DNA polymerases contain active sites that are structurally superimposable and conserved in amino acid sequence. To probe the biochemical and structure-function relationship of DNA polymerases, a large library (200,000 members) of mutant Thermus aquaticus DNA polymerase I (Taq pol I) was created containing random substitutions within a portion of the dNTP binding site (Motif A; amino acids 605–617), and a fraction of all selected active Taq pol I (291 out of 8000) was tested for base pairing fidelity; seven unique mutants that efficiently misincorporate bases and/or extend mismatched bases were identified and sequenced. These mutants all contain substitutions of one specific amino acid, Ile-614, which forms part of the hydrophobic pocket that binds the base and ribose portions of the incoming nucleotide. Mutant Taq pol Is containing hydrophilic substitution I614K exhibit 10-fold lower base misincorporation fidelity, as well as a high propensity to extend mismaps. In addition, these low fidelity mutants containing hydrophilic substitution for Ile-614 can bypass damaged templates that include an abasic site and vinyl chloride adduct ethenoA. During polymerase chain reaction, Taq pol I mutant I614K exhibits an error rate that is >20-fold higher relative to the wild-type enzyme and efficiently catalyzes both transition and transversion errors. These studies have generated polymerase chain reaction-proficient mutant polymerases containing substitutions within the active site that confers low base pairing fidelity and a high error rate. Considering the structural and sequence conservation of Motif A, it is likely that a similar substitution will yield active low fidelity DNA polymerases that are mutagenic.

Prolonged survival of individual species depends on the accurate transmission of genetic material from one generation to the next (1). However, in times of stress, the propensity to mutate and to rapidly create variants that can escape selection pressures facilitates survival of a small fraction of the original population (2). Thus, evolution may be characterized by periods of high fidelity DNA replication, as well as by the presence of transient mutators, which have a selective growth advantage during adverse conditions (3). Identifying mechanisms of generating potential mutators is crucial toward understanding the dynamic processes that govern evolution, as well as toward devising effective chemotherapeutic strategies against pathogenic bacteria (4, 5) and cells (6) that mutate at elevated rates.

Cells have evolved multistep mechanisms to guarantee the exceptionally high fidelity of DNA replication that is required for the maintenance of species. The genetic sequence of organisms is maintained over prolonged evolution by the fidelity of DNA replication (7), the efficiency of DNA repair processes (8), and the recombination-mediated lateral transfer events (9). Quantitatively, nucleotide selection at the active site of DNA polymerases is the most significant contributor to the fidelity of DNA replication (10). Nucleotide selection includes correct Watson-Crick base pair formation between complementary bases; further discrimination of base selection occurs by a conformational change at the active site during each nucleotide addition step (11) and preferential extension of the correct base pair by the addition of the next complementary nucleotide (12). Together, these processes contribute ~100,000-fold to the overall accuracy of DNA replication (one base change per $10^{9–10}$ bases per generation (13)). Inefficient extension of mispaired bases in vivo would facilitate 3′-5′ exonuclease removal of the nascent nucleotide. Exonucleolytic (3′-5′) proofreading activity of most DNA polymerases occurs on a separate domain (alternatively, this activity could reside in a separate protein) and contributes, on average, 10-fold to the overall mutation rate (14). In addition, errors in catalysis by DNA polymerases are subsequently corrected by a mismatch repair system, which contributes an additional 2–3 orders of magnitude to the overall accuracy of DNA replication (15). Disruption of either mismatch repair system or polymerase 3′-5′ exonuclease function within cells leads to a mutator phenotype (16, 17). Mice harboring disruption in mismatch repair (18) or in the 3′-5′ exonuclease of DNA polymerase δ develop cancer in multiple organs with elevated frequency. These studies provide direct evidence linking deficits in the fidelity of DNA synthesis with increased incidence of cancer.

The structure of a DNA polymerase resembles the human right hand and contains three distinct subdomains (finger, palm, and thumb (19)). High resolution crystal structures of DNA polymerase within the pol I$^*$ family of enzymes indicate that the base of the incoming nucleotide stacks with the hydrophobic planar amino acids located in the fingers subdomain (Motif B), and the triphosphate portion is bonded through metal cations by ionic interactions with Asp-610 located in the palm subdomain (Motif A (20, 21)). During nucleotide incorporation, DNA polymerases undergo a conformational change from open to a closed conformation, bringing the fingers subdomain.

