Goodhart, P. J. (1986) *Biochemistry 25*, 7271-7278.
Ljones, T., & Skotland, T. (1984) *Copper Proteins and Copper Enzymes* (Lontie, R., Ed.) pp 131-57, CRC, Boca Raton, FL.
Ljones, T., Skotland, T., & Flatmark, T. (1976) *Eur. J. Biochem. 61*, 521-533.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Makinnen, M. W., Kuo, L. C., Yim, M. B., Wells, G. B., Kukuyama, J. M., & Kim, J. E. (1985) *J. Am. Chem. Soc. 107*, 5245-5255.
Mangold, J. B., & Klinman, J. P. (1984) *J. Biol. Chem. 259*, 7772-9.
Markley, J. L. (1975) *Acc. Chem. Res. 8*, 70-80.
Markossian, K. A., Paitian, N. A., Mikaelyan, M. V., Nalbandyan, R. M., (1986) *Biochem. Biophys. Res. Commun. 138*, 1-8.
May, S. W., & Phillips, R. S. (1980) *J. Am. Chem. Soc. 102*, 5981-5983.
May, S. W., Phillips, R. S., Mueller, P. W., & Herman, H. H. (1981) *J. Biol. Chem. 256*, 8470-8475.
May, S. W., Herman, H. H., Roberts, S. F., & Cicarello, M. C. (1987) *Biochemistry 26*, 1626-1633.
Miller, S. M., & Klinman, J. P. (1985) *Biochemistry 24*, 2114-27.
Morpurgo, L., Agostinelli, E., Senepa, M., & Desideri, A. (1985) *J. Inorg. Biochem. 24*, 1-8.
Obata, A., Tanaka, H., & Kawazura, H. (1987) *Biochemistry 26*, 4962-4968.
Padgette, S. R., Wimalasena, K., Herman, H. H., Sirimanne, S. R., May, S. W. (1985) *Biochemistry 24*, 5826-5839.
Peisach, J., & Blumberg, W. E. (1974) *Arch. Biochem. Biophys. 165*, 691-708.
Rucicka, J., & Lamm, C. G. (1971) *Anal. Chim. Acta 53*, 206-8.
Rupp, H., Rao, K. K., Hall, D. A., & Cammack, R. (1978) *Biochim. Biophys. Acta 537*, 255-69.
Sharoyan, S. G., Shaldzhian, A. A., Nalbandyan, R. M., Bunitian, H. Kh. (1977) *Biochim. Biophys. Acta 493*, 478-87.
Sillen, L. G., & Martell, A. E. (1964) In *Stability Constants of Metal Ion Complexes*, Special Publication No. 17, Chemical Society, London.
Sillen, L. G., & Martell, A. E. (1971) in *Stability Constants of Metal Ion Complexes*, Special Publication No. 25, Chemical Society, London.
Skotland, T., & Ljones, T. (1979) *Eur. J. Biochem. 94*, 145-51.
Skotland, T., & Ljones, T. (1980a) *Biochim. Biophys. Acta 630*, 30-35.
Skotland, T., & Ljones, T. (1980b) *Arch. Biochem. Biophys. 20*, 81-7.
Skotland, T., Peterssen, L., Backstrom, D., Ljones, T., Flatmark, T., & Ehrenberg, A. (1980) *Eur. J. Biochem. 105*, 5-11.
Stewart, L. C., & Klinman, J. P. (1987) *Biochemistry 26*, 5302-5309.
Syvertsen, C., Gaustad, R., Schroeder, K., & Ljones, T. (1986) *J. Inorg. Biochem. 26*, 63-76.
Villafranca, J. J. (1981) in *Copper Proteins* (Spiro, T. G., Ed.) pp 263-289, Wiley, New York.
Walker, O. A., Kon, H., & Lovenberg, W. (1977) *Biochim. Biophys. Acta 482*, 309-322.
Wimalesena, K., & May, S. W. (1987) *J. Am. Chem. Soc. 109*, 4036-4046.
Zuberbuhler, A. D. (1981) *Met. Ions Biol. Syst. 12*, 133-189.

Fidelity of DNA Synthesis by the *Thermus aquaticus* DNA Polymerase

Kenneth R. Tindall* and Thomas A. Kunkel

Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

Received March 11, 1988; Revised Manuscript Received April 25, 1988

ABSTRACT: We have determined the fidelity of in vitro DNA synthesis catalyzed at high temperature by the DNA polymerase from the thermophilic bacterium *Thermus aquaticus*. Using a DNA substrate that contains a 3'-OH terminal mismatch, we demonstrate that the purified polymerase lacks detectable exonucleolytic proofreading activity. The fidelity of the *Taq* polymerase was measured by two assays which score errors produced during in vitro DNA synthesis of the *lacZα* complementation gene in M13mp2 DNA. In both assays, the *Taq* polymerase produces single-base substitution errors at a rate of 1 for each 9000 nucleotides polymerized. Frameshift errors are also produced, at a frequency of 1/41 000. These results are discussed in relation to the effects of high temperature on fidelity and the use of the *Taq* DNA polymerase as a reagent for the in vitro amplification of DNA by the polymerase chain reaction.

