The fidelity of DNA replication by *Escherichia coli* DNA polymerase I (pol I) was assessed *in vivo* using a reporter plasmid bearing a ColE1-type origin and an ochre codon in the β-lactamase gene. We screened 53 single mutants within the region Val700–Arg712 in the polymerase active-site motif A. Only replacement of Met, Asn, Phe, or Ala increasing the Km values for both insertion of non-complementary nucleotides and extension of mispaired primer termini. Abolishment of the 3′-5′ exonuclease activity of wild-type pol I increased mutation frequency 4-fold, whereas the combination of I709F and lack of the 3′-5′ exonuclease yield a 400-fold increase. We conclude that accurate discrimination of the incoming nucleotide at the polymerase domain is more critical than exonucleolytic proofreading for the fidelity of pol I *in vivo*. Surprisingly, the I709F polymerase enhanced mutagenesis in chromosomal DNA, although the increase was 10-fold less than in plasmid DNA. Our findings indicate the feasibility of obtaining desired mutations by replicating a target gene at a specific locus in a plasmid under continuous selection pressure.

DNA polymerases catalyze chain elongation reactions guided by complementary base pairings opposite a single-stranded DNA template. These reactions are highly accurate, exhibiting error rates of about one base substitution error per 10^4 to 10^7 nucleotides polymerized (1). However, errors made by the polymerase, if not subsequently excised, can become fixed as mutations during subsequent rounds of replication. As a result, errors by DNA polymerases can be a major source of spontaneous mutagenesis and can contribute to the multiplicity of mutations found in cancer cells (2, 3). Many DNA polymerases have intrinsic or associated 3′-5′ exonucleases that preferentially hydrolyze non-complementary nucleotides immediately after formation of the phosphodiester bond and contribute from a few- to 100-fold to the fidelity of DNA synthesis (4–6). In addition, errors introduced by DNA polymerases are subsequently corrected by a mismatch repair system, which contributes an additional 2–3 orders of magnitude to the accuracy of DNA replication (7). However, base selection at the polymerase active site during both the nucleotide insertion and subsequent extension reactions, including Watson-Crick base pair formation between complementary bases and a conformational change of the active site during each incorporation step, is likely the most significant contributor to the fidelity of DNA polymerization (1, 8–10). We have investigated the relationships between structure and function at the active site of DNA polymerases by substituting random sequences for nucleotides that encode residues at the active site and monitoring the effects of these substitutions on the fidelity of DNA synthesis (11, 12).

*Escherichia coli* DNA polymerase I (pol I) is involved in DNA replication, DNA repair, and genetic recombination (13); it is the most extensively studied of all DNA polymerases. Evidence indicates that pol I functions in DNA replication by removal of RNA primers and resynthesis of the resulting gaps between Okazaki fragments on the lagging strand (14, 15). In addition, pol I participates in DNA repair by filling gaps resulting from the excision of damaged bases (16, 17). Moreover, pol I is required for the initiation of synthesis at the origin of replication in certain plasmids (13, 18). The crystal structure of the Klenow fragment of pol I (which lacks the 5′–3′ exonuclease domain) reveals an architecture that is common among DNA polymerases and has been likened to a human right hand, with a fingers subdomain (which binds the incoming dNTP and interacts with the single-stranded DNA template), a thumb subdomain (which binds double-stranded DNA), and a palm subdomain (which harbors the catalytic amino acids and also interacts with the incoming dNTP) (19, 20). Several mutant forms of the Klenow fragment of pol I (in which single amino acid substitutions have been introduced into the active-site fingers or palm subdomain (21–23) or a large 24-amino acid segment in the thumb subdomain has been deleted (24)) that exhibit altered fidelity in DNA synthesis *in vitro* have been investigated. Although many such mutant enzymes exhibit reduced fidelity *in vitro*, none has been shown to alter accuracy *in vivo*.

In a recent study, we examined the mutability of motif A, extending from Val700 to Arg712, in the palm subdomain of *E. coli* pol I using random mutagenesis and a genetic complementation system (25). We established a library of 500,000 transfectants and sequenced 232 of 37,500 mutants that were active in the complementation assay. *E. coli* strains harboring the active mutants were fit to replicate repetitively, and the mutant polymerases, when purified, displayed 20–190% of the wild-type specific activity. Thus, motif A is highly mutable.

