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Proofreading-defective DNA polymerase II increases adaptive mutation in *Escherichia coli*

(*polB* gene/3′ exonuclease/mismatch repair/DNA polymerase III/antimutator)

PATRICIA L. FOSTER*, GUDMUNDUR GUDMUNDSSON†, JEFFREY M. TRIMARCHI*, HONG CAI†, AND MYRON F. GOODMAN†‡

*Department of Environmental Health, Boston University School of Public Health, Boston, MA 02118-2394; and †Department of Biological Sciences, Hedco Molecular Biology Laboratories, University of Southern California, Los Angeles, CA 90089-1340

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ABSTRACT **The role of *Escherichia coli* DNA polymerase (Pol) II in producing or avoiding mutations was investigated by replacing the chromosomal Pol II gene (*polB*$^+$) by a gene encoding an exonuclease-deficient mutant Pol II (*polBex1*). The *polBex1* allele increased adaptive mutations on an episome in nondividing cells under lactose selection. The presence of a Pol III antimutator allele (*dnaE915*) reduced adaptive mutations in both *polB*$^+$ cells and cells deleted for *polB* (*polBΔ1*) to below the wild-type level, suggesting that both Pol II and Pol III are synthesizing episomal DNA in nondividing cells but that in wild-type cells Pol III generates the adaptive mutations. The adaptive mutations were mainly −1 frameshifts occurring in short homopolymeric runs and were similar in wild-type, *polBΔ1*, and *polBex1* strains. Mutations produced by both Pol III and Pol II ex1 were corrected by the *mutHLS* mismatch repair system.**

There are three DNA polymerases (Pols) present in *Escherichia coli*. Pol I and Pol III have well-defined functions *in vivo* (1). Pol III is required for replication of the genome (2) and may also be required for gap filling during postreplication mismatch repair (3). Evidence also suggests that Pol III must be active for SOS-induced mutagenesis to occur (4, 5). Pol I is responsible for excising the RNA primers used to initiate Okazaki fragments and also plays an important role in the repair of damaged DNA (1). Although Pol II is known to be induced in response to DNA damage as part of the SOS regulon in *E. coli* (6, 7), its cellular roles are not well established. An early study suggested that Pol II was involved in repair of UV-damaged DNA (8), and a recent study concluded that Pol II was required to synthesize past abasic lesions in the absence of heat shock proteins (9). Recently, we found that cells with a *polB* deletion had a mutator phenotype for adaptive mutation of a frameshift allele carried on an episome, implying a role for Pol II in nondividing cells (10). We have used a proofreading-deficient Pol II mutant to further investigate the roles of Pol II and Pol III in generating these episomal mutations.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media. NR11555 (*ara, leu*::Tn*10*, Δ*prolac*), NR9915 (*dnaE915 zae502*::Tn*10*, *zae*::Tn*10*dCam), and NR9918 (as NR9915 but *dnaE*$^+$) (11) were provided by R. M. Schaaper, National Institute of Environmental Health Sciences. The wild-type HfrH strain, RM1094, was from M. Susskind, University of Southern California. HC101 is RM1094 carrying *polBex1*, encoding a 3′-exonuclease-deficient mutant Pol II. HC203 is NR11555 but *leu*$^+$ *ara*$^+$ and carries *polBex1*. The revertible F′ *lacI33*::*lacZ* strain, FC40, its F$^-$ parent, FC36, the scavenger F′ Δ*laclZ* strain, FC29, and the *polBΔ1* derivative of FC40, FCB60, have been described (10, 12, 13). A *polBex1* derivative of FC40 was made from HC203 by transduction to arabinose utilization. The *dnaE915* or control *dnaE*$^+$ strains were made from NR9915 or NR9918 by transducing to tetracycline resistance and then screening for chloramphenicol resistance. In all cases the F′ *lacI33*::*lacZ* was mated into the strain last, and then the phenotypes of several independent isolates were tested. No differences were found among isolates, and the results of only one isolate of each genotype are presented. Plasmid pHC700 is pPROK-1 carrying the *polBex1* allele (see below), pMQ315 is pBR322 carrying an active *mutS*$^+$ gene, and pMQ339 is pACYC184 carrying an active *mutL*$^+$ gene (14). Control strains carried the vectors. M9 minimal media have been described (12, 15, 16). Antibiotic concentrations in minimum media were as follows: kanamycin, 30 μg/ml; spectinomycin, 70 μg/ml; carbenicillin, 50 μg/ml; chloramphenicol, 17 μg/ml; tetracycline, 10 μg/ml; twice as much carbenicillin, chloramphenicol, and tetracycline were added to rich medium. Genetic and molecular procedures were standard (15, 17).

