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Role of *Escherichia coli* DNA Polymerase I in Conferring Viability upon the *dnaN159* Mutant Strain▽

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The *Escherichia coli dnaN159* allele encodes a mutant form of the β-sliding clamp (β159) that is impaired for interaction with the replicative DNA polymerase (Pol), Pol III. In addition, strains bearing the *dnaN159* allele require functional Pol I for viability. We have utilized a combination of genetic and biochemical approaches to characterize the role(s) played by Pol I in the *dnaN159* strain. Our findings indicate that elevated levels of Pol I partially suppress the temperature-sensitive growth phenotype of the *dnaN159* strain. In addition, we demonstrate that the β clamp stimulates the processivity of Pol I in vitro and that β159 is impaired for this activity. The reduced ability of β159 to stimulate Pol I in vitro correlates with our finding that single-stranded DNA (ssDNA) gap repair is impaired in the *dnaN159* strain. Taken together, these results suggest that (i) the β clamp-Pol I interaction may be important for proper Pol I function in vivo and (ii) in the absence of Pol I, ssDNA gaps may persist in the *dnaN159* strain, leading to lethality of the *dnaN159* Δ*polA* strain.

The *Escherichia coli dnaN*-encoded β-sliding clamp functions as a homodimer and is "loaded" onto primed DNA by the multisubunit DnaX clamp loader complex (5, 21). Once loaded, the β clamp slides freely along duplex DNA and functions to tether a variety of different proteins involved in replication and repair to the DNA (4, 6, 7, 9, 19, 23, 24, 26, 28, 41–43). The *dnaN159* allele encodes a mutant form of the β-sliding clamp (β159) that bears two amino acid substitutions: G66E (glycine at position 66 replaced with glutamic acid) and G174A (14, 35, 38). The G174A substitution appears to impair the ability of a hydrophobic cleft in β to interact with the eubacterial clamp-binding motif (QL[S/D]LF) that is conserved in most partner proteins reported to interact with the β clamp (8, 38). *E. coli* strains bearing the *dnaN159* allele display temperature-sensitive growth (14, 35, 38) and altered DNA polymerase (Pol) usage (29, 38–40). These phenotypes appear to result, at least in part, from impaired interactions of the mutant β159 clamp protein with the replicative DNA polymerase, Pol III (38). As a result of the impaired β159-Pol III interaction, *E. coli* strains bearing the *dnaN159* allele display increased utilization of the three SOS-regulated DNA polymerases, Pol II (*polB*), Pol IV (*dinB*), and Pol V (*umuDC*) (29, 38–40). In contrast to these three Pols, which under certain conditions impede growth of the *dnaN159* strain (29, 39, 40), presumably by impairing DNA replication, the catalytic DNA polymerase activity of Pol I (*polA*) is essential for viability of the *dnaN159* strain (38). Importantly, Pol I function is not required by the isogenic *dnaN*$^+$ strain (38), indicating that Pol I plays one or more essential roles in the *dnaN159* strain. Since Pol I is a multifunctional protein that participates in DNA replication, as well as numerous DNA repair pathways, several possibilities exist, including Okazaki fragment maturation (21, 31) and single-stranded DNA (ssDNA) gap repair (16, 22).

Since Pol III replication is abated in strains bearing the *dnaN159* allele (14, 35, 38), due to the impaired ability of β159 to interact with the α catalytic subunit of Pol III (38), we hypothesized that the requirement for Pol I function in the *dnaN159* strain might stem from its ability to augment Pol III function in DNA replication. The experiments discussed in this report were designed to test this hypothesis. Our findings indicate that elevated levels of Pol I partially suppress the temperature-sensitive growth phenotype of the *dnaN159* strain. In addition, we provide evidence that ssDNA gap repair is impaired in the *dnaN159* strain. Moreover, we determined that the β clamp confers a modest degree of processivity upon Pol I replication in vitro, while the mutant β159 clamp protein is impaired for this function, suggesting that the β clamp-Pol I interaction is important for ssDNA gap repair in vivo. Finally, we provide evidence that the fidelity of lagging-strand replication is impaired in the *dnaN159* strain, consistent with altered Okazaki fragment maturation in the *dnaN159* strain. Taken together, these findings suggest that (i) the β clamp-Pol I interaction may be important for proper Pol I function in vivo and (ii) in the absence of Pol I, ssDNA gaps may persist in the *dnaN159* strain, leading to lethality.

MATERIALS AND METHODS

***E. coli* strains, plasmid DNAs, and bacteriological techniques.** All *E. coli* strains used in this study are derivatives of either RW118 [*thr-1 araD139* Δ(*gpt-proA*)*62 lacY1 tsx-33 glnV44*(AS) *galK2*(Oc) *hisG4*(Oc) *rpsL31 xylA5 mtl-1 argE3*(Oc) *thi-1 sulA211*] (15), CJ278 [Δ(*gal-bio*) *thi-1 relA1 spoT1* Δ*polA*::*kan*] (18), or EC3144 and EC3138 {*ara* Δ(*lac-proB*)$_{XIII}$ *attB*::*lacI*$^+$*Z*[GGG→ GAG]*Y*$^+$*A*$^+$} (10). The strains were constructed using P1*vir*-mediated transduction (30), and the presence of the desired allele was confirmed by either diagnostic PCR or automated nucleotide sequence analysis of the PCR-amplified allele, as described previously (38). Transduction of the *dnaN*$^+$ and *dnaN159* alleles was

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achieved by selection for the closely linked (~80%) *tnaA300*::Tn*10* allele. *E. coli* strains were made competent for transformation with plasmid DNA by rubidium chloride treatment (36). All *E. coli* strains were grown in either LB or M9 medium (30), as indicated. When necessary, the following antibiotics were added at the indicated concentrations: ampicillin, 150 mg/ml; chloramphenicol, 20 mg/ml; kanamycin, 60 mg/ml; rifampin (Rif), 100 mg/ml; and tetracycline, 10 mg/ml.

