypes. In these assays, DNA polymerase errors are detected as blue 'revertant' phage plaques. Base substitution errors can be monitored in the A89 opal codon reversion assay (10,14), which detects eight of nine possible single-base substitution errors at this codon. The +T70 frameshift reversion assay measures the loss of a single base, either as a single thymine residue in a sequence of five consecutive thymine bases or as one base at 36 sites elsewhere in the *lac* Zα sequence (15).

In all M13mp2 assays, polymerase errors can be quantitated by the mutant frequency, defined as the proportion of mutant plaques to the total number of plaques scored. To confirm the mutants as true phenotypic changes in plaque color rather than artifacts of plating conditions, the mutant phage are replated in the presence of wild-type M13mp2 phage. After DNA sequence analysis, the polymerase error rate per detectable nucleotide (ER) can be calculated from the mutant frequency (MF) as follows: ER = 1/ {[(observed MF−background MF) / 0.6)] ÷ no. detectable sites} (see above), where 0.6 is the frequency of expression of the viral minus strand in *E. coli* (10). The background frequency for the opal codon reversion assay was determined to be 4×10^{-6}, while that for the +T70 frameshift reversion assay is 1.4×10^{-5}. The background frequencies that were used to calculate error rates for the forward mutation assay are 3×10^{-4} for base substitution mutations and 0.6×10^{-4} for single base frameshifts (16).

RESULTS

Fidelity versus $MgCl_2$ concentration

The effect of Mg^{++} ion concentration on the base substitution and frameshift error rates of *Taq* polymerase was examined by varying the total amount of $MgCl_2$ present in the DNA synthesis reactions. Using 250 μM each of four (1 mM total) deoxynucleotide triphosphates and 1 mM $MgCl_2$, we observed a base substitution reversion frequency of 40×10^{-6} (Figure 1). The mutant frequency increased as the $MgCl_2$ concentration was increased, with an observed value of 340×10^{-6} at 20 mM $MgCl_2$ (Figure 1). A similar response to $MgCl_2$ was observed in the frameshift reversion assay: at equimolar $MgCl_2$ and dNTPs, the reversion frequency was near the spontaneous frequency (1.4×10^{-5}) but increased to $\sim20\times10^{-5}$ at 10 mM $MgCl_2$ (Figure 1). When $MgCl_2$ and dNTPs are present in equimolar concentrations relative to excess $MgCl_2$ a six-fold

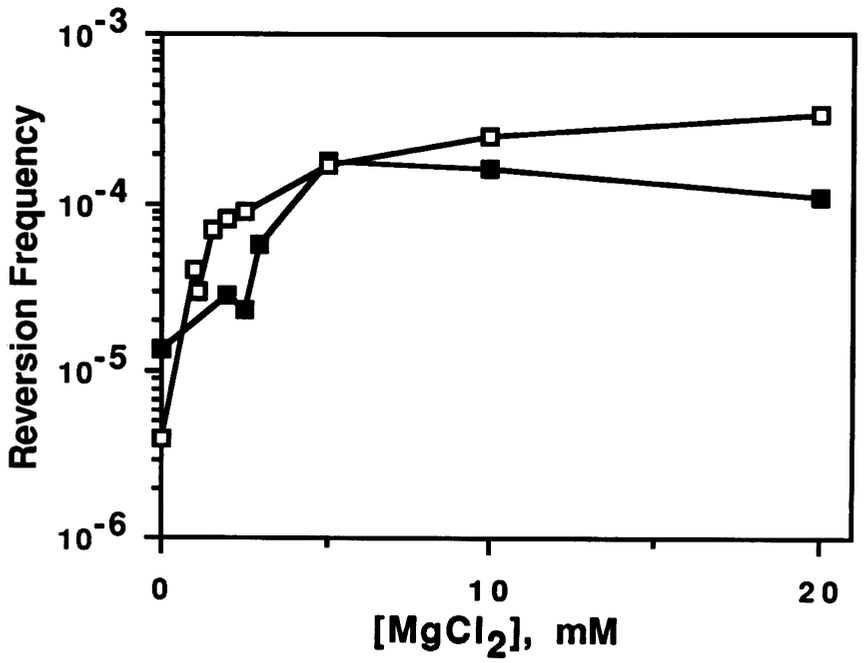

Figure 1. Fidelity of *Taq* polymerase versus magnesium chloride concentration. The mutagenic response of *Taq* polymerase to increasing $MgCl_2$ concentration is shown in the M13mp2 base substitution (□) and frameshift (■) reversion assays. Polymerase reactions were performed as described in Methods, varying the [$MgCl_2$] as indicated. The deoxynucleotide triphosphate concentrations were 250 μM each dNTP (1 mM total) in the base substitution assay and 500 μM each dNTP (2 mM total) in the frameshift assay. Synthesis reactions at equimolar $MgCl_2$ and dNTP concentrations were incubated for 30 min at 70°C. The data points for '0' $MgCl_2$ indicate the background frequencies for the assays, obtained by transfection of *E. coli* with the viral template DNA.