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1 The abbreviations used are: pol I, DNA polymerase I; exo−, exonuclease-deficient; 3′exo−, 3′–5′ exonuclease-deficient; pol III, DNA polymerase III.
while preserving wild type-like DNA polymerase activity in vitro and in vivo. The ease of substitutability of motif A residues revealed in this work, yielding highly functional variants, stands in sharp contrast to the marked conservation of the motif A sequence observed among prokaryotic DNA polymerases (26, 27). Interestingly, we also found that certain substitutions of Ile709 permit more efficient utilization of rNTPs as substrates in vitro.

In this study, we screened 53 mutations in motif A for fidelity of DNA synthesis in vivo and found that mutant enzymes harboring Ile709 substitutions exhibited less accurate DNA replication. The mutant phenotype was enhanced when the Ile709 substitutions were combined with deficiency of 3'–5' exonuclease-lytic proofreading activity. In subsequent in vitro experiments, we determined that the I709F substitution increased both insertion of non-complementary nucleotides as well as extension from primers with mismatched 3'-OH termini. To our knowledge, this is the first analysis of the effects of mutation in the polymerase active site of E. coli pol I on the fidelity of DNA synthesis both in vivo and in cells.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The wild-type and mutant E. coli pol I genes were inserted into pHSG576 (28), placing them under the control of the lac promoter. pHSG576 is low copy number plasmid that has a pol I-independent origin. To modify the gene, the wild-type pol I gene of E. coli DH5α was amplified by colony polymerase chain reaction and inserted into pHSG576 to create pECpol I as described previously (25). Site-directed mutagenesis was performed on pECpol I to introduce an A-to-C transition at position 1271, thus changing Asp424 to Ala and inserted into pHSG576 to create pECpol I-3'exo, pECI709F-3'exo, and pECI709F-3'exo, as indicated. The amplified fragment was cloned into pCRII; the nucleotide sequence of the entire trpE gene was then determined.

**Kinetic Analysis**—Steady-state kinetic analysis of misincorporation frequency was performed based on the method of Bossalis et al. (33). A 47-mer template (5'-CGCGCGCTTTAGGGCGATCTGATCGCCCT-3'; the relevant template bases are underlined) was hybridized with a 32P-labeled 23-mer primer (5'-CCGCCCGATTCCTGCTAGCAAT-3') for analysis of misincorporation efficiency opposite dT and with a 25-mer primer (5'-GGGGCGATCA-3') for analysis opposite dG. Primer-template (5 nM) was incubated for 5 min at 37 °C in a reaction mixture containing limiting amounts of purified recombinant Klenow (exo-) protein (5 nM) prepared as described previously (25) and varying concentrations of each dNTP in 10 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, and 7.5 mM diithiothreitol. The ranges of nucleotide substrate concentrations used for measuring incorporation opposite template dT were 0.5–7.5 μM dATP, 0.5–50 μM dGTP, and 10–300 μM dCTP and dTTP for the wild-type enzyme and 0.5–7.5 μM dATP, 0.05–5 μM dGTP, 2–14 μM dCTP, and 1–7 μM dTTP for the I709F mutant enzyme. The concentrations of the nucleotide substrates opposite template dG were 10–70 μM dATP, 1–50 μM dGTP, 2–300 μM dCTP, and 10–300 μM dTTP for the wild-type enzyme and 0.1–5 μM dATP, 0.1–5 μM dGTP, 2–30 μM dCTP, and 0.1–50 μM dTTP for the I709F mutant enzyme. Following termination of the reaction by addition of 2.5 μl of formamide solution, the products were analyzed by 14% polyacrylamide gel electrophoresis and quantified by phosphor image analysis (34).

Mismatch extension frequency was determined using a similar protocol, except that the sequence of the 24-mer primer was 5'-CCGCACGATTCCTGCTAGCAAT-3' and with a 25-mer primer (5'-GGGGCGATCA-3') for analysis opposite dG. Extended mismatch 5'oxanthanase was measured for each primer-template construct. The concentrations of the dNTP substrate mixture were 0.5–3.5 μM for the T:A mismatch.