Plasmid pRM100 is a pWSK29 (44) derivative that contains the complete *polA*$^+$ gene (coding sequence and promoter) and was described previously (29). Plasmid pRM104 is a pRM100 derivative that expresses the Klenow fragment of Pol I (lacking amino acids 1 to 322) from the native *polA* promoter. It was constructed by introducing NcoI restriction sites at nucleotide positions −2 through +4 (where +1 corresponds to the A in the ATG initiation codon) and +965 through +970 of the *polA* coding sequence using the Quickchange mutagenesis kit (Stratagene) with primers *polA*-Klenow-1 (5′-CAC GGA CAC CAT GGT TCA GAT CCC-3′) and *polA*-Klenow-2 (5′-GGG ATC TGA ACC ATG GTG TCC GTG-3′) and with *polA*-Klenow-3 (5′-GTG ACG GCC ATG GTG ATT TCT TAT GAC-3′) and *polA*-Klenow-4 (5′-GTC ATA AGA AAT CAC CAT GGC CGT CAC-3′), respectively (the NcoI sites are underlined). After confirming the correct nucleotide sequence (Biopolymer Facility, Roswell Park Cancer Institute, Buffalo, NY), the double-mutant plasmid was digested with NcoI, gel purified, and ligated, resulting in a plasmid expressing the Klenow fragment of Pol I from the native *polA*$^+$ promoter.

Plasmids pRM105 and pRM106 express untagged Pol I and N-terminally His_{10}-tagged Klenow, respectively. Plasmid pRM105 was cloned by PCR amplification of the *polA*$^+$ gene from RW118 genomic DNA using primers *polA*-forward (5′-CCG ACA CAT ATG GTT CAG ATC CCG CAG AAT CC-3′) and *polA*-reverse (5′-GGG ACA CCT AGG TTA GTG CGC CTG ATC CCA G-3′). Primer *polA*-forward introduces an NdeI site overlapping the ATG initiation codon (underlined). The PCR fragment was cloned into plasmid pCR-BluntII-Topo (Invitrogen), and the complete nucleotide sequence of the *polA*$^+$ reading frame was verified by automated nucleotide sequence analysis (Biopolymer Facility, Roswell Park Cancer Institute, Buffalo, NY). The *polA*$^+$ coding sequence was then subcloned into the overexpression vector pET11a (Novagen) using NdeI and BamHI (the site was present in the multiple cloning site of pCR-BluntII-Topo) restriction, placing it under the control of the T7 promoter. Plasmid pRM106 was created by PCR amplifying the Klenow fragment of Pol I from the *polA*$^+$ gene, using primers *Klenow*-forward (5′-CCA GAA GTG ACG GCA CAT ATG ATT TCT TAT GAC AAC TAC-3′, which introduces an NdeI restriction site [underlined] in place of residues 322 and 333) and *polA*-reverse (see above). The resulting PCR fragment was cloned similarly to *polA*$^+$, except that the overexpression vector pET16b (Novagen) was used to introduce an N-terminal His_{10} tag. As for pRM105, the sequence of the Klenow coding sequence was confirmed by automated nucleotide sequence analysis (Biopolymer Facility, Roswell Park Cancer Institute, Buffalo, NY).

UV sensitivity and mutation frequency measurements. Sensitivity to UV light (254 nm) was measured using a germicidal lamp (General Electric) as described previously (38). Briefly, cultures were grown at 30°C in M9 minimal medium supplemented with glucose (0.2%) and Casamino Acids (0.5%) until they reached mid-exponential growth (an optical density at 595 nm of ~0.6). The cultures were then transferred to a sterile 100-mm glass petri dish and irradiated with the indicated UV dose. Appropriate dilutions of each sample were plated onto LB plates supplemented with the appropriate antibiotics. Colonies were counted after overnight incubation at 30°C, and survival was expressed as percent viability relative to control samples that were mock UV irradiated.

Spontaneous-mutation frequencies were determined as previously described (29). Briefly, cultures were grown overnight at 30°C in LB medium and appropriate dilutions were plated onto LB plates or LB plates supplemented with Rif (100 μg/ml). The mutation frequency was calculated by dividing the number of Rif^r colonies observed by the number of viable cells.

Protein purification and in vitro primer extension assays. Purification of β, β159, and the $\gamma_3\delta\delta'$ clamp loader complex will be described elsewhere (R. W. Maul, S. K. Scouten-Ponticelli, J. M. Duzen, and M. D. Sutton, submitted for publication). Recombinant Pol I and N-terminally His_{10}-tagged Klenow were purified as described previously (17). Primer SP20 (5′-ACG CCT GTA GCA TTC CAC AG-3′) was gel purified (Sigma Genosys) and 5′-end labeled using polynucleotide kinase and [γ^{32}P]ATP as described previously (1). SP20 was annealed to M13mp18 ssDNA (New England Biolabs) in a reaction mixture (100 μl) containing 15 pmol ^{32}P-labeled SP20, 5 pmol M13mp18 ssDNA, and annealing buffer (25 mM Tris-HCl [pH 7.0], 100 mM NaCl, 15 mM $MgCl_2$) by incubation at 95°C for 3 minutes, followed by cooling to room temperature for 1 hour. To remove unannealed SP20, the annealing reaction mixture was passed over an S-400 microspin column (GE Healthcare) following the manufacturer's recommendations.

