A Thumb Subdomain Mutant of the Large Fragment of *Escherichia coli* DNA Polymerase I with Reduced DNA Binding Affinity, Processivity, and Frameshift Fidelity*

Dana T. Minnick‡, Mekbib Astatke§, Catherine M. Joyce§, and Thomas A. Kunkel¶

From the §Laboratory of Molecular Genetics, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709 and the ¶Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520-8114

In Klenow fragment DNA polymerase, a flexible 50-amino acid subdomain at the tip of the thumb which includes two α helices has been suggested to interact with the duplex template-primer (Beese, L.S., Derbyshire, V., and Steitz, T.A. (1993) *Science* **260**, 352–355). The present study investigates the properties of Klenow polymerase containing a 24-amino acid deletion (residues 590–613) that removes a portion of the tip of the thumb. The mutant polymerase has relatively normal dNTP binding and catalytic rate. However, its DNA binding affinity is reduced by more than 100-fold relative to the intact polymerase and its ability to conduct processive synthesis is also reduced. Although the mutant polymerase has relatively normal base substitution fidelity, it has strongly reduced frameshift fidelity, being especially error-prone for single nucleotide addition errors in homopolymeric runs. The addition error rate increases as the length of the reiterated sequence increases, indicative of errors initiated by template-primer strand slippage. These observations suggest a role for the tip of the thumb of Klenow polymerase in determining DNA binding, processivity and frameshift fidelity, perhaps by tracking the minor groove of the duplex DNA. The results are discussed in light of remarkably similar observations with T7 DNA polymerase in the presence or absence of thioredoxin, an accessory subunit that affects these same properties.

The Klenow fragment,1 a 68-kDa carboxy-terminal fragment of *Escherichia coli* DNA polymerase I, contains both DNA polymerase and 3′ → 5′ exonuclease activity. The polymerase active site is on a 45-kDa COOH-terminal domain of Klenow fragment, and the 3′ → 5′ proofreading exonuclease active site is on the smaller NH2-terminal domain. Structural information obtained from crystal structures of Klenow fragment demonstrates that the polymerase domain resembles a partially open right hand with three subdomains that form a cleft (for review, see Joyce and Steitz (1994)). The palm is located at the base of the cleft and contains the catalytically important carboxylates. The fingers form one wall of the cleft and the thumb another. The functional importance of the cleft is made apparent by the large number of residues on the exposed surfaces in this region that are highly conserved or invariant among the polymerase families (Delarue et al., 1990). The x-ray crystal structures of HIV-1 reverse transcriptase (Kohlstaedt et al., 1992; Jacobo-Molina et al., 1993; for a review, see Arnold et al., 1995), T7 DNA polymerase (Sousa et al., 1993), human DNA polymerase β (Pelletier et al., 1994; Sawaya et al., 1994; Davies et al., 1994) and *Thermus aquaticus* DNA polymerase (Kim et al., 1995; Korolev et al., 1995) reveal a similar polymerase subdomain organization.

Co-crystals of Klenow fragment with duplex DNA bound in an editing complex suggest the DNA is oriented such that the template-primer approaches the catalytic site from the side adjacent to the 3′ → 5′ exonuclease (Beese et al., 1993). Biochemical studies including cross-linking and footprinting experiments have suggested that 5–8 base pairs of duplex DNA are covered by Klenow fragment when the primer terminus is in the polymerase active site (Joyce and Steitz, 1987; Allen et al., 1989; Catalano et al., 1990; Guest et al., 1991). The fingers subdomain has been suggested to contact the template strand and possibly to interact with the incoming dNTP (for a review, see Joyce and Steitz (1994)). Numerous interactions are seen between highly conserved residues in the thumb subdomain and the phosphate backbone of the duplex DNA (Beese et al., 1993). Similar interactions have also been reported recently in a co-crystal of *Taq* DNA polymerase (a homolog of *E. coli* DNA polymerase I) with a blunt-ended duplex DNA (Eom et al., 1996).

Klenow fragment catalyzes high fidelity DNA synthesis (Kuchta et al., 1988; Bebenek et al., 1990), having rates of approximately 10⁻⁶ and 10⁻⁸ errors per nucleotide polymerized, respectively, for single-base substitution and single-base deletion errors. Although exonuclease activity contributes somewhat to this high fidelity, most of the discrimination results from the high selectivity of the polymerase. Polymerase errors arise by either of two initiating events, misinsertion of a dNMP or slippage of the template-primer (reviewed by Kunkel (1992)). For the former, Klenow fragment discriminates against incorporation of an incorrect dNMP during both dNTP binding and catalysis (Kuchta et al., 1988). Thus, it seems likely that side chains located in the fingers and palm subdomains at or near the polymerase active site may provide some of the key interactions responsible for high base substitution fidelity. For example, a previous study showed that substituting serine for tyrosine at residue 766, located on the exposed surface of the O helix in the fingers subdomain, resulted in reduced nucleotide discrimination (Carroll et al., 1991).

---

*This work was supported in part by a grant to (T. A. K.) from the National Institutes of Health Intramural AIDS Targeted Antiviral Program and by National Institutes of Health Grant GM-28550 (to C. M. J. and M. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Laboratory of Molecular Genetics, NIEHS, P.O. Box 12233, Research Triangle Park, NC 27709. Tel.: 919-541-2644; Fax: 919-541-7613; E-mail: kunkel@niehs.nih.gov.