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A Single Highly Mutable Catalytic Site Amino Acid Is Critical for DNA Polymerase Fidelity*}

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1 B. Preston, personal communication.
2 The abbreviations used are: pol I, polymerase I; Taq, Thermus aquaticus; WT, wild-type; PCR, polymerase chain reaction; kb, kilobase(s).
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corporation opposite template dA was measured for each primer-template construct as described (12).

**Template Bypass**—A 36-mer template (3'-gcg cgg ctt aag ggc gat cgt tat aac aag Xcg gtt-5') was hybridized with primer 23-mer (5'-cgc gcc gaa ttc cgg ctg cga at), where X is either ethenoA, abasic site, or dT residue. Incubations were done under standard conditions noted above with 250 μM each of the 4 dNTPs for 10 min at 55 °C in the presence of either WT Taq pol I and purified mutant 53, 265, 75, or 212 (at one of two concentrations: 2 or 20 fmol/μl). The site of the lesion is marked by an X.

**Error Spectrum**—The error spectrum of WT and mutant Taq pol I was determined using iterative replication of a 1.3-kb target using human thymidylate synthase. PCR samples contained 0.03 pmol of vector containing the target gene, 50 mM KCl, 10 mM Tris-HCl (pH 8), 0.1% Triton X-100, 1.5 mM MgCl₂, and 1 μM of purified Taq pol I (5 units) in 100-μl volumes in the presence of 250 μM of each of the dNTPs. All PCR samples were incubated at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 3 min for 45 cycles. Products (1.3 kb) from these amplification reactions were isolated by electrophoresis and cloned into the Topo T.A cloning vector. The cloned vector was transformed into bacteria, vectors from randomly chosen colonies were purified and sequenced, and 630 identical bases were analyzed for each clone. The error rate was determined by calculating the mutation frequency per base per duplication. The mutation frequency of each polymerase was determined by calculating the average number of mutations per clone per bases sequenced (630) following PCR. The error rate was determined by dividing the mutation frequency by the number replication cycles (as determined after calculating the product yield after PCR). The average number of replication cycles under PCR conditions were as follows: 8 (reflecting ~250-fold DNA amplification) by WT and mutant 346, 6 for mutant 75, and 5 for mutant 53.

**RESULTS**

**Identification of Mutant Polymerases with Altered Fidelity**—DNA polymerases contain active sites that are conserved in three-dimensional structure and amino acid sequence. Previously, we used random sequence mutagenesis coupled with genetic selection to demonstrate all active site amino acids, except those directly involved in the catalytic mechanism and/or protein tertiary structure, can be substituted by diverse amino acids (9). These data suggest active sites are highly plastic and can accommodate numerous amino acid substitutions without compromising protein function and viability of the organism. To determine whether substitutions within Motif A can affect the fidelity of DNA synthesis, we analyzed all 291 selected Taq pol I for the ability to incorporate noncomplementary nucleotides. Briefly, this analysis involved incubation of Taq pol I with 32P-labeled oligonucleotide primer-templates in the absence of the next complementary nucleotide. Products were separated by polyacrylamide gel electrophoresis and analyzed by autoradiography. Primer elongation in the absence of 1 or 2 correct dNTPs provides an effective initial screen for mutant enzymes with alterations in the fidelity of DNA synthesis (29, 30, 33).

Each of the 291 selected active Taq pol IIs, including 27 with wild-type amino acid sequence, was analyzed for the distribution of products synthesized in at least 10 different conditions (with combinations of 3 and 2 nucleotides using two separate primer-template constructs). Representative results with purified mutants (3 polymerases with low fidelity, 1 with high fidelity, 1 with normal fidelity, and WT Taq pol I) are shown in Fig. 2. In the presence of four dNTPs, all polymerases efficiently extend the primer to a position opposite the 5' template terminus; this result is consistent with the ability of the selected polymerases to complement E. coli polA12 strain and function in vivo. In the absence of a single dNTP, WT Taq pol I can misincorporate nucleotides and extend a mismatched primer to a limited extent. Both mismatch formation and subsequent extension are less efficient at repetitive template sequences of single nucleotides (i.e. positions at which Taq pol I must misincorporate opposite at least two successive identical nucleotides). Similar results were obtained for all 27 WT Taq pols containing silent mutations. A diverse distribution of elongation products was observed for the active mutants. Seven mutants consistently misincorporated and/or misextended bases at a higher frequency and synthesized longer products relative to WT Taq pol I in most conditions tested in which complementary nucleotides were lacking and thus are presumed to exhibit low fidelity. These low fidelity mutants can copy past sites at which the complementary nucleotide is missing, whereas the WT enzyme pauses at these sites. Several other polymerases synthesized a distribution of products significantly shorter than the WT Taq pol I (e.g. mutant 212) and thus potentially exhibit high fidelity.