Table 1. *Taq* DNA polymerase error rates under various reaction conditions. DNA synthesis reactions were performed at 70°C as described in Methods and the legends to Figures 1 and 2, varying individual reaction components as indicated by bold face type. The error rate per detectable nucleotide can be calculated from the observed mutant frequency as described in Methods.

	High Fidelity Conditions				Low Fidelity Conditions				
DNA Substrate	$[MgCl_2]$, mM	[dNTP], μM[a]	pH	Error Rate	$[MgCl_2]$, mM	[dNTP], μM[a]	pH	Error Rate	Relative Increase in Fidelity[b]
A. Base Substitutions									
A89 Opal Codon	**1**	250	7.2	1/50,000	**20**	250	7.2	1/5,400	9
	4	1000	7.2	1/300,000	**20**	1000	7.2	1/4,200	71
	10	1000	**5.1**[c]	1/180,000	10	1000	**8.2**[d]	1/3,200	56
Wild-type M13mp2	**1**	**250**	7.2	1/58,000	**10**	**1000**	7.2	1/7,500[e]	8
B. −1 Base Frameshifts									
+T70 Frameshift[f]	**2**	500	7.2	< 1/210,000	**10**	500	7.2	1/21,000	> 10
	4	1000	7.2	< 1/210,000	**10**	1000	7.2	1/24,000	≥ 9
	10	1000	**5.7**[d]	< 1/210,000	10	1000	**8.2**[d]	1/19,000	> 11
Wild-type M13mp2	**1**	**250**	7.2	1/1,800,000	**10**	**1000**	7.2	1/35,000[e]	51

[a] The total deoxynucleotide triphosphate concentration is four times the indicated value.
[b] High fidelity error rate ÷ Low fidelity error rate
[c] MES buffer
[d] Tris buffer
[e] Error rates were calculated from the mutant frequencies reported in reference 1.
[f] Error rates were calculated assuming all mutations occurred within the run of five consecutive thymine bases. However, the phenotypes of all revertants were not confirmed by plaque purification; thus,the reported error rate may over estimate the true value.

increase in the reaction incubation time was required for complete gap-filling synthesis.

For both base substitution and frameshift errors, fidelity was highest at a $MgCl_2$ concentration that was equimolar to the total concentration of dNTP substrates present in the reaction. Additional experiments were performed to determine whether the error rate varied as a function of total $MgCl_2$ or $MgCl_2$ in excess over deoxynucleotide triphosphates. For example, high base substitution fidelity (1/300,000) was also observed for reactions containing 4 mM total dNTPs (1 mM each of dATP, dTTP, dGTP and dCTP) and 4 mM $MgCl_2$ (Table 1). However, a 12-fold higher base substitution error rate (1/24,000) was observed when DNA synthesis reactions contained a two-fold lower $MgCl_2$ concentration (2 mM) but only 1mM total dNTPs (Figure 1). High excess $MgCl_2$ resulted in an error rate of ~1/5,000 at both dNTP concentrations (Table 1). Similar trends were observed for analogous frameshift assays (Table 1). The changes in dNTP concentration alone cannot account for the observed fidelity differences (see below). Therefore, these data strongly suggest that the fidelity of *Taq* polymerase responds not to the absolute $MgCl_2$ concentration, but rather to the amount of $MgCl_2$ in excess over the total deoxynucleotide triphosphates present during DNA synthesis.

