Side Chains That Influence Fidelity at the Polymerase Active Site of Escherichia coli DNA Polymerase I (Klenow Fragment)*

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To investigate the interactions that determine DNA polymerase accuracy, we have measured the fidelity of 26 mutants with amino acid substitutions in the polymerase domain of a 3’-5’-exonuclease-deficient Klenow fragment. Most of these mutant polymerases synthesized DNA with an apparent fidelity similar to that of the wild-type control, suggesting that fidelity at the polymerase active site depends on highly specific enzyme-substrate interactions and is not easily perturbed. In addition to the previously studied Y766A mutant, four novel base substitution mutants were identified; they are R668A, R682A, E710A, and N845A. Each of these five mutant alleles results from substitution of a highly conserved amino acid side chain located on the exposed surface of the polymerase cleft near the polymerase active site. Analysis of base substitution errors at four template positions indicated that each of the five mutant polymerases has its own characteristic error specificity, suggesting that the Arg-668, Arg-682, Glu-710, Tyr-766, and Asn-845 side chains may contribute to polymerase fidelity in a variety of different ways. We separated the contributions of the nucleotide insertion and mismatch extension steps by using a novel fidelity assay that scores base substitution errors during synthesis to fill a single nucleotide gap (and hence does not require mismatch extension) and by measuring the rates of polymerase-catalyzed mismatch extension reactions. The R682A, E710A, Y766A, and N845A mutations cause decreased fidelity at the nucleotide insertion step, whereas R668A results in lower fidelity in both nucleotide insertion and mismatch extension. Relative to wild type, several Klenow fragment mutants showed substantially more discrimination against extension of a T-G mismatch under the conditions of the fidelity assay, providing one explanation for the anti-mutator phenotypes of mutants such as R754A and Q849A.

Three steps are responsible for the high fidelity of DNA replication; they are nucleotide selectivity and exonucleolytic proofreading of errors by the polymerase and post-synthetic correction of mismatches. Among these, the DNA polymerase itself usually provides the greatest contribution to fidelity, producing on average only one substitution error for every 104 to 105 nucleotides incorporated, depending on the polymerase (1). Polymerase errors can be initiated either by misinsertion of a nucleotide or by misalignment of the template-primer. Either initiating event can ultimately result in base pair substitution, deletion, or addition. Using as a model system the extremely well characterized Klenow fragment of Escherichia coli DNA polymerase I, our approach for understanding the structural basis for polymerase fidelity has been to screen a battery of amino acid substitutions directed to the active site region, including side chains thought to be involved in nucleotide binding and interactions with primer and template.

Polymerases from different families share fundamental similarities in tertiary structure and catalytic mechanism (reviewed in Refs. 2–5). The polymerase domains resemble a partially open right hand, with a cleft formed by three subdomains that have been designated fingers, palm, and thumb. The palm subdomain, which forms the base of the polymerase cleft, contains important active site residues, in particular the cluster of carboxylate side chains that coordinate the pair of divalent metal ions that catalyze the polymerase reaction (6). The fingers subdomain also contributes side chains to the active site, especially those involved in nucleotide recognition (7, 8). The thumb subdomain interacts with the template-primer duplex upstream of the site of nucleotide addition (8–11). Many of the amino acid side chains that form the exposed surface of the polymerase cleft are highly conserved in their respective polymerase families.

Although the only structural information for Klenow fragment with duplex DNA is an editing complex with the DNA primer terminus at the 3’-5’-exonuclease active site (12), co-crystal structures with DNA at or close to the polymerase active site have been reported for other polymerases of the pol I family. Binary polymerase-DNA complexes have been described for the DNA polymerase I of Thermus aquaticus (Taq DNA polymerase) (10) and for the Klenow fragment analog derived from Bacillus stearothermophilus DNA polymerase I (11), and a ternary complex (polymerase-DNA-ddNTP) has been solved with the DNA polymerase of bacteriophage T7 (8). Because of the high degree of structural and sequence similarity, information from these three co-crystals can be used to interpret data obtained with Klenow fragment mutants.

Klenow fragment catalyzes DNA synthesis with high fidelity, having an average base substitution error rate of ∼2 × 10⁻⁸. The abbreviations used are: pol I, DNA polymerase I; Pu, purine.
10⁻⁵ and an average frameshift error rate of ~5 × 10⁻⁶, even in the absence of exonucleolytic proofreading (13). This polymerase discriminates against nucleotide misinsertion by having a lower binding affinity for the incorrect dNTP and a slower catalytic rate for misinsertion than for correct insertion (14); although the precise values vary from mispair to mispair, the rate discrimination is quantitatively larger than the nucleotide binding discrimination (15). To date, two mutation sites that influence fidelity in Klenow fragment have been described. Substitution of Ser or Ala for Tyr-766, at the carboxyl terminus of the long O-helix in the fingers subdomain (see Fig. 1), results in decreased nucleotide selectivity during polymerization (15) and a consequent increase in base substitution errors and frameshift errors at non-reiterated sequences (16). A Klenow fragment mutant that lacks 24 amino acids at the tip of the thumb subdomain has decreased DNA binding affinity and processivity and an increased rate of frameshift errors at homopolymeric sequences (17). These properties are consistent with the interactions of the thumb tip with the minor groove of the template-primer duplex that have been observed crystallographically (8, 10, 12). In this study we have screened a large number of mutations in the polymerase cleft region of an exonuclease-deficient Klenow fragment, for effects on polymerase fidelity, and have identified four new mutator DNA polymerases, each with its own distinct error specificity.

**EXPERIMENTAL PROCEDURES**

**Materials**

*Strains and Reagents—*Bacterial strains and reagents for polymerase fidelity measurements have been described (18). Oligonucleotides—Oligonucleotides were from Research Genetics. The 20-mer primers, 5'-GTAACCCAGGTTTTTCTCA and 5'-GTAACGCGGTTTTTCTCG, when annealed to the 33-mer template, (5')AACGTGACTGAGAAAACCTGGCGTTACCCA, give a correct (T'A) or an incorrect (TG) base pair, respectively, at the primer terminus. The template sequence corresponds to the region surrounding the opal codon introduced at positions 87–89 of the lacZα sequence, such that the primer terminal base is paired with the T of the TGA codon.