1 The abbreviations used are: Klenow fragment, large fragment of *E. coli* DNA polymerase I; HIV-1, human immunodeficiency virus, type 1; RT, reverse transcriptase.
In contrast, nucleotide addition and deletion errors occurring at reiterated sequences are thought to result from an initial template-primer slippage event (for a review, see Kunkel (1990)). DNA polymerases differ widely in their propensity to commit such errors, and frameshift error rates correlate with their ability to conduct processive synthesis. For example, DNA polymerase γ is highly processive and is also highly accurate for frameshift errors, whereas DNA polymerase α is somewhat less processive and has lower frameshift fidelity and DNA polymerase β is non-processive and highly error prone for frameshifts (Kunkel, 1985). Studies with the wild-type HIV-1 reverse transcriptase revealed that hot spots for frameshifts at reiterated sequences were also sites that exhibited a strong probability of termination of processive synthesis (Bebenek et al., 1989). Moreover, when the DNA sequence surrounding these sites was altered, especially in the duplex template-primer near the 3'-OH terminus, concomitant increases or decreases in both frameshift error rates and termination probabilities resulted (Bebenek et al., 1993). These correlations suggest that polymerase dissociation-reassociation allows formation and/or utilization of misaligned frameshift intermediates containing unpaired bases in the primer strand (for an addition) or the template strand (for a deletion). Thus, polymerase selectivity against these errors may depend on protein interactions with the duplex template-primer stem.

Studies of altered forms of two different DNA polymerases have supported this idea. T7 DNA polymerase contains a polymerase subunit and a smaller subunit, thioredoxin, that is proposed to clamp the polymerase to the template-primer. The holoenzyme conducts highly processive and highly accurate replication. However, DNA synthesis in the absence of thioredoxin was much less processive and highly error-prone for frameshift errors in homopolymeric runs (Kunkel et al., 1994; Kroutil et al., 1996). Moreover, while most DNA polymerases generally commit deletion errors at a substantially higher rate than addition errors (for review, see Kunkel (1990)), the reverse was true for synthesis by T7 DNA polymerase in the absence of thioredoxin, i.e., the addition error rate far exceeds the deletion error rate. This unusual error specificity is relevant to observations in the present study (see below).

Studies with mutants of HIV-1 reverse transcriptase have also suggested the importance of interactions between a polymerase and the duplex template-primer stem to processivity and frameshift fidelity. The structure of the RT-DNA complex (Jacob-Molina et al., 1993) has revealed that a helix H lies adjacent to the primer strand near the 3'-OH primer terminus, in the region where single-nucleotide changes were observed to affect the processivity (Abbotts et al., 1993) and the frameshift fidelity (Bebenek et al., 1993) of the wild-type RT. Two derivatives of the RT (G262A and W266A), which contained single amino acid changes on the side of helix H that faced the primer strand, had reduced DNA binding affinity (Beard et al., 1994) and reduced processivity and fidelity for errors initiated by strand slippage (Bebenek et al., 1995). These two amino acids are thought to make minor groove contacts with the duplex DNA within either three nucleotides (for W266A) or four to five nucleotides (for G262A) of the 3'-OH primer terminus.

The thumb domain of Klenow fragment contains two long anti-parallel α-helices, designated H and I, with a 50-amino acid connecting segment that was disordered in the initial crystals (Ollis et al., 1985a). Subsequent analysis of a Klenow fragment-DNA co-crystal (Beese et al., 1993), in which the tip of the thumb was apparently better ordered than in the apoenzyme structure, revealed that this region contains two short α-helices, designated H4 and H5, with random coils connecting these helices to each other and to helices H and I. Comparison of the native Klenow fragment structure with that of the enzyme bound to duplex DNA suggests that significant movement of the thumb subdomain occurs upon binding DNA, so that the NH2 terminus of helix H5 was seen to contact the minor groove of the duplex DNA in the co-crystal structure (Beese et al., 1993). In this paper, we describe the properties of a mutant derivative of Klenow fragment having a 24-amino acid deletion in the tip of the thumb. In comparison to the wild-type Klenow fragment polymerase, this mutant has lower DNA binding affinity and reduced processivity. It also has decreased fidelity for additions and deletions occurring in homopolymeric runs, whereas its base substitution fidelity is similar to that of the wild-type polymerase.

**EXPERIMENTAL PROCEDURES**

**Materials**

*Cell Strains and Reagents—E. coli strains, reagents, and preparation of gapped substrates were as described (Bebenek and Kunkel, 1995).*

*Mutant Klenow Fragment Polymerase—The Δ(590–613) mutation was made by oligonucleotide-directed mutagenesis using a uracil-containing M13 template, which contained the polymerase domain region of the polA gene (Polesky et al., 1990). The mutagenic oligonucleotide was a 30-mer capable of forming 15 base pairs at either end of the 72-nucleotide segment encoding residues 590–613 on the DNA polymerase I sequence (Joyce et al., 1982). A DNA fragment containing the mutation was subcloned into a high level expression plasmid for Klenow fragment, which also contained the D424A (3'→5' exonuclease-deficient) mutation, as described previously (Polesky et al. 1989, 1992). The mutant protein was purified by fast protein liquid chromatography (Joyce and Derbyshire, 1995). The purified D424A mutant derivative of Klenow fragment (Derbyshire et al., 1988) was used as a control for comparison purposes. The denaturation temperature (Tm) of the Δ(590–613) mutant protein was measured using a CD spectrophotometer assay that has been described elsewhere (Astatke et al., 1995).*