Primer extension reaction mixtures (10 μl) were assembled on ice and contained replication assay buffer (20 mM Tris-HCl [pH 7.5], 8.0 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiothreitol, 1 mM ATP, 5% glycerol, 0.8 μg/ml bovine serum albumin) supplemented with 0.133 mM deoxynucleoside triphosphates (dNTPs), 2 μM ssDNA-binding protein, and 2.5 nM ^{32}P-labeled SP20/M13mp18 ssDNA template. Reactions were initiated by addition of the indicated β clamp protein (40 nM) and $\gamma_3\delta\delta'$ clamp loader complex (10 nM). Following a 5-min preincubation at 30°C to allow loading of the clamp, Pol I or Klenow was added as indicated in the figure legends. After 10 min at 30°C, the reactions were quenched with 20 μl of quench buffer (95% formamide, 20 mM EDTA, 0.1% bromophenol blue), and 5 μl of each quenched reaction mixture was electrophoresed through an 8.0% urea-polyacrylamide gel. Replication products were visualized using a phosphorimaging screen K and personal FX imaging instrumentation (Bio-Rad).

For quantitation of replication products, reactions (20 μl) were performed as described above using either unlabeled SP20/M13mp18 as a template or 250 ng of a multiply nicked form of the double-stranded DNA (dsDNA) plasmid pBluescript KS II, together with a [^{3}H]dTTP-dNTP mixture (133 μM; 101 cpm/pmol). Reactions were quenched with 15% trichloroacetic acid, 100 mM sodium pyrophosphate. Acid-insoluble products were collected on 2.4-cm glass fiber filters (VWR) by vacuum filtration, and incorporation of [^{3}H]dTTP was quantitated by liquid scintillation counting, as described previously (34). The results shown represent the average of triplicates. The error bars represent the standard deviations. The nicked pBluescript template was generated by incubating 10 μg of pBluescript KS II dsDNA with 0.002 units of DNase I (Promega) for 30 min at room temperature. The reaction was quenched by extraction with a 50% phenol-chloroform suspension, followed by ethanol precipitation.

RESULTS AND DISCUSSION

Modest overexpression of Pol I partially suppresses the temperature-sensitive growth phenotype of the *dnaN159* strain. In contrast to Pol II, Pol IV, and Pol V, which confer lethality upon the *dnaN159* strain under certain conditions (29, 38, 39), the catalytic polymerase activity of Pol I is required for viability of the *dnaN159* strain (38). Since Pol III function is abated in the *dnaN159* strain (14, 35, 38), due to an impaired β159-Pol III interaction (38), we hypothesized that Pol I might act to augment Pol III function during DNA replication in the *dnaN159* strain. As a test of this hypothesis, we measured growth at different temperatures of the *dnaN159* strain MS105 bearing either the low-copy-number plasmid pWSK29 (44) or pRM100 (29), a pWSK29 derivative containing *E. coli* Pol I under the control of its native promoter. Consistent with our previous findings (29), strain MS105 bearing pWSK29 was severely impaired for growth at temperatures above 34°C (Fig. 1). In contrast, strain MS105 bearing the Pol I-expressing plasmid pRM100 grew normally at temperatures up to and including 39°C (Fig. 1), consistent with the idea that Pol I augments Pol III function during DNA replication in the *dnaN159* strain.

E. coli Pol I contains three distinct activities: (i) a 5′-to-3′ template-directed DNA polymerase activity, (ii) a 3′-to-5′ exonuclease proofreading activity, and (iii) a FEN1-like 5′-to-3′ exonuclease activity important for DNA repair functions, as well as Okazaki fragment maturation (21). Since growth of the *dnaN159* strain requires only the polymerase activity of Pol I (38), which, together with the 3′-to-5′ exonuclease proofreading activity, is contained within the Klenow fragment (residues 323 to 928 of Pol I), we asked whether plasmid-expressed Klenow fragment was sufficient to support growth of the *dnaN159* strain at elevated temperatures. To examine this, we utilized a pRM100 derivative named pRM104 that expresses the Klenow fragment of Pol I from the endogenous *polA* promoter. In contrast to Pol I, plasmid-expressed Klenow fragment was only able to support growth at temperatures up to

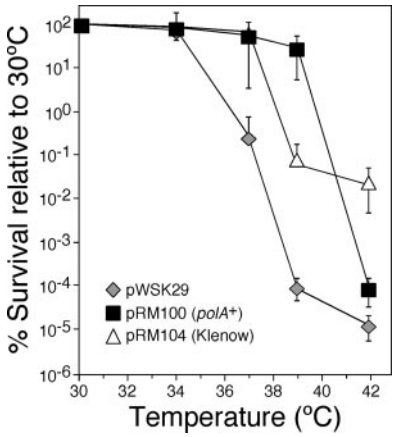

FIG. 1. Elevated levels of Pol I partially suppress the temperature-sensitive growth phenotype of the *dnaN159* strain. Strain MS105 [*thr-1 araD139* Δ(*gpt-proA*)*62 lacY1 tsx-33 glnV44*(AS) *galK2*(Oc) *hisG4*(Oc) *rpsL31 xylA5 mtl-1 argE3*(Oc) *thi-1 sulA211 lexA51*(Def) *tnaA300*::Tn*10 dnaN159*] was transformed with either pWSK29 (control), pRM100 (*polA*$^+$), or pRM104 (Klenow). We used strain MS105 for this experiment because it displays a more pronounced temperature-sensitive phenotype than many other *dnaN159* strains, thus increasing the range in which we could observe an effect by Pol I or Klenow. Temperature sensitivity was measured by plating out dilutions of overnight cultures grown at 30°C onto M9 minimal medium, followed by incubation at the indicated temperature overnight. The number of CFU observed at each indicated temperature is expressed as a percentage of that observed for the same strain at 30°C, which was set equal to 100%. The error bars indicate standard deviations. MS105 bearing pWSK29 had an average titer of 5.1×10^8 viable cells/ml, MS105 bearing pRM100 had an average titer of 1.5×10^8 viable cells/ml, and MS105 bearing pRM104 had an average titer of 0.1×10^7 viable cells/ml.

