DNA Polymerase III from Escherichia coli Cells Expressing mutA Mistranslator tRNA Is Error-prone*

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Translational stress-induced mutagenesis (TSM) refers to the elevated mutagenesis observed in Escherichia coli cells in which mistranslation has been increased as a result of mutations in tRNA genes (such as mutA) or by exposure to streptomycin. TSM does not require lexA-regulated SOS functions but is suppressed in cells defective for homologous recombination genes. Crude cell-free extracts from TSM-induced E. coli strains express an error-prone DNA polymerase. To determine whether DNA polymerase III is involved in the TSM phenotype, we first asked if the phenotype is expressed in cells defective for all four of the non-replicative DNA polymerases, namely polymerase I, II, IV, and V. By using a colony papillation assay based on the reversion of a lacZ mutant, we show that the TSM phenotype is expressed in such cells. Second, we asked if pol III from TSM-induced cells is error-prone. By purifying DNA polymerase III* from TSM-induced and control cells, and by testing its fidelity on templates bearing 3′,5′-ethenocytosine (a mutagenic DNA lesion), as well as on undamaged DNA templates, we show here that polymerase III* purified from mutA cells is error-prone as compared with that from control cells. These findings suggest that DNA polymerase III is modified in TSM-induced cells.

Autonomous organisms normally replicate its DNA accurately, but the fidelity of replication can be transiently decreased in response to environmental and physiological stimuli through a number of pathways (1). Although the Escherichia coli SOS response represents the best-described transient mutator response (2), emerging evidence indicates the existence of multiple inducible mutagenic pathways in E. coli (3). One especially intriguing pathway is provoked by increased translational errors resulting from mutations in tRNA genes (4, 5), in genes specifying tRNA-modifying enzymes (6), or from exposure to streptomycin, an antibiotic that promotes mistranslation (7). This pathway, dubbed translational stress-induced mutagenesis (TSM) (3), does not require the induction of lexA/napc-regulated SOS genes and is suppressed in cells defective for RecABC/RuvABC-dependent homologous recombination (5, 7–9). Available genetic (5, 7–9) and biochemical (10) evidence suggests that the TSM phenotype results from error-prone DNA replication rather than from defective DNA repair. On the basis of the effect of elimination of individual genes encoding “non-replicative” polymerases, we previously proposed that either DNA polymerase III or an unidentified new DNA polymerase is responsible for error-prone replication in TSM-induced cells.

DNA polymerase III holoenzyme (pol III HE) accounts for more than 90% of cellular DNA synthesis (11, 12) and is also required for the major post-replicative mismatch correction pathway (13, 14). Pol III HE was shown to effectively carry out translesion DNA synthesis past abasic sites, mostly producing −1-bp deletions (15), as contrasted to translesion synthesis carried out by Pol V, which mostly yields base substitutions at abasic sites. Pol III HE is a 10-subunit polymerase consisting of three main components (16, 17) as follows: 1) the core polymerase (αθ), responsible for DNA synthesis and proofreading (18, 19); 2) the processivity factor or β-sliding clamp (βp) (20) that tethers the polymerase to the DNA (21); and 3) the DnaX complex, containing δ′, χ, ψ, and either or both of two different DnaX proteins (γ and τ), that is responsible for loading the β-processivity clamp onto the DNA (22, 23). The largest subassembly of pol III HE is DNA polymerase III* (pol III*) composed of all the subunits of HE except the β-subunit (24). The HE complex is completed by the addition of βp to pol III* (22, 24).

In addition to pol III, four additional DNA polymerases (polymerses I, II, IV, and V) are known in E. coli. Even though these polymerases carry out important cellular functions, genes encoding each of these polymerases can be mutagenically inactivated, implying that considerable functional redundancy is built into the replication apparatus. DNA polymerase I (pol I; encoded by polA), known as a “repair polymerase,” normally functions to fill gaps that arise during lagging strand replication and during excision repair. The remaining three DNA polymerases, namely II (pol II; encoded by polB (25, 26)), IV (pol IV; encoded by dinB (27)), and V (pol V; encoded by umuC (28, 29)), are induced as a part of the SOS system. Pol II has been proposed to play a role in replication-restart following DNA damage, a process that bypasses DNA damage in both an error-free (30, 31) and error-prone manner (32, 33). Pol IV is involved in certain types of untargeted SOS mutagenesis (27, 34, 35), whereas pol V, working in conjunction with a number of other factors including the RecA protein, is believed to be responsible for translesion DNA synthesis (28, 29, 36). How-
ever, pol V may also be responsible for untargeted mutations at undamaged template sites (37, 38).

The individual loss of polA, polB, dinB, or umuDC genes does not affect the expression of the TSM response (5, 8, 10). However, an analysis based on cells with defects in individual genes leaves open the possibility that two or more of these four nonessential DNA polymerases may have nonexclusive (redundant) roles in error-prone DNA synthesis. Here we show that a strain simultaneously defective for pol I, pol II, pol IV, and pol V can be constructed, proving that loss of all four non-replicative polymerases is compatible with viability. Analysis of the TSM response in this strain confirms that the four non-replicative polymerases are not required collectively or individually for the TSM response. To address the question directly whether pol III HE or a unknown 6th DNA polymerase is responsible for mistranslation-induced mutagenesis, we purified pol III* from TSM-induced and uninduced cells and analyzed its replication fidelity on both damaged and undamaged template sites (37, 38).