*Mutant Polymerases—*Mutant derivatives of Klenow fragment were constructed and purified as described (19, 20). Biochemical properties of the majority of these proteins have been reported previously (7, 15, 19, 21, 22); exceptions are N579A, S582A, S608A, T609A, R631A, K635A, S670A, N675A, Q677A, N678A, S707A, and R835L. All polymerases in this study carried the D424A mutation, which eliminates the 3'-exonuclease activity (23).

**Methods**

**DNA Synthesis Reactions—*Reactions (25 μl) were performed as described previously (18), with 150 ng of gapped M13mp2 DNA in 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 2 mM dithiothreitol, and 1 mM each of dATP, dTTP, dGTP, and dCTP. Wild-type or mutant polymerase was added to initiate synthesis. The amounts (in picomoles) of each enzyme needed to fill the gap completely, determined by electrophoresis of a DNA synthesis condition were based on a slightly different short gap fidelity assay (27). Reactions (20 μl) contained 20 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 10 mM MgCl₂, 1 mM ATP, 150 fmol of gapped DNA, 1 mM each dATP, dTTP, dGTP, and dCTP, and 400 units of T4 DNA ligase. DNA synthesis was initiated by addition of wild-type or mutant Klenow fragment derivatives in the amounts described above. After incubation at 37 °C for 60 min, reactions were stopped by addition of EDTA to a final concentration of 15 mM. Reaction products were fractionated on a 0.8% agarose gel, and covalently closed circular products were isolated by electroelution from the agarose gel for transfection into E. coli strain MC1061 as above.

**RESULTS**

**Mutant Derivatives of Klenow Fragment—*The Klenow fragment mutations that we studied are indicated in Fig. 1. Wild-type residues were replaced by Ala (or, in one case, Leu) in order to remove potentially important side chain interactions. The majority of these 28 side chains are highly conserved in the pol I family of polymerases (Fig. 2) and all reside on or near the exposed surface of the cleft in the polymerase domain (Fig. 1). Most of the mutant proteins came from earlier studies on the role of side chains involved in catalysis and nucleotide binding within the polymerase active site region on the palm and fingers subdomains (7, 19, 21). Additional mutations were made in side chains, primarily in the thumb subdomain, that were identified as contacts to the DNA duplex in the Klenow fragment-DNA editing complex (12). In order to focus on fidelity-determining processes at the polymerase active site without complications due to exonucleolytic proofreading, all Klenow fragment derivatives in this study carried the 3'-5' exonuclease-deficient D424A mutation (23). For simplicity, each protein is described by the genotype of the polymerase domain; thus the D424A control is referred to as wild-type, and E710A denotes the double mutation D424A,E710A.

**Forward Mutation Assay—*We first measured the frequency of errors produced by each mutant polymerase during gap-filling synthesis across a lacZα target in phage M13mp2 (Fig. 2). The wild-type polymerase gave lacZα mutants at a frequency of 57 × 10⁻⁴ (Fig. 2), based on data from this and previous studies (13, 17). The D705A and D882A derivatives of Klenow fragment, which have very low catalytic activity, were unable to perform gap-filling synthesis even when a vast excess of polymerase was present in the reaction and were not investigated further. Of the remaining 26 proteins, the majority gave forward mutant frequencies similar to that of wild-type Klenow fragment. Several mutant derivatives, particularly R754A, Q849A, and H881A, gave lacZα mutants at frequencies lower than that observed for the wild-type enzyme. The R668A and Y766A polymerases gave mutants at 2.6- and 3.7-fold higher frequencies, respectively, than wild-type. The Y766A polymerase had previously been demonstrated to be a strong base

**Fidelity Mutants of Klenow Fragment**

TTTT run at positions 70–73. Plus one or −2 frameshifts were monitored in a substrate containing a run of 5 Ts at the same position but in the −1 reading frame (25).

**Single Nucleotide (TG“A”) Gap Fidelity Assay—*An M13mp2 substrate having a single nucleotide gap opposite the template A of a TGA codon artificially introduced within the lacZα codon, which have very low catalytic activity, were unable to perform gap-filling synthesis even when a vast excess of polymerase was present in the reaction and were not investigated further. Of the remaining 26 proteins, the majority gave forward mutant frequencies similar to that of wild-type Klenow fragment. Several mutant derivatives, particularly R754A, Q849A, and H881A, gave lacZα mutants at frequencies lower than that observed for the wild-type enzyme. The R668A and Y766A polymerases gave mutants at 2.6- and 3.7-fold higher frequencies, respectively, than wild-type. The Y766A polymerase had previously been demonstrated to be a strong base
substitution mutator (15, 16). The N678A, R682A, and E710A polymerases also gave smaller but reproducible increases in the frequency of *lacZ* mutants.

**FIG. 1.** Positions of amino acid side chains altered in this study. A stereo view of the α-carbon backbone of the polymerase domain (amino acids 548–928) of Klenow fragment (12). The palm subdomain (residues 648–717 and 848–928) is colored red, the fingers (residues 718–847) blue, and the thumb (residues 548–647) green. Residues 601–608, at the tip of the thumb subdomain, are missing because they were disordered in the co-crystal. The side chains shown are those mutated for this study. Mutations of the gray residues gave no change in fidelity (relative to wild-type Klenow fragment), mutations of the purple residues had an anti-mutator phenotype, and mutations of the green residues had a mutator phenotype. The carboxylate triad, Asp-705, Asp-882, and Glu-883, at the polymerase active site is shown in yellow. This figure was generated using Ribbons (36).

