Competition of *Escherichia coli* DNA Polymerases I, II and III with DNA Pol IV in Stressed Cells

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**Abstract**

*Escherichia coli* has five DNA polymerases, one of which, the low-fidelity Pol IV or DinB, is required for stress-induced mutagenesis in the well-studied Lac frameshift-reversion assay. Although normally present at ~200 molecules per cell, Pol IV is recruited to acts of DNA double-strand-break repair, and causes mutagenesis, only when at least two cellular stress responses are activated: the SOS DNA-damage response, which upregulates DinB ~10-fold, and the RpoS-controlled general-stress response, which upregulates Pol IV about 2-fold. DNA Pol III was also implicated but its role in mutagenesis was unclear. We sought *in vivo* evidence on the presence and interactions of multiple DNA polymerases during stress-induced mutagenesis. Using multiply mutant strains, we provide evidence of competition of DNA Pols I, II and III with Pol IV, implying that they are all present at sites of stress-induced mutagenesis. Previous data indicate that Pol V is also present. We show that the interactions of Pols I, II and III with Pol IV result neither from, first, induction of the SOS response when particular DNA polymerases are removed, nor second, from proofreading of DNA Pol IV errors by the editing functions of Pol I or Pol III. Third, we provide evidence that Pol III itself does not assist with but rather inhibits Pol IV-dependent mutagenesis. The data support the remaining hypothesis that during the acts of DNA double-strand-break (DSB) repair, shown previously to underlie stress-induced mutagenesis in the Lac system, there is competition of DNA polymerases I, II and III with DNA Pol IV for action at the primer terminus. Up-regulation of Pol IV, and possibly other stress-response-controlled factor(s), tilt the competition in favor of error-prone Pol IV at the expense of more accurate polymerases, thus producing stress-induced mutations. This mutagenesis assay reveals the DNA polymerases operating in DSB repair during stress and also provides a sensitive indicator for DNA polymerase competition and choice *in vivo*.

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**Introduction**

There are five replicative polymerases in *Escherichia coli* (reviewed [1]). The main replicative polymerase is Pol III. The catalytic subunit, designated Pol III*, is encoded by *dnaE*. Pol I, encoded by *polA*, plays roles in processing Okazaki fragments and also in gap-filling during excision-repair processes. The other three DNA polymerases are induced to higher levels of expression by the SOS DNA-damage response [2,3,4]. Two of them, Pol IV and Pol V (encoded by *dnaB* and *undC* respectively) are Y-family DNA polymerases [5]. These low-processivity error-prone polymerases play major roles in bypassing otherwise replication-blocking template lesions *via* trans lesion synthesis and also replicate undamaged DNA [6]. Y-family polymerases have large active sites, allowing higher frequencies of base misincorporation, and no proofreading-exonuclease subunits or domains to correct insertions of incorrect bases, both leading to their lower fidelity than “housekeeping” DNA polymerases [6]. When over-expressed, Pol IV gives a mutator phenotype, causing mutations predominantly in the lagging strand [3,7,8]. Pol V is present at significant levels only in SOS-induced cells, and its over-expression results in slowing of DNA synthesis [9].

Pol II is encoded by *polB*. It is an accurate DNA polymerase because, like Pol I, it has an editing or proofreading 3’-exonuclease domain that can erode a mispaired primer end and so remove incorrectly inserted bases. The role of Pol II is not well defined. It has been shown to play roles in DNA replication predominantly in the lagging strand, where it might edit errors made by Pol III [10,11]. It participates in some repair processes [12] (and reviewed by [11]) and in replication restart after DNA damage [13].
Pol III has its editing function provided by the dnaQ-encoded epsilon subunit of the Pol III holoenzyme. DNA polymerases are loaded onto DNA by a sliding clamp, the β-clamp, a homodimer that encircles the DNA molecule and serves as a processivity factor for DNA polymerases [reviewed by [14]]. The β-clamp is the structural homologue of eukaryotic PCNA, which plays a similar role in managing DNA polymerase traffic.