Table I

**Bacterial and plasmid strains**

| Strain | Relevant genotype | Source (Ref.) |
|--------|-------------------|---------------|
| **A. E. coli** | | |
| AM107  | ara Δ(lac-proB) xii| This laboratory (10) |
| AM109  | ΔumuDC955::Tn10, RW52 in AM107  | This study |
| AM132  | metE-3079::Tn10 (Tet') from CAG18491 in HMD83 | This study |
| AM134  | polA(Am), metE-3079::Tn10 (Tet') from AM123 in CC105  | This study |
| AM135  | polA(Am), metE-3079::Tn10 (Tet') from AM123 in CC105mutA  | This study |
| AM146  | ΔdinB - Kan' from YG7207 in AM130 | This study |
| AM147  | polA(Am), metE-3079::Tn10 (Tet') from AM123 in AM146  | This study |
| AM155  | ΔdinB - Kan' from YG7207 in CC104  | This study |
| CAG18491 | λ, rph-1, metE-3079::Tn10 (Tet') | M. Berlyn (43) |
| CC104  | ara Δ(lac-proB) xii F' lacIZ proB* | J. Miller (69, 70) |
| CC105  | ara Δ(lac-proB) xii F' lacIZ proB* | J. Miller (69, 70) |
| CC105mutA | mutA596C in CC105 | M. Berlyn (43) |
| HMD83  | polB100, lac53 (Am), λ-, thyA36, INvrnD-rrnE1, psL151 Sm*, polA1(Am), rha-5, deoC2?, lacI, tetr | R. M. Schaaper (71) |
| M1061  | hisD, rpsD+ Δ(ara-leu) Δ(araIPOZ) polV galK strA | R. M. Schaaper (60) |
| NR0699 | ara Δ(ara-pro) lacA56 thi F' (proAB lacIq ZM51) | R. M. Schaaper (71) |
| RW22   | ΔumuDC955 - Cm' arrA6 | R. M. Schaaper (60) |
| YG7207 | ΔdinB - Kan' in AB1157 | T. Nomh (35) |
| **B. Plasmids** | | |
| pHM11  | pSE380 with mutant glycine tRNA | This lab (5) |
| pHM22  | pSE380 with wild-type glycine tRNA | This lab (5) |
| pMV1  | Mutant glycine tRNA with lacP in pMW119 from pHM11 | This study |
| pMV22  | Wild-type glycine tRNA with lacP in pMW119 from pMV112 | This study |
| pMW119 | Amp' low-copy number (5–6 copies/cell) plasmid vector | A. M. Al Mamun (42) |
| pSE380 | Amp' vector | R. Maurer (73) |

**Materials and Methods**

Reagents, Enzymes, and Replication Proteins—Taq DNA polymerase was from Roche Molecular Biochemicals; restriction endonuclease BglII, T4 DNA ligase, Vent DNA polymerase, and T4 polynucleotide kinase were from New England Biolabs. The β subunit was purified as described by Johanson et al. (39). The primase DnaG was purified as described by Marinas (40). The E. coli single-stranded DNA-binding protein (SSB) was purified according to Minden and Marinas (41).

Plasmid and Strain Construction—Plasmid pMV22 was constructed from plasmid pHM22 (5) as follows. The Spfi-HindIII 1604-bp fragment containing the lacP* gene and the wild type gIVV RNA gene under Pr promoter was inserted into the low copy number plasmid pMW119 (40). The EcoRI and HindIII sites. Similarly, plasmid pMV11 was constructed by inserting the Spfi-HindIII 1604-bp fragment containing the gIVV tRNA gene (mutA) from pHM11 (5) into the Spfi-HindIII sites of pMW119.

To enable the subsequent transduction of the polA1 allele in strain HMD83 (see Table I for genotypes), the metE-3079 marker from strain CAG18491 (43) was first transferred to HMD83 (44) by P1 transduction. The transductants were selected on LB agar containing 30 μg/ml tetracycline (Sigma). One isolate, named AM132, was characterized by its inability to grow in minimal medium without methionine. *E. coli* strains AM134 and AM135 were constructed by co-transducing the polA1 allele along with the marker metE::Tn10 from strain AM132 into strains CC105 and CC105mutA, respectively. Strains AM134 and AM135 were characterized for the presence of polA1 allele by testing their sensitivity to 0.04% methyl methanesulfonate in LB plates and to UV irradiation at 7 J/m2 as described previously (45). The *E. coli* strain AM130 was created by P1 transduction of the ΔumuDC955::Cm' allele from strain RW22 to strain AM107 (CC105 polB (10)). The presence of the ΔumuDC955::Cm' allele was confirmed by UV sensitivity at 30 J/m2.