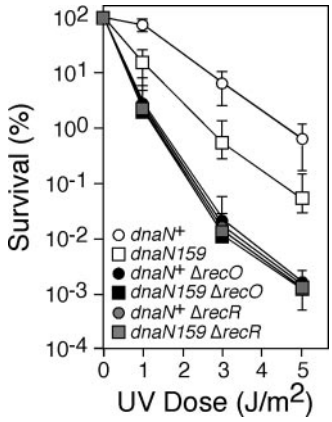

FIG. 2. The UV sensitivity of the *dnaN159* strain is epistatic with mutations that impair *recFOR* function. Survival following the indicated dose of UV irradiation was measured by plating serial dilutions as previously described (29). The strains examined are derivatives of RW118 [*thr-1 araD139* Δ(*gpt-proA*)*62 lacY1 tsx-33 glnV44*(AS) *galK2*(Oc) *hisG4*(Oc) *rpsL31 xylA5 mtl-1 argE3*(Oc) *thi-1 sulA211*] (15): RM149 (*dnaN*$^+$ Δ*uvrB*::*cat*), RM150 (*dnaN159* Δ*uvrB*::*cat*), RM151 (*dnaN*$^+$ Δ*uvrB*::*cat* Δ*recO1504*::Tn*5*), RM152 (*dnaN159* Δ*uvrB*::*cat* Δ*recO1504*::Tn*5*), RM163 (*dnaN*$^+$ Δ*uvrB*::*cat* Δ*recR252*::Tn*10*~*9*), and RM164 (*dnaN159* Δ*uvrB*::*cat* Δ*recR252*::Tn*10*~*9*). The results shown represent the average of two independent experiments, each done in duplicate. The error bars represent the range.

and including 37°C (Fig. 1). In addition, the titer of this strain was ~100-fold lower than those of the other strains examined (see the legend to Fig. 1). This finding suggests that elevated levels of the Klenow fragment are deleterious to the *dnaN159* strain. Taken together, these results indicate that both the DNA polymerase and the FEN1-like 5′-to-3′ exonuclease activities of Pol I are required for maximal suppression of temperature-sensitive growth of the *dnaN159* strain.

***The dnaN159* strain is impaired for ssDNA gap repair.** Our finding that the FEN1-like 5′-to-3′ exonuclease activity of Pol I is required for maximal suppression of temperature-sensitive growth in the *dnaN159* strain (Fig. 1) suggests that temperature sensitivity is the result, at least in part, of the accumulation of ssDNA nicks and/or gaps (hereafter referred to jointly as ssDNA gaps for simplicity). Consistent with this idea, the *dnaN159* strain is sensitive to UV light at the permissive temperature of 30°C, and this sensitivity is enhanced ~3-fold at the semipermissive temperature of 37°C (38). Therefore, we hypothesized that the UV sensitivity of the *dnaN159* strain would be epistatic with mutations that impair the RecFOR-mediated pathway of ssDNA gap repair, which is reported to utilize both Pol I and Pol III for gap filling (16, 37). Since the *recF* allele is located immediately downstream of *dnaN* (32, 33), we chose to construct isogenic *E. coli* strains bearing *dnaN*$^+$ or *dnaN159*, together with either the *recO*$^+$, Δ*recO1504*::Tn*5* (20), *recR*$^+$, or Δ*recR252*::Tn*10*~*9* (25) alleles, and subsequently compared their respective levels of sensitivity to UV irradiation. Consistent with our previous findings (38), the *dnaN159* strain was ~10-fold more sensitive to UV irradiation than was the isogenic *dnaN*$^+$ strain (Fig. 2). This UV sensitivity was completely epistatic with both the Δ*recO1504*::Tn*5* and the Δ*recR252*::Tn*10*~*9* alleles (Fig. 2), suggesting that the UV sensitivity of the *dnaN159* strains is a result of impaired ssDNA gap repair. However, our finding that the *dnaN159* strain was less sensitive to UV irradiation than were the isogenic *dnaN*$^+$ Δ*recO1504*::Tn*5* and *dnaN*$^+$ Δ*recR252*::Tn*10*~*9* strains indicates that the *dnaN159* strain is only partially impaired for ssDNA gap repair. Furthermore, our finding that the UV sensitivity of the *dnaN159* strain was suppressed by (not epistatic with) inactivation of Pol IV [Δ(*dinB-yafN*)::*kan*] (38) suggests that Pol IV competes with Pol I and/or Pol III for access to ssDNA gaps to impede repair in the *dnaN159* strain.

The β159 clamp is impaired for stimulating Pol I and Klenow replication on both a singly primed ssDNA and a nicked dsDNA template in vitro. The β clamp is reported to interact with, and to confer processivity upon, the Klenow fragment of Pol I in vitro during replication of singly primed M13 ssDNA (24) (Fig. 3). However, due to the fact that a consensus clamp-binding motif has not yet been identified in Pol I, it remains unclear exactly how Pol I interacts with the β clamp. As a result, it is currently unknown whether the Pol I-β clamp interaction is required for proper Pol I function in vivo. We hypothesized that the ssDNA gap repair defect of the *dnaN159* strain could be the result of an impaired ability of the β159 clamp protein to stimulate Pol I function, leading to a reduced capacity for ssDNA gap repair. As a test of this hypothesis, we cloned and purified recombinant forms of Pol I and Klenow for in vitro replication assays. In our first experiment, we utilized a M13 ssDNA template bearing a ^{32}P-labeled primer. Using this assay, the wild-type β clamp conferred a modest degree of processivity upon both Pol I and Klenow, as