It is of considerable interest to know the mechanism by which the active polymerase is chosen at any time or place during DNA synthesis. The β-clamp plays a major role in this decision, as shown by the isolation of a strain carrying a mutation in the dnaN gene, encoding the β-clamp, that exhibits altered preferences for DNA polymerases [15,16]. All five DNA polymerases bind to the β-clamp, and more than one may be present on the clamp at the same time [17]. This has given rise to the concept of a toolbelt, namely that more than one polymerase is attached to the β-clamp but with only one working at a time [17,18,19]. Because the β-clamp is a dimer, there are two polymerase binding pockets present on a single clamp. However, there is evidence that only one polymerase is bound to a pocket at one time, and that this is the active polymerase [20]. DNA polymerases also bind to the β-clamp at sites on the clamp rim, and it is the interplay between attachment at these two sites that is believed to control polymerase choice [18,20].

The availability of DNA polymerases also has a dramatic effect on DNA-polymerase choice, as shown by experiments in which over-expression of Pol IV stops DNA replication by displacing Pol III* [21,22]. This is suggested to represent a Pol IV- and (SOS-) mediated checkpoint [21], as was proposed earlier for Pol V [23].

Similarly, the proofreading activity of Pol III prevents lesion bypass by translesion DNA polymerases if the translesion polymerase is not sufficiently processive [12]. This is explained as being a reaction of the proofreading activity to the persistent presence of a lesion, which will cause Pol III to remove the strand opposite the lesion whenever Pol III* attempts to extend from a primer end that is too close to the lesion [24].

The interplay of high-fidelity and low-fidelity DNA polymerases is expected to have a profound impact on mutation rates and spectra, and conversely changes in mutagenesis can provide clues about the nature of the competition between DNA polymerases. The ability of a cell to modulate the activity of low-fidelity translesion polymerases is critical to the maintenance of genomic integrity [25], but also functions to increase the mutability of stressed cells [26]. Spontaneous generation-dependent base-substitution mutagenesis has been studied in strains deficient in different polymerases in cells that lack mismatch repair so that mismatch repair does not change the spectrum of mutations observed [27]. The surprising finding was that all DNA polymerases were involved at different sites and for different basepair substitutions. For example, Pols I, III, IV or V were required for base substitutions at specific hotspots.

In this study, we looked at the roles of different DNA polymerases in stress-induced frameshift mutagenesis in the E. coli Lac assay [28]. Mutagenesis in this assay occurs in starving cells by −1 basepair (bp) deletions that compensate for a +1 bp frameshift mutation in a lacI fusion gene. Unlike normal DNA replication and spontaneous mutagenesis in rapidly growing cells, this mutagenesis strongly requires RpoS, the starvation- and general-stress-response regulator, and is stress-induced [29,30]. The mutagenesis also requires single-strand DNA nicking or double-strand breakage of DNA in the general vicinity of the lac genes [31], the proteins of double-strand-break (DSB) repair [32,33,34] and DNA polymerase IV [35,36]. Stress-induced Lac reversion also requires the SOS response [37], which is required only for producing SOS-induced levels of Pol IV [38]. The mutagenesis in this assay is an important model for mutagenesis that produces antibiotic resistant E. coli [39,40], bile-resistant Salmonella [41] and several other stress-induced mutagenesis processes [26]. The working model for this mechanism of mutagenesis is that Pol IV errors occur during replication restart at sites of homologous recombinational DSB repair [26,31]. See [26,42] for discussion of alternatives.

In this assay, in which most frameshift reversion is clearly Pol IV-dependent, we have studied the effects of altering potential DNA polymerase competition among non-replicative as well as replicative DNA polymerases. Previous work showed that Pol V is not needed for mutagenesis in this assay [28,37] in which frameshift reversion is measured, though it is required in a related assay for DSB-repair-protein, RpoS- and DinB-dependent mutagenesis in which base substitutions dominate [40], probably reflecting the error profiles of Pols IV and V. For Lac frameshift reversion, Pol I- [43] and Pol II- [44] defective strains both show increased mutagenesis, suggesting that these DNA Pols might compete with low-fidelity Pol IV. The involvement of Pol III* is unknown except that an antimutator allele, dnaE915, reduces stress-induced mutagenesis [35,36,44,45], which could be caused by competition of the high-fidelity DnaE915-Pol III protein with low-fidelity Pol IV. Alternatively, it could be that (1) DNA Pol III is required for making Pol IV-dependent mutations; (2) the DnaE915-Pol III corrects [proofreads] Pol IV-generated errors; or (3) the DnaE915-Pol III* mutant protein diminishes SOS induction relative to wild-type cells, as reported [46].