**Methods**

*Steady-state Kinetic Measurements of the Polymerase Reaction—The steady-state parameters, K0.5(M prim), and kcat for the Δ(590–613) mutant polymerase were determined using poly(dA)poly(dt) as substrate, as described elsewhere (Astatke et al., 1995). Because the Δ(590–613) mutant protein has a much lower affinity for DNA compared to the wild-type protein, the concentration of DNA (20 μM in primer termini) was approximately 10-fold higher than in our previous experiments. The kcat for the reaction was found to be identical within experimental error, when the primer concentration was raised to 40 μM, verifying that a concentration of 20 μM was indeed saturating.*

*Determination of DNA Binding Affinity—The dissociation constant, KD(ΔDNA), for binding to a 68-mer hairpin oligonucleotide was determined using a gel mobility shift assay (Astatke et al., 1995). In recent experiments, the gel conditions have been modified slightly from those in our previous work; 10% polyacrylamide gels were used instead of 8%, and the gels were run at higher ionic strength (50 mM Tris borate). The increase in buffer concentration avoids the need for circulating the reservoir buffers during the run, thus increasing the convenience of the procedure. These changes had no effect on the observed KD(ΔDNA) values.*

*Processivity Analysis—The probability of termination of processive synthesis was analyzed using a primed single-stranded M13mp2 DNA substrate as described previously (Abbotts et al., 1993). 5'-32P-labeled primers were complementary to lacZ positions 141–161 (designated lacZ141 and ΔlacZ to l71 (lacZ137). Reaction conditions were the same as those for DNA synthesis fidelity measurements described below, except that the DNA (160 fmol) was in 300-fold molar excess over the wild-type Klenow fragment DNA polymerase (0.53 fmol) or in 15-fold molar excess over the Δ(590–613) derivative (11 fmol). In these, as in earlier reactions containing an excess of template-primer DNA over polymerase (Eckert and Kunkel, 1993), the amount of reinitiation on previously extended template-primer molecules relative to the total pool of product DNA was negligible, such that termination probabilities reflected one cycle of processive synthesis. Thirty-microliter reactions were incubated at 37 °C, and 10-μl aliquots were removed at 5, 15, and 30 min and quenched by addition of an equal volume of formamide dye (99% (v/v) formamide, 5 mM EDTA, 0.1% (w/v) xylene cyanol, and 0.1% (w/v) bromphenol blue). Products were analyzed by electrophoresis through a 16% denaturing polyacrylamide gel, and the amount of radioactivity in the product bands was quantified using a Molecular.*
Properties of Klenow Polymerase Thumb Mutant

Dynamics PhosphorImager (Sunnyvale, CA). The probability of the polymerase terminating at a specific site along the DNA template is defined as the ratio of the number of product molecules of a given length to the sum of that number plus the number of all longer products.

Fidelity Measurements—DNA synthesis fidelity was measured as described previously (Bebenek and Kunkel, 1995) using an M13mp2 DNA substrate with a single-stranded gap containing a portion of the lacZ α-complementation gene target. DNA synthesis reactions (25 µl) contained 20 mM Tris, pH 8.0, 10 mM MgCl₂, 2 mM dithiothreitol, 150 ng of gapped DNA, 0.17 pmol of wild-type Klenow fragment or 6.8 pmol of mutant Klenow fragment, and 1 µM each of the four dNTPs. Reaction mixtures were incubated at 37 °C for 10 min, quenched by addition of EDTA to 15 mM, and analyzed for complete gap-filling synthesis by agarose gel electrophoresis. All reactions filled the gap to the extent that products migrated coincident with nicked circular duplex DNA (Bebenek and Kunkel, 1995).

Products were introduced into E. coli and plated and synthesis errors identified by plaque phenotype. The forward mutation assay scores a variety of errors as light blue or colorless plaques. The DNAs of mutant phage were sequenced to define the polymerase error specificity, as described previously (Bebenek and Kunkel, 1995). For reversion assays, polymerase errors were scored as blue-plaque revertants of M13 substrates containing mutations encoding colorless plaques phenotypes. One-base deletions were scored in a substrate containing 5 consecutive Tₜₜₜₜₜ base pairs, with the coding sequence of the starting nucleotides (see below), each with the coding sequence of the starting transcript. Two additional M13-derived substrates and with poly(dA) were transmitted to the polymerase active site region, neither dNTP changes associated with the 24-amino acid deletion are not influenced by DNA sequence context, we wanted to determine whether the mutator phenotype of the (590–613) polymerase is a mutator for frameshift errors. In contrast, the mutation decreased the DNA binding affinity by at least 100-fold, as might be expected given the contacts seen between this region of the thumb subdomain and duplex DNA in a co-crystal of Klenow fragment with DNA (Beece et al., 1993).

