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In Vivo Mutagenesis by *Escherichia coli* DNA Polymerase I

ILE[709] IN MOTIF A FUNCTIONS IN BASE SELECTION*

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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 Val[700]–Arg[712] in the polymerase active-site motif A. Only replacement of Ile[709] yielded mutator polymerases, with substitution of Met, Asn, Phe, or Ala increasing the β-lactamase reversion frequency 5–23-fold. Steady-state kinetic analysis of the I709F polymerase revealed reductions in apparent K_m 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 yielded 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)[1] 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 Val[700] to Arg[712], 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 Ile^{709} permit more efficient utilization of rNTPs as substrates *in vitro*.

In this study, we screened 53 mutations in motif A for infidelity of DNA synthesis *in vivo* and found that mutant enzymes harboring Ile^{709} substitutions exhibited less accurate DNA replication. The mutator phenotype was enhanced when the Ile^{709} substitutions were combined with deficiency of 3′–5′ exonucleolytic 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 vitro* 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 lactose 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 transversion at position 1271, thus changing Asp^{424} to Ala and inactivating the 3′–5′ exonuclease activity (29), to construct pECpol I-3′exo$^-$, which carries the 3′–5′ exonuclease-minus pol I gene. Plasmids pECI709M, pECI709N, pECI709F, and pECI709A, which carry Ile^{709} mutant pol I genes, were isolated from a mutant pol I library by genetic selection as described previously (25). Plasmids pECI709M-3′exo$^-$, pECI709N-3′exo$^-$, pECI709F-3′exo$^-$, and pECI709A-3′exo$^-$ were constructed by substituting the 1.1-kb *Sac*I-*Eco*RI fragment of pECpol I-3′exo$^-$ for the corresponding fragment of pECI709M, pECI709N, pECI709F, and pECI709A, respectively.

The reporter plasmids for measuring the reversion frequency of the β-lactamase gene were constructed by modifying plasmid pGPS3 (New England Biolabs Inc., Beverly, MA), which contains a ColE1-type origin derived from pUC19. Site-directed mutagenesis was performed on pGPS3 to introduce a G-to-T transversion at position 76 of the β-lactamase gene, changing the codon GAA for Glu^{26} to the ochre codon TAA. The resulting plasmid was designated pLA2800. The mutant β-lactamase gene containing its own promoter was amplified by polymerase chain reaction with the synthetic oligonucleotides 5′-GCACCCGACATACATGTCCTATTTGTTTATT-3′ and 5′-AAACTTGGTCGGATCCTTACCAATGCTTAATC-3′ as primers and pLA2800 as a template, and the amplified fragment was cloned into pCRII (Invitrogen, Carlsbad, CA). The 1-kb *Afl*III-*Kpn*I fragment containing the mutant β-lactamase gene was excised and cloned into the *Afl*III-*Kpn*I site ~60 bp distant from the origin of pGPS3ΔLA (see below) to create pLA230. Plasmid pGPS3ΔLA was constructed by replacing the 1.2-kb *Bgl*II-*Bgl*I fragment of pGPS3 with the synthetic oligonucleotides 5′-GATCTGATCGCCCTTC-3′ and 5′-GGGCGATCA-3′. A schematic representation of the reporter plasmids pLA230 and pLA2800 is shown in Fig. 1*A*.

β-Lactamase Reversion Assay—A schematic representation of this assay is shown in Fig. 1*B*. *E. coli* JS200 (*recA718 polA12 uvrA155 trpE65 lon-11 sulA1*) (30, 31) harboring pLA230 or pLA2800 was transformed with plasmids carrying wild-type or mutant pol I genes. The recombinant strains were cultured at 30 °C for 16 h in nutrient broth containing 50 μg/ml kanamycin, 12.5 μg/ml tetracycline, and 30 μg/ml chloramphenicol. A 0.01 volume of the pre-cultured broth was inoculated into fresh medium; cultured at 37 °C until an A_{600} of ~1.0 was attained; and then plated onto LB agar plates supplemented with 50 μg/ml kanamycin, 12.5 μg/ml tetracycline, and 30 μg/ml chloramphenicol in the presence or absence of 80 μg/ml carbenicillin. After incubation at 37 °C for 16 h, colonies were counted, and reversion frequencies were calculated as the ratio of carbenicillin-resistant to total colonies.