Construction of a 3′ → 5′ Exonuclease-Deficient Mutant Pol II. The *polBex1* mutant used in this study was constructed by allele replacement (18). Two point mutations were introduced into the exonuclease domain of a wild-type *polB* (D155A and E157A) by site-directed mutagenesis (19). A silent point mutation was also introduced 8 bp upstream of the D155A mutation, creating an *Afl* II restriction site. *polBex1* was cloned onto the suicide vector pBIP3 (18), generating pHC205, which was used to transform *E. coli* JM109 and then crossed onto the chromosome in RM1094 as described (18). A PCR product containing the relevant region was amplified from several sucrose-resistant Km^r colonies and then digested with *Afl* II endonuclease. Chromosomal DNA digested with *Afl* II was sequenced to confirm the presence of the *polBex1* replacement in HC101. P1 transduction with selection for the Leu$^+$ Ara$^+$ phenotype was used to move *polBex1* from HC101 into NR11555, creating HC203.

Adaptive-Mutation Experiments. Reversion rates of *lacI33*::*lacZ* strains to Lac$^+$ were determined in fluctuation tests (12, 13, 16). For every experiment, 5–50 independent cultures of the relevant strain were grown to saturation in M9 glycerol medium, and the cells were mixed with 10^9 scavenger cells and plated on M9 lactose medium. The number of revertible cells plated was controlled by the amount of glycerol in the medium (0.1% or 0.01%) and the volume of the culture (1 or 0.1 ml). The number of revertible cells plated ranged from 10^7 to 10^9 and was chosen to give 0–5 Lac$^+$ colonies per plate on day 2 and 5–20 new Lac$^+$ colonies per plate each day during the rest of the experiment. Scavengers, used to prevent cell growth on the lactose plates, were FC29 cells or FC29 cells carrying

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Abbreviation: Pol, DNA polymerase.

‡To whom reprint requests should be addressed.

control plasmids, with the following exceptions. Both the *dnaE*$^+$ control and *dnaE915* derivatives constructed from NR9915 and NR9918, respectively, and strains carrying both pMQ315 and pMQ339 grew poorly. To prevent these cells from being outcompeted by healthier cells, scavengers were used that had exactly the same genetic makeup as the revertible strains except for their *lac* allele. The DNA polymerase mutants used here had no apparent effect on the ability of the strains to engage in F′ conjugation (unpublished data). Preplating generation-dependent mutation rates were calculated from the number of Lac$^+$ colonies appearing 2 days after plating on lactose plates, either by the maximum-likelihood method or by setting the proportion of cultures with no mutants equal to e^{-m} (20); in both cases m, the number of mutants per culture, was then divided by twice the final number of cells to give mutations per cell per generation. Postplating time-dependent mutation rates were calculated as the mean number of Lac$^+$ colonies appearing per day during days 3–5 divided by the number of cells plated. The number of viable cells on the lactose plates was determined by taking plugs off the plates and titering the cells, as described (16); there was no cell growth or loss of viability in any genetic background during the course of the experiments.

Mutational Spectra. For each genetic background, 20–50 independent cultures were plated, and one Lac$^+$ mutant was isolated each day from each plate. PCR and sequencing methods were as described (21). Mutations were identified by colony hybridization (22) with oligonucleotides complementary to the reverted and the unreverted sequence of the major mutational hotspot (5′-GTG-AGA-CGG-GCA-ACA-GCT-3′ and 5′-GTG-AGA-CGG-GGCA-ACA-GC-3′). A few of the Lac$^+$ mutants that hybridized to the reverted probe were sequenced to confirm the specificity of the oligonucleotides. Many of the Lac$^+$ mutants that hybridized to the unreverted probe were further analyzed by PCR and sequencing; most proved to carry simple frameshifts at sites other than the hotspot. A variable number of mutants hybridized to neither probe, and, when further analyzed, most of these proved to have size changes detectable by PCR. A few mutants hybridized to both probes, and most of these proved to be unstable when grown nonselectively, as would be expected if the Lac allele were amplified (23).