Kinetic and Equilibrium Parameters of the (590–613) Mutant Protein—Consistent with the view that the structural changes associated with the 24-amino acid deletion are not transmitted to the polymerase active site region, neither dNTP binding, measured by Kₗₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜ$_{\text{mut}}$(DNA) reflects the inclusion of new data.

<table>
<thead>
<tr>
<th>Protein$^{a}$</th>
<th>$K_{\text{cat}}$(ATTP)</th>
<th>$k_{\text{cat}}$(ATTP)</th>
<th>$K_{\text{DINN}}$</th>
<th>$T_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type$^{b}$</td>
<td>1.7 ± 0.4</td>
<td>1.0 ± 0.7</td>
<td>0.2 ± 0.1</td>
<td>56</td>
</tr>
<tr>
<td>(590–613)$^{c}$</td>
<td>3.7 ± 0.7</td>
<td>0.3 ± 0.1</td>
<td>42 ± 6</td>
<td>53</td>
</tr>
</tbody>
</table>

$^{a}$ The proteins in this study carry the D424A mutation and are therefore deficient in 3’ → 5’ exonuclease activity.

$^{b}$ Data for the wild-type polymerase have been taken from Astatke et al. (1995). The small change from the previously published value of $K_{\text{DINN}}$ reflects the inclusion of new data.

$^{c}$ The $K_{\text{cat}}$(ATTP), $k_{\text{cat}}$(ATTP), and $K_{\text{DINN}}$ values for the (590–613) mutant protein were from at least three independent determinations and are reported as mean ± standard deviation.

Kinetic and Equilibrium Parameters of the (590–613) Mutant Polymerase—The consequences of the decreased DNA binding affinity of the (590–613) derivative of Klenow fragment were readily seen when the processivity of the mutant polymerase (Fig. 1A, lane 2) was compared to that of the wild-type polymerase (Fig. 1A, lane 3). The processivity of the mutant was reduced such that, when averaged over 18 positions with two different template-primeras (Fig. 1, B and C), the probability of termination of processive synthesis increased 4-fold. The increase in termination probability varied from almost none at nucleotide 137 (Fig. 1C) to 9-fold at nucleotide –44 (Fig. 1B). Similar results have been obtained with two additional M13-derived substrates and with poly(dA) primed with oligo(dT).

Fidelity Measurements—The accuracy of DNA synthesis catalyzed by the wild-type and mutant Klenow fragment was first examined in reversion assays that monitor substitution or deletion errors at specific template positions in the lacZ reporter gene. Using a substrate that scores frameshift errors, the reversion frequency was 9-fold higher for the (590–613) mutant polymerase than for the wild-type enzyme. Thus, the (590–613) polymerase is a mutator for frameshift errors. In contrast, with a substrate that detects base substitution errors, gap-filling synthesis by the mutant Klenow fragment resulted in an average reversion frequency that was similar to that observed with wild-type Klenow fragment (Table II).

Because polymerase frameshift error rates are strongly influenced by DNA sequence context, we wanted to determine whether the mutator phenotype of the (590–613) enzyme is general, i.e. manifest at multiple template sites, and whether the (590–613) enzyme is a mutator for frameshift errors not scored in the above reversion assay (e.g. one-base additions). We, therefore, examined the error specificity in a forward mutation assay that scores frameshifts at 199 positions in the lacZ target sequence, 241 different base substitutions at 125 sites, and additions and deletions of larger numbers of bases as well as complex mutations (Bebenek and Kunkel, 1995).

2 J. Jäger and T. A. Steitz, personal communication.

3 C. M. Joyce, unpublished observations.
Properties of Klenow Polymerase Thumb Mutant

24957

Fidelity of wild-type and mutant Klenow fragment polymerase

The background mutant frequencies for uncopied DNA were $0.5 \times 10^{-5}$, $0.8 \times 10^{-5}$, and $6 \times 10^{-4}$, respectively, for the assays shown.

<table>
<thead>
<tr>
<th>Polymerase</th>
<th>Mutant frequency</th>
<th>Plaques scored</th>
<th>Total</th>
<th>Mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base substitution reversion assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>$270 \times 10^3$</td>
<td>91</td>
<td>34</td>
<td>$10^5$</td>
</tr>
<tr>
<td>$\Delta$(590–613)</td>
<td>$260 \times 10^3$</td>
<td>91</td>
<td>36</td>
<td>$10^5$</td>
</tr>
<tr>
<td>Frameshift reversion assaya</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>$20 \times 10^3$</td>
<td>63</td>
<td>32</td>
<td>$10^5$</td>
</tr>
<tr>
<td>$\Delta$(590–613)</td>
<td>$270 \times 10^3$</td>
<td>739</td>
<td>280</td>
<td>$10^5$</td>
</tr>
<tr>
<td>Forward mutation assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type$^b$</td>
<td>$10,312$</td>
<td>97</td>
<td>94</td>
<td>$10^4$</td>
</tr>
<tr>
<td>$\Delta$(590–613)</td>
<td>$11,710$</td>
<td>134</td>
<td>110</td>
<td>$10^4$</td>
</tr>
</tbody>
</table>

a The lacZ coding sequence is in plus-one reading frame (see “Methods”), permitting detection of $3n - 1$ errors (e.g. $-1$, $-4$, $+2$, $+5$, etc.). As shown previously (Bebenek et al., 1990), the majority of the errors by wild-type Klenow polymerase with this assay are loss of A-T in the run of five consecutive template T residues.

b The mutant frequency is the average of six independent determinations.