Studies on the fidelity of DNA synthesis by purified DNA polymerases have provided useful insights into the factors that influence mutation rates. In addition, DNA polymerases are valuable reagents for a variety of molecular techniques which require in vitro DNA synthesis. Depending on the anticipated use of synthesized DNA, the fidelity of polymerization may be a significant factor in the choice of polymerase. The fidelity of DNA synthesis, at least for base substitution errors at single codons, has been described for several DNA polymerases useful in molecular biology, including *Escherichia coli* DNA polymerase I, T4 DNA polymerase, and reverse transcriptase from avian myeloblastosis virus (AMV)[1] [for review, see Loeb

* Author to whom correspondence should be addressed.

and Kunkel (1982) and Loeb and Reyland (1987)].

Our interest in the DNA polymerase isolated from the thermophilic bacterium *Thermus aquaticus* (*Taq*) (Chein et al., 1976; Kaledin et al., 1980) stems from its ability to polymerize DNA at elevated temperatures, thus allowing an examination of high-temperature effects on polymerase fidelity under conditions where there is a high rate of synthesis and a decreased stability of the DNA helix. Furthermore, the thermal stability of the *Taq* polymerase and its ability to polymerize at elevated temperatures are properties suitable for application to the recently developed polymerase chain reaction (PCR) technique which is becoming widely used for the in vitro amplification of low-abundance DNA sequences (Saiki et al., 1985; Saiki et al., 1988). Indeed, the increasing use of PCR for a variety of analyses in molecular biology underscores the need to assess the degree to which mutations are induced by the *Taq* polymerase during the amplification process. The temperature optimum for DNA synthesis with the *Taq* polymerase is 80 °C (Chein et al., 1976); however, PCR amplification is performed with *Taq* at temperatures ranging from 50 to 70 °C (Kogen et al., 1987; Chehab et al., 1987; Saiki et al., 1988). In this study, we have examined the fidelity of the *Taq* polymerase during catalysis at both 55 and 70 °C.

Materials and Methods

Materials. *Taq* DNA polymerase (lots 2A and 3) was purchased from New England Biolabs. *E. coli* DNA polymerase I, large (Klenow) fragment (Kf), was purchased from Boehringer Mannheim Biochemicals. The AMV DNA polymerase was obtained from Pharmacia. The source of all other materials has been described (Kunkel, 1984, 1985a; Kunkel et al., 1987).

Methods. Preparation of M13mp2 DNA substrates, product analysis, transfection of competent cells, plating and scoring of α-complementation mutants, and DNA sequence analysis were performed as described (Kunkel, 1984, 1985a; Kunkel et al., 1987). DNA polymerase reactions are described in the legends to the tables.

3′→ 5′ Exonuclease Assay. The assay (Kunkel et al., 1987) measures the 3′ → 5′ exonucleolytic removal of a mismatched base from a primer terminus. Briefly, DNAs from two mutant derivatives of bacteriophage M13mp2, containing a single-base difference at position 103 in the *lacZ*α region, are used to construct a double-stranded heteroduplex with a 363-nucleotide gap of single-strand DNA. This molecule contains a 3′-terminal cytosine residue in the primer (minus) strand opposite an adenine residue in the template (plus) strand. The minus-strand cytosine encodes a medium blue plaque phenotype whereas expression of the plus-strand adenine yields faint blue plaques. Polymerization to fill the gap without excision of the cytosine will produce a complete heteroduplex that, upon transfection, will yield 50% medium blue and 50% faint blue plaques. However, if the mispaired cytosine is excised prior to gap-filling synthesis by a polymerase, subsequent correct incorporation of thymidine opposite the template adenine will yield a homoduplex molecule that will yield exclusively faint blue plaques. Medium blue and light blue plaques have been isolated and sequenced to confirm the mutant phenotype (data not shown). Thus, for any given reaction condition, the proportion of medium blue and faint blue plaques observed upon transfection of the reaction products and plating to score α-complementation is a measure of terminal mismatch excision activity.