RESULTS

Chromosomal Replacement of Wild-Type *polB* by *polBex1*, Coding for a Proofreading-Deficient Pol II. An exonuclease-deficient *polB* mutant, *polBex1* (D155A/E157A) was constructed by analogy to an exonuclease-deficient bacteriophage T4 Pol (D112A/E114A) (24). Pol II and T4 Pol have been classified as group B "α-like" polymerases (25) sharing five conserved domains (6). Both enzymes contain highly active proofreading exonuclease activities (26) and T4 Pol mutants that have decreased nuclease/polymerase ratios *in vitro* have been shown to possess mutator phenotypes *in vivo* (27). The D155A/E157A-substituted Pol II has a 10^3-fold reduction in exonuclease activity (26). A phagemid-based method for generating precise allele replacements in *E. coli* (18) was used to replace the wild-type *polB* gene by *polBex1*. All the normal control elements of Pol II remain, such as the LexA box, and the overall size of the replacement matches that of the parent. *polBex1* contains the two mutations described above and a silent mutation that created a restriction site for *Afl* II endonuclease, used in screening for the presence of the mutant gene. Fig. 1 shows an *Afl* II digestion used to distinguish between the *polBex1* mutant (HC101) and its parent strain (RM1094). Restriction patterns for the two are different because of the presence of an *Afl* II site in the mutant (lanes 4 and 5) but not in the parent (lanes 2 and 3). The DNA was sequenced to confirm the presence of the three mutations in *polBex1* and to show that no errors were introduced during PCR. Lanes 6 and 7 in Fig. 1 show the restriction pattern of pHC205, which carries the *polBex1* allele. The mutant Pol II reacted in Western blots with antibody prepared against purified wild-type Pol II (10) and was induced 7-fold by nalidixic acid (data not shown), which was similar to SOS induction for the wild-type enzyme (28).

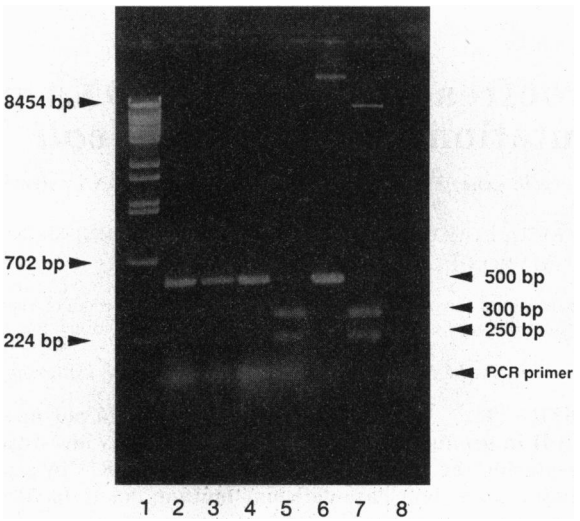

FIG. 1. Amplification and *Afl* II digestion of a 500-bp fragment carrying the *polBex1* mutation. When the mutated sequence is present, *Afl* II digestion produces two bands of 300 and 250 bp. Lanes: 1, *Bst*EII-digested λ DNA; 2, *polB*$^+$ strain RM1094; 3, *Afl* II-digested RM1094; 4, *polBex1* strain HC101; 5, *Afl* II-digested HC101; 6, pHC205, containing *polBex1*; 7, *Afl* II-digested pHC205; 8, control sample (no DNA added).

The Exonuclease-Defective Pol II Is a Mutator for Adaptive Mutation of an Episomal Allele in Nondividing Cells. Deletion of Pol II increased the spontaneous reversion rate of an episomal frameshift allele, *lacI33*::*lacZ*, when cells were under prolonged selection for lactose utilization (adaptive mutations) (10). This result suggested that Pol II was active in nondividing cells but that another, less accurate Pol could substitute for it. In support of this hypothesis, *polBex1* present on a multicopy plasmid (pHC700) conferred a mutator phe-

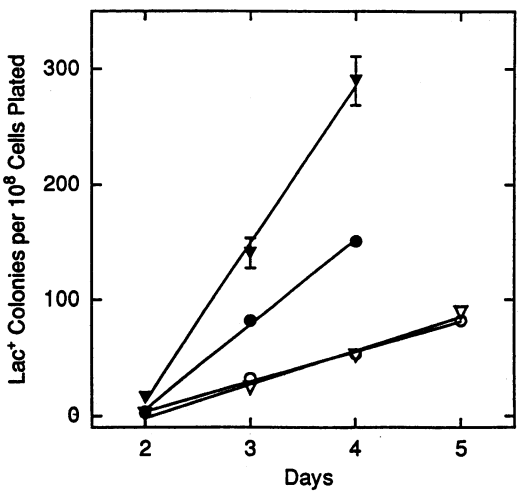

FIG. 2. Reversion of the *lacI33*::*lacZ* allele in various backgrounds. ○, Wild-type (*polB*$^+$) carrying the control plasmid (pPROK-1); ●, *polBΔ1* carrying pPROK-1; ▽, wild-type (*polB*$^+$) carrying *polBex1* on a plasmid (pHC700); ▼, *polBΔ1* carrying pHC700. Each point is the mean of 10 independent cultures, except for *polBΔ1* carrying pPROK-1, for which 20 cultures were used. Error bars show the standard error of the mean (bars are smaller than the symbols in many cases).