The *E. coli* strain AM146 was constructed by transferring the ΔdinB::Kan' allele from strain YG7207 to strain AM130 by P1 transduction. The presence of the ΔdinB::Kan' allele in AM146 was confirmed by PCR amplification (forward primer 5'-GGTGTATCAATACGTTGCCAG-3' and reverse primer 5'-AGGCGAATAAGTTCCTTGGTCA; reverse primer 5'-AGGGCAATTAGTTTTGTTTTGTGA; forward primer 5'-CGCGGTATCATAGTTGGTCA) followed by analysis of restriction digestion patterns of the PCR products. The *E. coli* strain AM147 was made by co-transducing the polA1 allele along with the marker metE::Tn10 from strain AM132 to AM146 by P1 transduction. Strain AM147 was characterized by sensitivity to 0.04% methyl methanesulfonate and to UV irradiation at 7 J/m2.

The F' factors contained in strains CC104 and CC107 (two strains related to CC105, the parental strain for AM147) contain a second copy of the dinB gene (46, 47). To show that AM147 does not have a second (wild type) copy of dinB, we carried out a PCR-based analysis as summarized in Fig. 1. This analysis shows that an "external" primer set (F1/R1 in Fig. 1) yields a single band corresponding to a disrupted version of dinB in AM147 cells (lane 2) and that two "internal" primer sets (F2/R2 and F3/R3) do not yield a band (lanes 4 and 6), indicating the loss of sequences corresponding to dinB. To verify that we could have detected a second copy of dinB, we repeated the analysis with AM155 (CC104 ΔdinB::Kan'), a strain harboring an F' factor that was previously shown to have a second, episomal copy of dinB. Our analysis shows that the F1/R1 primer set yields two bands (lane 8), one corresponding to a disrupted allele (1862 bp) and the other corresponding to the wild type allele (1603 bp). As expected, internal primer sets F2/R2 and F3/R3 amplify the second copy of dinB in AM155 cells. Thus, these data confirm that AM147 does not have an undisrupted dinB allele.

Papillation Assay—Papillation assays were performed with slight modifications of the procedures described by Miller (48). Cultures were spread on minimal A medium containing 0.2% glucose, 500 μg/ml phenyl-β-D-galactopyranoside (a non-inducing lactose analog that serves as a carbon source after exhaustion of glucose; Sigma), 40 μg/ml of the β-galactosidase inhibitor 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-gal; Gold Biotechnologies), and 1 mM isopropyl-β-D-thio-galactopyranoside (IPTG; Sigma). Plates were incubated at 37°C for 4–5 days at which time blue papillae (lacZ'-revertants) are distinctly visible against the white background of lacZ' cells.

Construction of Site-specific cE Lesion-bearing ssDNA Constructs—M13 single-stranded DNA (ssDNA) molecules bearing an cE residue were constructed following the procedures described elsewhere (49–51).
Fidelity of pol III from E. coli mutA Cells

The terms eC-ssDNA and C-ssDNA, respectively, refer to the primed ssDNA bearing a site-specific eC lesion or normal cytosine (as a control).

Buffers Used for Polymerase Purification—Buffers contain the following: "Tris-back extraction" buffer (TBEB): 50 mM Tris-Cl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 20% glycerol, and 5 mM DTT; "0.20 TBE" is TBE containing 0.2 g/ml of ammonium sulfate; "0.17 TBE" is TBE containing 0.17 g/ml of ammonium sulfate (52). Buffer A is 50 mM Tris-Cl (pH 7.5), 0.5 mM EDTA, 30% glycerol, and 5 mM DTT. Buffer B is buffer A supplemented with 5 mM MgCl₂, 0.2 mM EDTA and 0.4 mM ATP.

Purification of pol III*—pol III* was purified following the procedures described previously (23), as summarized below. Cells were grown in 200 liters of LB medium, supplemented with ampicillin (50 μg/ml) and IPTG (1 mM) as appropriate, and cells were harvested by centrifugation at 4 °C in a Sharples centrifuge. The cell pellet was resuspended in an equal volume of 50 mM Tris-Cl (pH 7.5), 10% sucrose solution, quick-frozen in liquid nitrogen, and stored at −80 °C.

The cell suspension (2 kg) was thawed on ice, and the A₅₅₀ was adjusted to 200 with 50 mM Tris-Cl (pH 7.5), 10% sucrose solution. One-ninth volume of lysis buffer (1.5 mM NaCl, 0.2 mM EDTA, 0.2 mM spermidine, and 50 mM DTT) was added, and the pH was adjusted to 8.5 by adding solid Tris base. Lysozyme (10 mg/ml in water) was added to 0.2 mg/ml, and the contents were incubated for 30 min on ice, followed by 10 min in a 37 °C water bath. The lysed cells were distributed into 250-ml Sorvall GSA bottles, kept on ice for 5 min, and centrifuged for 60 min at 13,000 rpm in a Sorvall SLA-1500 rotor. The supernatant (fraction 1a) was collected by gentle decantation. To precipitate nucleic acid, polyvinyl P (1% v/v; Amersham Biosciences) was added slowly, with gentle stirring, to fraction 1a to a final concentration of 0.06%. Stirring was continued for an additional 30 min at 4 °C. The suspension was centrifuged at 13,000 rpm for 30 min at 4 °C in a Sorvall SLA-1500 rotor to collect the supernatant (fraction 1b).