**FIG. 2.** Amino acid conservation and forward mutant frequencies for mutant derivatives of Klenow fragment. The approximate positions of the Klenow fragment mutations studied are indicated relative to the linear sequence of the polymerase domain, together with the corresponding forward mutant frequencies for DNA synthesis across the *lacZα* target (n.s., no synthesis.). Where measurements were made in duplicate, both values are listed; results from three or more determinations are given as mean ± S.D., with the number of determinations in parentheses. The background frequency (not subtracted) for uncopied DNA is 5 to 7 × 10⁻⁶. Additional information was obtained by sequencing the *lacZ* mutations recovered from three experiments; these were from R631A (47 mutations), H734A (48 mutations), and R841A (100 mutations). In all three cases the error distribution was not significantly different from that of wild-type Klenow fragment. Also shown is the extent to which each mutated side chain is conserved in the pol I family of DNA polymerases; where the side chain is not invariant, conservative substitutions are listed in parentheses. For residues from Asp-705 to the carboxyl terminus the sequence conservation information is taken from a partial alignment of the sequences of 23 bacterial, 7 bacteriophage, 8 mitochondrial, and 2 eukaryotic DNA polymerases (J. Jaeger and C. M. Joyce, unpublished work). For residues amino-terminal to Asp-705, the information is derived from the 23 bacterial sequences since the sequence motifs in this part of the protein are less well conserved and are therefore harder to identify with confidence in the more distantly related polymerase sequences.

| Pol I Family | Conservation | Mutant Frequency (x 10⁻⁴) |
|-------------|--------------|---------------------------|
| wild-type   |              |                           |
| N579A       | 22 (1H)      | 57 ± 14 (13)              |
| S582A       | 23           | 39                        |
| S608A       | 21 (2I)      | 39                        |
| T609A       | 22           | 39                        |
| R631A       | 23           | 32                        |
| R635A       | 21 (2R)      | 79                        |
| R668A       | 23           | 44                        |
| S670A       | 21 (1R)      | 160 ± 16 (4)              |
| N675A       | 23           | 59                        |
| Q677A       | 23           | 56                        |
| N678A       | 23           | 20                        |
| R682A       | 20 (3R)      | 120, 84                   |
| D705A       | 40           | 90 ± 28 (4)               |
| R707A       | 26 (2T)      | n.s.                      |
| Q708A       | 26           | 46                        |
| K710A       | 40           | 83, 110                   |
| H734A       | 38           | 120 ± 6 (3)               |
| R754A       | 40           | 39                        |
| K758A       | 40           | 5, 19                     |
| P762A       | 25 (14Y, 1L) | 24, 34                    |
| Y766A       | 40           | 30                        |
| R835L       | 6 (1K)       | 210, 220                  |
| R841A       | 26 (1K, 2H)  | 79                        |
| N845A       | 40           | 47                        |
| Q849A       | 40           | 66 ± 13 (3)               |
| H881A       | 40           | 9 ± 2 (4)                 |
| D882A       | 40           | 15                        |
| R883A       | 38           | n.s.                      |

Substitution mutator (15, 16). The N678A, R682A, and E710A polymerases also gave smaller but reproducible increases in the frequency of *lacZ* mutants.

Reversion Assays—Because the forward mutation assay detects many different types of errors in a variety of sequence contexts, substantial changes in particular subsets of errors
may not be apparent unless large numbers of lacZ mutants are sequenced. For example, although the forward mutant frequency for the Y766A derivative was increased only 3.7-fold overall (Fig. 2), a detailed analysis of error specificity in the lacZ forward mutant assay indicated that this enzyme exhibited a 60-fold increase in misincorporation of dTMP opposite template G and greater than 10-fold increases in several other base substitution and deletion errors (16). In the present study of 26 mutant polymerases, it was clearly impractical to sequence large collections of lacZ mutants generated by each enzyme (although this was done for a few enzymes, see legend to Fig. 2). We therefore decided to use a series of reversion assays that monitor base substitution or frameshift errors at specific template positions. These assays, which are highly sensitive due to low background mutation frequencies, were used to examine a number of different Klenow fragment derivatives, including those having forward mutant frequencies greater than the wild-type value.

**Base Substitution Reversion**—The first reversion substrate was an M13mp2 DNA with a 361-base gap containing a TGA which behaved as anti-mutators in both the forward mutation could certainly apply to the R754A and Q849A derivatives, but we have studied (see below), then this may either mask the enzyme's ability to extend particular mispairs will lead to their under-representation in the spectrum of mutations recovered from the assay. Moreover, if a mutant polymerase is more discriminating than the wild-type enzyme in mismatch extension under the assay conditions (as are several of the Klenow fragment mutants we have studied (see below)), then this may either result in an anti-mutator phenotype or limit the ability to detect reduced fidelity in the insertion step. This reasoning could certainly apply to the R754A and Q849A derivatives, which behaved as anti-mutators in both the forward mutation and the TGA₉₉ reversion assay (Fig. 2 and Table I). To separate the effects of nucleotide insertion selectivity and mismatch extension, we developed the TGₐₐ reversion substrate

| Enzyme           | Plaque number | Reversion frequency (× 10⁻⁵) |
|------------------|---------------|------------------------------|
| TGₐ₉₉ reversion substrate |               |                              |
| Wild-type        | 32            | 270                          | 12 (17 ± 5; n = 6) |
| R631A            | 103           | 540                          | 19                              |
| R675A            | 29            | 300                          | 10                              |
| R675A            | 36            | 310                          | 12                              |
| N678A            | 32            | 270                          | 12                              |
| Q708A            | 19            | 230                          | 8                               |
| R835L            | 34            | 160                          | 21                              |
| R841A            | 16            | 140                          | 11                              |
| H881A            | 17            | 120                          | 14                              |
| R668A            | 120           | 420                          | 29 (34)                         |
| R682A            | 163           | 450                          | 36 (29 ± 6; n = 3)               |
| E710A            | 68            | 100                          | 68 (80)                         |
| Y766A            | 157           | 140                          | 110                             |
| N845A            | 55            | 180                          | 31 (30 ± 2; n = 3)               |
| R754A            | 12            | 300                          | 4                               |
| Q849A            | 2             | 250                          | 0.8 (2)                         |

