Thermus aquaticus DNA Polymerase I Mutants with Altered Fidelity

INTERACTING MUTATIONS IN THE O-HELIX*

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Phe[667] in the conserved O-helix of *Thermus aquaticus (Taq)* DNA polymerase I (pol I) is known to be important for discrimination against dideoxy-NTPs. We show here that Phe[667] is also important for base selection fidelity. In a forward mutation assay at high polymerase concentration, wild type pol I catalyzed frequent A → T and G → T transversions and −1 frameshifts at nonreiterated sites involving loss of a purine immediately downstream of a pyrimidine. The mutants F667L and A661E,I665T, F667L exhibited large decreases in A → T and G → T transversions, and the triple mutant displayed reduction in the aforementioned −1 frameshifts as well. Kinetic analysis showed that the F667L and A661E,I665T, F667L polymerases discriminated against synthesis of A:A mispairs more effectively and catalyzed less extension of A:A mispairs than the wild type enzyme. These data indicate that Phe[667] functions in maintaining the error frequency and spectrum, and the catalytic efficiency, of wild type pol I. We also found that the strong general mutator activity conferred by the single A661E substitution was entirely suppressed in the A661E, I665T,F667L polymerase, exemplifying how interactions among O-helix residues can contribute to fidelity. We discuss the mutator and anti-mutator mutations in light of recently obtained three-dimensional structures of *T. aquaticus* pol I.

If DNA polymerases simply polymerized the nucleotides that align and form a stable complex with the template in solution, one mispaired nucleotide would be predicted for every ∼10–100 nucleotides incorporated (1). Processes that occur at the polymerase active site reduce this error frequency to 10^{-4}–10^{-5} (2). These processes assure both discrimination between correct and incorrect base pairs in insertion of the incoming nucleotide and discrimination between matched and mismatched base pairs in extension of the newly formed primer terminus. It has long been a goal to understand the structure-function relationships that govern this discrimination (3).

Amino acid sequence alignments (4) and x-ray crystallographic studies indicate that DNA polymerase I (pol I)[1] family members share highly homologous polymerase domains. Three-dimensional structures are now available for the prototypical large fragment of *Escherichia coli* pol I (5), for *Thermus aquaticus (Taq)* pol I (38% sequence identity with the *E. coli* enzyme) (6–9), for *Bacillus stearothermophilis* pol I (49% identical to the *E. coli* enzyme) (10), and for the more distantly related T7 DNA polymerase (11). In these homologous structures, the polymerase active site is similarly located within a deep, DNA-binding cleft whose architecture has been likened to a right hand. The evolutionarily conserved motif A with its catalytically essential asparatic acid, along with other structural elements of the active site, lies in the palm subdomain that forms the floor of the cleft. The conserved motif B comprises most of the long α-helix O in the fingers subdomain that defines part of one wall of the cleft.

A variety of experimental approaches have established that the O-helix is important in binding the incoming dNTP as well as the template-primer. A pioneering model developed for the *E. coli* pol I Klenow fragment, based on structural, biochemical, and mutagenesis studies, includes roles for four strictly conserved, catalytically crucial amino acids located on the face of the O-helix that lines the polymerase cleft. In this model, Arg[754] interacts with either the β or γ phosphate of the dNTP, and Lys[758] may interact with the α phosphate (12). The aromatic side chain of Phe[762] positions the nucleotide to allow discrimination against substrates containing 2′,3′-dideoxyribose (13, 14). Tyr[766] at the C terminus of the helix plays a lesser role in positioning the nucleotide and also interacts with the template-primer (12). Recent crystallographic studies of catalytically competent complexes of T7 DNA polymerase (11), *B. stearothermophilis* pol I (10) and the large fragment of *Taq* pol I (Klentaq1) (8, 9) support and extend this model. In particular, analysis of Klentaq1 complexed with DNA and ddCTP indicates that, during binding and catalysis, Arg[659], Lys[663], Phe[667], and Tyr[671] interact with the dNTP (note that these residues are homologous to the Klenow fragment residues mentioned above), whereas Arg[660], Thr[664], Gly[668], and also Tyr[671] interact with the DNA.

The participation of numerous O-helix residues in dNTP and template-primer binding suggests a concomitant role in governing polymerase fidelity. This premise is borne out by the altered accuracy of certain site-directed mutants of *E. coli* pol I, namely Y766A and Y766S, which exhibit reduced fidelity (15, 16), and R754A (17), which exhibits increased fidelity. To augment the supply of informative mutants, we have undertaken

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[1] The abbreviations used are: pol I, DNA polymerase I; *Taq*, *T. aquaticus*.

another approach to determining the effects of mutation in the O-helix on base selection fidelity. We have introduced randomized sequences into the O-helix of *Taq* pol I and selected for catalytically active mutants by genetic complementation; interestingly, no amino acid substitutions or only highly conservative substitutions were observed for the four evolutionarily conserved, catalytically crucial residues described above (18). Subsequent biochemical screening of extracts of active mutants allowed us to identify numerous O-helix variants with presumptively reduced fidelity, two of which we purified and showed to be authentic mutators (19).

Here, we describe two O-helix mutants of *Taq* pol I that display anti-mutator activity. Both contain a mutation at Phe[667], a residue that when changed to Tyr greatly alters sugar discrimination (13) but not previously known to be involved in base selection. Our results indicate that Phe[667] plays a role in regulating mutational frequency and specificity and maintaining catalytic efficiency. Together with our previous description of low fidelity mutants (19), our findings suggest that the wild type mutational frequency and spectrum may reflect the contributions and interactions of numerous O-helix residues, including those that directly contact the incoming dNTP and/or template-primer and those that do not.

EXPERIMENTAL PROCEDURES

DNA Polymerases—Wild type *Taq* pol I and its mutant derivatives were selected by complementation of the temperature-sensitive growth of an *E. coli* strain harboring temperature-sensitive pol I, as previously reported (18). The DNA sequence of the entire wild type gene and the polymerase domain of each of the mutants was determined. All mutations were within the randomized region, except that each construct lacked the N-terminal 3 amino acids. The mutant F667L was made by site-directed mutagenesis (18). Expression and purification of *Taq* pol I and its derivatives were as described previously (19). DNA polymerase activity was determined in assays measuring incorporation of label from [³H]dATP into activated calf thymus DNA at 72 °C (19); 1 unit represents incorporation of 10 nmol of dNMP in 1 h, corresponding to 0.1 unit as defined by PerkinElmer Life Sciences.