All low fidelity mutants contain amino acid substitutions at a single position, Ile-614. This Ile-614 residue can be replaced with amino acids that differ in size, charge, shape, and hydrophilicity while maintaining near WT activity. Diverse amino acid substitutions at position 614, occurring alone or in concert with secondary substitutions, confer low fidelity (Fig. 2 and Table I). In contrast, a putative high fidelity polymerase with six substitutions, mutant 212 (L605K/L606M/V607K/A608S/L609I/S612R), does not contain a substitution at position 614.

**Measurement of Polymerase Fidelity**—DNA polymerase fidelity is conferred by 1) the ability of the active site to incorporate the proper templated nucleotide relative to noncomplementary nucleotides, and 2) the ability to extend a properly matched relative to a mismatched 3' primer termini (12). To investigate the efficiency of misinsertion, we measured the kinetics of incorporation of a single complementary and non-complementary nucleotide using WT and mutant Taq polymerases. Efficiency of nucleotide misinsertion was determined opposite a DNA template dT residue primed with a 23-nucleotide oligomer that was labeled at the 5'-end with 32P. Apparent Michaelis constant (Kₘ), apparent maximum velocity (Vₘₙₐₓ), and relative insertion frequency were measured for each dNTP (Table II). Each of the mutant polymerases incorporates the complementary nucleotide with a maximum velocity and cata-
lytic efficiency \( (V_{\text{max}}/K_m) \) similar to that of the WT. These enzymes formed base pairs opposite template dT in the following order: A:T > G:T > T:T > C:T. The catalytic efficiency for misincorporation of each of the noncomplementary nucleotides by mutant 53 (I614K) is \(~10\) times greater than that of the WT enzyme. This propensity by mutant 53 (I614K) to misincorporate nucleotides is largely due to lower \( K_m \) for improperly base paired dNTPs relative to the WT enzyme. Mutant 346 (A608D/E615D) exhibits a “mixed” fidelity, with a high propensity to catalyze some mispairs (e.g. dT:dC primer:template) and a lower tendency to catalyze other errors. In contrast, mutant 212, containing 6 substitutions within the catalytic site, appears less error-prone relative to WT enzyme. Furthermore, the kinetic data indicate mutant 53 (I614K) to misincorporate and misextend nucleotides at efficiencies comparable to known recently discovered polymerases, including those in the UmuC group, which typically misinsert 1 per 1000 bases (34, 35).

These results indicate that hydrophilic substitution at position Ile-614 confers low base pairing fidelity.

To determine the efficiency of mispair extension, we constructed a series of primer(24-mer)-template pairs, each containing a 3’ terminal A:T, G:T, C:T, or T:T base pairs, and measured the frequency of extension at increasing concentration of the next correct dNTP (dTTP) in incubations containing limiting amounts of WT or mutant enzymes. All plots of velocity relative to substrate (dTTP) concentration exhibited saturation kinetics. WT Taq pol I efficiently extended 3’ matched and mismatched primer-template termini in the following order: A:T > G:T > C:T > T:T (Table IV). The \( V_{\text{max}} \) values for misincorporations and misextensions for specific base pair by the WT enzyme are very similar (except T:T); in contrast, the \( K_m \) values for misincorporation by the WT enzyme are significantly greater than that for misextension of specific base pairs. Mutant 53 (I614K) is up to 50 times more efficient at extending mispaired nucleotides opposite template dT than the WT enzyme. Whereas the kinetic data indicate mutant 53 (I614K) Taq pol I extends C:T mispairs more efficiently than the WT enzyme. Furthermore, the kinetic data indicate mutant 53 (I614K) Taq pol I exhibits an elevated propensity to catalyze misincorporation and misextension as well as misextension mutations.

**Template Bypass**—These low fidelity mutant Taq pol I misinsert and misextend nucleotides at efficiencies comparable to known recently discovered polymerases, including those in the UmuC group, which typically misinsert 1 per 1000 bases (34, 35).