**RESULTS**

**Screening of pol I Mutants for Mutator Activity**—To measure errors in DNA synthesis by pol I in vitro, we established a two-plasmid system. The reporter plasmid pLA230 (Fig. 1 A) contains a β-lactamase gene harboring an ochre mutation near the 5' terminus. Since evidence indicates that pol I is involved in initiation of DNA synthesis in ColE1-type plasmids (13, 18), we introduced the ochre mutation ~250 bp from the ori sequence. The reporter plasmid, together with a second plasmid encoding the wild-type or mutant pol I gene, was transfected into E. coli JS200 (30, 31), a strain that contains a temperature-sensitive pol I. The reversion frequency at the β-lactamase locus was determined by measuring colony formation in the presence and absence of carbenicillin (Fig. 1 B).

The pol I gene encoded by the second plasmid corresponds to the intact enzyme in the presence of 25 μg/ml carbenicillin and encodes both 5'-3' and 3'-5' exonuclease activities as well as DNA polymerase activity (13). To identify amino acids in active-site motif A that affect the fidelity of DNA synthesis, we tested 53 different single motif A mutations within the segment spanning Val700 to Ile709, which was amplified from chromosomal DNA by colony polymerase chain reaction and excised and cloned into the pLA2800 to create pECI709M, pECI709N, pECI709F, and pECI709A, respectively.

**Plasmid Construction**—As described previously (25) for analysis opposite dG. Primer-template (5 nM) was incubated for 5 min at 37 °C in a reaction mixture containing limiting amounts of purified recombinant Klenow (exo-) protein (5 nM) prepared as described previously (25) and varying concentrations of each dNTP in 10 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, and 7.5 mM diithiothreitol. The ranges of nucleotide substrate concentrations used for measuring incorporation opposite template dT were 0.5–7.5 μM dATP, 0.5–50 μM dGTP, and 10–300 μM dCTP and dTTP for the wild-type enzyme and 0.5–7.5 μM dATP, 0.05–5 μM dGTP, 2–14 μM dCTP, and 1–7 μM dTTP for the I709F mutant enzyme. The concentrations of the nucleotide substrates opposite template dG were 10–70 μM dATP, 1–50 μM dGTP, 2–300 μM dCTP, and 10–300 μM dTTP for the wild-type enzyme and 0.1–5 μM dATP, 0.1–5 μM dGTP, 2–30 μM dCTP, and 0.1–50 μM dTTP for the I709F mutant enzyme. Following termination of the reaction by addition of 2.5 μl of formamide solution, the products were analyzed by 14% polyacrylamide gel electrophoresis and quantified by phosphor image analysis (34).

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The pol I gene encoded by the second plasmid corresponds to the intact enzyme in the presence of 25 μg/ml carbenicillin and encodes both 5'-3' and 3'-5' exonuclease activities as well as DNA polymerase activity (13). To identify amino acids in active-site motif A that affect the fidelity of DNA synthesis, we tested 53 different single motif A mutations within the segment spanning Val700 to Arg212 (25).

Representative reversion frequencies obtained for mutants containing substitutions at each of the positions analyzed are
shown in Table I. The reversion frequency observed for wild-
type pol I was $\sim 1 \times 10^{-7}$, as was that for all the mutants tested 
except the four with substitutions at position 709. Substitution 
of Met, Asn, Phe, or Ala for Ile$^{709}$ yielded 5.3–23 times higher 
reversion frequencies than that for wild-type pol I (Tables I and 
II). We analyzed the nucleotide sequence of the $\beta$-lactamase 
reporter gene from five independent revertants harboring 
I709F mutant pol I and confirmed that the ochre mutation was 
converted twice to TTA, twice to TCA, and once to CAA. The 
enhanced mutagenesis observed for the Ile$^{709}$ mutants provides 
new evidence that E. coli DNA polymerase I is involved in 
plasmid replication by copying nucleotides near the ori 
sequence and demonstrates that Ile$^{709}$ is critical for accurate 
plasmid replication in vivo.