Ammonium sulfate (0.25 g/ml) was added to fraction 1b over a 10-min interval while stirring. The contents were stirred for an additional 30 min at 4 °C, transferred into Sorvall GSA bottles, and centrifuged at 27,000 × g in a Sorvall SLA-1500 rotor for 45 min at 4 °C. The precipitate was resuspended in 0.2 TBEB (1/40th of the fraction 1a volume), and the insoluble fraction was collected by centrifugation as above. This procedure was repeated with 0.17 TBEB (1/40th of the fraction 1a volume), and the final recovered insoluble fraction was dissolved in a small volume of TBEB (fraction II). Fraction II was quick-frozen in liquid nitrogen and stored at −80 °C.

Fraction II was thawed and dialyzed overnight against 4 liters of buffer A + 40 mM NaCl at 4 °C. The dialysate was centrifuged by centrifugation for 20 min at 48,200 × g at 4 °C. The clarified solution was diluted with buffer A to the conductivity of buffer A + 50 mM NaCl. The diluted fraction II was applied to a heparin-agarose (Sigma) column (1 ml of resin per 10 mg of protein) equilibrated with buffer A + 50 mM NaCl. The column was washed with 2 column volumes of equilibration buffer, and the activity was eluted with a 10-column volume of NaCl gradient (50–400 mM) in buffer A. Fractions were pooled, and ammonium sulfate was added to 0.26 g/ml. The suspension was stirred for 2 h at 4 °C and centrifuged at 37,000 rpm for 60 min at 4 °C in a Sorvall AS41 rotor. The precipitate was resuspended in a small volume (300 μl) of buffer B (fraction III).

Fraction III was clarified by centrifugation for 5 min at 13,000 rpm in a microcentrifuge. The clarified fraction III was gel-filtered through a fast protein liquid chromatography Superose 6 column (HR 10/30; Amersham Biosciences) equilibrated with buffer B at a flow rate of 0.1 ml/min. Peak fractions were pooled, and glycerol was added to achieve a final concentration of 38% (fraction IV) and stored in aliquots at −80 °C.

pol III* Assay—The assay mix (25 μl) containing 50 mM HEPES-KOH (pH 8.0), 50 mM potassium glutamte, 10 mM magnesium acetate, 10 mM DTT, 10 μg/ml rifampicin, 100 μg/ml bovine serum albumin (BSA), 24 μg/ml SSB, 3.2 μg/ml M13 Gt7 ssDNA, 100 μg each of CTP, GTP, UTP, 80 μM each of [α-32P]ATP, 20 mM DTT, 200 μM each of MnCl₂, MgCl₂, and 2% glycerol. After incubation for 200–400 rpm (μmol), and 1 mM ATP was pre-warmed for 5 min at 30 °C. Twenty ng of DnaG was added, and the contents were incubated for 5 min at 30 °C. Thirty ng of the β subunit were added, and the reactions were started by the addition of pol III*. If necessary, the products were diluted with 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM DTT, 500 μM each of dGTP, dCTP, and dTTP. After incubation for 5 min at 30 °C, the reaction was stopped by chilling and addition of 2 drops of 0.2 M sodium pyrophosphate, 2 drops of calf thymus DNA (1 mg/ml), and 4 μl of 5% trichloroacetic acid. Acid-insoluble radioactivity was then measured. One unit of activity is the amount and summarized below. M13mp7L2 ssDNA was linearized by cutting with the restriction endonuclease EcoRI that cuts a hairpin DNA structure within the polycoding site of the vector. The linearized DNA was annealed to a 57-nt “scaffold” and a 5’-phosphorylated 17-nt insert containing a single site-specific eC lesion. Annealing of the 57-mer draws the two ends of the linear M13 ssDNA together to form a non-covalently closed circular DNA with a 17-nt “gap” complementary to the lesion-containing 17-mer. The annealed DNA is subjected to DNA ligation to generate a covalently closed ssDNA circle containing the lesion-bearing 17-nt insert. After the ligation step, the scaffold is removed by heat denaturation in the presence of a 10-fold molar excess of an lesion-containing 17-mer. The annealed DNA is subjected to DNA ligation to generate a covalently closed circular DNA with a 17-nt “gap” complementary to the lesion-containing 17-mer. The annealed DNA is subjected to DNA ligation to generate a covalently closed ssDNA circle containing the lesion-bearing 17-nt insert, and the insoluble fraction was collected by centrifugation as above. The procedure was repeated with 0.17 TBEB (1/40th of the fraction 1a volume), and the final recovered insoluble fraction was dissolved in a small volume of TBEB (fraction II). Fraction II was quick-frozen in liquid nitrogen and stored at −80 °C.