We wished to determine the role of Pol III in stress-induced mutagenesis. We also wished to distinguish whether it and the other DNA polymerases compete directly with DNA Pol IV during stress-induced lac frameshift reversion, or cause their mutant phenotypes via other indirect means. At least two other explanations are possible. First, mutations affecting other DNA polymerases might alter induction of the SOS DNA-damage response, and so indirectly affect expression of dnaB, which is required at SOS-induced levels for mutagenesis [38], thereby affecting Pol IV-dependent mutagenesis. Second, another polymerase might proofread and correct replication errors made by Pol IV. Here, we provide genetic evidence against these indirect models, and support a model in which DNA polymerases I, II, and III compete with Pol IV at sites of DNA synthesis during stress-induced mutagenesis.

Results and Discussion

Pol II Interferes with Pol IV-Dependent Mutagenesis by an SOS-Independent Mechanism

Strains deficient for DNA Polymerase II (Pol II, encoded by polB) display an increase in stress-induced Lac− mutagenesis compared with Pol+ strains [44,47,48]. Therefore, the presence of Pol II inhibits stress-induced frameshift reversion. Pol II could inhibit mutagenesis by competition with the error-prone Pol IV at the site of DNA synthesis on an undamaged template. In a Pol II− strain, Pol IV might gain better access to the DNA, resulting in a higher frequency of Pol IV-generated frameshift mutations than in wild-type cells. Alternatively, cells lacking Pol II might be sufficiently impaired that they induce a LexA-regulated SOS response, resulting in up-regulation of Pol IV expression, thereby indirectly increasing Pol IV-mediated mutagenesis. We tested this hypothesis using a strain with constitutive SOS induction due to a defective (null) lexA allele, lexA51(Def), and found that the polBΔ/lexA51(Def) strain retains a hypermutagenesis phenotype equivalent to the polBΔ/strain

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Figure 1A. The difference in mutation rate (per day) between polBΔ lexA51(Def) and its isogenic PolB+ control is 6.3±1.1-fold. The same fold increase is observed for polBΔ1 compared with PolB+ in the Lex⁺ background (5.6±0.5-fold) (Table 1, detailed in Table S1). That is, the absence of Pol II still increases mutagenesis even when there is no LexA/SOS repressor and the SOS genes are maximally induced. Therefore, the effect of polB-defect does not promote mutagenesis by indirectly promoting cleavage of LexA and induction of the SOS genes. Whereas in principle, it might have been possible that even in the lexA51(Def) background, the absence of Pol II might somehow cause Pol IV levels to increase by an as-yet-unknown, SOS-independent mechanism, Western analyses show that this is not the case (Figure 2, compare lexAΔ lexBΔ with lexAΔ). Rather, these data support a direct competition model in which the absence of Pol II permits greater access of Pol IV to the template for DNA synthesis, promoting frameshift mutagenesis.

In support of this conclusion, we found that the increased stress-induced mutagenesis observed in the ΔpolB strain remains Pol IV-dependent (Figure 1B; also see [36]). The dinB10 allele, encoding catalytically inactive Pol IV, reduced mutagenesis in polBΔ cells to the level of the dinB10 strain (Figure 1B; Table 2). These data support the hypothesis that Pol II competes with Pol IV during stress-induced mutagenesis.