Kinetic Parameters—K_m and V_{max} for incorporation of dAMP into DNA were measured in assays containing 200 μg/ml activated calf thymus DNA, 10 ng of polymerase, 50 μM each dGTP, dCTP, dTTP, and either 0.5, 1, 2, 4, 8, or 16 μM [³H]dATP in 50 mM Tris-HCl (pH 8.0), 2 mM MgCl$_2$; incubation was for 5 min at 72 °C. Incorporation of radioactivity into acid-insoluble products was determined (19), and total nucleotide incorporation was calculated for each reaction. K_m and V_{max} values were determined from Lineweaver-Burk plots.

Efficiencies of nucleotide insertion and primer extension were determined by using the following oligonucleotides: 5′-AGG CAC CCC AGG CTT **T**AC ACT TTA TGC TTC CGG CTC GTA T (template for T:G mismatch measurements (target T in bold type) corresponding to positions −49 to −10 in the *lacZα* mutational target; see Fig. 1), 5′-ATA CGA GCC GGA AGC ATA AAG TGT (primer for correct/incorrect insertion), 5′-ATA CGA GCC GGA AGC ATA AAG TGT A (primer for extension of matched terminus), 5′-ATA CGA GCC GGA AGC ATA AAG TGT G (primer for extension of mismatched T:G terminus); 5′-CAT CCC CCT TTC GCC **A**GC TGG CGT AAT AGC GAA GAG GCC T (template for A:A mismatch measurements corresponding to positions +129 to +168 in the *lacZα* mutational target), 5′-GGG CCT CTT CGC TAT TAC GCC AGC (primer for correct/incorrect insertion), 5′-GGG CCT CTT CGC TAT TAC GCC AGC T (primer for extension of matched terminus), and 5′-GGG CCT CTT CGC TAT TAC GCC AGC A (primer for extension of mismatched A:A terminus). Measurements were performed essentially as described previously (20). ³²P-Labeled primer was annealed with 3-fold excess of template. A final concentration of 5 nM was used in 20-μl reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 2 mM MgCl$_2$, 50 mM KCl. To measure correct incorporation/extension, reactions were carried out at 45 °C for 5 min with 0.063–0.4 nM polymerase and 0.2–200 μM dNTP to achieve less than 20% template-primer utilization. To measure misincorporation/misextension, reactions were carried out 45 °C for 10–60 min with 0.16–25 nM polymerase and 1.6–400 μM dNTP. For extension of the A:A mismatched primer, 1300 nM F667L polymerase or 1000 nM A661E,I665T,F667L polymerase was incubated with 5 mM dGTP at 45 °C for 60 min. After the reactions were terminated by adding 20 mM EDTA, extension products were

analyzed in a 12% polyacrylamide gel containing 8 M urea. The results were quantified by using a Laser Image Analyzer (BAS 2000, Fuji Film, Japan). K_m and V_{max} values obtained from Hanes-Woolf plots are the averages of triplicate determinations.

Forward Mutation Assay and DNA Sequence Analysis—Mutant frequencies and spectra were determined as described previously (19) with minor modifications. The noncoding strand of the *lacZα* gene contained within 400 ng of gapped M13mp2 DNA (21, 22) was copied by using the specified amount of wild type or mutant *Taq* pol I in a reaction mixture containing 200 μM each dNTP, 50 mM Tris-HCl (pH 8.0), 7 mM MgCl$_2$, 50 mM KCl. After incubation at 72 °C for 5 min, the DNA was transfected, and plaques were scored. Nucleotide sequences were determined by using a Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems).

RESULTS

Generation of Catalytically Active O-helix Mutants and Screening for Increased Fidelity—We have previously created a large library of O-helix mutants by substituting random nucleotides for the 13 codons that specify amino acids Arg[659]–Tyr[671] in plasmid-borne *Taq* pol I. Functional mutants were then selected by complementing the temperature-sensitive growth of an *E. coli* strain harboring a temperature-sensitive host pol I. We obtained 75 active mutants with unique O-helix sequences that supported bacterial growth at nonpermissive temperature (18). To identify mutations that confer increased fidelity, extracts of *E. coli* expressing mutant *Taq* pol Is were screened in a primer extension assay in the presence of only three, rather than four complementary dNTPs (19). Relative to wild type *Taq* pol I, mutants with increased fidelity would extend a smaller proportion of primers up to and beyond the first template position for which a complementary dNTP is lacking. Following screening, we purified candidate high fidelity polymerases to apparent homogeneity, measured their specific activities, and determined which of the mutant enzymes were able to amplify a 500-base pair fragment in a polymerase chain reaction assay (data not shown). After re-examining the fidelity of the purified, polymerase chain reaction-competent polymerases in the primer extension assay, we chose one mutant, the triply substituted variant A661E,I665T,F667L, for more detailed study. For reference, we also examined the three singly substituted polymerases A661E and I665T, which we previously recovered by genetic complementation (18), and F667L, which we constructed by site-specific mutagenesis. The specific activities of the homogeneous *Taq* pol Is and their catalytic efficiencies (V_{max}/K_m for incorporation of dAMP into activated DNA) relative to wild type are: wild type, 66,000 units/mg protein, 100%; A661E,I665T,F667L, 27,000 units/mg protein, 19%; F667L, 27,000 units/mg protein, 7.2%; I665T, 30,000 units/mg protein, not determined; A661E, 45,000 units/mg protein, 65%.