One-nucleotide gap TGₐₐ reversion substrate

| Enzyme           | Plaque number | Reversion frequency (× 10⁻⁵) |
|------------------|---------------|------------------------------|
| Wild-type        | 104           | 210                          | 50 (50 ± 10; n = 6)             |
| R631A            | 48            | 130                          | 37                              |
| R675A            | 194           | 280                          | 70                              |
| N678A            | 112           | 150                          | 75                              |
| Q708A            | 31            | 61                           | 51                              |
| H734A            | 13            | 52                           | 25                              |
| K758A            | 15            | 51                           | 29                              |
| F762A            | 8             | 46                           | 17                              |
| R835L            | 8             | 23                           | 35 (79)                         |
| R841A            | 36            | 100                          | 36                              |
| H881A            | 35            | 270                          | 13                              |
| E883A            | 5             | 82                           | 60                              |
| R668A            | 119           | 170                          | 70 (35)                         |
| R682A            | 323           | 360                          | 90 (80)                         |
| E710A            | 369           | 260                          | 140 (140)                       |
| Y766A            | 325           | 120                          | 270                             |
| N845A            | 229           | 100                          | 230 (280)                       |
| R754A            | 12            | 39                           | 31 (28)                         |
| Q849A            | 73            | 440                          | 17 (10)                         |

TABLE I

lacZa revertants generated during synthesis by wild-type and mutant derivatives of Klenow fragment

a All the proteins in this study carry the D424A (exonuclease-deficient) mutation.

b The first three columns show complete data from a single experiment. Where available, reversion frequencies from a second independent experiment are in parentheses. Data from three or more independent experiments are given as average ± S.E. The background reversion frequency for uncopied DNA was 0.5 × 10⁻⁵ for the TGₐ₉₉ substrate and 0.6 × 10⁻⁵ for the TGₐₐ substrate.

assay and the TGA₉₉ reversion assay (Fig. 2 and Table I). To separate the effects of nucleotide insertion selectivity and mismatch extension, we developed the TGₐₐ reversion substrate that requires only insertion of a single nucleotide and does not depend on mismatch extension to score an error. With this substrate, all three possible misinsertions opposite the template A in the gap are scored as revertants. In the TGₐₐ assay, wild-type Klenow fragment gave an average reversion frequency of 50 × 10⁻⁵ (Table I), which corresponds to an increase of >16-fold over the frequency of errors recovered at the template A position in the TGA₉₉ gap-filling assay. The substantial increase in recovery of A and G misinsertions opposite the template A (Table II) was as expected since purine-purine mismatches are particularly poor substrates for extension by Klenow fragment (28). Removing the requirement for mismatch extension also unmasked a strong preference of wild-type Klenow fragment for dATP insertion errors; again, this was as expected from earlier biochemical studies (28).

Sequence analysis of the revertants obtained from the four newly identified mutator polymerases showed that each mutator has its own characteristic error specificity and that the
results of the TGA<sub>op</sub> and TG"A" assays can differ in informative ways (Table II). As indicated above, AdCTP errors dominated the revertants generated by the E710A protein; this contrasts with wild-type Klenow fragment and the other mutator derivatives, which make predominantly TdT errors. From the small number of light blue plaques obtained with E710A in the TGA<sub>op</sub> assay, we can infer that the frequency of TdT errors must actually be lower than the wild-type value. The R668A, R682A, and N845A mutants all gave ~2–3-fold increases in TdT errors; because TdT is such a common error, these changes were largely responsible for the increased reversion frequency in the TGA<sub>op</sub> assay. However, other changes, which had less effect on the overall reversion frequency, are probably more interesting mechanistically because the change relative to wild-type was greater. Thus, R682A and N845A both gave ~10-fold increases in TdT errors. Intriguingly, R668A, R682A, and N845A changed the distribution of errors opposite the template G in the TGA<sub>op</sub> opal codon; all three preferentially formed GdGTP mismatches rather than GdATP mismatches, whereas the opposite was true for wild-type Klenow fragment.

The errors recovered opposite the template A positions in the two reversion assays gave information about misinsertion specificity and, by comparing the two assays, about mispair extension. Thus, N845A was a stronger mutator in the TG"A" single nucleotide insertion assay than in the TGA<sub>op</sub> assay, because this enzyme tends to make A-dATP insertion errors (~6-fold increase over wild type) but is very inefficient at extending the resulting A-A mispairs. R668A, on the other hand, does not appear to be a mutator in the TG"A" assay (Table I) because the most dramatic increase is in a relatively uncommon error (A-dGTP), and there is little change in the frequency of the dominant A-dATP error (Table II). In contrast with the results of the TG"A" assay, the R668A mutant polymerase gave no A-Pu errors in the gap-filling TGA<sub>op</sub> assay, suggesting that this protein has particular difficulty extending A-Pu mismatches. The R682A mutation caused an increase in A-dATP errors in both assays; the increase in the TGA<sub>op</sub> assay may indicate an improvement in extension of A-A mispairs.

The two anti-mutators examined in the single nucleotide assay gave slightly different results from one another. The reversion frequency obtained with the R754A polymerase in the TG"A" assay was similar to the value for the wild-type enzyme, suggesting that the anti-mutator phenotype of this protein in the gap-filling assays is due to increased discrimination at the mismatch extension step. The reversion frequency obtained with the Q649A derivative was about 3-fold lower than the wild-type value in the TG"A" assay, whereas the value in the TGA<sub>op</sub> gap-filling assay was barely above the assay background (Table I). These results suggest that the anti-mutator phenotype of Q649A is largely determined by mismatch extension, although there may also be a small contribution from increased selectivity at the misinsertion step.