We note that here, as previously, when Pol IV is produced at levels equivalent to a fully derepressed SOS response, either in cells carrying the lexA51(Def) mutation [37,49] or in cells with a dinB-operator-constitutive mutation which produces Pol IV to levels equivalent to those in lexA51(Def) cells [38], we observed normal, not higher-than-wild-type, levels of stress-induced mutagenesis. As concluded previously [38], this implies that cells undergoing stress-induced mutation are either fully SOS induced or are induced to levels at which some component other than Pol IV becomes rate-limiting for mutagenesis.
Pol I Interferes with Pol IV-Dependent Mutagenesis by an SOS-Independent Mechanism

Strains deficient for DNA polymerase I (polA) display elevated levels of stress-induced point mutagenesis with respect to PolI strains ([43] and Figure 1C, in which a temperature-sensitive polA allele was used at semi-permissive temperature), and this enhanced mutagenesis is completely Pol IV-dependent [43]. One explanation for this effect could be that Pol I, a high-fidelity DNA polymerase, could compete with Pol IV reducing its opportunities for mutagenesis. Alternatively, cells lacking Pol I are SOS induced constitutively [50], and this might increase mutagenesis indirectly by up-regulation of LexA-controlled dinB, as proposed [36]. We were unable to test the comparison of a polI mutant to PolI in a lexA(Def) background (that would allow constitutive dinB induction) because we found that this combination is not viable in this strain background. We demonstrate that the effect of mutating Pol I is not caused by enhanced SOS induction by showing that cells unable to induce the SOS genes, due to a dinB background, still show increased mutagenesis when Pol I-deficient, using the polIATS allele at semi-permissive temperature (Figure 1C, Table 1). A null allele of polA renders a cell inviable in this strain background [48]. The increase in mutation rate in the polIATS strain over PolI is 2.8±0.3-fold per day (Table 1). The same comparison in the lexA(Ind) background, lexA(Ind) polIATS versus lexA(Ind), yields a similar result: 3.1±0.6-fold (Table 1). Therefore the effect of polIATS is independent of SOS induction. The unlikely possibility that the increased mutation caused by polIATS results from an as-yet-undescribed SOS-independent upregulation of Pol IV levels in polIATS cells is ruled by Western

| Relevant genotypes compared* | Mutant | Isogenic control | Experiment identification # | Mean difference in mutation rate from control ± S.E.M. | P-value  

Figure 2. Pol IV protein levels are not increased SOS-independently in cells carrying the ΔpolB, polIATS or dnaE915 mutations. Numbers represent densitometer readings of band intensity normalized to dinB*. Strains, from left to right: SMR562, SMR5889, SMR10308, SMR868, PH306, SMR5400, SMR8913, SMR7767. Two separate experiments gave similar results. doi:10.1371/journal.pone.0010862.g002
Table 2. *Escherichia coli* K-12 strains used in this study.