Fidelity in a Forward Mutation Assay—To quantitate the fidelity of *in vitro* DNA synthesis, we used an M13mp2 forward mutation assay that measures the sum of many different types of polymerase errors in diverse sequence contexts (21, 22). The mutational target was 214 nucleotides in the *lacZα* gene located within a single-stranded region in gapped circular double-stranded DNA. As in most other gap filling assays, detection of base substitution errors requires insertion of an incorrect nucleotide and extension of the resulting, mispaired primer terminus. Failure to extend mispairs, and the consequently incomplete gap filling, would result in underestimation of overall mutant frequencies and, presumably, in disproportional loss of particular types of errors. This is so because, upon transfection of partially filled molecules into reporter *E. coli*, gap filling is completed by the highly accurate host replication machinery, and mispaired termini formed *in vitro* are subject to exonucleolytic proofreading (23). Particularly prone to loss would be mutations resulting from mispairs that are difficult to

TABLE I

Mutant frequencies observed for wild type and mutant Taq DNA polymerases in the M13mp2 forward mutation assay

DNA polymerase	Units/μl[a]	Plaques scored		Mutant frequency			Relative mutant frequency
		Total	Mutant	Observed ($\times 10^3$)[b]	Observed average ($\times 10^3$)	Corrected average ($\times 10^3$)[c]	
Wild type	2.50	6353	194	29.7			
	2.50	3633	76	20.1			
	2.50	3111	28	8.1	20.2	16.5	1.0
	1.25	8720	208	23.0			
	0.25	12049	71	5.0			
A661E,I665T,F667L	2.50	13524	99	6.5	6.8	6.0	1/2.8
	1.25	14591	116	7.1			
F667L	2.50	16065	65	5.7	9.6	6.0	1/2.7
	1.25	4560	106	13.4			
I665T	2.5	4487	113	29.6	26.9	21.5	1.3
	1.25	8671	264	24.3			
A661E	2.50	2251	444	196	196	156	9.5
	1.25	ND[d]	ND	ND			

[a] 1 unit is equivalent to 0.1 unit of AmpliTaq (PerkinElmer Life Sciences).

[b] Background frequency (0.87×10^{-3}) was subtracted.

[c] Corrected for the percentage of false positive plaques scored for each polymerase, estimated by sequencing a representative sample of at least 50 mutant plaques: wild type, 19%; A661E,I665T,F667L, 12%; F667L, 37%; I665T, 20%; A661E, 21%.

[d] ND, not determined.

extend, *e.g.* A:A in the case of *Taq* pol I (24). In our case, if partial gap filling resulted in underestimation of the capacity of wild type pol I for error synthesis, our ability to document reduced error synthesis by more faithful mutant polymerases might be compromised.

To estimate the extent of gap filling, we monitored the reaction products by using agarose gel electrophoresis (data not shown); customarily, the reaction is judged to be complete when the product has the mobility of double-stranded circular M13 DNA (22). However, a small minority of partially filled target molecules may escape detection, particularly if heterogeneous in mobility. To avoid this potential problem, we titrated the amount of wild type polymerase in the DNA synthetic reactions and measured the resulting mutant frequencies (Table I). The frequencies found at 2.5 and 1.25 units/μl of wild type *Taq* pol I were indistinguishable, the average of the four determinations being 17×10^{-3}; 2.5 units/μl was five times the concentration required to convert all detectable reaction products to molecules migrating as double-stranded circles (data not shown). The mutant frequency at 0.25 units/μl was lower (5.0×10^{-3}), because of incomplete gap filling; as documented below, the lower mutant frequency was accompanied by a markedly different mutational spectrum. The observed frequencies bracket the value of 10×10^{-3} found by Tindall and Kunkel (25) at a lower temperature (55 °C). The average mutant frequency observed at 2.5 and 1.25 units/μl of the A661E,I665T,F667L triple mutant polymerase was 6.0×10^{-3}, a ~3-fold lower value than for wild type pol I (Table I). The average for the F667L polymerase, 6.0×10^{-3}, was also 3-fold lower than for the wild type enzyme, whereas the average for the I665T polymerase (22×10^{-3}) was similar to wild type. In striking contrast, the frequency for the A661E polymerase (16×10^{-2}) was 9.5-fold higher than for wild type pol I, in accord with previous results (19).

Mutational Specificity of Wild Type Taq pol I—Before comparing the error spectra of the wild type and mutant polymerases, we will first describe the mutational specificities obtained with different concentrations of wild type pol I in the DNA synthetic reactions. At a low polymerase concentration (0.25 units/μl), our results resemble the published data of Tindall and Kunkel (25). In both cases, transitions outnumbered

transversions by at least 2:1, with T $\rightarrow$ C transitions comprising 67 and 56% of the base substitutions, respectively; no other single mutation approached these proportions. In the Tindall and Kunkel study, all frameshifts occurred in runs of two or more identical bases, suggestive of a direct slippage mechanism; similarly, all but one of our frameshifts occurred in runs.

We observed a very different pattern of error synthesis at high pol I concentration (2.5 units/μl) (Table II and Fig. 1*A*). There was a profound shift among base substitutions, such that transversions now outnumbered transitions by nearly 2:1. A $\rightarrow$ T transversions (34%) and G $\rightarrow$ T transversions (21%) together constituted over half of the base substitutions, and T $\rightarrow$ C transitions (23%) no longer predominated. Moreover, there was a remarkably high fraction (13%) of frameshifts at nonreiterated sites. As shown in the mutational spectrum (Fig. 1*A*), all were deletions of a single purine immediately downstream of a pyrimidine; seven were deletion of an A adjacent to a 3'-T, and three were deletion of G adjacent to a 3'-C or -T. Despite the increases in transversions and frameshifts, the frequencies of T $\rightarrow$ C transitions at low and high pol I concentrations did not differ (3.4×10^{-3} *versus* 3.1×10^{-3}, respectively). These data suggest that: 1) synthesis and extension of T:G mismatches was relatively facile and reached a maximum, even at low polymerase concentration; and 2) at high *Taq* pol I concentration where gap filling was forced closer to absolute completion, difficult-to-extend A:A and G:A mispairs that were abandoned by the polymerase at lower concentration were utilized for synthesis of transversion and frameshift mutations.

Mutational Specificity of Mutant Taq pol Is—The types of errors catalyzed by the mutant polymerases in reactions containing 2.5 units/μl are recorded in Table II. As in the case of wild type pol I, the mutant frequencies observed at this polymerase concentration were no greater than found at 1.25 units/μl (Table I).

A661E,I665T,F667L Polymerase—The error spectrum observed for the triple mutant polymerase (Table II and Fig. 1*B*) was strikingly different from wild type, consisting almost entirely (63/70, 90%) of transitions, mainly T $\rightarrow$ C and C $\rightarrow$ T. Importantly, the frequency of transitions was essentially the same as observed for wild type pol I (5.4×10^{-3} *versus* 4.8×10^{-3}, calculated from the data in Tables I and II). However, the

TABLE II

Types of errors observed for wild type and mutant Taq DNA polymerases in the M13mp2 forward mutation assay

Nucleotide sequences were obtained for mutant plaques collected from copying reactions containing 2.5 units/µl of polymerase. The number of occurrences of each error type (*N*) and the percent of total errors observed are recorded. The error frequencies observed for the wild type polymerase and the error frequencies relative to wild type observed for the mutant polymerases were calculated from the sequence data and the mutant frequencies of Table I.