**Frameshift Reversion Substrates**—We examined some of the mutant Klenow fragment derivatives for single base frameshift fidelity, using a pair of frameshift reversion substrates containing a run of 5 template thymidine residues in the lacZ<sub>loc2a</sub> target. These assays score primarily single nucleotide deletion or addition errors, respectively. We focused mainly on Klenow fragment derivatives having mutations in the thumb subdomain because previous experiments with Klenow fragment and human immunodeficiency virus-1 reverse transcriptase have suggested that contacts between this region of the polymerase and the template-primer duplex are important in controlling frameshift fidelity (17, 29). Although the Δ590–613 mutant of Klenow fragment, lacking 24 residues of the thumb tip, is a frameshift mutator (Table III; Ref. 17), single alanine substitutions at highly conserved amino acids in the thumb tip (Asn-579, Ser-582, Ser-608, and Thr-609) had little effect on frameshift fidelity with either substrate (Table III). Since there are extensive sequence-independent interactions between the thumb tip and the sugar phosphate backbone of the duplex template-primer (8, 10), removal of a single interaction may not be

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**Table II**

**Base substitution error specificity for mutator polymerases in two opal codon reversion assays**

| Mutation                | Wild-type | R668A | R682A | E710A | N845A |
|-------------------------|-----------|-------|-------|--------|--------|
|                         | Rev. E.R. (<10<sup>−6</sup>) | Rev. E.R. (<10<sup>−6</sup>) | Rev. E.R. (<10<sup>−6</sup>) | Rev. E.R. (<10<sup>−6</sup>) | Rev. E.R. (<10<sup>−6</sup>) |
| TGA<sub>op</sub> reverse substrate<sup>a,b</sup> | | | | | |
| T → C TdTTP             | 49        | 136   | 74    | 390↑   | 35    | 230   | 67    | 340↑   |
| T → A TdTTP             | 5         | 14    | 1     | 5      | 3     | 19    | 6     | 30     |
| T → G TdCTP             | 1         | 2.8   | 1     | 5      | 6     | 39↑   | 7     | 35     |
| G → C GdGTP             | 7         | 19    | 12    | 64↑    | 4     | 26    | 9     | 45     |
| G → T GdATP             | 22        | 61    | 5     | 27↓    | 3     | 19↓   | 0     | ≤5↓    |
| A → T AdATP             | 12        | 33    | 0     | ≤5↓    | 16    | 103↑  | 7     | 35     |
| A → G AdCTP             | 6         | 17    | 7     | 37↑    | 8     | 52↑   | 4     | 20     |
| A → C AdGTP             | 0         | ≤2.8  | 0     | ≤5     | 0     | ≤5    | 0     | ≤5     |
| Total analyzed          | 102       | 100   | 75    | 136    | 100    |
| One-nucleotide gap TG"A" reverse substrate<sup>c</sup> | | | | | |
| A → T AdATP             | 41        | 680   | 28    | 600    | 38    | 1100  | 17    | 800    | 47    | 4200↑ |
| A → G AdCTP             | 1         | 17    | 3     | 65↓    | 0     | ≤30   | 32    | 1500↑  | 0     | ≤88   |
| A → C AdGTP             | 1         | 17    | 19    | 190    | 0     | ≤30   | 0     | ≤47    | 1     | 88    |
| Total sequenced          | 50        | 41    | 47    | 50     | 49     |
Frameshift reversion frequencies for Klenow fragment mutants

| Enzyme<sup>b</sup> | Plaque number | Reversion frequency (× 10<sup>3</sup>) |
|-------------------|--------------|-------------------------------------|
|                  | Revertant    | Total                               |
| TTTTT in the plus-one reading frame (scores −1 frameshifts) |             |                                      |
| Wild-type         | 24 ± 12 (n = 6) | 270                                  |
| Δ(590–613)<sup>b</sup> | 739          | 270                                  |
| N579A             | 48           | 480                                  |
| S582A             | 20           | 280                                  |
| S608A             | 143          | 380                                  |
| T609A             | 63           | 310                                  |
| R631A             | 34           | 130                                  |
| K635A             | 59           | 140                                  |
| R668A             | 14           | 170                                  |
| N675A             | 52           | 330                                  |
| N678A             | 462          | 950                                  |
| R682A             | 38           | 200                                  |
| Q708A             | 50           | 250                                  |
| E710A             | 2            | 150                                  |
| N845A             | 57           | 230                                  |
| Q849A             | 1            | 170                                  |
| Background<sup>c</sup> | ≤0.8       |                                      |

| Enzyme<sup>b</sup> | Plaque number | Reversion frequency (× 10<sup>3</sup>) |
|-------------------|--------------|-------------------------------------|
|                  | Revertant    | Total                               |
| TTTTT in the minus-one reading frame (scores +1/−2 frameshifts) |             |                                      |
| Wild-type         | 31 ± 10 (n = 6) | 83                                  |
| Δ(590–613)<sup>b</sup> | 225          | 270                                  |
| N579A             | 20           | 99                                   |
| S582A             | 27           | 80                                   |
| S608A             | 14           | 78                                   |
| T609A             | 21           | 63                                   |
| R631A             | 21           | 50                                   |
| K635A             | 12           | 34                                   |
| R668A             | 5            | 130                                  |
| N675A             | 45           | 94                                   |
| R682A             | 249          | 490                                  |
| Q708A             | 9            | 62                                   |
| E710A             | 1            | 73                                   |
| Background<sup>c</sup> | ≤0.8       |                                      |

<sup>a</sup> All the proteins in this study carry the D424A (exonuclease-deficient) mutation.  
<sup>b</sup> Apparent first-order rate constants for extension of a matched (T<sub>A</sub>) or mismatched (T<sub>G</sub>) primer terminus under reaction conditions similar to those used in the fidelity assays. At the dNTP concentration used (1 mM), the rate constants probably reflect k<sub>ext</sub>. All measurements were carried out at least twice.

The processes that take place at the polymerase active site of Klenow fragment are extremely accurate, allowing on average only one error for every 10<sup>4</sup> to 10<sup>6</sup> nucleotides incorporated. Which parts of the protein are responsible for maintaining this high fidelity? Our analysis of 26 mutations in the polymerase domain of Klenow fragment has identified four novel base substitution mutators (R668A, R682A, E710A, and N845A) to add to the Y766A and Y766S mutators, previously described (15, 16). It has also identified several mutations in conserved residues that result in an anti-mutator phenotype.