| Strain(s)       | Relevant genotype                                                                               | Reference or source |
|-----------------|-------------------------------------------------------------------------------------------------|---------------------|
| CM5407          | polA6 Tn10                                                                                        | [52]                |
| FC29            | Δ(lac-proAB)lux ara thi [F' ΔlacZ proAB*]                                                        | [28]                |
| FC36            | Δ(lac-proAB)lux ara thi Rif^                                                                      | [28]                |
| FC40            | FC36 [F' lacI33lacZ proAB*]                                                                       | [28]                |
| GW2100          | umuC122::Tn5                                                                                     | [66] CGSC*          |
| MG1655          | wild-type                                                                                        | [67]                |
| NR9779          | dnaE486 zae::Tn10Cam                                                                              | [68]                |
| NR9915          | dnaE915 zae::Tn10                                                                                 | [53]                |
| PJH305          | FC40 fadA83165::Tn10Kan lexA3(TS)                                                                   | SMR3490 x P1(SMR868) |
| PJH306          | FC40 polA1(TS) fadA83165::Tn10Kan lexA3(TS)                                                       | SMR3491 x P1(SMR868) |
| PJH354          | SMR4562 polA1 zih35::Tn10F'                                                                      | independent construct of PJH399 |
| PJH373          | SMR4562 dinB10 polA1 zih35::Tn10F' dinB10                                                       | [51]                |
| PJH399          | SMR4562 polA1 zih35::Tn10F'                                                                      | [51]                |
| PJH491          | SMR4562 polA1 zih35::Tn10 polB::Δ53-55                                                        | PJH354 x P1(SMR3661) |
| PJH510          | SMR4562 dinB10 polA1 zih35::Tn10 polB::Δ53-55                                                       | PJH373 x P1(SMR3661) |
| PJH601          | FC40 ΔumuDC::cat dinB10F' dinB10                                                                  | SMR3530 x P1 SMR3525 |
| RM3980          | MG1655 ΔmutQ903::tet spq-2                                                                       | [69]                |
| RW120           | ΔumuDC::cat                                                                                       | Roger Woodgate       |
| SH2101          | polA1::Δ53-55                                                                                    | [47]                |
| SMR540          | FC40 dnaE486 zae::Tn10Cam                                                                          | FC40 x P1(NR9779)   |
| SMR688          | FC40 lexA3(TS)                                                                                    | [37]                |
| SMR1547         | FC40 ΔmutQ903::tet spq-2 zae::Tn10Cam                                                               | FC40 x P1(SMR3640)  |
| SMR3490         | FC40 fadA83165::Tn10Kan                                                                           | [43]                |
| SMR3491         | FC40 polA1(TS) fadA83165::Tn10Kan                                                                  | [43]                |
| SMR3525         | FC40 ΔumuDC::cat                                                                                  | FC40 x RW120        |
| SMR3640         | MG1655 ΔmutQ903::tet spq-2 zae::Tn10Cam                                                              | RM3980 x P1(SMR3640) |
| SMR3661         | FC40 polA1::Δ53-55                                                                                 | FC40 x P1(SH2101)   |
| SMR4562         | independent construction of FC40                                                                   | [37]                |
| SMR5400         | SMR4562 lexA51(Def) sulA211 psib::cat                                                              | [37]                |
| SMR5830         | SMR4562 dinB10F' dinB10                                                                          | [35]                |
| SMR5889         | SMR4562 ΔdinB10::FRT [F' ΔdinB10::FRT]                                                              | [35]                |
| SMR6263         | MG1655 leu::Tn10                                                                                   | MG1655 x P1(ZK2146) |
| SMR7518         | SMR4562 umuC122::Tn5                                                                              | SMR4652 x P1(GW2100) |
| SMR7767         | SMR4560 dnaE915 zae::SO2::Tn10                                                                      | SMR4560 x P1(NR9915) |
| SMR7768         | SMR4560 zae::SO2::Tn10                                                                             | SMR4560 x P1(NR9915) |
| SMR8363         | SMR4562 zae::SO2::Tn10                                                                             | SMR4562 x P1(NR9915) |
| SMR8365         | SMR4562 dnaE915 zae::SO2::Tn10                                                                      | SMR4562 x P1(NR9915) |
| SMR8913         | SMR4562 polA1::Δ53-55                                                                             | SMR4562 x P1(SMR3661) |
| SMR8949         | SMR4562 dinB10 polA1::Δ53-55                                                                      | SMR5830 x P1(SMR3661) |
| SMR8950         | SMR4562 dinB10 leu::Tn10 ara5' [F' ΔdinB10]                                                        | SMR9949 x P1(SMR6263) |
| SMR8951         | SMR4562 dinB10F' dinB10F'                                                                          | SMR8950 x P1(4562)  |
| SMR9023         | SMR4562 polA4 Tn10                                                                                 | SMR4562 x P1(CMS407) |
| SMR9024         | SMR4562 polA* Tn10                                                                                 | SMR4562 x P1(CMS407) |
| SMR10308        | SMR4562 [F' loxU2-FRT::cat FRT dinB10]                                                              | [38]                |
| ZK2146          | leu::Tn10                                                                                         | S.E. Finkel         |

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analyses that show similar Pol IV levels in lexA Ind−) and lexA Ind− poltTS cells (Figure 2).