Plasmids prepared from revertants were re-transformed into *E. coli* BL21 and selected on LB plates containing 50 μg/ml carbenicillin. The plasmids were prepared from the recombinant BL21 strain, and the nucleotide sequence of the entire β-lactamase gene was determined.

Trp$^+$ Reversion Assay—The Trp$^+$ reversion assay was performed basically following the method of Washington *et al.* (32). *E. coli* JS200 was transformed with plasmids pECpol I, pECpol I-3′exo$^-$, pECI709F, and pECI709F-3′exo$^-$, as indicated. The transfectants were cultured at 30 °C for 16 h in nutrient broth supplemented with 30 μg/ml chloramphenicol and 12.5 μg/ml tetracycline. A 0.01 volume of each culture was inoculated into fresh medium, cultured at 37 °C until an A_{600} of ~1.0 was attained, and then plated onto M9 minimum agar plates supplemented with 30 μg/ml chloramphenicol in the presence or absence of 40 μg/ml tryptophan. After incubation at 37 °C for 20 h, colonies were counted, and the frequency of appearance of the Trp$^+$ strain was calculated.

The *trpE* gene in JS200 and in Trp$^+$ revertant strains was amplified by colony polymerase chain reaction with 5′-CCATGCGTAAAGCAATCAGATACCC-3′ and 5′-TTATCGAGCAGCAGAATGTCAGCCA-3′ as primers, and 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 Boosalis *et al.* (33). A 47-mer template (3′-GCGCGGCTTAAGGGCGATCGTTATAGCTTAAGGCCTTTAAAGGGCCC-5′; the relevant template bases are underlined) was hybridized with a ^{32}P-5′-end-labeled 23-mer primer (5′-CGCGCCGAATTCCCGCTAGCAAT-3′) for analysis of misinsertion efficiency opposite dT and with a 25-mer primer (5′-CGCGCCGAATTCCCGCTAGCAATAT-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 $MgCl_2$, and 7.5 mM dithiothreitol. The ranges of nucleotide substrate concentrations used for measuring incorporation opposite template dT were 0.5–7.5 nM dATP, 0.5–50 μM dGTP, and 10–300 μM dCTP and dTTP for the wild-type enzyme and 0.5–7.5 nM 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, 2–30 nM dCTP, and 10–300 μM dTTP for the wild-type enzyme and 0.1–5 μM dATP, 0.1–5 μM dGTP, 2–30 nM dCTP, and 0.1–30 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′-CGCGCCGAATTCCCGCTAGCAAT*X*-3′ (where *X* was A, G, C, or T). Reaction mixtures contained dTTP, *i.e.* the correct dNTP for insertion opposite the next template base. The efficiency of dTTP incorporation opposite template dA was measured for each primer-template construct. The concentrations of the dTTP substrate were 0.5–3.5 nM for the T:A matched pair, 5–1000 nM for the T:G and T:C mismatches, and 0.025–14 μM for the T:T mismatch.

RESULTS

Screening of pol I Mutants for Mutator Activity—To measure errors in DNA synthesis by pol I *in vivo*, 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 ~230 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 and hence 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 Val^{700}–Arg^{712} (25). Representative reversion frequencies obtained for mutants containing substitutions at each of the positions analyzed are