Error type	Wild type			A661E,I665T,F667L			F667L			I665T			A661E		
	N	%	Frequency ($\times 10^3$)	N	%	Frequency (M/WT)	N	%	Frequency (M/WT)	N	%	Frequency (M/WT)	N	%	Frequency (M/WT)
All errors	75	100	16.5	70	100	1/2.8	59	100	1/2.7	63	100	1.3	50	100	9.5
Base substitutions	61	81	13.4	68	97	1/2.3	44	75	1/3.0	46	73	1.2	42	84	9.8
Transitions	22	29	4.8	63	90	1.1	32	54	1/1.5	11	18	1/1.3	19	38	12
T→C	14	19	3.1	47	67	1.3	19	32	1/1.6	3	5	1/3.0	11	22	11
C→T	6	8	1.3	14	20	1/1.1	9	15	1/1.4	5	8	1.3	5	10	12
G→A	1			2			2			0			0		
A→G	1			0			2			3			3		
Transversions	39	52	8.6	5	7	1/20	12	20	1/7.0	35	56	1.4	23	46	8.4
T→G	1			2			1			2			2		
T→A	3			1			3			2			2		
C→G	0			0			1			0			0		
C→A	0			0			0			0			0		
G→T	13	17	2.9	1	1	1/33	2	3	1/14	17	27	2.0	10	20	11
G→C	1			0			2			1			0		
A→C	0			1			1			1			0		
A→T	21	28	4.6	0		<1/54	2	3	1/23	12	19	1/1.1	9	18	6.1
Frameshifts	14	19	3.1	2	3	1/18	15	25	1/2.0	17	27	1.9	8	16	8.1
In runs	4	5	0.88	2	3	1/5.1	8	14	1/1.1	6	10	2.3	4	8	14
In non-runs	10	13	2.2	0	0	>1/26	7	12	1/3.1	11	17	1.7	4	8	5.7
Δ-pur, 5'-pur	10	13	2.2	0	0	>1/26	7	12	1/3.1	10	16	1.6	4	8	5.7
Δ-A, 5'-T	7	9	1.5	0	0	>1/18	6	10	1/2.5	9	14	2.0	3	6	6.1

frequency of transversions was reduced 20-fold, due to >50- and >30-fold reductions, respectively, in A → T and G → T substitutions. Only two frameshifts were observed (3% of total mutations) in a run of either C or T; no frameshifts were found at nonreiterated sites. Thus, the 3-fold decrease in overall mutant frequency found for the A661E,I665T,F667L polymerase was conferred by large reductions in A → T and G → T transversions and by a greater than 20-fold reduction in the distinctive −1 frameshifts consisting of loss of a purine residue located 3′ of a pyrimidine at nonreiterated sites. These data suggest that the triple mutant polymerase catalyzes less synthesis of A:A and G:A mispairs and/or less extension of these mispairs than wild type pol I via either direct elongation or elongation in conjunction with misalignment.

I665T Polymerase—The mutational specificity of the I665T polymerase (Table II), like the overall mutant frequency, was similar to wild type with respect to the types of errors synthesized, the distribution of base substitutions, and the high proportion of −1 frameshifts involving loss of a purine downstream of a pyrimindine (16% *versus* 13%). As a single mutation, then, the I665T substitution had no notable effect on fidelity.

F667L Polymerase—The F667L polymerase, which exhibited an overall mutant frequency about one-third of wild type (Table I), displayed an error specificity comprising elements of both the wild type and A661E,I665T,F667L specificities. The frequency of transitions was similar to that of wild type (3.3 × 10^{-3} *versus* 4.8 × 10^{-3}), consisting largely of T → C and C → T mutations in wild type-like proportions. Akin to the triple mutant polymerase, the frequency of transversions was greatly reduced, comprising >10-fold reductions in A → T and G → T mutations, respectively (Table II). The frequency of frameshifts in runs was like wild type (0.81 × 10^{-3} *versus* 0.88 × 10^{-3}, respectively), and the frequency of the distinctive purine deletions downstream of a pyrimidine at nonreiterated sites was slightly lower (0.71 × 10^{-3} *versus* 2.2 × 10^{-3}, respectively). Interestingly, different mutational patterns at template A were observed for the wild type, F667L, and A661E,I665T,F667L polymerases. Three times *more* A → T transversions than ΔA

frameshifts were found for wild pol I; three times *fewer* A → T transversions than ΔA frameshifts were found for the F667L polymerase; and neither error was found among mutations synthesized by the A661E,I665T,F667L polymerase.

A661E Polymerase—The A661E polymerase, which exhibited a 9.5-fold elevation in overall mutant frequency (Table I), displayed a mutational specificity like wild type, with 6–14-fold increases in all the major error categories observed (Table II). The A661E polymerase may thus be a general mutator that exaggerates the overall error synthesis characteristic of wild type pol I. Remarkably, all of the excess error synthesis observed for the A661E polymerase is abolished in the A661E,I665T,F667L triple mutant polymerase, with A → T and G → T transversions and the distinctive purine deletions at nonreiterated sites being reduced to frequencies much lower than wild type.

Fidelity in a Gel-based Kinetic Assay—To better understand the basis of the reduction in A → T transversions observed for the A661E,I665T,F667L and F667L polymerases, we analyzed the synthesis and extension of A:A mispairs in a steady state, gel-based kinetic assay (20, 24). For comparison, we also examined formation and extension of T:G mispairs, because the mutational product (T → C transitions) was equally frequent in the wild type, A661E,I665T,F667L, and F667L error spectra. We used oligonucleotide template-primers containing either a target A in the template, corresponding to nucleotide +144 in the target in the forward mutation assay, or a target T corresponding to nucleotide −34 (Fig. 1). Table III records K_m and V_{max} values for insertion and extension reactions catalyzed by the wild type, triple mutant, and F667L polymerases. The ratio V_{max}/K_m is a measure of the efficiency of nucleotide incorporation in each reaction. The ratio of efficiencies for insertion of correct *versus* incorrect nucleotides is a measure of discrimination against insertion of mispaired bases, indicated in Table III as misinsertion efficiency. The ratio of efficiencies for addition of nucleotides to matched *versus* mismatched primer termini is a measure of discrimination against mispair extension, indicated in the table as mismatch extension efficiency.