Table 1. Preplating and adaptive reversion rates of *lacI33*::*lacZ* in different genetic backgrounds relative to wild type

Genotype				Ratio of reversion rates*	
Chromosomal					
Pol II	Pol III	Plasmid	*n*	Preplating†	Adaptive‡
polBΔ1	+	Vector	20	0.9	3.0 ± 0.8
+	+	*polBex1*	10	0.5	1.1 ± 0.2
polBΔ1	+	*polBex1*	10	5.4	6.1 ± 1.0
polBex1	+	−	50	3.5	4.3 ± 0.9
+	+	*mutS*$^+$ *mutL*$^+$	5	0.3	0.2 ± 0.1
polBΔ1	+	*mutS*$^+$ *mutL*$^+$	5	0.1	0.2 ± 0.04
polBex1	+	*mutS*$^+$ *mutL*$^+$	10	0.6	0.5 ± 0.1
+	*dnaE915*	−	5	0.6	0.3 ± 0.1
polBΔ1	+	−	5	1.6	2.7 ± 0.3
polBΔ1	*dnaE915*	−	5	0.4	0.2 ± 0.01
polBex1	+	−	10	3.4	2.8 ± 0.3
polBex1	*dnaE915*	−	10	5.5	2.8 ± 0.2

*The values for each mutant strain were divided by the values for the isogenic wild-type strain, where *n* is the number of independent cultures in the experiment.

†The ratio of Lac$^+$ mutations per cell per generation, calculated from the numbers of Lac$^+$ colonies appearing 2 days after plating (see *Materials and Methods*). When 10^8 wild-type cells were plated, most plates had 1–3 Lac$^+$ colonies on day 2. The mean preplating mutation rate of the wild-type strain (± the standard error of the mean) was 0.41 (± 0.10) × 10^{-8} mutation per cell per generation.

‡The ratio of the number of Lac$^+$ colonies appearing per cell per day, 3–5 days after plating. The means of the ratios for days 3–5 are shown with the standard errors of the mean. When 10^8 wild-type cells were plated, 10–20 Lac$^+$ colonies appeared per plate each day, except for experiments with *dnaE915*, where the numbers were lower (see *Materials and Methods*). Excluding the *dnaE*$^+$ control, the mean adaptive mutation rate of the wild-type strain (± the standard error of the mean) was 16.5 (± 3.2) × 10^{-8} mutation per cell per day.

notype upon *polBΔ1* cells (Fig. 2). The postplating mutation rate to Lac$^+$ was increased 4- to 6-fold when Pol II ex1 was the only Pol II present, whether Pol II ex1 was expressed from a plasmid or from the chromosome (Fig. 2; Table 1). Thus, *polBex1* produces episomal adaptive mutations in nondividing cells and outcompetes the Pol that can substitute for Pol II. But, even when in excess, Pol II ex1 cannot outcompete wild-type Pol II (Fig. 2).

Adaptive Mutations on the Episome Appear to Be Caused by Pol III. The polymerase subunit of Pol III (α subunit) is encoded by *dnaE* (2). In cells with an antimutator allele of *dnaE, dnaE915* (11), the adaptive mutation rate of *lacI33*::*lacZ* was about one-third that of wild-type cells (Table 1). The adaptive mutation rate in *polBΔ1 dnaE915* cells was as low as that of *polB*$^+$ *dnaE915* cells (Table 1)—i.e., virtually all the excess mutations that appeared when *polB* was deleted were eliminated by the *dnaE915* allele. This suggests that Pol III is normally responsible for 70% of the episomal adaptive mutations. The residual 30% of the mutants may result from errors made by another polymerase or from mutations produced by another mechanism. However, the excess adaptive mutations produced when *polBex1* was present were not affected by the *dnaE915* allele (Table 1).