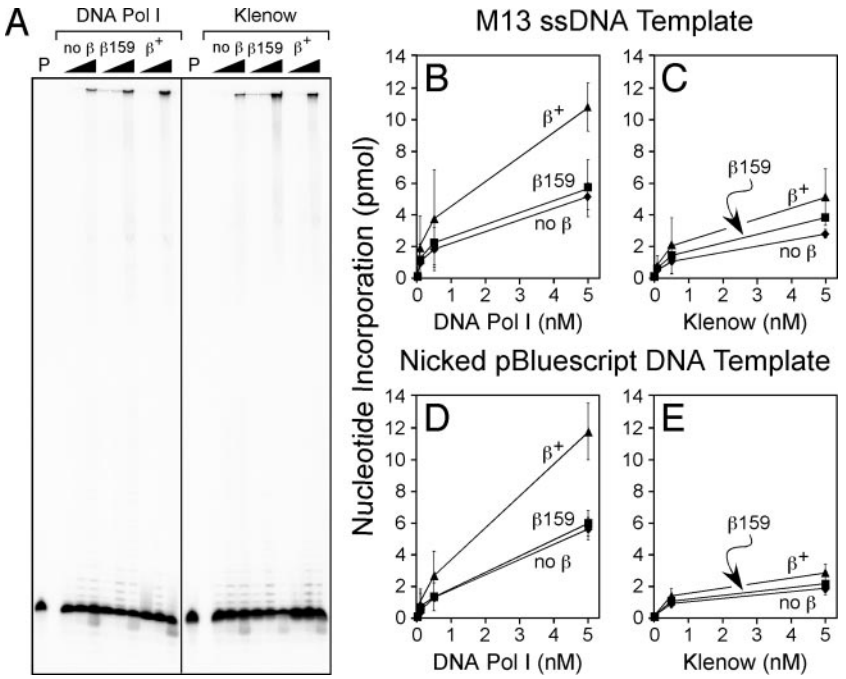

FIG. 3. The wild-type β clamp stimulates Pol I replication in vitro. (A) Reaction mixtures containing M13 ssDNA bearing a 5′-^{32}P-labeled primer as a template, together with either 0.05, 0.5, or 5 nM of the indicated polymerase, as described in Materials and Methods. After being quenched, aliquots of each reaction mixture were electrophoresed through an 8% urea-polyacrylamide gel. The lanes labeled P represent control reactions lacking clamp and polymerase and serve to indicate the position of free primer. Replication products were visualized by phosphorimager analysis. The faint full-length bands visible in reactions containing β159 and 0.05 or 0.5 nM Pol I or Klenow are due to a trace polymerase contamination in the β159 preparation. Alternatively, replication utilizing either the same M13 ssDNA template lacking the 5′ ^{32}P label (B and C) or a multiply nicked form of pBluescript KS II dsDNA (D and E), together with [^{3}H]dTTP-dNTPs, was quantified by liquid scintillation spectroscopy of acid-insoluble products collected on 2.4-cm glass fiber filters as described in Materials and Methods. Reaction mixtures contained the indicated amounts (0.05, 0.5, or 5 nM) of Pol I (B and D) or the Klenow fragment of Pol I (C and E).

judged by the increase in the abundance of full-length product near the top of the gel with 5 nM Pol I or Klenow in a reaction mixture containing the β clamp relative to those lacking the clamp (Fig. 3A). Our finding that the wild-type β clamp conferred processivity upon Klenow confirms an earlier report (24). Although β159 was impaired for conferring processivity upon Pol I as measured by this assay, it did confer modest processivity upon Klenow (Fig. 3A).

In order to quantify the extent to which the wild-type and β159 clamp proteins influenced Pol I and Klenow replication in vitro, we repeated the experiments described above using the same singly primed M13 ssDNA template, but instead of the 5′-^{32}P-label, we utilized [^{3}H]dTTP-dNTPs and measured nucleotide incorporation by liquid scintillation spectroscopy of acid-insoluble [^{3}H]dTTP retained on glass fiber filters (34). Consistent with the results shown in Fig. 3A, addition of the wild-type β clamp and γ complex stimulated both Pol I and Klenow replication ~2-fold at all levels of enzyme (0.05, 0.5, and 5 nM) examined (Fig. 3B and C). In contrast, β159 failed to stimulate Pol I replication under these conditions (Fig. 3B) and stimulated Klenow only moderately (Fig. 3C).

We next asked whether the β clamp might stimulate Pol I or Klenow more effectively on a nicked DNA substrate. For these experiments, we used a multiply nicked form of the double-stranded plasmid pBluescript KS II as the DNA template and again measured nucleotide incorporation by Pol I or Klenow by liquid scintillation spectroscopy. Although the wild-type β clamp stimulated Pol I replication ~2-fold on the multiply nicked template, β159 failed to stimulate Pol I to a detectable level on this DNA template (Fig. 3D). Consistent with a need for the FEN1-like 5′-to-3′ exonuclease domain of Pol I for nick translation (21), the Klenow fragment inefficiently replicated the nicked dsDNA template regardless of whether the reaction mixture contained the wild-type β or β159 clamp protein (Fig. 3E). These biochemical findings, taken together with the genetic results discussed above (Fig. 2), suggest that ssDNA gap repair is abated in the *dnaN159* strain due, at least in part, to the diminished ability of the mutant β159 clamp protein to stimulate Pol I function.