Reversion Assay for Base Substitution Fidelity. Single-base substitution errors occurring during in vitro DNA synthesis were measured by the previously described (Kunkel et al., 1987) opal codon reversion assay. Briefly, an M13mp2 DNA molecule is constructed having a 361-nucleotide gap containing a single-base change [G → A in the viral (plus) template strand at position 89 of the *lacZ*α coding sequence]. This change creates an opal (TGA) codon, resulting in a colorless plaque phenotype under the appropriate plating conditions. The gap is filled by a single cycle of in vitro DNA synthesis using the desired DNA polymerase and appropriate reaction conditions. A portion of the product is then analyzed to confirm complete synthesis (Kunkel, 1985a), and the remainder is used to transfect competent α-complementation host cells to ascertain the colors of the resulting M13mp2 plaques (Kunkel, 1984). Base substitution errors at the opal codon during gap-filling DNA synthesis are detected as blue plaques. The reversion frequency (the proportion of blue to total plaques) reflects the error rate for the single round of gap-filling DNA synthesis. Eight of nine possible base substitution errors at the TGA opal codon yield a detectable blue plaque phenotype.

Forward Mutational Assay. A large variety of errors produced during in vitro DNA synthesis can be quantitated and subsequently recovered for DNA sequence analysis with the M13mp2 forward mutational assay (Kunkel, 1985a). As described above, a gapped DNA substrate is used (in this case the single-strand gap is 390 nucleotides), but the target for mutations is the 250-nucleotide wild-type M13mp2 DNA sequence, and the assay scores the loss of α-complementation of β-galactosidase activity; i.e., mutants are identified as light blue or colorless. Since α-complementation activity is not essential for M13mp2 plaque production, over 200 different base substitution errors at over 100 different sites can be scored within the 250-nucleotide *lacZ*α sequence (Kunkel & Alexander, 1986). In addition, frameshift mutations (Kunkel, 1986), deletions (Kunkel, 1984, 1985b), and more complex errors (Kunkel et al., 1986) can be scored. The mutant frequency, i.e., the number of light blue and white plaques relative to the total number of plaques scored, reflects the error rate, which can be precisely calculated as described (Kunkel & Alexander, 1986; Kunkel, 1986). Following confirmation of the mutant phenotype, the precise nature of the mutation can be determined by DNA sequence analysis (Kunkel, 1984). Note that in both the reversion and in the forward mutational assays the Pol I (Kf) reactions were performed in the presence of 1000 μM dNTPs which substantially reduce the associated 3′ → 5′ exonuclease activity.

Results

Lack of 3′→ 5′ Exonuclease Activity. *Taq* DNA polymerase was examined for the presence of a proofreading exonuclease activity with a highly sensitive assay for excision of a single mispaired base from a 3′-OH primer terminus. An M13mp2 heteroduplex substrate was used which contained 6832 nucleotides of double-strand DNA and a single-base mismatch [A(template)·C(primer)] on the 3′-OH end of a 363-nucleotide single-strand gap. This molecule is sufficiently stable to provide an acceptable substrate for gap-filling DNA synthesis at the reaction temperature of 70 °C used for the *Taq* polymerase studies. Synthesis reactions were performed with the *Taq* polymerase, with AMV reverse transcriptase, which lacks an associated 3′ → 5′ exonuclease activity (Battula

[1] Abbreviations: *Taq*, *Thermus aquaticus*; PCR, polymerase chain reaction; Pol I (Kf), large fragment *E. coli* DNA polymerase I; AMV, avian myeloblastosis virus; Pol, DNA polymerase.

Table I: Absence of 3′ → 5′ Exonuclease Activity Associated with the *T. aquaticus* DNA Polymerase[a]

DNA polymerase	reaction temperature (°C)	plaques scored		% expression of mismatched base	% terminal mismatch excised
		total	medium blue		
AMV Pol	37	897	458	51	0
Pol I (Kf)	37	952	59	6.2	88
Taq Pol	70	824	465	56	0
Taq Pol +5-min preincubation	70	954	494	52	0

[a] *Taq* DNA polymerase reactions (30 μL) contained 20 mM Tris (pH 8.5), 2 mM dithiothreitol, 10 mM $MgCl_2$, 50 mM KCl, 100 μg/mL gelatin, 10 μM each of dATP, dTTP, dGTP, and dCTP, 300 ng of gapped M13mp2 DNA containing the A·C terminal mispair (Kunkel et al., 1987), and 0.6 unit (lots 2A and 3 as defined by New England Biolabs) of *Taq* polymerase. Reactions were performed in 1.5-mL polycarbonate microcentrifuge tubes. The reactions were placed in a 70 °C H_2O bath for 3 min prior to the addition of enzyme. Enzyme (1.2 μL) was added, and the reaction mixture was quickly vortexed and placed at 70 °C for an additional 5 min. Lot 2A was assayed in a preincubation reaction in which all components except the enzyme and dNTPs were added and prewarmed. As previously described, the enzyme was then added, and the reaction mixture was incubated at 70 °C for an additional 5 min; then, dNTPs were added, and the 70 °C incubation was continued for an additional 5 min. The AMV polymerase and the Pol I (Kf) reactions were performed in a similar manner with 10 μM dNTPs [exactly as described in Kunkel et al. (1987)] but at 37 °C. All reactions were terminated by the addition of EDTA to a final concentration of 15 mM. Agarose gel electrophoresis analysis of 20 μl of these reactions (as well as those reactions in Tables II and III) was performed as described in Kunkel (1985a) (data not shown). In all cases the gap was filled to the extent that the DNA migrated coincidentally with completely double-stranded (RF II) M13mp2 DNA [see Figure 2 in Kunkel (1985a)]. Transfection of competent cells with 1 μL of the remaining reaction volume followed by plating on the appropriate host to score M13mp2 mutant plaques was performed as described (Kunkel, 1984, 1985a). The background frequency of medium blue mutants was determined to be 0.37%, which represents a lower limit of the assay for 100% excision prior to polymerization. To calculate percent excision, we have used this background as well as a value of 50% minus-strand expression for no excision (the approximate value obtained with AMV DNA polymerase which lacks exonuclease activity). Thus, Pol I (Kf) excises $[1 - \{(6.2 - 0.37)/(50 - 0.37)\}] \times 100 = 88\%$ of the cytosine prior to filling the gap.