Mutations Having No Apparent Effect on Polymerase Fidelity—The majority of the Klenow fragment mutations in our study had little or no observable effect on polymerase fidelity. Although nucleotide binding and catalysis might be considered crucial to nucleotide misinsertion, several mutations that affect these processes (e.g. F762A, K758A, and E883A, Refs. 7 and 21) did not influence fidelity. We also did not detect any substantial frameshift mutators, even among polymerases having mutations in regions that we had expected to play a role in controlling misalignment-mediated errors.

The small number of mutator polymerases revealed by our survey suggests that fidelity is determined by a limited number of interactions. However, it is possible that some interesting mutator polymerases may have escaped detection, even with the use of multiple assays, since there are several factors that can conceal a mutator effect. One factor is the broad error

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**Table III**

Frameshift reversion frequencies for Klenow fragment mutants

| Enzyme<sup>b</sup> | Plaque number | Reversion frequency (× 10<sup>3</sup>) |
|-------------------|--------------|-------------------------------------|
|                  | Revertant    | Total                               |
| TTTTT in the plus-one reading frame (scores −1 frameshifts) |             |                                      |
| Wild-type         | 24 ± 12 (n = 6) | 270                                  |
| Δ(590–613)<sup>b</sup> | 739          | 270                                  |
| N579A             | 48           | 480                                  |
| S582A             | 20           | 280                                  |
| S608A             | 143          | 380                                  |
| T609A             | 63           | 310                                  |
| R631A             | 34           | 130                                  |
| K635A             | 59           | 140                                  |
| R668A             | 14           | 170                                  |
| N675A             | 52           | 330                                  |
| N678A             | 462          | 950                                  |
| R682A             | 38           | 200                                  |
| Q708A             | 50           | 250                                  |
| E710A             | 2            | 150                                  |
| N845A             | 57           | 230                                  |
| Q849A             | 1            | 170                                  |
| Background<sup>c</sup> | ≤0.8       |                                      |

**Table IV**

Rates of extension of matched and mismatched primer termini

| Enzyme<sup>b</sup> | k<sub>TA</sub> (s<sup>−1</sup>) | k<sub>TG</sub> (s<sup>−1</sup>) | k<sub>TA/kTG</sub> |
|-------------------|-------------------------------|-------------------------------|------------------|
| R668A             | 3.3                          | 0.14                          | 24               |
| Y766S             | 28                           | 0.16                          | 180              |
| Y766A             | 24                           | 0.068                         | 350              |
| Q708A             | 110                          | 0.31                          | 360              |
| N845A             | 36                           | 0.097                         | 370              |

<sup>a</sup> All the proteins in this study carry the D424A (exonuclease-deficient) mutation.  
<sup>b</sup> Apparent first-order rate constants for extension of a matched (T<sub>A</sub>) or mismatched (T<sub>G</sub>) primer terminus under reaction conditions similar to those used in the fidelity assays. At the dNTP concentration used (1 mM), the rate constants probably reflect k<sub>ext</sub>. All measurements were carried out at least twice.
specificity of the M13mp2 forward mutation assay used here (Fig. 2), which means that a mutator effect at a subset of errors will be averaged over a large target and may be hard to detect simply by measuring the forward mutant frequency. Additionally, a mutator polymerase could increase the frequency of some errors while decreasing the frequency of others, giving little net change in the overall forward mutant frequency. The TG₈₆₉₉₉ reversion assay addresses some of these concerns by focusing on a smaller number of errors at a smaller target. It has the potential to amplify the signal from base substitution mutators (compare Fig. 2 with Table I), but it will fail to detect a putative mutator polymerase that has an error specificity that does not result in a large number of mutations at the opal codon site. Even in the reversion assays, frequencies are dominated by the most common errors, so that a dramatic increase in a rare error may not be apparent until the revertants are sequenced; consider, for example, the quantitatively rather small contributions of the T₉₈₁₈₉₈ and the A₉₈₄₉₈₉ promotors (which requires mispair extension) and the single gap TG₈₉₆₇ assay (Tables I and II).

The single nucleotide (TG₈₉₆₇) gap assay is an important addition to the repertoire of fidelity assays because it does not require mispair extension, which could limit detection of a mutator effect in the gap-filling fidelity assays. As expected, this assay allowed a greater recovery of errors resulting from purine-purine mispairs, which are usually refractory to extension (28), and revealed features of the error specificity (for both wild-type and mutant Klenow fragment derivatives) that would not have been suspected from the results of the two gap-filling assays.⁶

Anti-mutators and the Role of Mismatch Extension—A few Klenow fragment derivatives, particularly R₇₅₄₉₈, Q₈₄₉₉₈, and H₈₈₈₉₈, gave mutant frequencies in the fidelity assays that were substantially lower than the values for the wild-type enzyme. Several mutations, including these three, result in increased discrimination (relative to wild-type) against extension of a T-G mispair under conditions similar to those used in the fidelity assays (Table IV).⁷ Therefore, it seems likely that the anti-mutator behavior of these proteins is due largely to inefficient mispair extension, and this is consistent with the data for R₇₅₄₉₈ and Q₈₄₉₉₈ in the opal codon reversion assay (which requires mispair extension) and the single gap TG₈₉₆₇ assay (which does not) (Table I).