Effect of Exonucleolytic Proofreading Activity by pol I on 
Plasmid Replication—To analyze the contribution of the 3’–5’ 
exonuclease (“proofreading”) activity of pol I to the fidelity of 
plasmid replication, we mutated the 3’–5’ exonuclease in the 
wild-type enzyme and in the Ile$^{709}$ variants by substituting Ala 
for Asp at position 424 in the exonuclease domain. When in-

| pol I | No. of colonies $\times 10^{9}$ (±carbenicillin) | No. of colonies/ml (+carbenicillin) | Reversion frequency $\times 10^{8}$ |
|-------|-----------------------------------------------|-----------------------------------|-------------------------------|
| Wild-type | 8.7 | 90 | 1.0 |
| V700I | 10 | 140 | 1.4 |
| I701N | 9.4 | 120 | 1.3 |
| V702A | 9.4 | 70 | 0.7 |
| S703R | 8.5 | 90 | 1.1 |
| A704S | 8.8 | 110 | 1.3 |
| Aasp$^{705}$ | 9.0 | 60 | 0.7 |
| Y706F | 8.6 | 70 | 0.8 |
| Q708H | 9.2 | 80 | 0.9 |
| I709N | 9.1 | 2100 | 23 |
| E710D | 8.6 | 30 | 0.3 |
| L711V | 8.9 | 160 | 1.8 |
| R712S | 9.6 | 80 | 0.8 |

* The polymerases listed exhibited the highest reversion frequencies 
  observed among mutants at each position in motif A.

* Aasp$^{705}$ is inmutable (25).

introduced into the wild-type construct, the D424A substitution 
enhanced the reversion frequency of the $\beta$-lactamase gene by 
4.4-fold (Table II). Larger increases (up to 22-fold) were ob-
served for specific amino acid substitutions of Ile$^{709}$, indicating 
that discrimination at the active site can be more important for 
fidelity than exonucleolytic proofreading. Abolishment of the 
3’-exonuclease activity in the mutants harboring the I709M,
I709N, I709F, or I709A substitution resulted in a 29–416-fold 
increase in mutation frequency relative to the wild-type en-
zyme. For each of the mutants, the increase in reversion fre-
quency associated with inactivation of the exonuclease was 
greater than that observed for the wild-type enzyme. In the 
case of I709F, the increase was substantially greater than 
multiplicative, suggesting a functional interaction between the 
exonuclease domain and motif A.

To further evaluate the mutant polymerases, we measured 
the reversion frequency of the same $\beta$-lactamase gene on an-
other plasmid, pLA2800 (Fig. 1A and Table III). In this con-
struct, the $\beta$-lactamase gene is located $\sim 2.8$ kb downstream 
of the origin of replication and thus is $\sim 10$-fold more distant from 
the origin than in pLA230. Introduction of the 3’ exo mutation 
into the wild-type construct or substitution of Met, Asn, Phe, or 
Ala for Ile$^{709}$ in separate constructs resulted in at most 
1.8-fold increase in reversion frequency. In contrast, pol I har-
boring both the 3’ exo mutation and an Ile$^{709}$ substitution 
showed 10–87-fold higher reversion frequency than wild-type 
pol I; the elevations were not as large, however, as those ob-
served for pLA230 (Table II and III). These results suggest that 
DNA synthesis by pol I is not necessarily limited to nucleotides 
near the origin, but can occur much farther downstream.

Effect of Mutator pol I on Replication of Chromosomal 
DNA—E. coli JS200 cannot grow in the absence of tryptophan 
since it carries the trpE65 (ochre) allele in the host chromo-
some. We investigated the effects of the mutant polymerases on 
the reversion frequency at the trpE locus (Table IV). The re-
version frequency observed for wild-type pol I was $2.0 \times 10^{-8}$. 
Neither the 3’ exo mutation nor the I709F substitution signif-
icantly increased this frequency. In contrast, the mutant pol I 
with both the 3’ exo mutation and the I709F substitution 
exhibited a 40-fold increase in reversion frequency. We ana-
alyzed the nucleotide sequence of the trpE gene from three 
independent revertants and determined that the ochre muta-
tion was converted once to TAC and twice to TCA. These 
results indicate that the mutant pol I bearing both an Ile$^{709}$ 
substitution and the 3’ exo mutation participates in replica-

FIG. 1. Schematic representation of reporter plasmids (A) 
and the $\beta$-lactamase reversion assay (B). A, the mutant 
$\beta$-lactamase gene harboring an ochre codon was inserted $\sim 0.23$ kb (pLA230) or $\sim 2.8$ kb (pLA2800) from the ColE1-type origin on pGPS3 as described under 
“Experimental Procedures”. B, a pol I-deficient temperature-sensitive 
(ts) E. coli strain was transformed by a reporter plasmid carrying a 
$\beta$-lactamase mutant, after which the wild-type or mutant pol I gene on 
a plasmid was introduced into the bacteria. The recombinant strain was 
cultured in liquid medium containing kanamycin (Km), tetracycline (Tet), 
and chloramphenicol (Cm) and then plated on agar plates containing 
kanamycin, tetracycline, and chloramphenicol with or without 
carbenicillin (Carb). Colonies were counted after 16 h at 37°C, 
and reversion frequency was determined.