Table II: Fidelity of the *T. aquaticus* DNA Polymerase in a Base Substitution Reversion Assay[a]

DNA polymerase	reaction temperature (°C)	plaques scored		reversion frequency ($\times 10^{-6}$)
		total ($\times 10^6$)	blue	
none		1.8	2	1.1
Taq Pol				
lot 2A	55	1.2	177	150
lot 3	55	0.74	103	140
lot 2A	70	1.3	344	260
lot 3	70	0.59	184	310
Pol I (Kf)	37	0.50	20	40

[a] *Taq* reactions (100 μl) were performed at the indicated temperatures as described in the legend to Table I, but with 1000 μM dNTPs and gapped DNA (600 ng) containing the TGA opal codon. Pol I (Kf) reactions were performed at 37 °C as described (Kunkel et al., 1987) with 1000 μM dNTPs. Product analyses, transfections, and plating were performed as described (Kunkel et al., 1987). The *Taq* lot numbers are those of New England Biolabs.

Table III: Fidelity of the *T. aquaticus* DNA Polymerase in the Forward Mutational Assay[a]

DNA polymerase	reaction temperature (°C)	plaques scored		mutant frequency ($\times 10^{-4}$)[b]
		total	mutant	
none		32922	22	6.7
Taq Pol				
lot 2A	55	5698	68	120
lot 3	55	5558	45	81
lot 2A	70	8510	108	130
lot 3	70	7492	89	120
Pol I (Kf)	37	13473	59	44

[a] Polymerase reactions (50 μL) were performed as described in the legend to Table II, but with 300 ng of gapped wild-type M13mp2 DNA. In this experiment, the plaque counts shown were derived from transfection of 1.5 μL of the resulting reaction products. The negative control (no polymerase) value was determined as described (Kunkel & Alexander, 1986). [b] Not all mutants were verified by plaque purification and DNA sequence analysis; therefore, the reported mutant frequency represents an upper limit to the absolute mutant frequency.

& Loeb, 1976), and with the large (Klenow) fragment of *E. coli* DNA polymerase I, which contains a well-characterized proofreading exonuclease (Kornberg, 1980). All reactions generated products that migrated coincident with a completely double-strand RF II DNA standard [data not shown; see Kunkel (1985a) for a similar analysis]. A second aliquot of each reaction was used to transfect competent cells which were then plated to score the colors of the resulting M13 plaques, thus measuring the extent of excision of the mispaired cytosine prior to polymerization. The results are presented in Table I. The 51% minus-strand expression value for the AMV polymerase reaction reflects the expected result for no excision before polymerization. As expected, the proofreading exonuclease activity associated with the Klenow fragment excised most (88%) of the mismatched bases before polymerase activity filled the gap. The *Taq* polymerase reaction yields a value similar to that of AMV polymerase, demonstrating the absence of a proofreading exonuclease activity. This was true even when the *Taq* polymerase was preincubated with the mismatched DNA for 5 min at 70 °C prior to the addition of deoxynucleotide triphosphates (dNTPs) to permit gap-filling synthesis. These results are consistent with earlier reports on the absence of nuclease activity capable of rendering radiolabeled *E. coli* DNA acid soluble (Chein et al., 1976; Kaledin et al., 1980) and demonstrate that, under these conditions, the *Taq* polymerase lacks an associated 3′ → 5′ exonuclease activity capable of excising a 3′-terminal mismatched base.

Fidelity of Taq DNA Polymerase. The base substitution fidelity of the *Taq* polymerase was first determined by the opal codon reversion assay (Table II). *Taq* polymerase produces base substitution errors that revert the opal codon at a frequency more than 100-fold above the background reversion frequency of uncopied DNA. The reversion frequency is about 2-fold higher for reactions performed at 70 °C rather than at 55 °C. For comparison, synthesis by the Klenow fragment at 37 °C is 4–8-fold more accurate, despite the fact that the reaction conditions used (1000 μM dNTPs) substantially diminished the contribution of proofreading to fidelity (Kunkel et al., 1987).