Pol I and Pol V compete with each other in the *dnaN159* strain. The results discussed above are consistent with a model in which the ssDNA gap repair defect of the *dnaN159* strain is the result, at least in part, of the impaired ability of β159 to stimulate Pol I (Fig. 3) and Pol III (38). We hypothesized that ssDNA gap repair might be further abated in the *dnaN159* strain by the actions of the SOS-regulated Pols via their ability to compete with Pol I and/or Pol III for access to ssDNA gaps. It was previously reported that spontaneous Pol V-dependent mutagenesis was enhanced in *E. coli* strains either bearing the Δ*polA* allele or expressing the Klenow fragment of Pol I in place of the full-length Pol I (2). The ability of Pol V to contribute to spontaneous mutagenesis under these conditions presumably results from the ability of Pol V to gain access to ssDNA gaps in the absence of Pol I or in the presence of a Pol

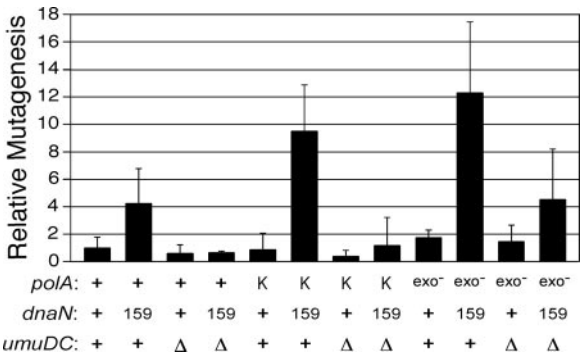

FIG. 4. Spontaneous mutation frequencies in *dnaN*$^+$ and *dnaN159* strains expressing either Pol I or the Klenow fragment of Pol I. All strains used were derivatives of CJ278 [Δ(*gal-bio*) *thi-1 relA1 spoT1* Δ*polA*::*kan*] and expressed either the *polA*$^+$ allele, the Klenow fragment of Pol I, or the *polA-exo* mutant allele from an F′ episome (18). The strains examined were RM137, *dnaN*$^+$ (F′ *polA*$^+$); RM138, *dnaN159* (F′ *polA*$^+$); RM139, *dnaN*$^+$ Δ*umuDC595*::*cat* (F′ *polA*$^+$); RM140, *dnaN159* Δ*umuDC595*::*cat* (F′ *polA*$^+$); RM141, *dnaN*$^+$ (F′ Klenow); RM142, *dnaN159* (F′ Klenow); RM143, *dnaN*$^+$ Δ*umuDC596*::*ermGT* (F′ Klenow); RM144, *dnaN159* Δ*umuDC596*::*ermGT* (F′ Klenow); RM145, *dnaN*$^+$ (F′ *polA-exo* mutant); RM146, *dnaN159* (F′ *polA-exo* mutant); RM147, *dnaN*$^+$ Δ*umuDC596*::*ermGT* (F′ *polA-exo* mutant); RM148, *dnaN159* Δ*umuDC596*::*ermGT* (F′ *polA-exo* mutant). The mutation frequency was calculated following growth at 30°C by dividing the number of Rif^r colonies by the total number of viable cells, as described previously (29, 38, 40). Mutation frequencies are expressed relative to the *dnaN*$^+$ *polA*$^+$ strain (9.7 ± 7.5 Rif^r CFU/10^9 total viable cells), which was set equal to 1.0, and are the averages of at least five independent determinations. The error bars represent the standard deviations. Abbreviations: +, *polA*$^+$, *dnaN*$^+$, or *umuD*$^+$*C*$^+$, as indicated; exo$^-$, Pol I *exo* mutant; K, Klenow fragment; 159, *dnaN159*; Δ, Δ*umuDC596*::*ermGT* or Δ*umuDC595*::*cat*.

I mutant (Klenow) impaired for proper function. Based on our finding that Pol V plays a more prominent role in DNA replication in the *dnaN159* strain in response to UV irradiation (38), we hypothesized that Pol V would likewise play a larger role in replication in the *dnaN159* strain bearing mutations impairing Pol I function. Due to the fact that Pol V is error prone, we further reasoned that if Pol I and Pol V do in fact compete with each other for access to ssDNA gaps in the *dnaN159* strain, then we should be able to manipulate the frequency of spontaneous mutagenesis by using different combinations of *polA* (Pol I) and *umuDC* (Pol V) alleles.

Although a *dnaN159* Δ*polA* strain is nonviable (38), it is possible to construct a *dnaN159* strain bearing a Δ*polA* allele on the chromosome that is complemented by the Klenow fragment of Pol I expressed from an F′ episome (38). We therefore utilized Rif^r as a measure of the spontaneous mutation frequencies of isogenic *dnaN*$^+$ and *dnaN159* strains bearing either the *umuD*$^+$*C*$^+$ (Pol V) or Δ*umuDC* alleles together with the Δ*polA* allele on their chromosome and expressing either the *polA*$^+$ allele or the Klenow fragment of Pol I in order to gauge the respective effects of β159, Klenow, and Pol V. Although we observed little effect on the frequency of spontaneous mutagenesis in the *dnaN*$^+$ strain expressing the Klenow fragment of Pol I relative to the same strain expressing Pol I (Fig. 4), the *dnaN159* strains expressing Pol I or the Klenow fragment of Pol I displayed ~4-fold- and ~9-fold-higher spontaneous mutation frequencies, respectively, than did the *dnaN*$^+$ Pol I control strain. Consistent with our hypothesis, the elevated frequency of spontaneous mutagenesis in these strains was completely dependent upon Pol V, as the mutation frequencies of the *dnaN159* Δ*umuDC* strains expressing either Pol I or Klenow were comparable to that of the *dnaN*$^+$ Pol I strain (Fig. 4). Our observation that the *dnaN159* Klenow strain displayed a higher Pol V-dependent spontaneous-mutator phenotype than the isogenic *dnaN159* Pol I strain correlates with our finding that the β clamp stimulated Pol I replication more robustly than it did Klenow (Fig. 3), consistent with the β clamp-Pol I interaction being important for proper Pol I function in vivo.