**Mutator Mutants of E. coli DNA Polymerase I**

| pol I | Reversion frequencya | Relative frequency |
|-------|----------------------|--------------------|
|       |                      |                    |
| Wild-type | (1.2 ± 0.7) × 10⁻⁷ | 1                  |
| 3' exo | (3.3 ± 1.6) × 10⁻⁷ | 4.4                |
| I709M | (2.2 ± 0.3) × 10⁻⁷ | 5.3                |
| I709M/3' exo⁻ | (3.5 ± 0.3) × 10⁻⁶ | 29                 |
| I709N | (2.6 ± 0.6) × 10⁻⁶ | 22                 |
| I709N/3' exo⁻ | (3.3 ± 1.5) × 10⁻⁵ | 280                |
| I709F | (1.6 ± 0.1) × 10⁻⁵ | 13                 |
| I709F/3' exo⁻ | (5.0 ± 0.1) × 10⁻⁵ | 416                |
| I709A | (1.5 ± 0.2) × 10⁻⁶ | 13                 |
| I709A/3' exo⁻ | (1.2 ± 0.1) × 10⁻⁵ | 100                |

**Values represent means ± S.D. obtained by plating three independent clones.**

| pol I | Reversion frequencya | Relative frequency |
|-------|----------------------|--------------------|
|       |                      |                    |
| Wild-type | (1.5 ± 0.4) × 10⁻⁷ | 1                  |
| 3' exo | (1.3 ± 0.9) × 10⁻⁷ | 0.9                |
| I709M | (2.7 ± 0.5) × 10⁻⁷ | 0.7                |
| I709M/3' exo⁻ | (1.0 ± 0.5) × 10⁻⁶ | 6.7                |
| I709N | (2.7 ± 1.1) × 10⁻⁶ | 1.3                |
| I709N/3' exo⁻ | (5.2 ± 0.9) × 10⁻⁶ | 3.5                |
| I709F | (1.4 ± 0.6) × 10⁻⁷ | 0.9                |
| I709F/3' exo⁻ | (1.3 ± 0.4) × 10⁻⁵ | 87                 |
| I709A | (1.9 ± 0.7) × 10⁻⁷ | 1.3                |
| I709A/3' exo⁻ | (2.9 ± 1.0) × 10⁻⁶ | 19                 |

**Values represent means ± S.D. of three determinations.**

| pol I | Reversion frequencya | Relative frequency |
|-------|----------------------|--------------------|
|       |                      |                    |
| Wild-type | (2.0 ± 0.8) × 10⁻⁸ | 1                  |
| 3' exo | (3.0 ± 2.2) × 10⁻⁸ | 1.5                |
| I709F | (2.3 ± 1.3) × 10⁻⁸ | 1.2                |
| I709F/3' exo⁻ | (0.8 ± 0.2) × 10⁻⁶ | 40                 |

**Values represent means ± S.D. obtained by analyzing three clones.**

**DISCUSSION**

We have used random mutagenesis to establish a library of mutations in motif A of E. coli DNA polymerase I and utilized genetic complementation of a pol I-deficient temperature-sensitive E. coli strain to identify active mutants (25). By screening portions of this library with a reporter plasmid, we determined here that pol I mutants with an I709M, I709N, I709F, or I709A substitution in the catalytic palm subdomain exhibit a mutator phenotype. Enhanced mutagenesis was observed during both plasmid and chromosomal DNA replication. Thus, we have obtained pol I mutants that display low fidelity of DNA replication in vivo. We know of no other active-site mutants of pol I that exhibit reduced replication accuracy in cells, although Minnick et al. (23) have reported that a mutant Klenow (exo⁻) enzyme with the single amino acid substitution E710A in motif A efficiently incorporates mismatched nucleotides in vitro. We have not detected the E710A mutation in the E. coli pol I motif active mutant library (25) or in a corresponding Thermus aquaticus pol I library (35), presumably because the catalytic activity of the mutant is insufficient to permit complementation.