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catalyzing the incorporation of 1 nmol of deoxyribonucleotide in 30 min at 37 °C. Replication of DNA-primed M13 ssDNA by Purified Proteins—Replication of M13 ssDNA primed with an annealed 60-mer by purified proteins was carried out following the procedures of Geider and Kornberg (53) and of Livneh (54) as follows. The standard reaction mixture (25 μl) contained 20 μM Tris-Cl (pH 7.5), 4% glycerol, 0.1 mM EDTA, 8 mM MgCl2, 50 mM potassium glutamate, 80 mM KCl, 10 μg/ml rifampicin, 1 mM ATP, 80 μM each of the dNTPs, 200 fmol 50-mer primed C ssDNA or C ssDNA, 32 μg/ml SSB, 20 ng of β subunit, and 4 units of pol III*. Reactions were allowed to proceed for 20 min at 37 °C and were stopped by adding EDTA to 5 mM, Tris-Cl (pH 8.0) to 10 mM, and SDS to 0.5% (w/v), followed by heating at 65 °C for 10 min. Proteinase K was added to a final concentration of 50 μg/ml, and the mixture was heated at 55 °C for 30 min. Then the reaction mixture was subjected to phenol extraction, followed by standard ethanol precipitation. The precipitated DNA was dissolved in a minimum volume of H2O, and an aliquot was checked by electrophoresis on an agarose gel (0.8%). Ethidium bromide staining revealed that about half of the input ssDNA was isolated using a Qiagen plasmid mini kit following the procedures described elsewhere (56, 57) with some modifications. Double-stranded form I (RF-I) DNA of phage M13mRS65 was subjected to 0.5% (w/v) SDS to 0.5% (v/v), followed by heating at 65 °C for 3 min, cooled in an ice bath, incubated at 70 °C for 5 min, and allowed to slow-cool to room temperature over several hours by switching off the water bath.

Gap-filling DNA Synthesis and Mutation Frequency Determination—Gap-filling DNA synthesis was performed following the procedures described previously (56, 57). The 25-μl reaction mixture contained 30 mM HEPES-KOH (pH 7.6), 10 mM MgCl2, 8 mM DTT, 100 μg/ml BSA, 200 μM each of deNTPs, 1 mM ATP, 20 mM β subunit, 50 fmol of gapped DNA, and 5–15 units of DNA polymerase III* (pol III*). Where indicated, replication products were radiolabeled by including 7 μCi of [α-32P]dATP (10 μCi/ml; 6000 Ci/mmol; Amersham Biosciences). The reaction mix was incubated at 37 °C for 10 min and quenched by adding EDTA to 15 mM. To assess gap-filling, 10 μl of each reaction were mixed with 2.5 μl of SDS/dye mix (20 mM Tris-Cl (pH 7.5), 5 mM EDTA, 5% SDS, 0.5% bromphenol blue, 25% glycerol) and subjected to electrophoresis in a 5% agarose gel in TAE buffer containing 0.5% (v/v) SDS to 0.5% (w/v), followed by heating at 65 °C for 3 min, cooled in an ice bath, incubated at 70 °C for 5 min, and allowed to slow-cool to room temperature over several hours by switching off the water bath.

RESULTS

The TSM Phenotype Is Expressed in an E. coli mutA Strain Simultaneously Deficient for pol I, pol II, pol IV, and pol V—In contrast to previous results showing that gapped DNA serves as the template for incorporation of an error-prone DNA polymerase that is demonstrable in cell-free extracts. This activity is detected as increased in vitro mutation fixation at a site-specific mutagenic lesion (3,N’-ethenocytosine; εC) borne on phage M13 ssDNA (10). In this system, primed ssDNA bearing an εC residue (εC ssDNA) is replicated with cell-free extracts from mutA cells, and the newly synthesized complementary (minus) strand is selectively amplified using ligation-mediated PCR (LM-PCR). The amplified products are subjected to multiplex sequencing to characterize mutation at the lesion. Interestingly, the error-prone polymerase activity is undetectable in cell-free extracts prepared from recA mutA cells (10), consistent with the observations that expression of the TSM phenotype requires a functional recA gene (5, 8).

We have shown previously, through a series of in vivo experiments, that the TSM phenotype does not require the individual function of pol I, pol II, pol IV, or pol V (5, 8, 10). This observation, together with the crude extract results considered above, argued that the TSM phenotype is mediated by pol III. However, the available data did not exclude the possibility of a redundant function for the nonessential DNA polymerases (pol I, pol II, pol IV, and pol V), for the loss of any one polymerase. To address this possibility, we constructed a strain (AM147) that is defective for all four nonessential DNA polymerases (pol I, pol II, pol IV, and pol V), and we asked if the TSM phenotype, detected as increased lacZ → lacZ” papillation (58), is still displayed in such a strain in response to the expression of the mutA tRNA gene. When E. coli AM147 is transformed with pMV11, a plasmid bearing mutA, a strong mutator phenotype (increased papillation) is observed in the presence of IPTG, a condition in which the mutA allele is expressed (Fig. 2). The intensity of the papillation is comparable with that in strain CC105/pMV11, the positive control with wild type polA, polB, and
umlDC alleles. Increased papillation is not observed for strains CC105/pMV22 (plasmid-borne wild type glyV) and AM147/pMV22 (plasmid-borne wild type glyV) in the presence of IPTG. These observations rule out a requirement for the individual or collective function of polA, polB, dinB, and umuDC for the expression of the TSM phenotype. It may be noted that a second copy of dinB has been found on the F factor of some E. coli strains (46, 47). We have tested for a second dinB allele in AM147 by PCR amplification using both an external primer set and two internal primer sets, and we confirmed that the strain did not have a second dinB allele (see “Materials and Methods”; Fig. 1).

Purification and Characterization of pol III* from Mutator and Control Strains—To address the question whether DNA polymerase III is error-prone in TSM-induced E. coli cells, we purified pol III* from two pairs of strains. The first pair consisted of strains AM134 (polA; non-mutator control) and AM135 (polA mutA; mutator); the second set consisted of strains AM147/pMV22 (polA polB dinB umuDC; non-mutator control) and AM147/pMV11 (polA polB dinB umuDC mutA; mutator). Purification was carried out as described under “Materials and Methods.” Table II summarizes some characteristics of the four purified enzymes and shows that all four enzymes had similar specific polymerase activities by the M13Gori polymerization assay (59).