The forward mutational assay allows the detection of a variety of errors (e.g., transitions, transversions, frameshift, and deletion mutations) occurring during in vitro DNA synthesis. The fidelity of synthesis by the *Taq* polymerase was assayed at both 55 and 70 °C, the temperature range useful for PCR. Errors produced during a single round of gap-filling DNA synthesis gave rise to mutant frequencies ranging from 81×10^{-4} to 130×10^{-4} (Table III), which is 12–19-fold higher than the mutant frequency of the uncopied control DNA. As in the reversion assay, even under conditions of diminished proofreading, the Klenow fragment is more accurate than the *Taq* polymerase.

Table IV: *Taq* DNA Polymerase Base Substitution Specificity[a]

template mutation	mismatch formed (template: dNMP)[b]	observed number of occurrences: mutants	observed number of occurrences: sites[c]
T → C	T:G	18	6
T → G	T:C	0	
T → A	T:T	3	3
C → T	C:A	2	2
C → G	C:C	1	1
C → A	C:T	0	
G → A	G:T	2	2
G → T	G:A	2	1
G → C	G:G	2	1
A → G	A:C	1	1
A → T	A:A	0	
A → C	A:G	1	1

[a] Plaque-purified mutants were confirmed by replating on an indicator plate and were then analyzed by DNA sequence analysis as previously described (Kunkel, 1984, 1985a). [b] Because of the single-stranded nature of the target region, the mismatch formed can be inferred from the mutant DNA sequence analysis. [c] Sites reported indicate the number of different sites in which a specific mutation was observed.

Mutant Sequence Specificity. In order to determine the nature and position of mutations produced by the *Taq* polymerase, a small collection of independent and randomly chosen mutants was characterized by DNA sequence analysis. Of the 42 mutants with sequence changes within the single-stranded DNA target, 32 contained single-base substitutions (Table IV). Transitions were predominant (23/32) with T → C transitions most common. The remaining base substitutions were observed at roughly equivalent frequencies.

From these data, the average base substitution fidelity or error frequency (EF) per nucleotide incorporated by the *Taq* DNA polymerase can be calculated according to

$$EF = [mf(MF_o - MF_b)/f_0]/N_d$$

where mf = the mutant fraction as determined by DNA sequence analysis, MF_o = the observed mutation frequency following replication, MF_b = the background mutation frequency, f_0 = the frequency of expression of the newly synthesized strand, which has been measured to be 0.6 in this system (Kunkel & Soni, 1988), and N_d = the number of nucleotides within the 250-nucleotide target region known to yield a mutant phenotype with a specific mutational change. For base substitutions, N_d = 110, and for frameshift mutations, N_d = 160.

Thus, for example, the calculation of the error frequency for base substitutions for lot 3 of the *Taq* polymerase at 55 °C (Table III) is $EF = [(32/42)(81 \times 10^{-4} - 6.7 \times 10^{-4})/0.6]/110 = 8.6 \times 10^{-5}$ or 1 error per 11 700 nucleotides [i.e., $1/(8.6 \times 10^{-5})$]. The data in Table III indicate that the base substitution mutation rate per detectable nucleotide polymerized ranged from 1/7000 (at a mutation frequency of 130×10^{-4}) to 1/11 700 nucleotides (at a mutation frequency of 81×10^{-4}), the average being 1/9000.

The remaining 10 mutations represented the loss (9 mutants) or gain (1 mutant) of a single base. Thus, the frameshift error frequency per detectable nucleotide polymerized can be calculated as above to range from 1/31 000 to 1/51 000, the average being 1/41 000.

Discussion

Using M13mp2-based fidelity systems, we have established the absence of a conventional 3′ → 5′ proofreading exonuclease associated with the *Taq* polymerase and determined its fidelity at high temperature for both base substitution errors and frameshift errors. The inability to proofread errors may partly explain why the *Taq* polymerase is less accurate than the Klenow polymerase in both fidelity assays. Whether a proofreading exonuclease that could provide a proofreading function in vivo has been dissociated from the *Taq* polymerase during purification remains to be determined. In the commercially available preparations of the *Taq* polymerase assayed here, however, there is no detectable proofreading activity.

It is interesting to compare the fidelity of the *Taq* polymerase at elevated temps. with the fidelity of polymerization at 37 °C of other exonuclease-deficient DNA polymerases. The *Taq* polymerase is more accurate than DNA polymerase β purified from several different eukaryotic sources (Kunkel, 1985a) and is most similar with regard to fidelity to purified preparations of eukaryotic replicative DNA polymerase α (Kunkel, 1985b).