### Table I: Sequences of low fidelity Taq polymerases

| Mutants | 605 | L | V | A | L | Y | S | Q | 614 | I | 615 | E | L | 617 | R |
|---------|-----|---|---|---|---|---|---|---|-----|---|-----|---|---|---|---|
| 53      | 605 | L | V | A | L | Y | S | Q | 614 | I |       |   |   |   |   |
| 94      | 605 | L | V | A | L | Y | S | Q | 614 | I |       |   |   |   |   |
| 164     | 605 | L | V | A | L | Y | S | Q | 614 | I |       |   |   |   |   |
| 187     | 605 | L | V | A | L | Y | S | Q | 614 | I |       |   |   |   |   |
| 198     | 605 | L | V | A | L | Y | S | Q | 614 | I |       |   |   |   |   |
| 205     | 605 | L | V | A | L | Y | S | Q | 614 | I |       |   |   |   |   |
| 212     | 605 | L | V | A | L | Y | S | Q | 614 | I |       |   |   |   |   |

### Table II: Misinsertion efficiency

| Enzyme     | dNTP | \( K_m \) (app) \( ^{a} \) | \( V_{\text{max}} \) | \( V_{\text{max}}/K_m \) | Relative frequency \( ^{b} \) | \( \mu \text{m} \) | \( /\text{min} \) |
|------------|------|---------------------|-----------------|------------------|------------------------------|-----------------|-------------|
| WT Taq pol I | A    | 0.11                | 8.4             | 76               | 1                           | 1/17,000        |
|            | G    | 1300               | 12.0            | 0.0092           | 1/8300                      |                 |
|            | C    | 2000               | 1.3             | 0.00065          | 1/120,000                   |                 |
|            | T    | 930                | 5.6             | 0.0060           | 1/13,000                    |                 |
| Mutant 53 (I614K) | A    | 0.09               | 7.4             | 82               | 1                           |                 |
|            | G    | 110                | 7.4             | 0.067            | 1/1200                      |                 |
|            | C    | 630                | 6.0             | 0.0095           | 1/8600                      |                 |
|            | T    | 110                | 9.4             | 0.086            | 1/950                       |                 |
| Mutant 346 (A608D/E615D) | A    | 0.041              | 9.2             | 224              | 1                           |                 |
|            | G    | 1200               | 7.7             | 0.0064           | 1/35,000                    |                 |
|            | C    | 870                | 2.8             | 0.0032           | 1/70,000                    |                 |
|            | T    | 630                | 9.4             | 0.015            | 1/15,000                    |                 |
| Mutant 212 | A    | 0.14               | 5.6             | 40               | 1                           |                 |
|            | G    | 840                | 2.2             | 0.0026           | 1/15,000                    |                 |
|            | C    | 3000               | 0.39            | 0.00013          | 1/310,000                   |                 |
|            | T    | 450                | 0.97            | 0.0022           | 1/18,000                    |                 |

\( ^{a} \) All values have \( \pm <20\% \) error.

\( ^{b} \) Relative frequency equals efficiency \( (V_{\text{max}}/K_m) \) relative to that of dATP incorporation.

### Table III: Misinsertion efficiency opposite template dC

| Enzyme     | dNTP | \( K_m \) (app) \( ^{a} \) | \( V_{\text{max}} \) | \( V_{\text{max}}/K_m \) | Relative frequency \( ^{b} \) | \( \mu \text{m} \) | \( /\text{min} \) |
|------------|------|---------------------|-----------------|------------------|------------------------------|-----------------|-------------|
| WT Taq pol I | G    | 0.051              | 14              | 280              | 1                           | 1/17,000        |
|            | A    | 0.019              | 8               | 0.016            | 1/17,000                    |                 |
| Mutant 265 (I614N/L616I) | G    | 0.019              | 8               | 0.016            | 1/17,000                    |                 |
|            | T    | 0.39               | 1               | 1/1100           | 1/4300                      |
|            | C    | 0.099              | 0.014           | 1/3900           | 1/30,000                    |

\( ^{a} \) All values have \( \pm <20\% \) error.

\( ^{b} \) Relative frequency equals efficiency \( (V_{\text{max}}/K_m) \) relative to that of dGTP incorporation.