Table III

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Mutant frequency</th>
<th>Error rates</th>
<th>Error rates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type$^c$</td>
<td>$\times 10^{-6}$</td>
<td>$\times 10^{-6}$</td>
</tr>
<tr>
<td></td>
<td>$\Delta$(590–613)</td>
<td>$\times 10^{-6}$</td>
<td>$\times 10^{-6}$</td>
</tr>
<tr>
<td>Substitutions</td>
<td>64</td>
<td>63</td>
<td>28</td>
</tr>
<tr>
<td>Frameshifts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+1 in run</td>
<td>1</td>
<td>3</td>
<td>52</td>
</tr>
<tr>
<td>-1 in run</td>
<td>8</td>
<td>22</td>
<td>6</td>
</tr>
<tr>
<td>-1 in non-run</td>
<td>7</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Other</td>
<td>14</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>94</td>
<td>98</td>
<td></td>
</tr>
</tbody>
</table>

a Data are from Bebenek et al. (1990).

b Error rates are errors per detectable nucleotide incorporated, calculated by multiplying the mutant frequencies by the proportion of mutants for each class, dividing by 0.6 to correct for expression of errors upon transcription, and dividing by the number of known detectable sites for each class of mutations. Mutant frequencies used to calculate the error rates were the average of six independent experiments for the wild-type and two independent experiments for the $\Delta$(590–613) polymerase (from Table II).

c The most striking observation was that 52 of 98 mutations in the mutant polymerase collection contained a single-base addition (Table III). The result was quite unexpected because, like other DNA polymerases (Kunkel, 1990), the wild-type Klenow polymerase is much more prone to generate deletions and only rarely makes addition errors; note that only one +1 mutant was observed in the wild-type polymerase error collection (Table III). The mutant polymerase is thus a 70-fold mutator for addition errors. A sequence-specific increase in one-base deletions by the $\Delta$(590–613) polymerase was also observed, since 4 of 6 one-base deletions in the $\Delta$(590–613) polymerase collection occur at a single TTTT run, while none were recovered at this site for the wild-type polymerase. This result is consistent with the result of the reversion assay (Table II), which scores loss of a base within a run of 5 T nucleotides at this same position. Thus, the data reveal that the $\Delta$(590–613) polymerase has a mutator phenotype for single-base additions and some single-base deletions.

The addition errors by the $\Delta$(590–613) polymerase all occurred within runs of three or more consecutive identical bases, and the addition error rate increased with increasing homopolymeric run length (Fig. 3). This is consistent with a model wherein frameshift errors occur through misaligned intermediates. In contrast, the $\Delta$(590–613) polymerase error rates for substitutions and for deletions at non-reiterated sequences were similar to those of the wild-type polymerase (Table III).

Forty-two of 52 additions by the $\Delta$(590–613) polymerase were in a run of five consecutive C-G base pairs (Fig. 2), representing a 60-fold increase in error rate relative to the wild-type polymerase. Since errors at this site dominate the mutant polymerase error collection, removing this site from the analysis would result in a lower estimate of the apparent mutator phenotype of the $\Delta$(590–613) polymerase. To examine if this is a unique hot spot or if the mutant polymerase is a strong mutator for additions in other runs that differ in base composition, length, and surrounding sequence, we examined error rates using reversion substrates which score single base additions at runs of 4, 5, 6, or 7 consecutive Ttemplate:Aprimer base

---

Properties of Klenow Polymerase Thumb Mutant

The results of two independent experiments with this assay yielded lacZ mutant frequencies among the products of gap-filling that were slightly higher for the $\Delta$(590–613) polymerase than for the wild-type enzyme (Table II). DNA samples from 98 independent mutants generated by the $\Delta$(590–613) polymerase were isolated and sequenced to determine the error specificity. These data were compared to 94 independent mutants generated by the wild-type enzyme (Table II).

The most striking observation was that 52 of 98 mutants from the $\Delta$(590–613) polymerase collection contained a one-base addition (Table III). The result was quite unexpected because, like other DNA polymerases (Kunkel, 1990), the wild-type Klenow polymerase is much more prone to generate deletions and only rarely makes addition errors; note that only one +1 mutant was observed in the wild-type polymerase error collection (Table III). The mutant polymerase is thus a 70-fold mutator for addition errors. A sequence-specific increase in one-base deletions by the $\Delta$(590–613) polymerase was also observed, since 4 of 6 one-base deletions in the $\Delta$(590–613) polymerase collection occur at a single TTTT run, while none were recovered at this site for the wild-type polymerase. This result is consistent with the result of the reversion assay (Table II), which scores loss of a base within a run of 5 T nucleotides at this same position. Thus, the data reveal that the $\Delta$(590–613) polymerase has a mutator phenotype for single-base additions and some single-base deletions.

The addition errors by the $\Delta$(590–613) polymerase all occurred within runs of three or more consecutive identical bases, and the addition error rate increased with increasing homopolymeric run length (Fig. 3). This is consistent with a model wherein frameshift errors occur through misaligned intermediates. In contrast, the $\Delta$(590–613) polymerase error rates for substitutions and for deletions at non-reiterated sequences were similar to those of the wild-type polymerase (Table III).