The slight differences in mutation frequency observed in the reversion assay (Table II) at reaction temperatures of 70 °C versus 55 °C or compared to the Klenow fragment in both the reversion and the forward (Table III) assays may provide insight regarding DNA synthesis by the *Taq* polymerase. The combined effects of the temperature-dependent increase in the rate of DNA synthesis, the decrease in the stability of the DNA duplex at higher temperatures, and the lack of an exonuclease–proofreading mechanism associated with the *Taq* polymerase should contribute to a significantly higher mutation frequency at elevated temperatures. Yet, the observed mutation frequency is increased only 2-fold at a temperature difference of 15 °C (Table II). In the forward mutation assay (Table III), there are no detectable differences on the resulting mutant frequency with a temperature difference of 15 °C. Thus, the observed 2-fold difference in revertant frequency, yet undetectable difference in the forward mutant frequency for reactions performed at 55 °C versus 70 °C, could represent a real temperature effect on specific mispairs at the opal codon that is not readily apparent in the forward assay since the forward assay monitors a wide spectrum of possible errors at a number of sites. Alternatively, the 2-fold effect in the reversion assay could simply reflect experimental variation that is more obvious in the more sensitive (i.e., lower background) reversion assay.

We can also compare the fidelity of the *Taq* polymerase at elevated reaction temperatures to the mutation frequency of the Klenow fragment under conditions (1000 μM dNTPs) that suppress the 5′ → 3′ exonucleolytic proofreading function. At a reaction temperature difference of 33 °C (37 °C for Klenow versus 70 °C for *Taq*), we observe only a 6–8-fold (Table II) or a 3–4-fold (Table III) difference in mutation frequency for these two DNA polymerases.

Thus, the data in Tables II and III reveal modest increases in mutation frequency with changes in reaction temperature, and these data imply that the rate of DNA synthesis as well as duplex stability has little effect on the nucleotide discrimination mechanisms of the *Taq* polymerase. It is notable that, at 70 °C, a 9000-fold selection against base substitution errors and a 41 000-fold selection against frameshift errors can be obtained. In addition, no large deletions or complex mutations were observed in the collection of mutants sequenced. These data may reflect the nature of the physical interaction of the polymerase with the DNA template. Future studies using the *Taq* polymerase should prove useful in evaluating the effects of reaction temperature on the fidelity of DNA synthesis as well as determining the specific protein–DNA interactions that may contribute to correct nucleotide discrimination.

Of equal interest is the error specificity of the *Taq* polymerase. While detailed specificity considerations are beyond the intent of this paper, the few mutants sequenced already reveal interesting results. For example, among the base pair substitutions, transitions predominate (23/32), and among the observed transitions, there is a strong bias for T → C transitions (18/23). Since we synthesize only one strand of DNA in this system, we can say with certainty that the T → C transitions obsereved are the result of the misincorporation of G opposite a template T. Moreover, the T → C transitions were observed at 6 sites, although there are 17 detectable sites for T → C transitions within the 250-nucleotide target. The predominance of T → C transitions is unique among polymerases studied thus far in the M13mp2 system and suggests specific mispair and site preferences for the *Taq* polymerase. Mutational specificity and site preferences have been examined for several other polymerases in the M13mp2 fidelity assay (Kunkel & Alexander, 1986). Such data support the notion that unique mutational biases and/or hot spots may reflect specific protein–substrate interactions for a given polymerase.

Approximately 20% (10/42) of the sequenced mutants were frameshift mutations. All of the frameshift mutations arose in runs of at least two bases. On the basis of observations with other polymerases in this system, it is likely that at least some of these frameshift mutations occurred via a slippage mechanism (Kunkel, 1986).

It is interesting that AT → GC transitions are the predominant mutational change observed in cloned HLA-DPβ sequences following 30 round of PCR with the *Taq* polymerase (Saiki et al., 1988). These data are consistent with the predominant bias for T → C transitions reported here for the *Taq* polymerase (Table IV). In addition, a mutation rate of 2×10^{-4} per nucleotide per generation is reported following 30 round of PCR and is derived from error rates observed in the cloned HLA-DPβ sequences (Saiki et al., 1988). This reported mutation rate is consistent with the rate we report for the *Taq*-directed synthesis using the M13mp2 assay. Notably, the mutation rate calculation derived with the *lacZ*α gene in the M13mp2 assay is calculated to average 1/9000 (1.1×10^{-4}) base pair substitutions per nucleotide synthesized from data derived in a single round of DNA synthesis and corrected for error rates per detectable nucleotide synthesized. It is remarkable that a precisely determined mutation rate for the *Taq* polymerase and a mutation rate determined following 30 round of PCR are within approximately 2-fold.