Evidence for a more prominent role for Pol I in DNA replication in the *dnaN159* strain. In addition to being impaired for stimulation of Pol I replication in vitro (Fig. 3), β159 is also impaired for interactions with the α catalytic subunit of Pol III (38). We hypothesized that the impaired ability of β159 to interact with Pol III might not only impair ssDNA gap repair, due to a role for Pol III in gap filling (11, 16), but in addition might actually contribute to the further formation of ssDNA gaps in the *dnaN159* strain by decreasing the efficiency of Pol III replication. We further hypothesized that if the *dnaN159* strain contains on average more ssDNA gaps than the isogenic *dnaN*$^+$ strain, then Pol I should play a more prominent role in DNA replication in the *dnaN159* strain due to its role, albeit impaired, in the repair of these gaps. We tested this hypothesis by measuring spontaneous-mutation frequencies in isogenic *dnaN*$^+$ and *dnaN159* strains expressing the *polA-exo* mutant allele from an F′ episome (18). The *polA-exo* mutant allele encodes D355A and E357A substitutions that essentially eliminate the 3′-to-5′ proofreading nuclease activity of Pol I, thereby reducing its polymerase fidelity ~4-fold, allowing a measure of its contribution to DNA replication in vivo (3). As shown in Fig. 4, the *dnaN159* Pol I *exo* mutant strain displayed an ~12-fold-higher spontaneous-mutation frequency compared to an ~2-fold increase for the *dnaN*$^+$ Pol I *exo* mutant strain relative to the *dnaN*$^+$ Pol I control, suggesting that Pol I plays a more prominent role in replication in the *dnaN159* strain. In light of our results discussed above indicating that Pol V contributes to spontaneous mutagenesis in the *dnaN159* strains expressing Pol I (Fig. 4), we questioned whether Pol V contributed to the mutation frequencies observed for the strains expressing the Pol I *exo* mutant. Inactivation of Pol V (Δ*umuDC*) had essentially no effect on the mutation frequency of the *dnaN*$^+$ Pol I *exo* mutant and produced only an ~3-fold reduction in the mutation frequency of the *dnaN159* Pol I *exo* mutant (Fig. 4), suggesting that the elevated mutation frequencies observed were largely the result of Pol I replication errors. Thus, these findings suggest that Pol I plays a more prominent role in DNA replication in the *dnaN159* strain than it does in the otherwise isogenic *dnaN*$^+$ strain, consistent with the idea that the *dnaN159* strain suffers from more numerous ssDNA gaps.

Lagging-strand replication is more severely impaired than leading-strand replication in the *dnaN159* strain. The lagging strand, by virtue of its discontinuous synthesis, contains ssDNA nicks between Okazaki fragments. These nicks are ultimately repaired by the combined actions of RNase H, Pol I, and DNA ligase as part of the Okazaki fragment maturation process (21). Given that the *dnaN159* strain is impaired for repair of ssDNA

TABLE 1. Leading- and lagging-strand mutation frequencies in *dnaN*$^+$ and *dnaN159* strains

Strain[a]	*dnaN* allele	Orientation of *lacZ*[GGG→ GAG] allele[b]	Mutants/10^6 CFU[c]			
			lacZ[GGG→ GAG]→ Lac$^+$		Rif^r	
			Mutation frequency	Effect (*n*-fold) (leading/lagging)	Mutation frequency	Effect (*n*-fold) (leading/lagging)
JL100	*dnaN*$^+$	Leading	0.60 ± 0.13	2.9	4.87 ± 1.77	1.3
JL101	*dnaN*$^+$	Lagging	0.21 ± 0.05		3.74 ± 0.22	
JL102	*dnaN159*	Leading	1.40 ± 0.47	0.9	7.31 ± 1.95	1.1
JL103	*dnaN159*	Lagging	1.59 ± 1.29		6.59 ± 1.23	

[a] Strains JL100 to JL103 are derived from strains EC3144 and EC3138 (10) and differ only in their respective *dnaN* alleles (*dnaN*$^+$ or *dnaN159*), as well as the orientation of the *lacZ*[GGG→ GAG] allele (leading or lagging) integrated at *attB*. Their remaining genotype is *ara* Δ(*lac-proB*)$_{XIII}$ *attB*::*lacI*$^+$*Z*[GGG→ GAG]*Y*$^+$*A*$^+$ *tnaA300*::Tn*10* Δ*mutL*::*cat*.

[b] The *lacZ*[GGG→ GAG] allele is integrated at *attB* so that the coding strand is replicated as part of the leading strand (Leading) or as part of the lagging strand (Lagging).

[c] Growth of strains bearing the *lacZ*[GGG→ GAG] allele on minimal media containing lactose as the sole carbon source requires a "true" reversion of the *lacZ* mutation, as no other mutation confers a Lac$^+$ phenotype. Mutation frequencies were measured as described previously (10). The results shown represent the averages of at least three independent determinations ± the standard deviation.

gaps, we hypothesized that it would likewise be impaired for proper processing of ssDNA nicks located between adjacent Okazaki fragments on the lagging strand. As a test of this hypothesis, we asked whether the fidelity of lagging-strand replication was reduced in the *dnaN159* strain.

Using a collection of isogenic *E. coli* strains bearing defined *lacZ* mutant alleles integrated in both orientations at the *attB* site (i.e., so that the *lacZ* coding sequence was replicated as part of either the leading or the lagging strand) to measure *lacZ* mutant→ *lacZ*$^+$ reversion, the fidelity of lagging-strand replication was reported to be ~2- to ~6-fold higher (depending upon the *lacZ* mutant allele analyzed) than that of the leading strand (10, 13, 27). Using this same approach, we measured the respective fidelities of leading- and lagging-strand replication in four isogenic *E. coli* strains bearing the *lacZ*[GGG→ GAG] allele integrated in both orientations at *attB* together with a Δ*mutL*::*cat* mutation to inactivate mismatch repair (to allow a more accurate measure of replication errors) and either the *dnaN*$^+$ or the *dnaN159* allele.