Forty-two of 52 additions by the $\Delta$(590–613) polymerase were in a run of five consecutive C-G base pairs (Fig. 2), representing a 60-fold increase in error rate relative to the wild-type polymerase. Since errors at this site dominate the mutant polymerase error collection, removing this site from the analysis would result in a lower estimate of the apparent mutator phenotype of the $\Delta$(590–613) polymerase. To examine if this is a unique hot spot or if the mutant polymerase is a strong mutator for additions in other runs that differ in base composition, length, and surrounding sequence, we examined error rates using reversion substrates which score single base additions at runs of 4, 5, 6, or 7 consecutive Ttemplate:Aprimer base
pairs, where the nucleotide sequences flanking the runs are identical on the 5' side and similar on the 3' side (see legend to Table IV; see also Fig. 2 in Kroutil et al. (1996)). The use of four substrates containing runs of increasing length also probes the nature of the interaction between the polymerase and frameshift intermediates containing extra nucleotides at different locations in the duplex template-primer stem (see "Discussion").

Gap-filling synthesis reactions catalyzed by the wild-type and mutant polymerases were performed with each of the four substrates, and the products were plated to score lacZ reversion frequencies. Since revertants (i.e. blue-plaques) result from any gain or loss of nucleotides that restores the correct reading frame, revertants from all eight polymerization reactions were sequenced in order to calculate reversion frequencies for the addition of a single TzA base pair within the homopolymeric runs. The results from this experiment are shown in Table IV.

The total reversion frequencies of products generated by the wild-type Klenow polymerase were above the uncopied DNA control values (see legend to Table IV). Most of the revertants resulted from deletion of two non-reiterated bases flanking the TzA runs, and only a few resulted from addition of a TzA base pair within the run (Table IV). This is consistent with the earlier observation that the error rate for two-base deletions was substantially higher than that for single-base additions (Bebenek et al., 1990). The overall reversion frequencies for the products of synthesis by the Δ(590–613) polymerase were higher than for wild-type Klenow for all four substrates, and all but three of the 38 revertants sequenced contained an additional TzA base pair in the run. Moreover, except for the substrate containing seven consecutive TzA base pairs, the addition error rate increased with increasing run length, consistent with the involvement of misaligned intermediates. Relative to wild-type Klenow, the addition error rate of the Δ(590–613) polymerase with the four substrates was elevated by 25–67-fold. These data clearly show that the strong frameshift mutator phenotype conferred by the Δ(590–613) mutation is not limited to a run of a particular length. Moreover, when combined with data from the forward mutation assay, they also show that this phenotype is not limited to a run of a particular nucleotide composition, or to a run surrounded by a particular flanking nucleotide sequence.

**Fig. 2. Spectrum of single base errors by the Δ(590–613) mutant.** Three lines of LacZ α-complementation template sequence are shown, with position +1 as the first transcribed nucleotide. Substitutions are shown above the sequence, while deletions (Δ) and additions (Å) are below. For frameshifts at reiterated sequences, the symbol is centered under the run, since it is not possible to determine which base was deleted or added.

**Fig. 3. Plus-one error rates versus homopolymeric run length.** Error rates were calculated by multiplying the overall mutant frequency by the proportion of mutants occurring at each run length (from Fig. 2), dividing by 0.6 (the probability of expressing an error upon transfection) and then dividing by the number of known detectable sites for each run length (from Bebenek and Kunkel (1995)).
DISCUSSION

Residues 558–637 of the thumb subdomain of Klenow polymerase contain two long anti-parallel \( \alpha \)-helices, \( H \) and \( I \), connected by 50 amino acids that were disordered in the initial crystals (Ollis et al., 1985a). These are now known to comprise two short \( \alpha \)-helices, \( H_2 \) and \( H_3 \), with random coils connecting these to each other and to helices \( H \) and \( I \) (Beese et al., 1993).

Upstream of the thumb's tip is a coiled structure that makes a cleft in which duplex DNA binds. The NH\(_4\)\( \)\textsuperscript{+} terminus of helix \( H \) fits into the minor groove, perpendicular to the DNA helix axis (Beese et al., 1993), and interactions are observed between side chains of specific residues and the DNA phosphate backbone of the primer strand. The residues involved are highly conserved in the polymerase I family (Beese et al., 1993).

Consistent with the functional importance of these interactions, the present study reveals that removal of residues in the thumb subdomain yields a polymerase with strongly reduced DNA binding affinity (Table I), reduced processivity (Fig. 1) and reduced frameshift fidelity at reiterated sequences (Fig. 3, Tables III and IV). The fact that most frameshift errors by the mutant polymerase were in homopolymeric runs (Fig. 2) and that the addition rate increased with increasing run length (Fig. 3) is consistent with errors initiated by strand slippage (Streisinger et al., 1966; reviewed by Kunkel (1990)). In contrast, the wild-type and mutant polymerases had similar error rates for substitutions and for deletions of non-reiterated bases (Table III). Our favored hypothesis for the latter errors is nucleotide misinsertion, followed by primer relocation to generate misaligned intermediates (Kunkel and Soni, 1988; Bebenek and Kunkel, 1990). This absence of a mutator effect for either type of error initiated by misinsertion, coupled with the fact that the \( \Delta \)(590–613) mutant polymerase has kinetic constants and a denaturation temperature similar to those of wild-type Klenow fragment (Table I), suggests that the structural changes resulting from the 24-amino acid deletion are confined to the tip of the thumb subdomain and therefore that interactions between the thumb's tip and the DNA do not strongly influence misinsertion fidelity.