Of a representative sample of 13 Klenow fragment mutants, eight were more discriminating than wild type in T-G mispair extension (Table IV), resulting either in an anti-mutator phenotype (R₇₅₄₉₈ and Q₈₄₉₉₈) or in diminishing the apparent magnitude of a mutator phenotype (E₇₁₀₉₈). The only two proteins that were significantly less discriminating than wild-type were both mutators (R₆₈₈₉₈ and Y₇₆₆₈₉). Why do so many mutations cause increased discrimination in extension of this mispair? There is no obvious correlation with biochemical properties since the eight mutant proteins listed in Table IV cover a wide range of biochemical phenotypes (7, 17, 19, 21). It is also hard to know how to view the role of a side chain whose removal makes the polymerase more accurate. Perhaps the loss of an interaction with the primer terminus has a more significant effect on a mismatched DNA, which is already somewhat destabilized. This explanation could apply to Q₈₄₉₉₈ and H₈₈₈₉₈, where the mutated side chains contact the primer terminus, but not to R₇₅₄₉₈, since Arg-754 contacts the dNTP phosphate tail (7, 8). An alternative possibility, which does not depend on the mutated side chains having a specific role in fidelity, is that an apparent increase in fidelity in the mispair extension reaction may correspond to a shift in rate-limiting step to one that is inherently more discriminating.

Base Substitution Mutators—From this and our previous study (16) we have identified a total of at least five side chains (Arg-668, Arg-682, Glu-710, Tyr-766, and Asn-845) that play an important role in preventing base substitution errors. An additional possibility is Asn-67₈, since N₆₇₈ gave a small increase in mutant frequency in the forward assay as well as marginal effects in three of the four reversion assays (Tables I and III). The mutator Klenow fragment derivatives have a variety of biochemical phenotypes (7, 19, 21, 22, 31); relative to wild-type Klenow fragment, R₆₈₈₉₈ and E₇₁₀₉₈ show substantial decreases in reaction rate, R₆₈₈₉₈ and R₆₈₈₉₈, and E₇₁₀₉₈ have decreased DNA binding affinity, and all show moderate changes in Kₚₛ₃₃. Not only is it hard to discern any correlation of mutator phenotype with a particular subset of biochemical properties, but one can also find other Klenow fragment mutants with similar biochemical properties but no mutator phenotype. The obvious attributes these five side chains have in common are that they are all highly conserved in the pol I family and that they are clustered very near the polymerase active site.

Each of the four base substitution mutations we describe here has its own distinct error specificity. For E₇₁₀₉₈, the most frequent error is the AdGTP mispair (Table II), an error not frequently made by wild-type Klenow fragment. By contrast, the frequency of TdGTP errors is reduced (relative to wild-type), presumably because of the difficulty the E₇₁₀₉₈ protein has in extending the T-G mispair (Table IV). R₆₈₈₉₈ appears to favor errors involving insertion of dGTP; R₆₈₈₉₈ and N₈₄₅₈ both gave an increase in TdCTP errors, and N₈₄₅₈ also gave an increase in AdATP errors. (For comparison, the previously characterized mutants with substitutions at Tyr-76₈ (Y₇₆₆₈₉ and Y₇₆₆₈₉) gave increases in several types of errors, particularly transition mispairs (16).) Although these error specificities provide an interesting basis for consideration of fidelity mechanisms, an important caveat is that the data in Table II are derived from mutations recovered at two small targets. Some types of errors cannot be detected at these targets, and the possible effects of the surrounding sequence have not been determined. A more comprehensive description of the error specificity of the four mutator polymerases identified in the present study must therefore await a full analysis of lacZ mutations recovered in the forward mutational assay, as was carried out previously for both Y₇₆₆₈₉ and Y₇₆₆₈₉ mutants (16).

Whereas it seems likely that the errors made by all these mutator polymerases reflect a loss in fidelity in the insertion step, our data provide relatively little information on the way in which mispair extension might augment or diminish the observed error rates. The data of Table II suggest the need for caution in generalizing the kinetic data obtained with the T-G mispair (Table IV) to other mispairs. Thus, the R₆₈₈₉₈ mutant protein, which is the least discriminating in T-G extension (Table IV), may be particularly bad at extending A-Pu mispairs. Conversely, R₆₈₈₉₈ discriminates more against T-G mispairs

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⁵ A good illustration of these factors is provided by our data for the E₇₁₀₉₈ derivative; this protein gave a lacZ mutant frequency 2-fold above wild type in the forward mutation assay (Fig. 2), yet it is an 80-fold mutator for insertion of dCMP opposite the template A in the opal codon (Table II), a strong anti-mutator for frameshift errors (Table III), and a possible anti-mutator for TdGTP insertion errors (Tables II and IV).

⁶ Interestingly, the recovery of AdCTP errors using the single nucleotide gap substrate was similar to the frequency in the opal codon reversion assay, suggesting, perhaps, that the reaction conditions used for gap filling (long reaction times, high dNTP concentrations, and exounuclease-deficient enzymes) allow extension of all but the most refractory mismatches.

⁷ An anti-mutator must affect TdGTP, the most common error made by wild-type Klenow fragment, in order to have a detectable phenotype.
but, perhaps, less against A:A. Given the different geometrical constraints posed by individual mispairs, one might expect to see mispair-specific effects in the extension reaction, and we are currently investigating this issue.

**Fidelity Mutants in Relation to Polymerase Structures**—What is the structural basis for the decrease in fidelity resulting from mutations at Arg-668, Arg-682, Glu-710, Tyr-766, and Asn-845? Co-crystal data for polymerases related to Klenow fragment, especially the ternary complex structure of T7 DNA polymerase (8), provide an excellent starting point to explore this issue.\(^8\) A limitation, however, is that we can only guess at the structural changes that may take place when an incorrect incoming dNTP or a mispaired primer terminus is bound at the polymerase site. Structural information on complexes containing mismatches will be crucial for a full understanding of polymerase fidelity mechanisms.

Three general mechanisms (which are not mutually exclusive) have been proposed to account for polymerase fidelity; they are water exclusion from the active site, steric complementarity between the active site binding pocket and a nascent Watson-Crick base pair, and hydrogen bonding to the minor groove of the template-primer duplex and the nascent base pair. Water exclusion provides a possible mechanism for the polymerase to magnify the free energy difference between correct and incorrect base pairs in the context of the enzyme active site (32). Given that all the mutator mutants we have identified involve substitution of a smaller side chain, they could act by allowing access of water to the active site. To account for the observed error specificities, however, each alanine substitution should allow water access to a restricted and specific part of the active site so that all mispairs are not equally affected. A mutation that allows general water access, reducing selectivity throughout the active site, might be expected to increase the frequency of all polymerase errors; thus far we have not identified any mutator mutants with this phenotype.