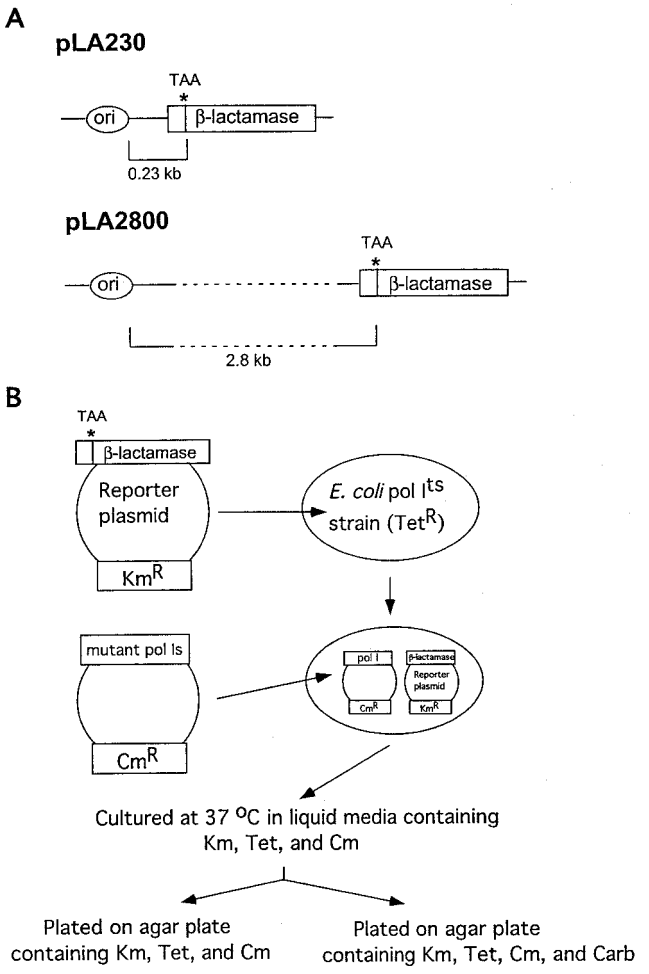

FIG. 1. **Schematic representation of reporter plasmids (*A*) and the β-lactamase reversion assay (*B*).** *A*, the mutant β-lactamase gene harboring an ochre codon was inserted ~0.23 kb (pLA230) or ~2.8 kb (pLA2800) from the ColE1-type origin on pGPS3 as described under "Experimental Procedures". *B*, a pol I-deficient temperature-sensitive (*ts*) *E. coli* strain was transformed by a reporter plasmid carrying a β-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.

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 β-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 introduced into the wild-type construct, the D424A substitution enhanced the reversion frequency of the β-lactamase gene by 4.4-fold (Table II). Larger increases (up to 22-fold) were observed 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 enzyme. For each of the mutants, the increase in reversion frequency 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.

TABLE I
Reversion frequency at an ochre codon in the β-lactamase gene in E. coli expressing wild-type and mutant pol I

pol I[a]	No. of colonies × 10^{-8}/ml (−carbenicillin)	No. of colonies/ml (+carbenicillin)	Reversion frequency × 10^{7}
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
Asp^{705}[b]			
Y706F	9.0	60	0.7
S707A	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

[a] The polymerases listed exhibited the highest reversion frequencies observed among mutants at each position in motif A.
[b] Asp^{705} is immutable (25).

To further evaluate the mutant polymerases, we measured the reversion frequency of the same β-lactamase gene on another plasmid, pLA2800 (Fig. 1*A* and Table III). In this construct, the β-lactamase gene is located ~2.8 kb downstream of the origin of replication and thus is ~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 a 1.8-fold increase in reversion frequency. In contrast, pol I harboring 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 observed 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 chromosome. We investigated the effects of the mutant polymerases on the reversion frequency at the *trpE* locus (Table IV). The reversion frequency observed for wild-type pol I was 2.0×10^{-8}. Neither the 3′exo$^-$ mutation nor the I709F substitution significantly 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 analyzed the nucleotide sequence of the *trpE* gene from three independent revertants and determined that the ochre mutation 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 replication of the *E. coli* genome with less accuracy than the wild-type