The concomitant decreases in DNA binding, processivity, and errors initiated by strand misalignment that result from the \( \Delta \)(590–613) mutation are consistent with observations with other DNA polymerases indicating a relationship between these three properties (see Bebenek et al. (1995), and references therein). In particular, studies of HIV-1 reverse transcriptase revealed that frameshift hot spots were sites that exhibited a strong probability of termination of processive synthesis (Bebenek et al., 1989) and that altering the template-primer sequence near the 3′-OH terminus resulted in concomitant increases or decreases in both frameshift error rates and termination probabilities (Bebenek et al., 1993). These correlations and the present results with the mutant Klenow polymerase suggest that dissociation and reassociation of the enzyme-DNA complex may allow formation and/or utilization of misaligned frameshift intermediates.

The idea that interactions between a DNA polymerase and the duplex template-primer stem may control the rate of misalignment-mediated errors is supported both by the present study and by studies showing that alanine substitution of two amino acid residues (Gly-262 and Trp-266) in \( \alpha \) helix \( H \) of the thumb subdomain of HIV-1 reverse transcriptase also resulted in decreased DNA binding, decreased processivity, and increased rates for errors resulting from template-primer slippage (Beard et al., 1994; Bebenek et al., 1995). These two amino acids in the RT are thought to make contacts in the minor groove, specifically with the primer strand of the duplex DNA within either three nucleotides (for W266A) or four to five nucleotides (for G262A) of the primer terminus. Thus, even though the thumb subdomains of Klenow polymerase and HIV-1 RT share little structural similarity (except that both contain mostly helical structures), they may serve common functions in binding the duplex template-primer upstream of the catalytic site.

There are two remarkable features of the mutator specificity of the \( \Delta \)(590–613) polymerase. One is the strong increase in single-base addition error rates, such that additions far exceed deletions (Tables III and IV). This is quite different from most other DNA polymerases, which generate deletions much more readily than additions (e.g. see Kunkel (1990)). This unusual specificity of the \( \Delta \)(590–613) polymerase may relate to perturbations of the interactions observed in the co-crystal structure between residues in the thumb's tip and the primer strand, since it is this strand, not the template strand, that would contain the unpaired nucleotide in the misaligned intermediate required for an addition error. A similar explanation may account for the 46-fold increase in single-base addition errors previously observed for synthesis by T7 DNA polymerase in the absence of its accessory subunit, thioredoxin (Kunkel et al., 1994). Although the structure of T7 DNA polymerase is not known, it is likely to be very similar to that of Klenow fragment because these two polymerases exhibit strong sequence homology, particularly within the polymerase domain (Ollis et al., 1985b; Braithwaite and Ito, 1993). Based on the location of mutations in T7 DNA polymerase that suppress thioredoxin

<table>
<thead>
<tr>
<th>Number of Ts in run</th>
<th>Total Plaques</th>
<th>Total Reversion Frequency</th>
<th>Plus-one mutations</th>
<th>Relative Reversion frequency (Mutant:WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Revertant</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \times 10^7 )</td>
<td>( \times 10^{-5} )</td>
<td>( \times 10^{-5} )</td>
<td></td>
</tr>
<tr>
<td>Wild-type Klenow polymerase</td>
<td>4</td>
<td>280</td>
<td>27</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>240</td>
<td>55</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>920</td>
<td>24</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>390</td>
<td>55</td>
<td>14</td>
</tr>
<tr>
<td>( \Delta )(590–613) polymerase</td>
<td>4</td>
<td>69</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>270</td>
<td>225</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>270</td>
<td>338</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>160</td>
<td>212</td>
<td>130</td>
</tr>
</tbody>
</table>

The reversion frequency for addition errors was calculated by dividing the number of revertants by the total number of plaques and then multiplying by the proportion of revertants containing one additional A base pair in the run, as determined by DNA sequence analysis of revertants as listed. The reversion frequencies of uncopied DNA are 1.3 \( \times 10^{-5} \), 0.8 \( \times 10^{-5} \), 0.7 \( \times 10^{-5} \), and 8.5 \( \times 10^{-5} \), respectively, for the substrates containing 4, 5, 6, or 7 consecutive T base pairs, as determined by DNA sequence analysis of the revertants containing one additional A base pair in the run.
point mutations, Himawan and Richardson (1992) have proposed that thioredoxin may contact the polymerase thumb subdomain at the junction with the flexible subdomain at the tip, and may act together with the polymerase to clamp the DNA into position. Together with previous structural information, the DNA binding, processivity, and fidelity data reported here are consistent with the possibility that the domain at the tip of the thumb in Klenow polymerase may serve a similar role to that proposed for thioredoxin in the T7 system. However, there may be differences between these two polymers in the precise nature of the interactions with DNA, as the site specificity of addition errors by the Δ(590–613) Klenow polymerase and T7 DNA polymerase lacking thioredoxin are different (compare Fig. 2 to Fig. 1 in Kunkel et al. (1994)).