Fig. 3 shows that the polymerization activity, based on an assay in which a 60-mer primer annealed to M13 ssDNA is elongated, is somewhat higher for pol III* from non-mutator strains as compared with that from mutator strains. Because polymerization activity for the four polymerase preparations is essentially similar using the DnaG-primed M13Gori ssDNA assay (Table II), it is not clear why the 60-mer primer elongation assay shows a slight reduction in polymerization by pol III* from mutator strains as compared with those from non-mutator strains.

Mutation Fixation at an eC Lesion by Purified pol III*—We have shown previously (5) that mutation fixation at an eC residue borne on transfected M13 ssDNA is significantly increased in mutA cells. Subsequently we showed that in vitro translesion DNA synthesis across a site-specific eC residue by crude extracts prepared from mutA cells is error-prone (10). To determine whether translesion synthesis across eC by purified pol III* from mutA strains is also error-prone, we used a modification of the previously described in vitro replication-in vitro mutation detection system (10, 55). In the modified assay system for purified pol III, we created a primed template (eC-ssDNA) consisting of M13 ssDNA bearing a site-specific lesion to which a 60-mer primer is annealed so that the 3′-OH terminus is situated 388 nt upstream of the lesion site. The primer is elongated by the DNA polymerase, followed by selective amplification of the newly synthesized complementary strand by LM-PCR and subsequent multiplex sequencing analysis as outlined in Fig. 4. In the multiplex sequence analysis strategy, the specificity of base insertion opposite the lesion is determined by elongation of a labeled sequencing primer in the presence dGTP, dCTP, and ddATP. Correct insertion of a guanine opposite eC yields a 23-mer limit elongation product, whereas insertion of a T (i.e. a C → T transition) opposite eC yields, respectively, 21- and 22-nt-long products. Densitometric analysis of the relative signal intensity of each band is used to calculate the frequency of each type of mutation as described previously.

Fig. 4C shows that when a control template-primer in which normal cytosine replaces eC (C-ssDNA) is replicated by pol III* from non-mutator strains (lanes 1 and 3) or from mutator strains (lanes 2 and 4), as expected, almost all of the signal is contained in the 23-nt band (i.e. no detectable 22- and 21-nt bands are observed). Lane 5 shows that eC-ssDNA, when replicated by pol III* from non-mutator strain AM134, displays detectable mutagenesis. Replication of eC-ssDNA by pol III* from mutator strain AM135 (lane 6) results in detectably increased mutagenesis, as indicated by the increased intensity of the bands corresponding to C → A (21 nt) and C → T (22 nt) events. Table III provides a quantitative summary of the results obtained from at least three independent replication for each of the pol III*, followed by mutation analysis. Translesion synthesis by pol III* from AM134 results in 17% mutagenesis, whereas translesion synthesis by the polymerase from AM135 results in 34% mutagenesis (Table III). A similar pattern of mutation is observed when eC-ssDNA is replicated with pol III* from non-mutator strain AM147/pMV22 (Fig. 4C, lane 7) or from the mutator strain AM147/pMV11 (lane 8) (9.5 versus 26%; Table III). In vivo mutagenesis at an eC lesion ranges from 4 to 13% for wild type strains and 40 to 60% in mutA cells (5, 8, 10). The lower fold elevation in mutagenesis by pol III* from mutator strains versus non-mutator strains (2–3-fold; Table III) is considered under “Discussion.”

To address the question whether the elevation of mutagenesis at eC lesion is mediated by elevated translesion DNA synthesis by pol III from mutator strains, we used a template-primer consisting of a 120-nt-long oligonucleotide bearing a site-specific eC lesion (or normal C in controls) at nucleotide position 51 annealed to a 5′-end-labeled 30-mer primer whose 3′-OH terminus is 14 nt downstream of the lesion site (Fig. 5). The primer was elongated by pol III following the procedures described under “Materials and Methods,” and the products were analyzed by high resolution gel electrophoresis. Fig. 5 shows that all of the tested pol III preparations were capable of significant levels of translesion DNA synthesis across eC. Table IV presents a quantitative analysis, based on densitometric analyses of the type of data represented by Fig. 5, and confirms that there are no significant differences in translesion DNA synthesis by pol III preparations from control and mutator strains. Therefore, elevated mutagenesis at eC lesion by pol III

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**Table II**

Purification of DNA polymerase III* For purification procedures see “Materials and Methods.”

| Source strain | Volume | Protein concentration | Total units | Specific activity |
|---------------|--------|-----------------------|-------------|------------------|
| AM134         | 2      | 0.22                  | 5,529       | 12,567           |
| AM135         | 1.8    | 0.59                  | 9,860       | 9,285            |
| AM147/pMV22   | 3.5    | 1.078                 | 38,805      | 10,285           |
| AM147/pMV11   | 2.7    | 0.45                  | 20,390      | 16,782           |

* One unit is defined as 1 nmol of nucleotide incorporation in 30 min at 30 °C using the procedures described under “Materials and Methods.”
from mutator strains appears to result from increased errors rather than from increased translesion DNA synthesis.