The fidelity results also have implications for the use of the *Taq* polymerase in PCR. PCR provides a method by which specific DNA sequences can be amplified from genomic sequences in vitro with appropriately positioned primers. Often the Klenow fragment of *E. coli* DNA polymerase I has been used. However, PCR requires multiple rounds of heat denaturation, rehybridization, and DNA synthesis. The heat stability of the *Taq* polymerase simplifies the procedure and makes it a particularly attractive polymerase for use in PCR amplification (Saiki et al., 1988). The data in Tables II and III suggest that a single round of synthesis at 70 °C with the *Taq* polymerase will yield one base substitution error per 9000 nucleotides and one frameshift error per 41 000 nucleotides polymerized. In addition, the manipulations requiring multiple rounds of heat denaturation and extended time periods at 55–70 °C are likely to increase the mutations occurring during PCR. Heat treatment of DNA is mutagenic [Drake and Baltz (1976) and references cited therein], generating both transitions (Baltz et al., 1976) and transversions (Bingham et al., 1976). These factors may significantly affect the mutation frequency in a population of amplified sequences depending upon experimental conditions. Because the target sequence is amplified exponentially during PCR, individual sequences that contain errors may become a significant portion of the reaction product, depending upon how early in the amplification process the error occurs. Thus, both polymerase-dependent and damage-induced mutations will occur during the multiple rounds of DNA synthesis required for PCR amplification. In spite of this potential limitation, experimental conditions can be designed (Saiki et al., 1988) to limit generation of mutations occurring during PCR resulting in a mutation rate that is only approximately 2-fold greater than that observed in a single round of DNA synthesis. These and our data suggest that most mutations generated during PCR are the result of replication errors during DNA synthesis by the *Taq* polymerase. However, reaction conditions may vary, and heat damage remains a potentially serious source of induced mutations. Thus, one should consider reaction conditions that limit incubation times at high temperatures in order to minimize the contribution of heat-induced DNA damage during PCR amplification.

In any case, one can often analyze the amplified sequences directly, minimizing or eliminating the effects of mutations arising during PCR amplification. Direct hybridization analyses of PCR-generated sequences have been used to detect specific point mutational changes in genomic sequences (Saiki et al., 1985; Kogen et al., 1987), and direct sequence analysis of PCR-amplified DNA generates a consensus sequence independent of errors that occur during amplification, therefore allowing detection of genomic mutations (McMahon et al., 1987; Engelke et al., 1988; K. R. Tindall, unpublished observations). However, the cloning of any single amplified sequence may yield an unacceptable alteration(s) within the isolated clone. Thus, the significance of errors that occur during PCR amplification will depend entirely upon the experimental requirement for nonmutant sequences within the amplified target region.

ACKNOWLEDGMENTS

We greatfully acknowledge the expert technical assistance of Aruna Soni. We also thank Drs. John W. Drake and John D. Roberts for their critical evaluation of the manuscript.

REFERENCES

Baltz, R. H., Bingham, P. M., & Drake, J. W. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1269–1273.

Battula, N., & Loeb, L. A. (1976) *J. Biol. Chem. 251*, 982–986.

Bingham, P. M., Baltz, R. H., Ripley, L. S., & Drake, J. W. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4159–4163.

Chehab, F. F., Doherty, M., Cai, S., Kan, Y. W., Cooper, S., & Rubin, E. M. (1987) *Nature (London) 329*, 293–294.

Chein, A., Edgar, D. B., & Trela, J. M. (1976) *J. Bacteriol. 127*, 1550–1557.

Drake, J. W., & Baltz, R. H. (1976) *Annu. Rev. Biochem. 45*, 11–37.

Engelke, D. R., Hoener, P. A., & Collins, F. S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 544–548.

Kaledin, A. S., Slyusarenko, A. G., & Gorodetskii, S. I. (1980) *Biokhimiya (Moscow) 45*, 644–651.

Kogen, S., Doherty, M., & Gitschier, J. (1987) *N. Engl. J. Med. 317*, 985–990.

Kornberg, A. (1980) *DNA Replication*, Freeman, San Francisco.

Kunkel, T. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1494–1498.

Kunkel, T. A. (1985a) *J. Biol. Chem. 260*, 5787–5796.
Kunkel, T. A. (1985b) *J. Biol. Chem. 260*, 12866–12874.
Kunkel, T. A. (1986) *J. Biol. Chem. 261*, 13581–13587.
Kunkel, T. A. & Alexander, P. S. (1986) *J. Biol. Chem. 261*, 160–166.
Kunkel, T. A., & Soni, A. (1988) *J. Biol. Chem. 263*, 4450–4459.
Kunkel, T. A., Gopinathan, K. P., Dube, D. K., Snow, E. T., & Loeb, L. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1867–1871.
Kunkel, T. A., Sabatino, R. D., & Bambara, R. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4865–4869.
Loeb, L. A., & Kunkel, T. A. (1982) *Annu. Rev. Biochem. 51*, 429–457.
Loeb, L. A., & Reyland, M. E. (1987) *Nucleic Acids Mol. Biol. 1*, 157–173.
McMahon, G., Davis, E., & Wogan, G. N. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4974–4978.
Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., & Arnheim, N. (1985) *Science (Washington, D.C.) 230*, 1350–1354.
Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., & Erlich, H. A. (1988) *Science (Washington, D.C.) 239*, 487–491.