A second interesting aspect of the mutator specificity of the Δ(590–613) polymerase is the extraordinary hot spot for addition of a C-G base pair in a run of five consecutive C-G base pairs at nucleotides 132–136 of the lacZ target sequence (Fig. 2). The addition error rate per detectable nucleotide polymerized at this run is 1.6 × 10^{-4} (Fig. 3), which is 60-fold greater than for the wild-type polymerase and by far the highest rate for the mutant polymerase among all the template positions monitored in the forward mutation assay. One factor contributing to this high rate is likely to be the run length. Since this is the longest homopolymeric run in the wild-type lacZ target, it may allow formation of the most stable misaligned intermediates. Other factors that could possibly contribute to the high error rate at this site include the base composition of the run (template C, incoming G) and the sequence of nucleotides flanking the run. However, comparison of the data from the forward assay to that from the reversion assays (Table IV) clearly demonstrates that the strong mutator phenotype for single-base-addition errors conferred by the Δ(590–613) mutation in Klenow polymerase is not limited to a particular run length, nucleotide composition, or flanking nucleotide sequence.

The experiment shown in Table IV was designed to examine the possibility that the magnitude of the frameshift mutator effect at the hot spot (Fig. 2) might partly reflect the ability of the Δ(590–613) polymerase to generate more frequently, or to use more efficiently, a subset of the possible misaligned intermediates that could form in that run. This concept emerged from previous observations with mutant derivatives of HIV-1 RT (Bebenek et al., 1985). For example, a mutator HIV-1 RT containing an alanine substituted for a tryptophan at residue 590–613 has frameshift fidelity that is reduced more in three- and four-base runs than in longer runs. RT-DNA co-crystal structural data (Jacobo-Molina et al., 1993) and modeling (Bebenek et al., 1995) suggest that Trp-266 interacts with the primer strand three base pairs back from the active site. Thus, removing this specific side chain interaction might more readily allow formation of or use of a misaligned intermediate with an unpaired nucleotide in this region of the duplex template-primer stem, but have little effect if the unpaired nucleotide were positioned further back, as is possible in a longer run. In the present study, the most stable intermediate at the CCCCC run (Fig. 2), formed by slippage after incorporation of the fifth dGMP residue, would have the unpaired base residing in the primer strand between the fourth and fifth nucleotides back from the terminus. Side chains from the thumb's tip interact with the duplex DNA in this region in both Klenow polymerase (see Table I in Beese et al. (1995)), and in Teg DNA polymerase (Eom et al., 1996). Loss of these interactions in the Δ(590–613) polymerase might permit more efficient use of misaligned intermediates with unpaired nucleotides in this region.

This model was probed here using substrates containing a run of TA base pairs of increasing run length but in similar sequence contexts. The error rate with the Δ(590–613) polymerase increases as the run length increases from 4 to 5 to 6, and the mutator effect is similar (50-, 56-, and 67-fold, respectively; Table IV) at these three run lengths. This suggests that addition errors can be generated at high rates via misaligned intermediates with extra primer strand nucleotides at any of several positions in the region, up to six base pairs from the active site. The slightly smaller mutator effect observed with the run of seven template T residues (25-fold, Table IV) may suggest lesser or different polymerase-DNA interactions when an extra base is positioned at a greater distance from the active site. Additional studies, including those using longer runs, would be required to test this possibility.

In conclusion, removal of residues 590–613 in the tip of the Klenow fragment thumb results in a protein with reduced DNA binding, processivity, and fidelity for frameshifts occurring at reiterated sequences. These observations are consistent with structural information on Klenow polymerase bound to DNA (Beese et al., 1993), indicating a role for these residues in duplex DNA binding. Since several of the residues in this region are highly conserved or invariant in the polymerase I family of polymerases (Delarue et al., 1990), it may be possible to identify specific residues that provide key interactions for determining template-primer binding, processivity, and frameshift fidelity.

Acknowledgments—We thank Xiaojun Chen Sun and Ruoying Tang for expert technical assistance. We also thank William C. Copeland and Joann Sweeney for their comments on the manuscript and Joe Jager, Soo Hyun Eom, Jimin Wang, and Tom Steitz for helpful discussions on polymerase structures.

REFERENCES

Edwards, K. A., J. Biol. Chem. 263, 13242–13247
Properties of Klenow Polymerase Thumb Mutant

A Thumb Subdomain Mutant of the Large Fragment of *Escherichia coli* DNA Polymerase I with Reduced DNA Binding Affinity, Processivity, and Frameshift Fidelity

Dana T. Minnick, Mekbib Astatke, Catherine M. Joyce and Thomas A. Kunkel

doi: 10.1074/jbc.271.40.24954

Access the most updated version of this article at [http://www.jbc.org/content/271/40/24954](http://www.jbc.org/content/271/40/24954)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 45 references, 21 of which can be accessed free at [http://www.jbc.org/content/271/40/24954.full.html#ref-list-1](http://www.jbc.org/content/271/40/24954.full.html#ref-list-1)