Fidelity of pol III* from Mutator and Control Strains during In Vitro Gap-filling DNA Synthesis—M13mp7L2 genome showing the position of the C lesion (or C in control constructs; position X at nucleotide 6240), the BglII restriction endonuclease site (at nucleotide 6402), and the 60-mer primer-annealing site (nt 6628–6687). DNA synthesis on the primed template was carried out as described under “Materials and Methods,” and a 319-nt sequence of the newly synthesized strand was selectively amplified by ligation-mediated PCR as described in detail elsewhere (10) and summarized briefly under “Materials and Methods.” B, summary of multiplex sequence analysis procedures for a 319-bp double-stranded DNA fragment derived by selective amplification of the newly synthesized strand as above. A 5′-32P-labeled 19-nt-long primer (50) was mixed with the 309-bp DNA fragment and subjected to 20 cycles of (linear) elongation by Tq DNA polymerase (Taq pol) in the presence of dGTP, dCTP, and ddATP as described under “Materials and Methods.” Under the described conditions, primer elongation on wild type (WT) and mutant templates results in dead-end products of characteristic lengths: 23-mer for wild type, 22-mer for C → T mutation, and 21-mer for C → A mutation. The elongation products were fractionated by high resolution gel electrophoresis and quantitated by computing densitometry. C, composite autoradiogram showing in vitro mutation fixation at C leisions (lanes 5–8) and, as controls, at normal cytosine (lanes 1–4). In vitro synthesized complementary (minus) strand was selectively amplified by LM-PCR followed by PCR, and the double-stranded DNA products were subjected to multiplex sequence analysis as outlined in B above. The resulting labeled elongation products were fractionated on 8% polyacrylamide, 8 μM urea gels followed by autoradiography. Lanes 1–4, C-ssDNA (control, C) replicated with 4 units of pol III* from the indicated strains. Lanes 5–8, C-ssDNA replicated with 4 units of pol III* from the indicated strains. We have previously demonstrated, by reconstruction experiments, that the in vitro mutation analysis system described here measures mutation frequency and specificity with reasonable accuracy (10).

observed against a background of colorless plaques. Fig. 6A outlines an in vitro assay system based on this phage. The assay consists of creating a 439-nt-long gap in the lacI region (−60 to +379) following the procedures described under “Materials and Methods.” The lacI gap is filled by DNA synthesis catalyzed by pol III* from non-mutator and mutator strains, and the resulting products are transfected into an appropriate host for analysis of mutagenesis that occurred during in vitro DNA synthesis.

Fig. 6B (ethidium bromide fluorescence) and Fig. 6C (autoradiography) show that the mobility of the gapped DNA changes to that of replicative form II (RF-II) DNA after a 10-min gap-filling reaction by pol III. Table V summarizes...
mutation frequencies obtained by transfection of the replication products into competent *E. coli* MC1061 cells, and shows that the mutation frequency for pol III from the non-mutator strain AM134 is $34.7 \times 10^{-4}$, whereas it is $83.4 \times 10^{-4}$ for AM135, a 2.4-fold elevation. Similar results were obtained for pol III from strains AM147/pMV22 (non-mutator) and AM147/pMV11 (mutator; Table V). Thus pol III* from mutator strains is error-prone against undamaged DNA also. Table V also shows that mutation frequencies for uncopied DNA, and for DNA replicated with wild type pol III (15–20) are due to differences in the preparation of the template region of the *lacI* gene (60 to +379 of the *lacI* gene) and covers the promoter region and the first 379 bp of the *lacI* gene. Gap-filling proceeds from the +379 position (right) to the –60 position (left). Gapped DNA was replicated with pol III*, and the replicated products were analyzed as described under "Materials and Methods." Ethidium bromide-stained agarose gel (B) and an autoradiogram of the same gel (C), showing replicated products, are shown. Lane 1, uncopied M13mRS65 gapped DNA; lane 2, replicated DNA with pol III* from strain AM134; lane 3, replicated DNA with pol III* from strain AM147/pMV22; lane 5, replicated DNA with pol III* from strain AM147/pMV11; and lane 6, RF II DNA marker (nickcd double-stranded M13mRS65 DNA).
observed for pol III from mutator strains in comparison to non-mutator strains (Table V) is similar to the 3-fold elevation in a lacZ forward mutagenesis assay based on replication of M13 ssDNA by crude extracts from mutA and wild type cells (10). Thus, pol III* from the mutator strains tested here is error-prone against undamaged DNA.

**DISCUSSION**

The present study was undertaken to investigate the mechanisms underlying the mutator phenotype observed in cells in which mismatch translation levels have been increased either due to mutations in genes for tRNAs or tRNA-modifying enzymes or as a result of exposure to mismatch-promoting agents such as streptomycin. Although mutator phenotypes can result from defects in repair, replication, or recombination, accumulating evidence suggests that mismatch translation-mediated mutagenesis is mediated by error-prone DNA replication. In particular, our previous work (10) showed that a transiently expressed novel DNA polymerase or an induced alteration of a constitutively expressed DNA polymerase might be responsible for the TSM phenotype.