Fidelity versus pH

High fidelity DNA synthesis by *Taq* polymerase was also observed at low reaction pH. In the opal codon reversion assay, low mutant frequencies ranging from $14-66\times10^{-6}$ were observed at pH 5−6 (70°C) using Tris and 4-morpholineethane-sulfonic acid (MES) buffers (Figure 2). Similar mutant frequencies were obtained using reactions buffered to pH 5.0 and 5.8 with 1,4-piperazinebis(ethane-sulfonic acid) (data not shown). As noted above for decreasing the concentration of $MgCl_2$, increased reaction times are required for *Taq* polymerase to complete gap-filling synthesis when the reaction pH is less than 5.8. The base substitution mutant frequency increased to 560×10^{-6} as the pH was increased to pH 8.2 (Tris buffer) (Figure 2). Thus, the base substitution error rate of *Taq* polymerase changes approximately 60-fold in response to a three unit pH change (Table 1). The frameshift reversion frequency of *Taq* polymerase also varied with changing reaction pH. Using Tris buffer at 70°C, a mean mutant frequency of 2.7×10^{-5} was observed at pH 5.7, a two-fold increase over the background frameshift frequency (Figure 2). The minus-one-base error rate was ≥11-fold lower at pH 5.7 than at pH 8.2 (Table 1).

Fidelity versus deoxynucleotide triphosphate concentration

In the opal codon reversion assay, the base substitution error rate of the *Taq* polymerase varied only two-fold between 1 mM dNTPs (1/6,000) and 1 μM dNTPs (1/12,000) at 10 mM $MgCl_2$ (pH 7.2). A similar decrease in mutant frequency over this range of dNTP concentrations was observed in the frameshift reversion assay (data not shown).

High fidelity DNA synthesis in the forward mutation assay

The reversion assay measurements indicate that the two most influential parameters affecting the fidelity of *Taq* polymerase are pH and the concentration of $MgCl_2$ relative to the total dNTP concentration. To determine if the effects on fidelity could be generalized to a larger number of template sites and types of errors than are monitored in the reversion assays, we determined the fidelity of *Taq* polymerase in the forward mutation assay as pH and $[MgCl_2]$ were varied independently and in combination. As shown in Table 2A, decreasing the reaction pH from 7.2 to 5.7 (70°C) with a high excess Mg^{++} concentration resulted in a two-fold decrease in the forward mutant frequency, while decreasing the $MgCl_2$ concentration from 10 mM to 1 mM resulted in a four-fold decrease in mutant frequency. At low pH and a 1 mM concentration of $MgCl_2$ and total dNTPs (250 μM each of four), the mutant frequency was only three-fold above

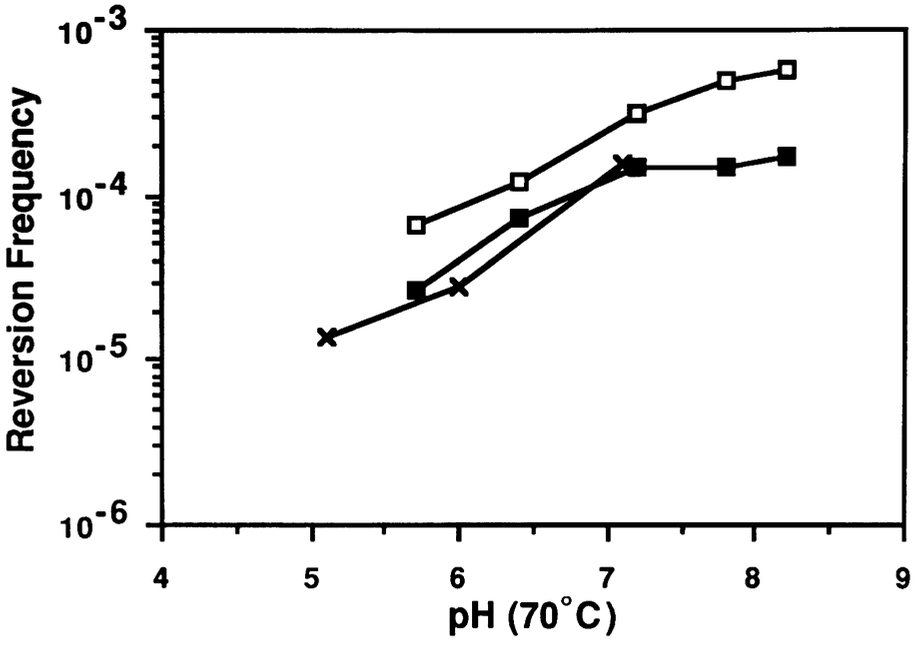

Figure 2. *Taq* polymerase fidelity versus reaction pH. The M13mp2 reversion frequencies of *Taq* polymerase reactions were measured as a function of pH using 20 mM Tris (□) or 20 mM MES (x) buffers in the base substitution assay, and 20 mM Tris buffer (■) in the frameshift assay. DNA synthesis reactions were performed as described in Methods, allowing the buffers to equilibrate to 70°C for 10 min prior to addition of the remaining reagents. Reactions performed at pH < 5.5 were incubated for 30 min to allow complete gap-filling synthesis. The plotted pH values are the average of the measured and calculated pH values at 70°C of mock reactions. (The differences between the measured and calculated pH values ranged from 0.31−0.50 pH units.)