TABLE II
Reversion frequency of the β-lactamase gene in E. coli expressing wild-type and mutant DNA pol I lacking 3′–5′ exonuclease activity

pol I	Reversion frequency[a]	Relative frequency
Wild-type	$(1.2 \pm 0.7) \times 10^{-7}$	1
3′ exo$^-$	$(5.3 \pm 1.6) \times 10^{-7}$	4.4
I709M	$(6.3 \pm 3.2) \times 10^{-7}$	5.3
I709M/3′ exo$^-$	$(3.5 \pm 0.3) \times 10^{-6}$	29
I709N	$(2.6 \pm 0.6) \times 10^{-6}$	22
I709N/3′ exo$^-$	$(3.3 \pm 1.5) \times 10^{-5}$	280
I709F	$(1.6 \pm 0.1) \times 10^{-6}$	13
I709F/3′ exo$^-$	$(5.0 \pm 0.1) \times 10^{-5}$	416
I709A	$(1.5 \pm 0.2) \times 10^{-6}$	13
I709A/3′ exo$^-$	$(1.2 \pm 0.1) \times 10^{-5}$	100

[a] Values represent means ± S.D. obtained by plating three independent clones.

TABLE III
Reversion frequency of the β-lactamase gene located distally from the origin of plasmid replication

pol I	Reversion frequency[a]	Relative frequency
Wild-type	$(1.5 \pm 0.4) \times 10^{-7}$	1
3′ exo$^-$	$(1.3 \pm 0.9) \times 10^{-7}$	0.9
I709M	$(1.0 \pm 0.1) \times 10^{-7}$	0.7
I709M/3′ exo$^-$	$(1.0 \pm 0.5) \times 10^{-6}$	6.7
I709N	$(2.7 \pm 1.1) \times 10^{-7}$	1.8
I709N/3′ exo$^-$	$(5.2 \pm 0.9) \times 10^{-6}$	35
I709F	$(1.4 \pm 0.6) \times 10^{-7}$	0.9
I709F/3′ exo$^-$	$(1.3 \pm 0.4) \times 10^{-5}$	87
I709A	$(1.9 \pm 0.7) \times 10^{-7}$	1.3
I709A/3′ exo$^-$	$(2.9 \pm 1.0) \times 10^{-6}$	19

[a] Values represent means ± S.D. of three determinations.

TABLE IV
Reversion frequency at an ochre codon in the chromosomal trpE65 gene in E. coli expressing wild-type and mutant pol I

pol I	Reversion frequency[a]	Relative frequency
Wild-type	$(2.0 \pm 0.8) \times 10^{-8}$	1
3′ exo$^-$	$(3.0 \pm 2.2) \times 10^{-8}$	1.5
I709F	$(2.3 \pm 1.3) \times 10^{-8}$	1.2
I709F/3′ exo$^-$	$(0.8 \pm 0.2) \times 10^{-6}$	40

[a]Values represent means ± S.D. obtained by analyzing three clones.

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 Ile^{709} 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 23- or 25-nucleotide oligomer that was labeled at the 5′-end with ^{32}P, 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 ColE1-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 exo$^-$ 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.

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 A 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 10^4 to 10^7 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

TABLE V
Misinsertion efficiency of wild-type and I709F Klenow (exo⁻) polymerases

Klenow (exo⁻)	Base pair	$k_{cat} \times 10^3$	K_m	k_{cat}/K_m	Relative frequency[a]
		s^{-1}	μM	$s^{-1}\,M^{-1}$	
Wild-type	T:A	2.2 ± 0.1	(2.1 ± 0.2) × 10^{-3}	1.0 × 10^6	1
	T:G	2.1 ± 0.1	6.4 ± 1.3	3.3 × 10^2	3.3 × 10^{-4}
	T:C	2.1 ± 0.1	70 ± 5.4	30	3.0 × 10^{-5}
	T:T	1.8 ± 0.1	64 ± 10	28	2.8 × 10^{-5}
	G:C	2.5 ± 0.1	(15 ± 1.4) × 10^{-3}	1.7 × 10^5	1
	G:G	2.4 ± 0.1	61 ± 6.7	39	2.3 × 10^{-4}
	G:T	1.9 ± 0.1	(1.0 ± 0.1) × 10^2	19	1.1 × 10^{-4}
	G:A	3.1 ± 0.1	55 ± 4.8	56	3.3 × 10^{-4}
I709F	T:A	2.0 ± 0.1	(1.7 ± 0.3) × 10^{-3}	1.2 × 10^6	1
	T:G	2.2 ± 0.1	0.5 ± 0.1	4.4 × 10^3	3.7 × 10^{-3}
	T:C	4.2 ± 1.0	19 ± 7.0	2.2 × 10^2	1.8 × 10^{-4}
	T:T	3.2 ± 0.3	4.2 ± 0.8	7.6 × 10^2	6.3 × 10^{-4}
	G:C	2.9 ± 0.8	(14 ± 6.5) × 10^{-3}	2.1 × 10^5	1
	G:G	2.7 ± 0.7	6.7 ± 2.6	4.0 × 10^2	1.9 × 10^{-3}
	G:T	2.2 ± 0.1	2.7 ± 0.5	8.1 × 10^2	3.9 × 10^{-3}
	G:A	2.8 ± 0.2	2.6 ± 0.5	1.1 × 10^3	5.2 × 10^{-3}