Using the two isogenic *dnaN*$^+$ strains bearing the *lacZ* [GGG→ GAG] allele in each orientation, we observed a 2.9-fold-lower spontaneous *lacZ*[GGG→ GAG]→ *lacZ*$^+$ reversion frequency on the lagging strand ($0.21 \times 10^{-6} \pm 0.05 \times 10^{-6}$) than on the leading strand ($0.60 \times 10^{-6} \pm 0.13 \times 10^{-6}$) (Table 1), indicating that the lagging strand displayed 2.9-fold-higher fidelity than the leading strand, consistent with previous reports (10, 13, 27). This difference was specific to the respective orientations of the *lacZ*[GGG→ GAG] allele, as it was not observed when measuring Rif^r arising from mutations within *rpoB*, which is present in the same native orientation in both strains (Table 1), also consistent with previous reports (10, 13, 27). In contrast, the fidelity of lagging-strand replication in the pair of *dnaN159* strains was more severely impaired than that of the leading strand: the frequency of *lacZ*[GGG→ GAG]→ *lacZ*$^+$ reversion on the leading strand was 2.3-fold higher in the *dnaN159* strain ($1.40 \times 10^{-6} \pm 0.47 \times 10^{-6}$) than it was in the isogenic *dnaN*$^+$ strain ($0.60 \times 10^{-6} \pm 0.13 \times 10^{-6}$), while that of the lagging strand in the *dnaN159* strain ($1.59 \times 10^{-6} \pm 1.29 \times 10^{-6}$) was 7.6-fold higher than that of the isogenic *dnaN*$^+$ strain ($0.21 \times 10^{-6} \pm 0.05 \times 10^{-6}$) (Table 1). Furthermore, the fidelities of the leading and lagging strands were comparable to each other in the *dnaN159* strain (Table 1). The frequencies of Rif^r for these two *dnaN159* strains were also comparable to each other, although they were ~2-fold higher than those observed in the isogenic *dnaN*$^+$ strains (Table 1).

In order to confirm that the predominant base substitution contributing to spontaneous mutations in the *dnaN159* strain resulted from G→ A transitions (the mutation that was measured by *lacZ*[GGG→ GAG]→ *lacZ*$^+$ reversion), as has been reported for the *dnaN*$^+$ strain (12), we sequenced 22 independent Rif^r mutants from the *dnaN159* strain and compared the spectrum to those observed for 15 independent Rif^r *dnaN*$^+$ strains (Table 2). Based on this analysis, Rif^r of all 15 *dnaN*$^+$ strains examined resulted from a single GC→ AT transition mutation affecting one of five different nucleotide positions (Table 2). Of the 23 mutations identified in the *dnaN159* strains, 17 (74%) corresponded to GC→ AT transition mutations, 2 corresponded to TA→ CG transition mutations, and 4 corresponded to CG→ AT transversion mutations (Table 2).

TABLE 2. Nucleotide sequence analysis of the *rpoB* alleles from independent Rif^r *dnaN*$^+$ and *dnaN159* isolates

Nucleotide position[b]	*rpoB* mutations identified in strain[a]:			
	JL100 (*dnaN*$^+$)		JL103 (*dnaN159*)	
	Base substitution	No. of occurrences	Base substitution	No. of occurrences
1455	ND	NA	TA→ CG	1
1534	ND	NA	TA→ CG	1
1546	GC→ AT	4	GC→ AT	10
1576	GC→ AT	5	CG→ AT	4
1586	GC→ AT	1	GC→ AT	4
1592	GC→ AT	1	GC→ AT	3
1691	GC→ AT	4	ND	NA

[a] Cultures of the indicated strains were spread on LB plates supplemented with Rif (100 μg/ml) and incubated overnight at 30°C. A single Rif^r isolate from each plate was colony purified prior to PCR amplification of the *rpoB* gene, as described previously (12). The nucleotide change(s) in *rpoB* alleles leading to Rif^r in independent Rif^r isolates of strains JL100 (*dnaN*$^+$; 15 isolates) and JL103 (*dnaN159*; 22 isolates) were determined by automated nucleotide sequence analysis (Biopolymer Facility, Roswell Park Cancer Institute, Buffalo, NY), as described previously (38). Each Rif^r *dnaN*$^+$ isolate examined contained a single *rpoB* mutation, while one of the Rif^r *dnaN159* isolates contained two mutations (TA→ CG at position 1455 and GC→ AT at position 1586). Abbreviations: ND, none detected; NA, not applicable.

[b] The position of each mutation identified is indicated, with position 1 referring to the A in the ATG initiation codon of *rpoB*.

Based on these results, we conclude that spontaneous mutations in both the *dnaN*$^+$ and *dnaN159* strains result largely from GC→ AT transition mutations. These results, taken together with our findings discussed above, indicate that the fidelity of lagging-strand replication is more severely impaired than that of the leading strand in the *dnaN159* strain, suggesting that Okazaki fragment synthesis/maturation is impaired.

Summary and conclusions. The results discussed above suggest that Pol I plays an important role in both DNA replication (Fig. 1) and ssDNA gap repair in the *dnaN159* strain (Fig. 2). In addition, our findings discussed above (Fig. 4), taken together with those discussed previously (29, 38, 39), suggest that the SOS-regulated Pol II (*polB*), Pol IV (*dinB*), and Pol V (*umuDC*) are able to compete with Pol I and Pol III for access to ssDNA gaps in the *dnaN159* strain, particularly on the lagging strand (Table 1). This apparent competition is presumably attributable in large part to impaired interactions of β159 with Pol I (Fig. 3) and Pol III (38). Finally, the observation that the β clamp confers processivity upon Pol I in vitro, and that β159 is impaired for this activity (Fig. 3), suggests that the β clamp might act to manage the actions of Pol I in vivo, as has been suggested previously for the lagging strand (24). Further work utilizing mutant forms of Pol I deficient for interaction with the β clamp will help to establish whether Pol I must interact with the β clamp in order to catalyze ssDNA gap repair and/or Okazaki fragment maturation in vivo.

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