\( ^{c} \) \( V_{\text{max}}/K_m \) for mispairs reflect the initial slope of velocity vs. dNTP concentration plots.
randomly picked clones, and the error rate was determined. The polymerases in this newly discovered group are characterized by relatively low specific activity on normal templates but robust bypass activity on templates with damaged bases. To determine whether the low fidelity Taq polymerase can also bypass damaged templates, polymerization across template containing either an abasic site or vinyl chloride alkylate product ethenoA was studied (Fig. 3). In these reactions, the primer-templates were constructed such that the primer 3′ terminus was 7 nucleotides from the damaged template site, and efficient bypass of the lesion would be accompanied by addition of 5 complementary nucleotides past the site of damage. WT Taq pol I and purified mutants were able to efficiently synthesize across undamaged templates. With the WT enzyme, significant pausing was observed opposite abasic residue in the template, and a nearly complete block was observed at the site of ethenoA lesion. In contrast, mutant Taq pol I is containing a hydrophilic substitution at position 614 exhibited a reduction in pausing or no pausing at the abasic site. Mutant 265 (I614K/L616I) exhibited no pausing at the abasic site and was able to bypass ethenoA lesion. Mutants 212 (L605R/L606M/L609I) and 75 (I614M) did not carry out synthesis across from abasic sites or ethenoA adducts in identical control incubations. These data suggest, at least with the mutants tested, that there is a direct relationship between fidelity and the ability to bypass template lesions.

**Error Spectrum**—The evolution of highly active polymerases with low fidelity suggests that subtle alterations within the DNA polymerase catalytic site can lead to enhanced error rates during DNA synthesis. In *vitro* iterative replication by such a polymerase could be useful in generating libraries containing multiple mutations. We carried out repetitive replication with WT and mutant Taq pol Is utilizing the 1.3-kb human *thy* gene encoding thymidylate synthase as a template. Both WT and 1614K were able to amplify low amounts of the starting material to generate abundant levels of a 1.3-kb PCR product, which was subsequently excised from an agarose gel, purified, and cloned. A stretch of 630 bases of *thy* gene was sequenced from randomly picked clones, and the error rate was determined. WT Taq pol I exhibited an error rate of 3.3 × 10⁻¹⁸, Mutant 53 (I614K) exhibited an error rate of 8.0 × 10⁻⁴, or >20-fold higher error rate relative to the WT enzyme. Following PCR with wild-type or mutant 346 (A608D/E615D), most clones lack any changes in DNA sequence. In contrast, clones synthesized by mutant 53 (I614K) contained multiple single base substitutions, with an average of 2–3 base changes per clone (Fig. 4).

![32pTAAGACGXXGGTT](image)

**FIG. 3. Bypass of damaged templates.** Primertemplates were constructed such that the primer 3′ terminus was 7 nucleotides from the damaged template site, and efficient bypass of the lesion (either abasic site or vinyl chloride adduct ethenoA) would be accompanied by addition of 5 complementary nucleotides past the site of damage. WT Taq pol I and purified mutants 53 and 265 were incubated in the presence of 5 pmol primer-template for 10 min at 55°C. For each primer-template, the lane on the left contains a lower polymerase concentration, and the lane on the right contains a higher concentration. A single concentration of WT Taq pol I (20 fmol/μl) was studied with a normal template. The site of the lesion is marked by an X. The DNA construct and the template sequence (5′-end) are diagrammed at the top.

An analysis of the mutation spectrum by WT, mutant 346 (A608D/E615D), and mutant 53 (I614K) shows that WT Taq pol I and mutant 346 are especially adept at producing the transition error A:T → G:C, whereas mutant 1614K makes a significant number of the other transition error G:C → A:T, as well as transversion error A:T → T:A (Table V). The error rate by mutant 75, which contains a nonhydrophilic substitution (I615M) and the WT enzyme are similar, although their error spectrum differ (Table V). The error spectrum of WT Taq pol I is consistent with published results, which show that WT Taq pol I predominantly catalyzes T → C transition errors (36), resulting from efficient dGTP:dT (incoming nucleotidetemplate) mismatch (Table II).