Polymerase co-crystal structures support the idea that selectivity could depend on a precise fit of the binding pocket to a correct nascent base pair at the active site. In the T7 DNA polymerase ternary complex, the incoming nucleotide is sandwiched tightly between the primer terminus and conserved side chains on the O-helix, with no flexibility to accommodate mismatched base pairs (8). The side chains corresponding to our mutator mutants are clustered around this binding region, so that replacement of one of these side chains by Ala might compromise the tight fit of the binding site. To account for the observed error specificities, the changes in the binding site in each mutator polymerase must be such that binding of only a subset of nascent mispairs is enhanced. In general terms, we can imagine how this might work for the E710A mutant. The Glu-710 side chain is known to be close to the 2' position of the deoxyribose of the incoming nucleotide (8, 31). When Glu-710 is replaced by Ala, the additional space could therefore accommodate any aberrant base pair that deviates from normal geometry in a way that would place extra bulk in this part of the active site.

In the binary enzyme-DNA complex of *B. stearothermophilus* Klenow fragment, a tight pocket is seen surrounding the terminal base pair of the DNA duplex (11). All five of the side chains implicated by our mutator mutants, as well as the homologs of Gln-849 and His-881, are part of this pocket. These co-crystals are able to carry out nucleotide addition to the bound DNA, indicating (at a minimum) that the complex can achieve a catalytically competent conformation; however, the relationship of the observed primer terminus position to the position of the incoming nucleotide remains to be established. Although many similar interactions are seen in the polymerase binary and ternary complexes, the data for the invariant tyrosine, corresponding to Tyr-766 of Klenow fragment, is puzzling. In both *Taq* and *B. stearothermophilus* binary complexes with DNA, the Tyr-766 homolog is stacked against the template base at the end of the DNA duplex (10, 11), consistent with the idea that this side chain could play a role in template positioning (and consequently in fidelity) (7). In the T7 DNA polymerase ternary complex, however, the corresponding Tyr is hydrogen-bonded to the homolog of Glu-710 (8).\(^9\) An intriguing possibility is that this side chain might be actively involved in the movement of the O-helix that appears to accompany ternary complex formation.

It has long been recognized that polymerases should be able to distinguish correct from incorrect base pairs by hydrogen bonding to the minor groove of the template-primer duplex (33, 34). The positions of the two minor groove hydrogen bond acceptors (N-3 of purines and O-2 of pyrimidines) are similar in Watson-Crick base pairs (35) but are not the same in mismatched base pairs. In all three polymerase co-crystals the A-like conformation of the DNA duplex close to the active site creates a wider and shallower minor groove, facilitating interactions of the minor groove hydrogen bond acceptors with several of the side chains shown to be involved in fidelity in the present study (8, 10, 11). In both the T7 DNA polymerase ternary complex and the *B. stearothermophilus* binary complex, the side chain equivalent to Arg-668 interacts with the acceptor on the primer strand, and the Gln-849 homolog interacts with the acceptor on the template strand, of the terminal base pair. The *Taq* DNA polymerase binary complex is slightly different, perhaps because the primer terminus is further from the active site; the Gln-849 homolog (as part of a hydrogen-bonding network with residues equivalent to the Arg-668 homolog and Glu-710) interacts with the primer strand, whereas the residue equivalent to Arg-841 forms a hydrogen-bonded network with the acceptor on the template strand and the Asn-845 homolog. In the T7 DNA polymerase ternary complex, the Arg-668 homolog also interacts with the sugar of the incoming nucleotide. The interactions of Arg-668 with both primer terminus and incoming nucleotide might perhaps correlate with the effect of the R668A mutation on both misinsertion and mispair extension fidelity. For the other side chains discussed above, we have postulated that interactions with the template-primer duplex can influence selection of the incoming dNTP. This seems intuitively reasonable since the loss of a binding contact to template or primer strand could allow more flexibility in positioning the primer 3'-OH and an increased tolerance of geometrically aberrant nascent mispairs. The side chains equivalent to Arg-682 and Tyr-766 are not involved in hydrogen-bonding interactions with the minor groove in any of the co-crystal structures. This does not rule out an indirect contribution of these side chains to fidelity via this mechanism since removal of a side chain close to the primer terminus could modify DNA binding geometry and thus influence minor groove recognition.

In conclusion, we have found that mutations that alter base substitution fidelity are clustered around the primer terminus at the polymerase active site. This contrasts with mutations that affect errors resulting from strand slippage that are located further from the primer terminus in the duplex DNA.

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\(^{8}\) For simplicity, amino acid side chains are described in terms of the Klenow fragment numbering.

\(^{9}\) As pointed out elsewhere (31), this interaction is hard to reconcile with the biochemical consequences of mutations at Tyr-766 and Glu-710, particularly with the negligible effect of the Y766F mutation on polymerase fidelity and other biochemical properties (15, 16, 22).
binding region. Based on the available structural data for complexes of DNA polymerases of the pol I family, plausible scenarios can be envisaged that account in general terms for the observed mutator phenotypes. Our data do not exclude any of the mechanisms that have previously been proposed to explain nucleotide insertion fidelity, although the need to account for the observed error specificities will place quite stringent demands on any structural explanation that emerges. It is conceivable that DNA polymerases may use a combination of strategies to ensure fidelity and that the different mutator polymerases may act in quite distinct ways. For example, the important attribute of the E710A mutation could be that it diminishes steric complementarity between the active site and the nascent base pair, whereas R663A could affect fidelity because it removes an important hydrogen bond donor to the minor groove at the primer terminus. Structural studies on polymerase complexes containing mismatches should lead to a more complete understanding of polymerase fidelity, including a full explanation for the characteristic error specificities of the mutator polymerases described here.

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