The Polymerase Domain of Pol I Inhibits Pol IV-Dependent Mutagenesis

Pol I inhibits Pol IV-dependent mutagenesis [43], but the Pol I enzymatic function responsible for inhibiting Pol IV-dependent mutagenesis was not established. Pol I has three enzymatic functions. In addition to a DNA polymerase, the protein also contains 5′-3′ (nick-translation) and 3′-5′ (proofreading) exonuclease activities. The 5′-3′ exonuclease activity is required for stress-induced gene amplification of lac, with no effect on point mutagenesis [51]. Mutation of both the polymerase and 3′-5′ exonuclease segments (polA1) results in a hypermutagenesis phenotype ([51], Figure 1D) as observed for poltTS (Figure 1C and [43]). One explanation for this phenotype could be that the 3′-5′ proofreading exonuclease is normally responsible for correcting errors generated by Pol IV, and in its absence, more Pol IV errors persist as mutations. We eliminated this possibility through use of the polA6 allele, which encodes a protein deficient in the polymerization function, while retaining exonuclease functions [52]. As shown in Figure 1D, polA6 cells retain the hypermutagenesis phenotype, supporting a model in which the polymerase portion of the protein inhibits Pol IV-independent mutagenesis. Both polA6 and polA1 cells have increased mutation rates with respect to their isogenic PolI strain (7.2±2.1-fold and 5.9±0.9-fold respectively, Table 1), and their rates do not differ from each other (Figure 1D; Table 1). We conclude that the 3′-5′ exonuclease of Pol I is not involved in correcting stress-induced errors generated by Pol IV, but that the polymerase domain inhibits Pol IV-dependent mutagenesis.

Pol I and Pol II Act Independently to Reduce Pol IV-Mediated Mutagenesis

Pol IV-dependent mutagenesis is greatly enhanced when either Pol I or Pol II are deficient. The mutation rate per day increases approximately 5-fold when either Pol I or Pol II is deficient (Table 1 and reviewed above). We report an additional approximately 5-fold increase in the polA1 polBΔ1 double mutant compared with each single mutant (Figure 1E, 1F; Table 1). This implies that both Pol I and Pol II exclude lower-fidelity Pol IV from the site of DNA synthesis, largely independently. When both are absent, Pol IV has the greatest access to the site of DNA synthesis. In support of this model, the hyper-mutagenesis observed in a polA1 polBΔ1 strain is completely dnaB-dependent. That is, there is no difference in mutation rate between the polA1 polBΔ1 dnaB10 and dnaB10 strains (Figure 1E, 1F; Table 1). This indicates that Pol IV is wholly responsible for the increased mutagenesis in the polA1 polBΔ1 cells. The data show that Pol I and Pol II reduce Pol IV-independent mutagenesis and that each does so independently of the other.

DNA Pol III Affects Pol IV-Dependent Mutagenesis Independently of SOS and Proofreading and Does Not Make the Mutations

Although Pol IV generates most of the stress-induced frameshift-reversion (“point”) mutations [35], surprisingly, a higher-fidelity “anti-mutator” mutant form of DNA polymerase Pol III, the major replicative DNA polymerase, also reduces stress-induced point mutagenesis strongly [35,36,44,45]. The dnaE915 antimitator Pol IIII protein [53] could have this effect via any of a few mechanisms. The possibility that dnaE915 resulted in increased availability of mismatch repair, and so less mutagenesis, was ruled out [45]. Other possibilities are that Pol III and Pol IV could work together to cause mutagenesis (perhaps one DNA polymerase causing the deletion and the other extending from the mispaired primer terminus [35]). Alternatively, reduced spontaneous SOS induction has been demonstrated in dnaE915 cells [46], and this might lower mutagenesis by reducing expression of dnaB encoding Pol IV. Yet another possibility is that the DnaE915-Pol III mutant protein might allow more efficient Pol III proofreading of Pol IV errors. Finally, the DnaE915-Pol IIII protein might exclude the more mutagenic Pol IV better than wild-type Pol IIII.

We investigated the possibility that dnaE915 reduces Pol IV-dependent mutagenesis by reducing SOS expression in stationary-phase, stressed cells. We examined the effect of dnaE915 in constitutively “SOS-on” lexA-defective strains. Constitutive derepression of the lex regulon did not alleviate the decreased mutagenesis phenotype of the dnaE915 allele relative to the lexA (Def) DnaE* control strain (Figure 1G, Table 1). The mutation rate in dnaE915 is ~5-fold lower than the isogenic DnaE* control, and this effect remains in the Lex-defective background (Figure 1G, Table 1). These results indicate that the decreased mutagenesis caused by dnaE915 is independent of documented effects [46] of dnaE915 on SOS induction.