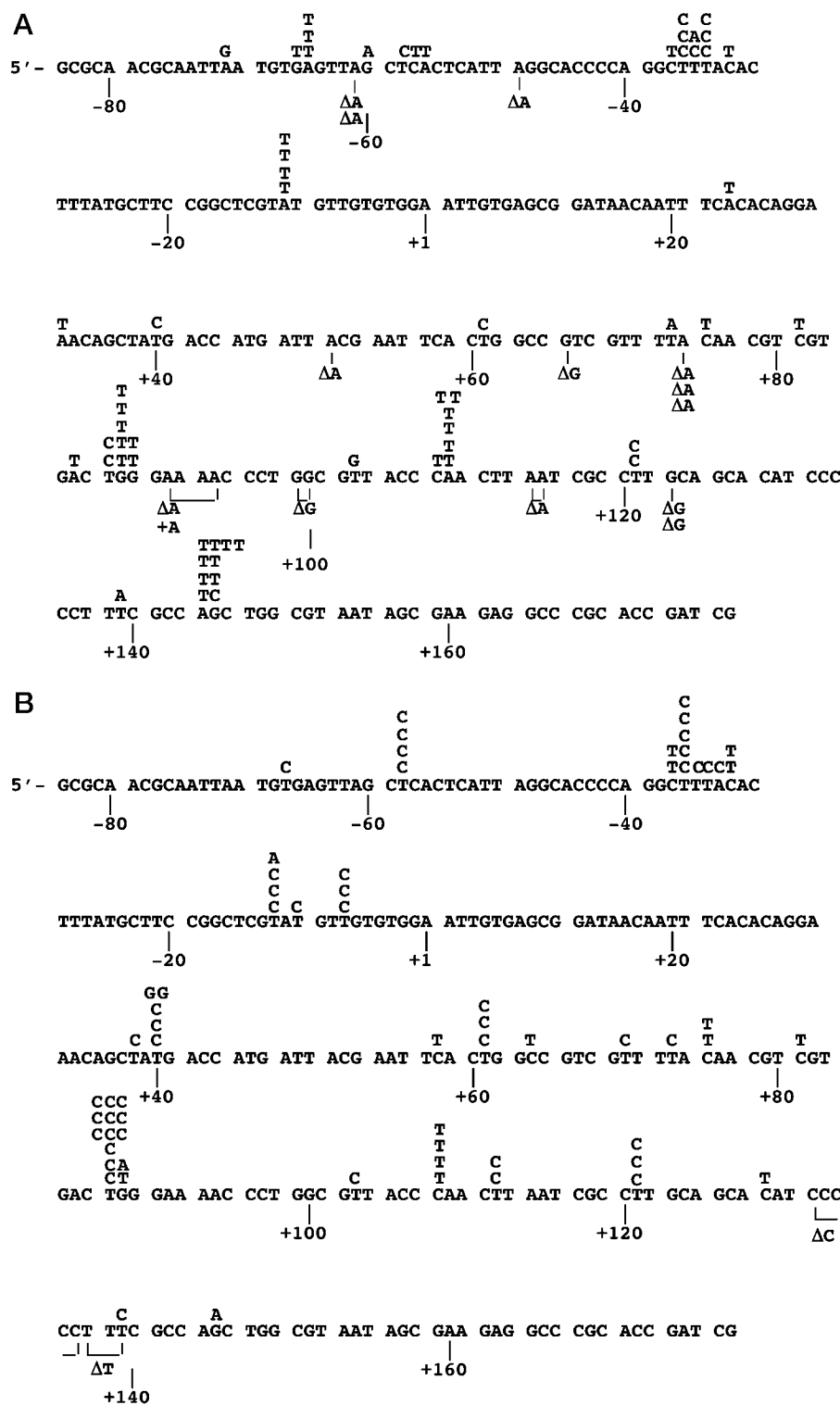T:G mismatches are synthesized and extended relatively

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A
                                        T                               C C
                                        T                               CAC
                        G               TT        A     CTT             TCCC T
5'- GCGCA ACGCAATTAA TGTGAGTTAG CTCACTCATT AGGCACCCCA GGCTTTACAC
            -80                     ΔA              ΔA        -40
                                    ΔA|
                                    -60
                        T
                        T
                        T
                        T
    TTTATGCTTC CGGCTCGTAT GTTGTGTGGA ATTGTGAGCG GATAACAATT TCACACAGGA
              -20                 +1                   +20                   T

T          C                                 C              A   T      T
AACAGCTATG ACC ATG ATT ACG AAT TCA CTG GCC GTC GTT TTA CAA CGT CGT
            +40              ΔA            +60        ΔG      ΔA      +80
                                                              ΔA
            T                                                 ΔA
            T                               TT
            T                               T
            CTT                             T
T           CTT                             TT              C
GAC TGG GAA AAC CCT GGC GTT ACC CAA CTT AAT CGC CTT GCA GCA CAT CCC
        ΔA              ΔG                      ΔA      +120  ΔG
        +A                                                   ΔG
         TTTT
         TT          |
         TT          +100
    A    TC
CCT TTC GCC AGC TGG CGT AAT AGC GAA GAG GCC CGC ACC GAT CG
    +140                          +160
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B
                                        C                               C
                                        C                               C
                                        C                               C
                                        C                               TC    T
                                C       C                               TC CCCT
5'- GCGCA ACGCAATTAA TGTGAGTTAG CTCACTCATT AGGCACCCCA GGCTTTACAC
            -80                     -60                    -40
                        A
                        C
                        C           C
                        C  C        C
    TTTATGCTTC CGGCTCGTAT GTTGTGTGGA ATTGTGAGCG GATAACAATT TCACACAGGA
              -20                 +1                   +20

            GG
            C                               C
            C                               C
            C  C            T   C   T           C   C   T T      T
AACAGCTATG ACC ATG ATT ACG AAT TCA CTG GCC GTC GTT TTA CAA CGT CGT
    CCC     +40                          +60                  +80
    CCC
    CCC
    C
    CA                              T
    CT                              T       C              C
GAC TGG GAA AAC CCT GGC GTT ACC CAA CTT AAT CGC CTT GCA GCA CAT CCC
                    +100                             +120              ΔC