Deoxyribose Ring Conformation of $[d(GGTATACC)]_2$: An Analysis of Vicinal Proton–Proton Coupling Constants from Two-Dimensional Proton Nuclear Magnetic Resonance†

Ning Zhou,‡,§ Sadasivam Manogaran,‡,∥ Gerald Zon,⊥ and Thomas L. James*,‡

Departments of Pharmaceutical Chemistry and Radiology, University of California, San Francisco, California 94143, and Applied Biosystems, Foster City, California 94404

Received December 14, 1987; Revised Manuscript Received April 4, 1988

ABSTRACT: Exchangeable and nonexchangeable protons of $[d(GGTATACC)]_2$ in aqueous cacodylate solution were assigned from two-dimensional nuclear Overhausser effect (2D NOE) spectra. With phase-sensitive COSY and double quantum filtered COSY (DQF-COSY) experiments, the cross-peaks resulting from deoxyribose ring conformation sensitive proton–proton vicinal couplings, i.e., all 1′–2′, 1′–2″, 2′–3′, and 3′–4′ couplings and six from 2″–3′ couplings, were observed. From the cross-peak fine structure, the 2′,2″ proton assignments can be confirmed; coupling constants $J_{1'2'}$ and $J_{1'2''}$ and sums of coupling constants involving H2′ and H2″ for all residues and H3′ for C8 were obtained. The DISCO procedure [Kessler, H., Muller, A., & Oschkinat, H. (1985) *Magn. Reson. Chem. 23*, 844–852] was used to extract individual 1′–2′ and 1′–2″ coupling constants. The sum of coupling constants involving H1′ or H3′ was measured from the one-dimensional spectrum where signal overlap is not a problem. Analysis of the resulting coupling constants and sums of coupling constants, in the manner of Rinkel and Altona [Rinkel, L. J., & Altona, C. (1987) *J. Biomol. Struct. Dyn. 4*, 621–649], led to the following conclusion: C2′-endo deoxyribose ring conformation is predominant for every residue, but a significant amount of C3′-endo conformation may exist, ranging from 14% to 30%.

X-ray diffraction and NMR studies provide evidence (Saenger, 1984; Altona, 1982) that deoxyribose sugar rings in DNA molecules occur as two distinct types of conformers, commonly denoted as N-type [with the five membered ring pseudorotation phase angle (Altona & Sundaralingam, 1972) $P = 0 \pm 90°$, including C3′-endo, C2′-exo, and C4′-exo] and S-type (with $P = 180 \pm 90°$, including C2′-endo, C3′-exo, and C1′-exo). In B-DNA, sugar rings are generally in S-type conformation; in A-DNA, they are in N-type; and in Z-DNA, N and S conformers alternate. However, significant deviations from the above have been found in single-crystal X-ray diffraction studies.

The two types of sugar ring manifest quite different proton–proton coupling constants. From sugar exocyclic proton (H1′, H2′, H2″, H3′, and H4′) vicinal coupling constants, one can deduce pseudorotational parameters describing sugar ring conformation and relative populations of these conformers (de Leeuw & Altona, 1982, 1983). Various techniques have been developed to obtain these coupling constants from NMR experiments and to analyze them in terms of deoxyribose conformation. The conventional one-dimensional (1D) double-resonance method is not very useful when signal overlap is significant. Two-dimensional (2D) *J*-resolved and *J*-correlated spectra (COSY) and their 1D versions (Bermel et al., 1986; Kessler et al., 1987) provide more powerful ways to resolve proton multiplet structure. A pulse sequence has been suggested to obtain simplified cross-peak fine structure in a 2D correlated spectrum (exclusive COSY; Griesinger et al., 1985) to facilitate coupling constant determination. Kessler et al.

† This work was supported by the National Science Foundation (Grant PCM 84-04198), the National Institutes of Health (Grants CA 27343 and RR01695), donors of the Petroleum Research Fund, administered by the American Chemical Society, and instrumentation grants from the National Institutes of Health (RR 016688) and the National Science Foundation (DMB 84-06826).

* Address correspondence to this author at the School of Pharmacy, UCSF, San Francisco, CA 94143.
‡ University of California.
§ Present address: Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada T2N 1N4.
∥ Present address: Department of Chemistry, Indian Institute of Technology, Kampur, India 208 016.
⊥ Applied Biosystems.

0006-2960/88/0427-6013$01.50/0