Pol III and the Exonuclease-Defective Pol II Produce the Same Mutations. We determined the spectra of growth-dependent and adaptive revertants of *lacI33*::*lacZ* in cells that were *polB*$^+$, *polBΔ1*, or *polBΔ1* with *polBex1* on a plasmid or in which *polBex1* replaced the chromosomal *polB* allele. Results from PCR, sequencing, and oligonucleotide probing were combined with the mutations already identified in the wild-type background (21). Adaptive revertants in wild-type cells were mainly −1-bp frameshifts at iterated bases, dominated by a strong hotspot at a run of four cytosines beginning at bp 1036 (21, 29) (Table 2). This was also the pattern of mutations found in *polBΔ1* cells (Table 2). The mutations in *polBΔ1* cells carrying the plasmid-encoded *polBex1* allele were even more specific—90% of the mutations were simple −1-bp frameshifts, and 70% of these were at the major hotspot (Table 2). Cells with the *polBex1* allele on the chromosome gave similar results (Table 3). There were no obvious differences among the strains in the distributions of mutations among the various sites (data not shown). Thus, both Pol III and Pol II can make the −1 frameshifts that dominate the spectrum, but in the case of Pol II, these are normally almost entirely eliminated by exonuclease editing.

polBex1 also changed the spectrum of mutations appearing on day 2, which mainly occur during nonselective growth prior to plating on lactose plates (12). In wild-type and *polBΔ1* cells, about 50% of these are gains or losses of bases, most detectable as size changes by PCR (21) (Table 2). In contrast, when Pol II ex1 was the only Pol II present, most of the early-arising mutants were the same 1-bp deletions as the late-arising mutants (Tables 2 and 3). The proportion of early-arising mutations that were −C at 1036 was significantly greater in the *polBex1* strains than in wild type (see Table 3; $\chi^2 = 32$ for the plasmid-borne *polBex1* and $\chi^2 = 12$ for the chromosomal *polBex1*; $P < 0.01$ in both cases). *polBex1* appeared to also increase the rate at which revertants of *lacI33*::*lacZ* arose during nonselective growth (Table 1). However, it is possible that in the *polBex1* background, adaptive mutations produced in the first few hours after plating contribute to the numbers of Lac$^+$ colonies appearing on day 2.

Table 2. Mutational spectra of *lacI33*::*lacZ* revertants determined by PCR, sequencing, and oligonucleotide probing

	No. of mutants (% total)					
	Wild type		*polBΔ1*		*polBΔ1* with *polBex1* plasmid	
Mutation	Day 2	Days 3–5	Day 2	Days 3–5	Day 2	Days 3–5
Size changes*	16 (36)	21 (13)	17 (65)	10 (11)	3 (10)	3 (10)
Add bases	4 (9)	3 (2)	4 (15)	5 (6)	1 (3)	0 (0)
Lose bases	12 (27)	18 (11)	13 (50)	5 (6)	2 (7)	3 (10)
Frameshift†	18 (41)	121 (75)	9 (35)	79 (88)	26 (90)	25 (83)
−C at 1036	9 (20)	83 (51)	2 (8)	44 (49)	20 (69)	18 (60)
Not −C at 1036‡	6 (14)	12 (7)	0 (0)	0 (0)	0 (0)	0 (0)
Others§	4 (9)	8 (5)	0 (0)	1 (1)	0 (0)	2 (7)
Total	44	162	26	90	29	30

*Loss of >1 bp or gain of >2 bp determined by PCR or sequencing.

†Loss of 1 bp or gain of 2 bp, determined by sequencing or oligonucleotide probing.

‡Mutants that hybridized to the probe with the unreverted sequence at 1036, which were not a size change detectable by PCR and which were not further identified.

§Mutants that hybridized to neither or both probes (see *Materials and Methods*) and were not further identified, plus extragenic revertants.

Table 3. Mutational spectra of *lacI33*::*lacZ* revertants determined by oligonucleotide probing only

	No. of mutants (% total)							
	Wild type		*polBΔ1*		*polBΔ1* with *polBex1* plasmid		Chromosomal *polBex1*	
Mutation	Day 2	Days 3–5	Day 2	Days 3–5	Day 2	Days 3–5	Day 2	Days 3–5
−C at 1036	5 (14)	52 (35)	12 (41)	78 (52)	31 (82)	83 (71)	11 (65)	97 (65)
Hybridized to probe with unreverted sequence at 1036*	18 (49)	65 (44)	12 (41)	63 (42)	4 (11)	27 (23)	6 (53)	31 (21)
Hybridized to neither probe†	14 (38)	25 (17)	5 (17)	5 (3)	3 (8)	6 (5)	0 (0)	9 (6)
Hybridized to both probes†	0 (0)	5 (3)	0 (0)	4 (3)	0 (0)	1 (1)	0 (0)	13 (9)
Total	37	147	29	150	38	117	17	150