**Measurements of the in vitro fidelity of the 3'exo⁻ Klenow fragment of E. coli pol I (9) and the 3'exo⁻ E. coli DNA polymerase III (pol III) holoenzyme (8) indicate that abolishment of the exonucleolytic activity increases the overall error frequency by 4–7-fold for pol I and by <10-fold for pol III. These relatively modest increases indicate that the major component of accuracy of these enzymes, one error per 10⁷ nucleotides polymerized, represents discrimination during polymerization, presumably including a conformational change in the enzyme at each nucleotide addition step (36, 37). Our results demonstrating that nucleotide selection at the polymerase active site is the major contributor to fidelity in vivo provide a novel enzyme. However, the effect of the mutator activity on chromosomal DNA synthesis was less than on plasmid DNA synthesis. In all of the in vivo situations examined, specific mutations at the polymerase active site and inactivation of the proofreading activity acted synergistically to increase the mutator activity of pol I.

**Measurement of Polymerase Fidelity in Vitro**—To establish in vitro correlates of the mutator phenotype of the Ile709 mutants, we purified the Klenow fragments of the wild-type and I709F exo⁻ polymerases to apparent homogeneity (25). These fragments lack both 5’–3’ and 3’–5’ exonuclease activities. The 5’–3’ exonuclease could remove the 5’-label from the primer, and the 3’–5’ exonuclease could remove added nucleotides in extension experiments. We then analyzed the efficiency of misinsertion using a steady-state gel-based assay (33) to measure the kinetics of single nucleotide addition opposite template dT or dG. The primer was a 62- or 25-nucleotide oligomer that was labeled at the 5’-end with ³²P, and the 3’-terminal nucleotide was one residue upstream from the target. The wild-type and mutant enzymes showed typical Michaelis-Menten saturation kinetics when initial velocity was plotted against the concentration of each nucleotide (data not shown). Apparent kinetic parameters and relative insertion frequencies were determined for each dNTP (Table V). The I709F polymerase incorporated complementary nucleotides with a catalytic efficiency indistinguishable from that of the wild-type enzyme. However, the catalytic efficiency of misincorporation of the non-complementary nucleotides was 6–35 times greater than that of the wild-type enzyme; the enhancement was 6–23-fold for misinsertion opposite template T and 8–35-fold opposite template G. Increased misincorporation by the mutant enzyme was due almost exclusively to a lower $K_m$ for mispaired dNTPs. Notably, misincorporation opposite dT parallels our in vivo finding of A-to-C or A-to-T transversions among the plasmid-borne β-lactamase revertants. Based on current models of initiation at ColEl1-type origins (13, 18), these transversions putatively arise from T:C or T:T mispairs catalyzed by pol I during leading strand synthesis.

Both incorporation of mispaired nucleotides and extension of mispaired primer termini are required for base substitution mutations in vivo. We determined the efficiency of mispair extension using a series of primer-templates containing a 3’-terminal T:A, T:G, T:C, or T:T base pair and measuring the frequency of incorporation of the next correct nucleotide, dTTP (Table VI). All plots of initial velocity versus dNTP concentration exhibited saturation kinetics (data not shown). The I709F exon⁻ polymerase extended the matched T:A pair with a catalytic efficiency indistinguishable from that of the wild-type enzyme. However, the catalytic efficiency of extension of the mismatched termini was 3–14 times greater than that of the wild-type enzyme, due predominantly to 7–17-fold lower $K_m$ values for the next correct nucleotide. The increases in catalytic efficiency and the reductions in $K_m$ are similar (i.e. are within a factor of ~2) to those observed for misincorporation opposite template T (Table V). These results indicate that the I709F mutation reduces discrimination against extension of mismatched primer termini as well as discrimination against incorporation of non-complementary nucleotides.
incorporation of non-complementary nucleotides and more efficient base substitution fidelity in vitro may make a smaller contribution to base substitution fidelity in vivo. The detailed mechanism and location of the isoleucine residue appears to result in loss of stable packing against incoming nucleotides, thus facilitating inaccurate polymerization.