**DISCUSSION**

The accuracy of DNA replication is crucial for maintaining genomic stability from one generation to the next (37, 38).
Nevertheless, in times of crisis, it is beneficial for cells to exhibit diversity and thus mutate at higher rates. The fidelity of DNA replication is largely determined at the DNA polymerase active site, which is responsible for 5–6 orders of magnitude of the overall mutation rate of cells (11). Thus far, the majority of the bacteria populations that mutate at high rates, which have been investigated, contain loss of specific DNA repair pathways (39). Studies with mutant DNA polymerases have mainly focused on analyzing the effects after the loss of 3′-5′ exonucleolytic proofreading activity. E. coli and yeast harboring DNA polymerases with loss of exonuclease activity exhibit a 10-fold elevated mutation rate (14, 40). Development of additional mutants, particularly those containing substitutions within the active site of polymerases, coupled with high resolution crystal structures, should advance our understanding of the determinants of polymerase accuracy, as well as facilitating studies of the phenotypes associated with mutated polymerases exhibiting poor fidelity. However, current structure-based site-directed mutagenesis studies have not been successful at producing mutant polymerases with WT-like activity that exhibit low fidelity.

We have found, following random mutagenesis of a portion of the polymerase active site and stringent selection, a single amino acid residue (Ile-614) that, when substituted to a variety of hydrophilic amino acids, reduces the fidelity by at least 10-fold. No other amino acid within Motif A, when substituted, consistently exhibited such low base pairing fidelity. In addition, nonhydrophilic substitutions, including mutant I614M, did not alter the error rate during DNA synthesis (Table V). In reactions containing 3 nucleotides (Fig. 2), mutants containing substitutions at Ile-614 are able to misincorporate a base opposite the template position for which there is not a complementary dNTP. In addition, these mutant polymerases can also extend nascent primers containing mismatched DNA termini more efficiently than can WT Taq pol I. The ability for polymerases containing hydrophilic substitutions at position 614 to efficiently catalyze misincorporation was tested kinetically. Mutant I614K was shown to misincorporate nucleotides 10-fold more efficiently relative to WT enzyme; in addition, kinetic experiments showed that I614K mutant is also efficient at forming transversion errors by misextending pyrimidine-pyrimidine base pairs at higher rates relative to WT. These kinetic data suggest the mutant I614K Taq pol I should produce both transition and transversion errors, and WT Taq pol I and mutant 53 (I614K) should exhibit unique error spectrums following DNA replication of a specific sequence. We tested these predictions by conducting PCR amplification of a homogeneous sequence and measured the spectrum of mutations produced by mutant 53 (I614K). The results show that whereas WT Taq pol I and mutant 346 (A608D/E615D) contain very similar distributions of errors, PCRs were carried out under nonmutagenic conditions containing Mg2+ (1.5 mM) and 250 μM each dNTP. Products (1.3 kb) from these amplification reactions were isolated by electrophoresis, cloned, and sequenced. The majority of clones produced by WT Taq pol I contained either no changes or a single nucleotide change. Similar results were obtained following PCR with mutant 346. Products generated following PCR with mutant 53 resulted in 0–6 nucleotide changes that were evenly distributed over the length of the template.

![Graphic](image_url)

**FIG. 4. Mutagenesis during PCR.** Iterative replication of a 1.3-kb target gene encoding human thymidine synthase by PCR using either WT Taq pol I, mutant 346 (A608D/E615D), or mutant 53 (I614K) resulted in a wide distribution of errors. PCRs were carried out under nonmutagenic conditions containing Mg2+ (1.5 mM) and 250 μM each dNTP. Products (1.3 kb) from these amplification reactions were isolated by electrophoresis, cloned, and sequenced. The majority of clones produced by WT Taq pol I contained either no changes or a single nucleotide change. Similar results were obtained following PCR with mutant 346. Products generated following PCR with mutant 53 resulted in 0–6 nucleotide changes that were evenly distributed over the length of the template.

**TABLE V**

| No. of analyzed clones | Average number of mutations in each clone | Error rateb | No. of individual substitutions | Transitions | Transversions | No. of deletions |
|-----------------------|------------------------------------------|-------------|---------------------------------|-------------|---------------|-----------------|
| WT                    | 36                                       | 0.2         | $3.3 \times 10^{-5}$ | AT → GC     | GC → AT       | 6 0 0 1 0 0 0 1 0 0 0 0 |
| Mutant 346            | 25                                       | 0.4         | $8.7 \times 10^{-5}$ | AT → TA     | AT → CG       | 8 1 2 0 0 0 0 0 0 0 |
| Mutant 53             | 42                                       | 2.5         | $8.0 \times 10^{-4}$ | AT → TA     | GC → TA       | 44 25 26 3 3 3 4 4 |
| Mutant 75             | 45                                       | 0.1         | $3.0 \times 10^{-5}$ | AT → TA     | GC → TA       | 2 0 2 0 2 0 0 0 0 0 |

a Number of mutations per 630 bases sequenced per clone.