[a] Values represent the efficiency (k_{cat}/K_m) relative to that of correct nucleotide incorporation.

TABLE VI
Mismatch extension efficiency of wild-type and I709F Klenow (exo⁻) polymerases

Klenow (exo⁻)	Terminus	$k_{cat} \times 10^3$	K_m	k_{cat}/K_m	Relative frequency[a]
		s^{-1}	nM	$s^{-1}\,M^{-1}$	
Wild-type	T:A	2.7 ± 0.3	1.7 ± 0.5	1.6 × 10^6	1
	T:G	2.0 ± 0.1	(1.9 ± 0.3) × 10^2	1.1 × 10^4	6.9 × 10^{-3}
	T:C	2.5 ± 0.2	(1.0 ± 0.2) × 10^2	2.5 × 10^4	1.6 × 10^{-2}
	T:T	2.7 ± 0.1	(3.3 ± 0.3) × 10^3	8.2 × 10^2	5.1 × 10^{-4}
I709F	T:A	2.6 ± 0.3	1.5 ± 0.4	1.7 × 10^6	1
	T:G	1.7 ± 0.1	27 ± 2.0	6.3 × 10^4	3.7 × 10^{-2}
	T:C	1.6 ± 0.1	22 ± 3.0	7.3 × 10^4	4.3 × 10^{-2}
	T:T	2.3 ± 0.1	(1.9 ± 0.2) × 10^2	1.2 × 10^4	7.1 × 10^{-3}

[a] Values represent the efficiency (k_{cat}/K_m) relative to that of T:A extension.

confirmation of the findings for pol I. Interestingly, our results indicate that exonucleolytic proofreading may make roughly the same contribution to base substitution fidelity *in vivo* (4-fold in this study) as *in vitro* (<10-fold) (9). In contrast, available data for the pol III holoenzyme suggest that proofreading may make a smaller contribution to base substitution fidelity *in vitro* (less than ~10-fold under most conditions) (8, 38) than *in vivo* (>50–400-fold) (39), perhaps due to interaction of the holoenzyme with other proteins at the replication fork.

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.* I709M, I709N, I709F, or I709A, together with 3′–5′ exonuclease deficiency, produces an increase in mutation frequency that is more than additive and, in the case of I709F, 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 vivo*. 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, I709F, 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 ~10-fold reduction of K_m values. We have previously observed that the same Ile^{709} mutant efficiently incorporates ribonucleotides *in vitro*, also mediated by ~10-fold decreased K_m values for incoming rNTPs (25). Taken together, our observations indicate that Ile^{709} contributes to both base and sugar discrimination in wild-type pol I. Results from substitution of the corresponding residue of *T. aquaticus* pol I, Ile^{614}, 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·ddNTP ternary structure, Ile^{614} 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 Ile^{709} 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 RNase 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 synthesis near the origin, but also can participate in replication at a distance 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 ColE1-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 ColE1-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 I709F/3′exo$^-$ mutator pol I displays 100 times more inaccurate DNA synthesis than 3′exo$^-$ pol I. Taken together, these findings suggest the possibility of creating more efficient systems for targeted random mutagenesis and for selecting specific mutations *in vivo*.

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