Table 2. The fidelity of *Taq* DNA polymerase in the M13mp2 forward mutation assay. DNA synthesis reactions were performed using the wild-type M13mp2 DNA substrate and 2 units of *Taq* polymerase as described in Methods, except that the reactions in Part A were incubated at 70°C for forty minutes. Other reaction conditions were varied as indicated in the Table.

Reaction Conditions (70°C)				
	$[MgCl_2]$, mM	[dNTP][a], μM	pH	Mutant Frequency[b] $\times 10^4$
	None[c]			6.6 (0.68)
A.	10	250	7.2	104 (17)
	10	250	5.7	50
	1	250	7.2	24 (6.6)
	1	250	5.7	22
B.	10	1000	7.2	75 (1.8)
	2	200	7.2	25 (6.7)

[a] The total deoxynucleotide triphosphate concentration is four times the indicated value.

[b] Values are the mean of two or three (standard deviation) independent experiments. Not all mutants were verified for changes in the *lac* Z target region by DNA sequence analysis; therefore, the reported mutant frequency represents an upper limit to the absolute mutant frequency.

[c] Value represents the background mutant frequency for this assay, obtained by transfection of *E. coli* with the viral template DNA.

the background mutant frequency of the assay (Table 2A). The same high fidelity for *Taq* polymerase was observed when gap-filling synthesis was carried out using 2 mM $MgCl_2$ and 200 μM dNTPs (Table 2B), reaction conditions close to standard PCR conditions (7).

A detailed characterization of the types of mutations which are detected in the *lacZ* forward mutation assay is available (see 'Experimental procedures'). This information together with DNA sequence analysis of independent mutants and control experiments can be used to calculate the average error rate per detectable nucleotide polymerized for the spectrum of base substitution and frameshift errors produced by the polymerase. We have determined the DNA sequence changes of 30 mutants obtained from two high fidelity (1 mM $MgCl_2$, 250 μM each dNTP, pH 7.2) *Taq* polymerase reactions with an average mutant frequency of 16×10^{-4}. In this collection are 27 single base substitution mutations, one minus-one-base frameshift mutation, and two other types of sequence changes. These data demonstrate that under high fidelity conditions the average minus-one base frameshift error rate of *Taq* polymerase is 1/1,800,000, a 50-fold higher frameshift fidelity than previously reported, while the average single base substitution error rate is 1/58,000, an eight-fold higher fidelity than previously described using higher pH and $MgCl_2$ (1, Table 1).

Lack of a detectable proofreading exonuclease activity under high fidelity reaction conditions

We have compared the 3′→5′ proofreading exonuclease activity of *Taq* polymerase at low and high fidelity reaction conditions to the activities of the Klenow fragment of *E. coli* DNA polymerase I and a 3′→5′ exonuclease-deficient derivative of the Klenow fragment (D355A, E357A) (12) in an M13mp2-based 3′-terminal mismatch excision assay (14). No 3′→5′ exonuclease activity of the *Taq* polymerase was detected using low reaction pH or equimolar concentrations of magnesium chloride and deoxynucleotide triphosphates after pre-incubation of 0.37 pmol of enzyme with the DNA substrate for three minutes (data not

shown). Assuming a detection limit of 10% excision for this assay, we can state that the 3′→5′ exonuclease activity of the *Taq* polymerase is at least 10-fold less than that of the Klenow fragment of wild-type *E. coli* DNA polymerase I under all conditions assayed.

DISCUSSION

We have demonstrated that the fidelity of DNA synthesis *in vitro* catalyzed by the *Thermus aquaticus* DNA polymerase responds strongly to changes in relative $MgCl_2$ concentration and pH. A base substitution error rate as low as 10^{-5} and a frameshift error rate of 10^{-6} can be achieved using equimolar concentrations of magnesium chloride and deoxynucleotide triphosphates in *Taq* polymerase reactions (Table 1). High fidelity DNA synthesis by a polymerase which lacks a 3′→5′ proofreading exonuclease was quite unexpected for two reasons. In the forward mutation assay, average fidelity is at least ten-fold lower for the exonuclease-deficient DNA polymerases a and β (12, 13). Furthermore, the accuracy of the *Taq* polymerase using high fidelity reaction conditions approaches that of polymerases capable of proofreading, including *E. coli* DNA polymerase I (5) and DNA polymerase γ (10). We are currently examining the question of whether effects of reaction pH and $MgCl_2$ concentration on fidelity similar to those reported here for *Taq* polymerase will be observed with other DNA polymerases.