b Error rate equals number of mutations per base per division.
the structure, this residue is not in contact with the nucleotide, but instead hydrogen bonds to Glu-615. In the open ternary form, this Tyr residue occupies the site of the template base, opposing the incoming nucleotide and hydrogen bonded to it. Substitutions of Phe-667 to Tyr within Taq pol I yields active enzymes capable of incorporating dideoxynucleotides (44). In the closed form of Taq pol I, Phe-667 is 3.7 Å from the base, but in the open form, this residue packs near the ribose, the base, and the middle phosphate oxygen of the incoming nucleotide. Ile-614 packs against the ribose ring and the other free oxygen of the middle phosphate in both the closed and open forms of the Taq pol I-DNA-ddNTP ternary structure (21). We propose that diverse substitutions for Ile-614, and especially hydrophilic substitutions, lead to a more "open" pocket that can accommodate damaged templates, non-Watson-Crick base pairs, and diverse nucleotide analogs. This model proposes that stable stacking/packing interactions with the base and ribose rings are crucial for polymerase fidelity and is consistent with a model for nucleotide incorporation proposed for HIV-1 reverse transcriptase (45) and other polymerases (46).

In summary, we have evolved a set of polymerases containing substitutions at a single amino acid with low base pairing fidelity, the ability to bypass template lesions, and the ability to incorporate nucleotide analogs. Substitution of Ile-614, an amino acid that is structurally conserved in all DNA polymerases, produces active DNA polymerase with very broad substrate specificity. These findings are consistent with models of adaptive evolution that 1) in times of stress, the inherent plasticity of enzyme active site facilitates the generation of beneficial mutants with altered substrate specificity, which could provide a selective advantage, and 2) following successful survival through periods of adverse conditions, WT nucleotide sequence (one that is fit and the most prevalent) can be generated through recombination-mediated lateral transfer (9). This study suggests that other populations of mutants may contain substitutions within the polymerase catalytic site that confer low fidelity. Considering the vital role of DNA polymerases during DNA replication, repair, and recombination, it may be important to genotype tumors characterized by elevated mutation rates for polymorphic differences within the polymerase catalytic site.

REFERENCES
1. Welch, D. M., and Meselson, M. (2000) Science 288, 1211–1215
2. Radman, M., Matic, I., and Taddei, F. (1999) Ann. N. Y. Acad. Sci. 870, 146–155
3. Mao, E. F., Lane, L., Lee, J., and Miller, J. H. (1997) J. Bacteriol. 179, 417–422
4. Oliver, A., Canton, R., Campo, P., Baquero, F., and Blazquez, J. (2000) Science 288, 1251–1254
5. LeClere, J. E., Li, B., Payne, W. L., and Cebula, T. A. (1996) Science 274, 1208–1211
6. Loeb, L. A. (1996) in Genetic Instability in Cancer (Lindahl, T., ed) Vol. 28, pp. 329–342, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
7. Kornberg, A., and Baker, T. (1992) DNA Replication, 2nd ed, W. H. Freeman and Co., New York
8. Lindahl, T., and Nyberg, B. (1972) Biochemistry 11, 3610–3618
9. Patel, P. H., and Loeb, L. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5095–5100
10. Kunkel, T. A., and Nyberg, B. (1972) J. Bacteriol. 116, 1468–1474
11. Johnson, K. A. (1993) Annus. Rev. Biochem. 62, 685–713
12. Perrino, F. W., Preston, B. D., Sandell, L. L., and Loeb, L. A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8343–8347
13. Drake, J. W. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7160–7164
14. Eichler, H., Lu, C., and Burgers, P. M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 2189–2192
15. Modrich, P. (1989) J. Biol. Chem. 264, 6597–6600
16. Fishel, R., Lescoe, M. K., Rao, M. R. S., Copeland, N. G., Jenkins, N. A., Garber, J., Kane, M., and Kolodner, R. (1993) Cell 75, 1027–1038
17. Bronner, C. E., Baker, S. M., Morrison, P. T., Warren, G., Smith, L. G., Lescoe, M. K., Kane, M., Earhamo, C., Lipford, J., Lindsblom, A., Tannergard, P. R. J., Godwin, A. R., Ward, D. C., Nordenskild, M., Fishel, R., Kolodner, R., and Liskay, R. M. (1994) Nature 368, 258–261
18. Reitmair, A. H., Schmitz, R., Ewel, A., Bapat, B., Redston, M., Mitri, A., Waterhouse, P., Mittrucker, H. W., Wakeham, A., Liu, B., Thomasen, A., Griesser, H., Gallinger, S., Ballhausen, W. G., Fishel, R., and Mak, T. W. (1995) Nat. Genet. 11, 64–70
19. Reese, L. S., Derbysht, V., and Steitz, T. A. (1993) Science 260, 352–355