    C              A
CCT TTC GCC AGC TGG CGT AAT AGC GAA GAG GCC CGC ACC GAT CG
    ΔT                            +160
    +140
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readily by a variety of polymerases (26). Wild type *Taq* pol I conformed to this pattern, exhibiting a misinsertion efficiency of 3.2×10^{-4} and a mismatch extension efficiency of 4.5×10^{-3}. The latter value is consistent with that found by Huang *et al.* (24) (7×10^{-4}) for a different sequence context. The F667L polymerase was greatly impaired in formation and extension of both T:G and T:A base pairs. However, the degree of impairment was comparable in the incorrect *versus* correct reactions, resulting in wild type levels of discrimination. The triple mutant polymerase also exhibited a wild type level of discrimination against synthesis of T:G mismatches; however

discrimination against mismatch extension was 10-fold higher than wild type, mainly because of a lower V_{max} for elongation of the mismatch. These data are consistent with the results of the forward mutation assay and illustrate an important point. The comparable T:G misinsertion efficiencies observed for the wild type, F667L, and triple mutant polymerases were associated with similar frequencies of T → C transitions, even though the triple mutant polymerase extended T:G mismatches 10 times less efficiently than wild type pol I. Thus, a lower misextension efficiency does not *necessarily* result in a lower error frequency in the forward mutation assay, if, by repeatedly reassociating

TABLE III

Steady state kinetic parameters for nucleotide incorporation by wild type and mutant Taq DNA polymerases

K_m and V_{max} were obtained for insertion of correct *versus* incorrect nucleotides and for extension of matched *versus* mismatched primer termini. The values are the average of triplicate determinations.

Polymerase	T:A synthesis			T:G synthesis			Misinsertion efficiency
	V_{max}	K_m dATP	V_{max}/K_m	V_{max}	K_m dGTP	V_{max}/K_m	
	min^{-1}	μM	$min^{-1}\,\mu M^{-1}$	min^{-1}	μM	$min^{-1}\,\mu M^{-1}$	
Wild type	1.7	0.18	9.6	0.16	53	3.1×10^{-3}	3.2×10^{-4}
F667L	1.3	13.0	0.10	1.6×10^{-3}	97	1.6×10^{-5}	1.6×10^{-4}
A661E,I665T,F667L	1.2	2.0	0.59	2.5×10^{-3}	120	2.0×10^{-4}	3.4×10^{-4}

Polymerase	T:A extension			T:G extension			Mismatch extension efficiency
	V_{max}	K_m dATP	V_{max}/K_m	V_{max}	K_m dATP	V_{max}/K_m	
	min^{-1}	μM	$min^{-1}\,\mu M^{-1}$	min^{-1}	μM	$min^{-1}\,\mu M^{-1}$	
Wild type	1.2	0.21	5.5	1.8	70	2.5×10^{-2}	4.5×10^{-3}
F667L	1.0	6.3	0.16	1.9×10^{-2}	70	2.7×10^{-4}	1.7×10^{-3}
A661E,I665T,F667L	0.87	0.68	1.3	6.1×10^{-2}	110	5.7×10^{-4}	4.4×10^{-4}

Polymerase	A:T synthesis			A:A synthesis			Misinsertion efficiency
	V_{max}	K_m dTTP	V_{max}/K_m	V_{max}	K_m dATP	V_{max}/K_m	
	min^{-1}	μM	$min^{-1}\,\mu M^{-1}$	min^{-1}	μM	$min^{-1}\,\mu M^{-1}$	
Wild type	2.3	0.35	6.5	0.84	120	7.1×10^{-3}	1.1×10^{-3}
F667L	4.2	21	0.20	1.6×10^{-3}	110	1.5×10^{-5}	7.5×10^{-5}
A661E,I665T,F667L	1.4	0.83	1.7	3.0×10^{-2}	130	2.4×10^{-4}	1.4×10^{-4}

Polymerase	A:T extension			A:A extension			Mismatch extension efficiency
	V_{max}	K_m dGTP	V_{max}/K_m	V_{max}	K_m dGTP	V_{max}/K_m	
	min^{-1}	μM	$min^{-1}\,\mu M^{-1}$	min^{-1}	μM	$min^{-1}\,\mu M^{-1}$	
Wild type	3.4	0.13	27	1.5×10^{-3}	47	3.3×10^{-5}	1.2×10^{-6}
F667L	3.9	5.3	0.73				[a]
A661E,I665T,F667L	2.7	2.2	1.3				[a]

[a] The maximum velocities measurable for the F667L and A661E:I665T:F667L polymerases were 1.2×10^{-6} and 1.3×10^{-6}, respectively. By using the maximum measurable velocity and the wild type K_m to estimate the efficiency of A:A extension, mismatch extension efficiencies for the F667L and A661E:I665T:F667L polymerases were estimated to be less than 3.5×10^{-8} and 2.2×10^{-8}, respectively.

with mispaired termini, an inherently more discriminating mutant polymerase can catalyze a wild type amount of misextension within the time allotted for *in vitro* DNA synthesis.

Wild type *Taq* pol I discriminated relatively modestly against synthesis of A:A mispairs and very stringently against extension, exhibiting a misinsertion efficiency of 1.1×10^{-3} and a mismatch extension efficiency of 1.2×10^{-6}. The latter value, which is at the limit of detection of the assay, agrees well with the value of 2×10^{-6} found by Huang *et al.* (24) for a template A in a different sequence context. Both the F667L and triple mutant polymerases discriminated more effectively against formation of A:A mispairs than the wild type enzyme by 15- and 8-fold, respectively. Both polymerases extended A:A mispairs so inefficiently that we could not obtain kinetic parameters for the reactions; only a very small percentage of primers was extended after 1 h of incubation with up to 5 mM dGTP and hundreds-fold excess of enzyme relative to template-primer. If we take V_{max} to be the highest velocities we were able to observe and assume that the K_m values are similar to that of wild type pol I (which seems reasonable based on the values found in the other reactions), we can estimate that the triple mutant and F667L polymerases discriminate against extension of the A:A mispair at least 30- and 60-fold better, respectively, than wild type pol I (see footnote to Table III). These findings suggest that reduced misinsertion and misextension were both involved in the reduction in A $\rightarrow$ T transversions observed in the forward mutation assay.

The data in Table III are also important with respect to the catalytic impairment of the mutant polymerases. The F667L polymerase exhibited 30–70-fold increases in K_m for incorporation of correct dATP, dTTP, and dGTP, whereas the A661E,I665T,F667L polymerase showed 2–17-fold increases; neither polymerase displayed significant changes in V_{max}. Apparently, the deleterious effects of the single F667L substitu-

tion are partially suppressed in the context of the triple mutant polymerase. With respect to insertion of mismatched nucleotides and extension of mismatched template-primers, both mutant polymerases yielded decreases in V_{max} for dNTPs, with little or no change in K_m.