At the present time, the mechanism(s) of increased fidelity by low reaction pH and low concentration of $MgCl_2$ relative to deoxynucleotide triphosphates is unknown. Given the precedent for uncovering a cryptic exonuclease activity of DNA polymerase α-primase by subunit dissociation (18), we considered the possibility that altering *in vitro* reaction conditions resulted in the activation of a proofreading exonuclease activity of the *Taq* polymerase. However, we have detected no 3′→5′ exonuclease activity in our *Taq* polymerase preparation under the high fidelity conditions of low reaction pH or equimolar concentrations of $MgCl_2$ and deoxynucleotide triphosphates. Thus, the observed high fidelity DNA synthesis appears to be due solely to polymerase discrimination, at either the initial misinsertion event and/or the extension of a mispaired or misaligned primer-terminus.

Low pH and low free Mg^{++} ion concentration need not affect fidelity by the same mechanism. The mutant frequencies observed in the forward mutation assay are not sufficiently greater than the spontaneous frequency of this assay to determine if pH and relative $MgCl_2$ concentration have an additive or synergistic effect on fidelity (Table 2). However, the data presented do demonstrate that both base substitution and frameshift error rates are substantially affected by changes in reaction conditions (Table 1). Furthermore, as 56% of the base substitution mutations produced by *Taq* polymerase in the forward assay under low fidelity conditions were T→C transition mutations (1), a greater than two-fold decrease in mutant frequency at high fidelity conditions indicates more than this single pathway is being affected. We are examining the specificity of mutations induced at high and low fidelity conditions to determine whether decreases in pH and Mg^{++} ion concentration equally suppress all classes of base substitution mutations.

Several lines of experimental evidence support a positive correlation between the processivity, i.e. the number of nucleotides added per association event, and the fidelity of DNA polymerases (19, 20 and references therein). Interestingly, the *in vitro* reaction conditions that increase the fidelity of *Taq* DNA polymerase are similar to those conditions that have been shown to affect the rate and processivity of other DNA polymerases. The processivities of the eukaryotic DNA polymerases α and δ as well as *E. coli* DNA polymerase I increase as either the $MgCl_2$ concentration and/or the reaction pH are decreased (3, 21, 22).

All DNA polymerases require the presence of a divalent metal cation for activation. A number of divalent metals are mutagenic and carcinogenic, and have been shown to decrease the fidelity of DNA polymerases *in vitro* (23). Mutagenesis by manganese activation of *E. coli* DNA polymerase I is a concentration-dependent phenomenon, with very low concentrations of free Mn^{++} resulting in high fidelity DNA synthesis (24). The enzyme-mediated manganese mutagenesis does not correlate with Mn^{++} occupation of sites on the polymerase, but rather has been suggested to result from Mn^{++} binding to the DNA template or, less likely, to the incoming dNTP substrates (24). Divalent metal cations have also been shown be involved in both template and primer binding by other DNA polymerases, steps likely to be important for polymerase discrimination of the incoming nucleotide triphosphates (4).

Due to its themostability, *Taq* polymerase is the enzyme of choice for PCR. The fidelity of this enzyme under any *in vitro* synthesis condition examined here is adequate to yield amplified DNA of sufficient quality for studies with biochemical endpoints, such as DNA sequencing or blot hybridization, where the rare error does not interfere with the analyses. However, the results presented in this communication suggest that PCR reactions wherein fidelity is of concern should employ low concentrations of dNTPs and low concentrations of $MgCl_2$ in excess over total dNTPs. The use of lower pH reactions may be of benefit for high fidelity synthesis under some circumstances, but the increased fidelity of the polymerase may be offset by the increased rate of DNA damage (depurination and/or deamination), particularly at high temperature (25). An important point to note is that conditions that are optimal for polymerase fidelity are not necessarily those that are optimal for synthetic rate and/or product yield in PCR (3, Figures 1 and 2). Nevertheless, in those instances where the characterization of individual molecules generated during PCR (7) or of rare DNA molecules present in the starting population (8) is required, high fidelity amplification by the *Taq* polymerase will be necessary for maintaining the integrity of the genetic information.

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