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3 P. H. Patel, H. Kawate, E. Adman, M. Ashbach, and L. A. Loeb, unpublished results.
20. Doublie, S., Tabor, S., Long, A. M., Richardson, C. C., and Ellenberger, T. (1998) *Nature* **391**, 251–258
21. Li, Y., Korolev, S., and Waksman, G. (1998) *EMBO J.* **17**, 7514–7525
22. Patel, P. H., and Preston, B. D. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 549–553
23. Carroll, S. S., Cowart, M., and Benkovic, S. J. (1991) *Biochemistry* **30**, 804–813
24. Joyce, C. M., Sun, X. C., and Grindley, N. D. (1992) *J. Biol. Chem.* **267**, 24485–24500
25. Desai, S. D., Pandey, V. N., and Modak, M. J. (1994) *Biochemistry* **11868–11874
26. Delarue, M., Poch, O., Tordo, N., Meras, D., and Argos, P. (1990) *Protein Eng.* **3**, 461–467
27. Wang, J., Sattar, A. K., Wang, C. C., Karam, J. D., Konigsberg, W. H., and Steitz, T. A. (1997) *Cell* **89**, 1087–1099
28. Grimm, E., and Arbuthnot, P. (1995) *Nucleic Acids Res.* **23**, 4518–4519
29. Preston, B. D., Poiesz, B. J., and Loeb, L. A. (1988) *Science* **242**, 1168–1171
30. Suzuki, M., Avicola, A. K., Hood, L., and Loeb, L. A. (1997) *J. Biol. Chem.* **272**, 11228–11235
31. Engelke, D. R., Krikos, A., Bruck, M. E., and Ginsburg, D. (1990) *Anal. Biochem.* **191**, 396–400
32. Bossa, M. S., Petruska, J., and Goodman, M. F. (1987) *J. Biol. Chem.* **262**, 14689–14699
33. Kim, B., Hathaway, T. R., and Loeb, L. A. (1998) *Biochemistry* **37**, 5831–5839
34. Johnson, R. E., Washington, M. T., Prakash, S., and Prakash, L. (2000) *J. Biol. Chem.* **275**, 7447–7450
35. Tang, M., Shen, X., Frank, E. G., O'Donnell, M., Woodgate, R., and Goodman, M. F. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 8919–8924
36. Tindall, K. R., and Kunkel, T. A. (1988) *Biochemistry* **27**, 6006–6013
37. Echols, H. (1982) *Biochimie (Paris)* **64**, 571–575
38. Loeb, L. A., and Kunkel, T. A. (1982) *Annu. Rev. Biochem.* **51**, 429–457
39. Miller, J. H. (1998) *Mutat. Res.* **409**, 99–106
40. Schaaper, R. M. (1989) *Genetics* **121**, 205–212
41. Friedberg, E. C., and Gerlach, V. L. (1999) *Cell* **98**, 413–416
42. Goodman, M. F. (2000) *Trends Biochem Sci.* **25**, 189–195
43. Dong, Q., Copeland, W. C., and Wang, T. S. (1993) *J. Biol. Chem.* **268**, 24163–24174
44. Tabor, S., and Richardson, C. C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 6339–6343
45. Patel, P. H., Jacobo-Molina, A., Ding, J., Tantillo, C., Clark, A. D. Jr., Raag, R., Nanni, R. G., Hughes, S. H., and Arnold, E. (1995) *Biochemistry* **34**, 5351–5363
46. Goodman, M. F., and Fygenson, K. D. (1998) *Genetics* **148**, 1475–1482
47. Kraulis, P. (1991) *J. Appl. Crystallogr.* **24**, 946–950
48. Merritt, E. A., and Murphy, M. E. P. (1994) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **50**, 869–873
49. Patel, P. H., and Loeb, L. A. (2000) *J. Biol. Chem.* **275**, 40266–40272