DISCUSSION

DNA polymerases perform highly accurate DNA synthesis even in the absence of exonucleolytic proofreading. This accuracy, maintained at the polymerase active site, comprises base discrimination at both the nucleotide insertion and subsequent extension steps. Correct nucleotide incorporation has been further divided kinetically into five steps including dNTP binding, a rate-limiting conformational change, phosphodiester bond formation, a second conformational change, and pyrophosphate release and translocation of the DNA (27–29). The rate-limiting conformational transition may correspond to the reorientation of the fingers domain, including the O-helix in pol I class polymerases, to assemble a closed ternary complex (8, 11). The contribution of each of these steps to discrimination between complementary and noncomplementary nucleotides may differ among different polymerases. Our understanding of DNA polymerase fidelity rests not only on biochemical and structural analysis of wild type polymerases, but on study of mutants with altered accuracy. We describe here two high fidelity mutants of *Taq* pol I bearing mutations in the conserved and catalytically important O-helix. The value of these mutants is greatly enhanced by the availability of detailed three-dimensional structures of wild type *Taq* pol I (8, 9, 11).

Concentration Dependence of Mutational Spectra—We used high concentrations of polymerase in the forward mutation assay to promote saturation of mutation synthesis and to thereby enhance detectability of reduced mutation synthesis by high fidelity polymerases. We observed that, at high concen-

trations, wild type *Taq* pol I yielded more transversion and frameshift mutagenesis than has been reported. It is important to note that the frequencies of T → C transitions remained the same at low and high pol I concentrations, suggesting that T:G mispairs were readily synthesized and extended and reached maximum levels at both concentrations. This inference is supported by our kinetic data (Table III) and that of Huang *et al.* (24) and is consistent with data showing that T:G mispairs are synthesized and extended relatively rapidly by the exo⁻ Klenow fragment of *E. coli* pol I (26). In contrast, extension of A:A and G:A mispairs was apparently much more frequent at high *Taq* pol I concentration, greatly increasing the proportion of A → T and G → T transversions among the total mutations. This inference is likewise supported by our data (Table III) and that of Huang *et al.* (24) and is also consistent with data showing that A:A mispairs are readily synthesized, but very poorly extended by the exo⁻ Klenow fragment of *E. coli* pol I (26). Observations related to ours have been reported by Minnick *et al.* (17), who found 21-fold more A → T transversions made by wild type Klenow fragment in a reversion assay that does not require misextension to score an error than in a reversion assay that is otherwise comparable but requires misextension to register the error. Enhanced utilization of A:A and G:A mispairs at high pol I concentration can also account for the >30-fold increase in the frequency of −1 frameshifts that involve deletion of a purine residue immediately downstream of a pyrimidine (2.2 × 10⁻³ *versus* 0.15 × 10⁻³). In the case of these distinctive frameshifts, realignment of the mismatched purine at the primer terminus with the adjacent T or C in the template would permit elongation of the incorrectly incorporated nucleotide (30).

A661E Taq pol I: A General Mutator—The A661E substitution confers general mutator activity while preserving the catalytic competence of wild type *Taq* pol I. The A661E polymerase yielded a 10-fold increase in overall mutant frequency (Table I) and elevated all quantifiable errors 6–14-fold (Table II). The mutator activity is at least in part due to reduced discrimination at the nucleotide insertion step. Thus, we have observed a 10-fold increase in $V_{\max}$ of the A661E polymerase for incorporation of incorrect dATP opposite template C in a primer extension assay; the $V_{\max}$ for incorporation of correct dGTP and the K_m for both dGTP and dATP were the same or very similar to wild type (data not shown).

High resolution crystal structures indicate that Ala[661] makes no direct contacts with either the template-primer or incoming ddCTP and faces away from the ddCTP in the binary and in the open and closed ternary complexes of wild type Klentaq1 (8). In accord, our random mutagenesis and genetic complementation analysis has shown that diverse replacements at Ala[661], including negatively and positively charged residues, permit high levels of catalytic activity when present as single substitutions *in vivo* (18). The crystal structure of the closed, ternary ddCTP-trapped complex also shows that the adjacent Arg[660] makes direct contact with the first phosphate at the 3′ end of the primer backbone and that Arg[587] makes direct contact with the second phosphate. A model of the A661E polymerase (Fig. 2) indicates that, with suitable rotation, the carboxylate side chain of Glu[661] could compete with the second primer phosphate for an electrostatic interaction with the guanidinium group of Arg[587]. A possible consequence would be formation of a new interaction between the O-helix and the primer that might stabilize the closed form, allowing more time for chemistry and increasing the probability of phosphoryl transfer involving incorrectly paired dNTPs. The model also shows that Glu[661] could compete with the first primer phosphate for an electrostatic interaction with Arg[660]; such inter-
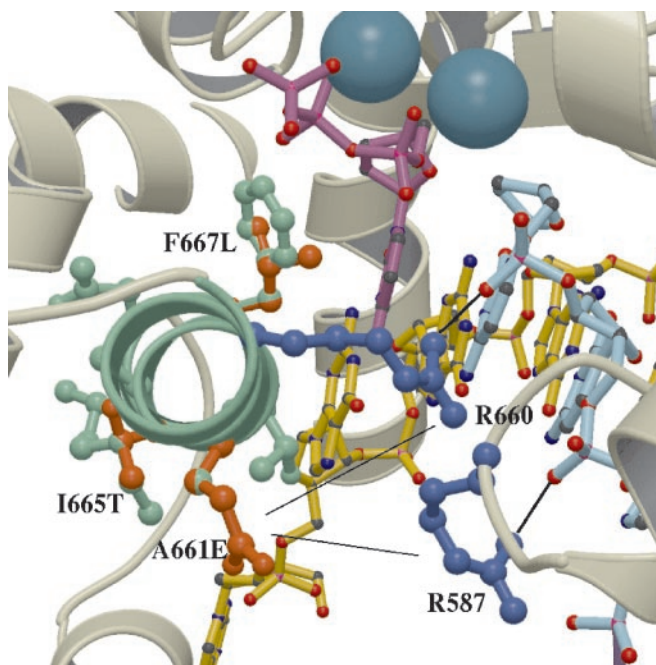