*This category includes size changes as well as frameshifts at other sites.
†See *Materials and Methods.*

Mutations Produced by Both Pol III and Pol II ex1 Are Corrected by Mismatch Repair. It might appear surprising that Pol III and Pol II ex1 produce the same mutations in the static cells. However, −1 frameshifts are frequent errors made by polymerases (30), and the *lacI33*::*lacZ* mutational target is not extensive, consisting of 120 bp within which a net −1 frameshift must occur (21). The mutations recovered are those that escape correction by the methyl-directed mismatch repair system, and it has been hypothesized that this repair pathway is deficient in nutritionally deprived cells (21, 29, 31, 32). If so, the polymerase errors that are normally most readily corrected by the mismatch repair system would dominate the late-arising mutations. In support of this hypothesis, the simultaneous overproduction of two enzymes of the methyl-directed mismatch repair system, MutS and MutL, by supplying them on separate but compatible plasmids, nearly eliminated late-arising mutants in wild-type, *polBΔ1*, and *polBex1* cells (Table 1).

DISCUSSION

Following the discovery of Pol II in 1970 (33), efforts to define its biological function have been hampered by the difficulty in finding phenotypes associated with *polB* mutants. In a recent study using a strain of *E. coli* carrying a Pol II deletion (*polBΔ1*), we observed a 3-fold increase in adaptive reversion of an episomal frameshift mutation, *lacI33*::*lacZ* (10). By adaptive mutation, we mean mutations arising in a static population of cells subjected to a nonlethal selection—e.g., the accumulation of Lac$^+$ revertants when lactose is the only energy source (12, 34, 35). In this study, we have investigated the effect of proofreading exonuclease-deficient *polB* mutant (*polBex1*) on these mutations.

When Pol II ex1 was the only Pol II enzyme in the cell, the rate at which the episomal adaptive mutants appeared was enhanced 4- to 6-fold (Fig. 2; Table 1), strongly suggesting that the exonuclease-defective Pol II synthesizes DNA in these nondividing cells. Yet when wild-type Pol II was present in the cell, the exonuclease-defective Pol II had no effect (Fig. 2). While it is formally possible that loss of its exonuclease activity permits Pol II to assume a role that it normally does not play, it is more likely that wild-type Pol II is actively synthesizing the episomal DNA in static cells but makes few mistakes because of its efficient exonuclease (26).

Both Pol III and the exonuclease-defective Pol II make the −1 frameshift mutations that account for most of the adaptive reversion of the *lacI33*::*lacZ* allele (Tables 2 and 3). These errors are subject to correction by the methyl-directed mismatch repair system (Table 1). While this result supports the hypothesis that mismatch repair is defective in starving cells (21, 29, 31, 32), it does not prove it. Supplying excess MutS and MutL proteins may simply improve the efficiency of error correction. Indeed, excess MutS and MutL also decreased the rate at which Lac$^+$ mutations appeared during nonselective growth (Table 1).

The *dnaE915* allele decreased adaptive reversion of *lacI33*::*lacZ* in both *polB*$^+$ and *poBΔ1* cells, but not in *polBex1* cells. These results suggest that Pol III is active in nondividing cells, and in wild-type cells Pol III, not Pol II, is responsible for most of the episomal mutations that occur. Pol II and Pol III have been shown to share polymerase accessory subunits, β and γ complex, *in vitro* (36), and it is probable that the two polymerases have overlapping functions during replication, repair, or both. Mutational spectra generated *in vitro* by a *lacZ* α-complementation assay (37) showed that wild-type Pol II was highly accurate whereas the proofreading-deficient Pol II ex1 catalyzed a significant number of −1 frameshifts in repetitive sequences (26), reminiscent of types of errors reported in Tables 2 and 3.

The results presented here show that Pol II is involved in episomal DNA synthesis in static cells. As adaptive reversion of *lacI33*::*lacZ* requires both conjugal and recombinational functions (12, 35, 38–41), this synthesis may be initiated at the conjugal origin (38–40), or it may be initiated by DNA repair or recombination events (21, 29). We have not, as yet, demonstrated that the *polBex1* influences chromosomal mutations.

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