The importance of communication between the polymerase and exonuclease active sites for proofreading has been suggested by in vitro data (40, 41). Our results show that an amino acid substitution in the polymerase active site, i.e. 1709M, 1709N, 1709F, or 1709A, together with 3'-5' exonuclease deficiency, produces an increase in mutation frequency that is more than additive and, in the case of 1709F, that is more than multiplicative. Thus, the polymerase and exonuclease active sites of E. coli pol I may cooperate to achieve accurate DNA polymerization in vitro. Recent studies on mutations in bacteriophage RB69 DNA polymerase also provide evidence for coupling between the exonuclease and polymerase sites (42). In this enzyme, the contribution of the exonuclease to accuracy is much greater than in E. coli pol I. Nevertheless, the mutation rate of the double mutant is greater than the sum of the components (42).

In vitro kinetic analysis showed that one of our Klenow (exo⁻) polymerase mutants, 1709F, exhibited more efficient incorporation of non-complementary nucleotides and more efficient extension of mismatched 3' termini than the wild-type enzyme. Increased efficiencies were due almost entirely to a 10-fold reduction of $K_m$ values. We have previously observed that the same Ile¹⁷⁰⁹ mutant efficiently incorporates ribonucleotides in vitro, also mediated by ~10-fold decreased $K_m$ values for incoming rNTPs (28). Taken together, our observations indicate that Ile¹⁷⁰⁹ contributes to both base and sugar discrimination in wild-type pol I. Results from substitution of the corresponding residue of T. aquaticus pol I, Ile⁶¹⁴, also indicate that this amino acid serves to maintain the fidelity of base selection and to exclude ribonucleotides in vitro (12, 34). However, in contrast to E. coli pol I, hydrophobic substitutions in T. aquaticus pol I at position 614 do not reduce base discrimination. As discussed previously with respect to the T. aquaticus pol I-DNA-dNTP ternary structure, Ile⁶¹⁴ packs near the sugar and base portions of the incoming nucleotide, and substitution of the isoleucine residue appears to result in loss of stable packing against incoming nucleotides, thus facilitating inaccurate polymerization (12, 34). Studies of the Klenow fragment of E. coli pol I have shown that the coordination between the polymerase and exonuclease sites can be affected by changing amino acids between or within the active sites (43, 44). The Ile⁶¹⁴ mutation in pol I might affect the ability of the mismatched primer terminus to slide into the exonuclease active site.

In ColE1-type plasmids, pol I initiates DNA synthesis from primers synthesized by RNA polymerase and R Nas H and is replaced by the pol III holoenzyme to complete the replication of the plasmid (13, 18). The detailed mechanism and location of the switch from pol I to pol III are not completely understood. We observed here that mutations occur in a β-lactamase reporter gene when the target is close to the origin at a frequency
4–6-fold greater than when the same gene is located 2.5 kb from the origin. This suggests that pol I not only catalyzes mismatch repair. Our results indicate that I709F/3exo/H11032 from the origin, possibly, the increased expression of pol I in our recombinant host cells favors substitution for pol III. In E. coli chromosomal DNA replication, pol III is responsible for synthesis of both the leading and lagging strands. The role of pol I is limited and estimated to be responsible for less than ~1% of chromosomal replication by acting in joining of Okazaki fragments and in DNA repair (13). Thus, the 40-fold enhancement of the reversion frequency of a single codon in the chromosomal trpE65 gene was unexpected. At least three mechanisms can be invoked for this enhancement of chromosomal mutagenesis. 1) The trpE65 gene is a hot spot for mutagenesis, possibly due to unusual secondary structure; 2) the mutated site corresponds to a segment involved in the synthesis of an RNA primer; and 3) the contribution of pol I to chromosomal replication is greater than previously surmised.

The high level of mutagenesis displayed by pol I mutants in copying genes located near the ColEI-type origin of replication suggests the feasibility of placing specific genes at this site and developing systems for progressive mutagenesis under continuous selection for mutants with desired properties. In the course of these investigations, Fabret et al. (45) reported a method for in vivo gene-targeted random mutagenesis. They showed that a targeted gene on a ColEI-type plasmid could be randomly mutated by 3’exo− pol I when that gene was lysogenized in an E. coli strain lacking both wild-type pol I and mismatch repair. Our results indicate that 1709F/3exo− mutator pol I displays 100 times more inaccurate DNA synthesis than 3’exo− pol I. Taken together, these findings suggest the feasibility of creating more efficient systems for targeted random mutagenesis and for selecting specific mutations in vivo.

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