FIG. 2. **Model of the *Taq* DNA polymerase I mutant A661E,I665T,F667L that contains three amino acid substitutions in the O-helix.** The figure shows part of the catalytic site in the closed ternary complex of *Taq* pol I with a primer/template DNA and ddCTP (8). The O-helix is indicated in *light green*, with both wild type (*light green*) and mutant (*orange*) amino acid side chains shown for comparison. The template DNA strand is indicated in *yellow*, the primer DNA strand is in *light blue*, and the incoming ddCTP is in *magenta*, with carbon atoms in *black*, nitrogen atoms in *blue*, oxygen atoms in *red*, and phosphorus atoms in *pink*. Metal ions are represented as *blue spheres*. In the mutant as well as the wild type (8) ternary complex, the side chains of Arg[660] and Arg[587] (*deep blue*) contact the terminal and penultimate phosphates at the 3′ end of the primer backbone, as indicated by *thick black lines*. Two possible electrostatic interactions of Glu[661] in the mutant polymerase with Arg[660] or Arg[587] are indicated by *thin black lines*. The coordinate set for the wild type complex (3KTQ) was obtained from Protein Data Bank (32); mutant amino acid side chains were inserted using the program O (33). We did not attempt to calculate alternative conformations, although E661 is free to rotate around side chain bonds. The drawing was made by using the programs Molscript (34) and Raster 3D (35).

action might create slightly more space in the catalytic pocket to accommodate incorrect base pairs. Interestingly, more recent data (9) indicate that the structure of the O-helix in Fig. 2, shown for incoming ddCTP, applies as well to ddTTP and ddATP.

F667L Taq pol I: A Transversion Anti-mutator—Phe[762] in *E. coli* pol I, which is homologous to Phe[667] in *Taq* pol I, promotes wild type levels of catalytic activity and sugar selectivity, apparently by binding and positioning dNTP substrates (14). The *E. coli* F762A mutant exhibits a much higher K_D and a lower k_{cat} for dNTPs, the latter presumably reflecting suboptimal positioning of the dNTP in the ternary complex; a massive loss of discrimination against dideoxynucleoside triphosphates supports the premise that Phe[762] constrains the dNTP to achieve accurate positioning of the 3′-OH within the active site. Preceding work (13) had shown that Phe[667] in *Taq* pol I is critical for sugar discrimination. Our data show that Phe[667] is also important for base discrimination and catalytic efficiency. The F667L mutant exhibited a higher K_m for correct dNTPs with no significant change in $V_{\max}$ (Table III), consistent with data for the *E. coli* F762A mutant (31). The F667L mutant also displayed anti-mutator activity, evidenced as a 3-fold reduction in mutant frequency (Table I) and greater than 10-fold reductions in A → T and G → T transversions (Table II). The reduction in A → T transversions was accompanied by increased ability to

discriminate against synthesis of A:A base pairs and reduced ability to extend the mispairs (Table III).

Direct interaction between Phe[667] and each of the four different incoming dideoxynucleoside triphosphates has been observed in the respective structures of the closed ternary Klentaq1 complexes, with the aromatic phenylalanine ring stacking against the nucleotide base (8, 9). It has been postulated that steric congruence between the active site and a complementary, Watson-Crick base pair contributes to fidelity. In fact, the tight binding pocket that contains the nascent base pair in the Klentaq1 closed ternary complex and is formed in part by Phe[667] is incompatible with a mismatched base pair (Ref. 8 and Fig. 2). The F667L mutant is particularly favorable for assessing the importance for fidelity of stacking interactions between Phe[667] and the incoming dNTP, because the substituted Leu[667] side chain provides hydrophobicity and most of the space filling function of Phe[667]. Our data indicate that removal of the stacking interactions has a disproportionately large effect on A → T transversion mutagenesis, likely because of reduction in both formation and extension of A:A mispairs. Animated visualization of three reported structures of Klentaq1 (8) gives the impression that, concomitant with rotation of the O-helix, the planar benzyl ring of Phe[667] acts as a sweep or paddle that aids delivery of the bound dNTP to the active site and helps to maintain the nucleotide within the catalytic pocket. Perhaps loss of this chaperoning effect and concomitant loss of stacking interactions in the closed complex reduces the overall catalytic efficiency of the F667L polymerase, disproportionately affecting bulky mispairs responsible for transversion errors.

A661E,I665T,F667L Taq pol I: A Transversion and Frameshift Anti-mutator—The A661E,I665T,F667L polymerase exhibited catalytic efficiency intermediate between that of the A661E and F667L single mutants in a standard incorporation assay employing a gapped DNA substrate. In accord, the high K_m values for incorporation of dNTPs into oligonucleotide template-primers exhibited by the F667L polymerase were much reduced in the triple mutant polymerase (Table I). With respect to fidelity, the triple mutant harbors amino acid substitutions that, when present singly, are a strong general mutator mutation (A661E), a mutation that is fidelity-neutral or nearly so (I665T), and a mutation that greatly reduces transversion mutagenesis (F667L). When present in the same polypeptide, these three mutations do not confer a fidelity that is the sum of the fidelities of the single mutants or even an intermediate fidelity. Rather, the mutations interact functionally to create a distinctive mutational specificity. Remarkably, the strong mutator effect of the A661E single mutation is entirely suppressed in the A661E,I665T,F667L polymerase, the error frequency being 25-fold lower than that for the A661E polymerase (Table I). The triple mutant retains the anti-transversion activity characteristic of the F667L polymerase (Table II) and yielded no frameshifts at nonreiterated sites, the reduction in frequency being at least 20-fold relative to wild type pol I. Thus, either F667L or I665T or both together act as intragenic suppressors of the A661E mutator activity, demonstrating that functional interactions among O-helix residues can greatly affect the capacity of *Taq* pol I for mutation synthesis. Most likely, the presence of Phe[667] is required for the increased mutation frequency conferred by Glu[661] as a single substitu-

tion. We conjecture that the stacking and space filling functions of Phe[667] that putatively promote delivery of the dNTP to the active site and help to position the nucleotide in the catalytic pocket are of primary importance and that Glu661 cannot promote mutation synthesis in their absence. Our findings for the A661E,I665T,F667L polymerase emphasize two apparent roles of Phe[667] in wild type *Taq* pol I, namely maintenance of the characteristic error frequency and spectrum and preservation of catalytic efficiency.

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