How exactly mistranslation activates error-prone replication is not understood. Two available hypotheses have focused on a modification of E. coli DNA polymerase III. Slupska et al. (4) have proposed that the phenotype results from direct mistranslation of the ε subunit protein of pol III to create a small fraction of dominant-negative ε proteins. Because Asp → Gly mistranslation by a missense suppressor tRNA is inefficient (1–2% (63)), a key prediction of this hypothesis is that the mutator phenotype is transient, being expressed in a small fraction (1–2%) of mutA cells at any one time. However, transfection of M13 ssDNA bearing a site-specific ε residues results in a 5–20-fold elevation in mutagenesis, a result that suggests that a majority of mutA cells must constitutively express a mutator phenotype (5). To allow for a constitutive expression of the phenotype, the alternative hypothesis suggested that mistranslation triggered an “upstream” event activating a pathway that ultimately led to the constitutive expression of an error-prone replication activity (5). The molecular signal that turns on error-prone replication is presumed to be increased protein turnover due to an accumulation of misfolded proteins generated by mistranslation. Alternatively, mistranslation may result in the production of very small amounts of “gain-of-function” mutant proteins that activate a signal cascade leading to the production of an error-prone polymerase.

The work reported here shows that efficient induction of the TSM response occurs in cells simultaneously defective for pol I, pol II, pol IV, and pol V (Fig. 2). The construction of a viable, multiply deficient strain (AM147 polA polB dinB umuDC) suggests that all essential replication functions in E. coli can indeed be carried out by the remaining polymerase, namely pol III.

To address the question whether a modified form of pol III can account for the mutator phenotype, we purified pol III* from two pairs of TSM-induced and uninduced cells (Fig. 3 and Table II) and analyzed the replication fidelity of the purified enzymes on both damaged (εC; Fig. 4 and Table III) and undamaged DNA templates (Fig. 6 and Table V). Our results show that mutagenesis is elevated over 2-fold on both damaged and undamaged DNA when they are replicated by pol III from mutator strains as compared with control strains (Tables III and V).

The enhanced mutagenesis by pol III* from TSM-induced cells is characterized by an increase in C → A and C → T mutations, with C → A mutations predominating over C → T mutations (Table III). This mutational specificity is in complete agreement with our in vivo studies (5, 8) as well as our previous in vitro studies with crude extracts (10). The data here show that pol III is capable of extensive DNA synthesis across an εC lesion (~35% translesion synthesis; Fig. 5 and Table IV). Thus, our results suggest that both base insertion opposite an εC residue as well as extension past the site can be carried out by pol III in E. coli cells and that “lesion-bypass polymerases” such as pol II, pol IV, and pol V are not required for some noninstructive mutagenic DNA lesions. This finding resolves a number of early and previously puzzling observations on the mutagenic properties of εC, including its noninstructive template characteristics (64), as well as its ability to induce mutations in SOS-deficient cells (65). Indeed, the unusual mutagenic properties of εC were instrumental in uncovering UVM, an SOS-independent DNA damage-inducible pathway in E. coli (3, 49, 50, 66).

Because essentially identical results are obtained with two different pol III* preparations from two different mutA strains (one of which was defective for all four of the remaining DNA...
polymerases thus ruling out any polymerase cross-contamination), these results are consistent with the conclusion that pol III from mutA cells is error-prone. Nevertheless, the apparent magnitude of the mutator effect at εC residues differs from that observed in vivo as well as previous in vitro replication studies based on crude extracts. Thus, mutagenesis is elevated 2-fold at εC by pol III from TSM-induced cells over controls, as compared with 5–20-fold in the observed εD, εE in vivo (5, 8) and in vitro with crude cell extracts (10). This magnitude of difference between in vivo and in vitro results appears to be an inherent, as yet unexplained, property of highly purified pol III preparations. It is instructive to compare these results with the in vivo and in vitro magnitude of differences for wild type dnaQ and mutDΔ pol III heloenzymes preparations (62). The dnaQ defect in mutDΔ cells reduces the editing activity εC to <2% of the wild type level and leads to a 10,000-fold elevation in mutagenesis. A part of the mutator effect is attributable to saturation of the mismatch repair system), there is only a limitation component of mismatch repair system), there is only a limit to the editing activity εC to <2% of the wild type level and leads to a 10,000-fold elevation in mutagenesis. A part of the mutator effect is attributable to saturation of the mismatch repair system), there is only a limitation component of mismatch repair system). In contrast to mutDΔ, mutA is a weaker mutator that elevates mutagenesis by about 5–20-fold depending on the assay (3, 7, 58), the most accurate determination being a 17-fold increase in mutation rate in streptomycin-induced rpsL1408 cells at a specific lacZ site (7). The above considerations suggest that the observed elevation in error levels mutA pol III is significant. These results, in conjunction with the demonstration that the mutA phenotype is expressed in cells defective for polA, polB, dinB, and umuDC (Fig. 2), suggest that the error-prone polymerase mediating TSM is a modified version of pol III. How exactly pol III is modified in TSM-induced cells is unknown. Among the possibilities are incorporation of one or more mistranslated subunits into the holoenzyme, chemical modification (such as phosphorylation or acetylation), and acquisition of a cofactor that can affect fidelity.

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DNA Polymerase III from *Escherichia coli* Cells Expressing *mutA* Mistranslator tRNA Is Error-prone

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