Similarly, the unlikely possibility the dnaE915 mutation somehow caused lower levels of Pol IV independently of effects on SOS induction is ruled by data that show similar Pol IV levels in lexA (Def) dnaE915 and lexA (Def) strains (Figure 2). Therefore, dnaE915 did not reduce Pol IV-dependent mutagenesis by decreasing Pol IV levels in these cells.

DnaE915-Pol III could decrease Pol IV-dependent mutagenesis by preventing Pol IV from making errors, promoting DNA Pol III proofreading of DNA Pol IV errors, reducing Pol III ability to extend synthesis from the mismatched primer terminus caused by Pol IV errors or by reducing Pol III errors that Pol IV might extend [35]. We exclude the possibility that Pol III makes a subset of the errors that become mutations by showing that a proofreading-defective Pol III (ΔdnaQ) does not increase stress-induced mutagenesis (Figure 1H, Table 1). Although the absolute number of Lac− colonies in Figure 1H is significantly higher in the dnaQ strain compared with dnaQ−, due to a high mutation rate during liquid growth of the cultures prior to starvation on the lactose plates, the rate of Lac− colony formation post-plating (the slope of the lines in Figure 1H) is the same for dnaQ− and dnaQ+ strains after day 4, after which time colonies from pre-existing mutant cells cease to arise. Thus, the stress-induced-mutation rate is no higher in the dnaQ strain. If Pol III generated the errors that become mutations, then proofreading-defective Pol III would produce more, which it does not. Moreover, these data also demonstrate that Pol III does not proofread errors made by Pol IV, or any other DNA polymerase generating stress-induced point mutations. It has been proposed that the exonuclease activity of Pol III might be down-regulated during SOS induction [24], which could render a ΔdnaQ mutation inconsequential in an assay in which SOS induction plays a role, which would be compatible with our results.

The data presented indicate that Pol III neither makes nor corrects DNA polymerase errors that become stress-induced point mutations and that the dnaE915 product does not suppress mutagenesis via suppression of the SOS response and down-regulation of dnaB. Rather, we suggest that cells carrying the dnaE915 allele show decreased stress-induced point mutagenesis because of the DNAE915-Pol IIII protein’s increased ability to exclude Pol IV (relative to wild-type Pol IIII) and other DNA polymerases (as suggested for Pol II [44]) from the site of
DNA synthesis during DSB-repair associated stress-induced mutagenesis.

**Little Effect of UmuDC on Stress-Induced Frameshift Reversion**

In previous studies, Pol V (encoded by *umuDC*) did not contribute to stress-induced reversion of the frameshift allele in the Lac assay [28,37], which we also observe here (Figure 1I, Table 1). The slightly higher rate observed in Figure 1I is not significant averaged over 5 experiments (Table 1). We find that mutagenesis in the *umuDC* strain requires dinB to about the same extent as the wild-type (Figure 1I and Table 1) indicating that there was no subtle effect of the absence of Pol V, such as changing the DNA polymerase responsible for the frameshift reversion measured in this assay.

Although we found no effect of Pol V in *lac* frameshift reversion in this study (Figure 1I, Table 1, and [28,37]), previous data indicate that Pol V is also present in the DSB-repair synthesis that leads to DSB-repair-associated stress-induced mutagenesis, but simply does not contribute to −1 bp deletions. In an assay measuring stress-induced β-lactam-resistance mutagenesis in the chromosomal *ampD* gene, as here the mutagenesis required DSB-repair proteins, Pol IV, and SOS and RpoS responses, implying a DSB-repair-associated stress-induced mutagenesis mechanism [40]. However, the mutagenesis also partially required Pol V [40]. The *ampD* β-lactam-resistance mutations were mostly base-substitutions, which are not detected in the Lac assay. Similarly, Cirié et al. found that base-substitution mutations conferring ciprofloxacin resistance required Pol II, Pol IV and Pol V as well as SOS induction and DSB-repair proteins [39]. The simplest interpretation is that all five DNA polymerases are routinely present at sites of stress-induced mutagenesis associated with DSB repair, but that Pol V contributes only to substitutions whereas Pol IV contributes to both frameshift and substitution mutagenesis.

**DNA Polymerases I, II and III Compete with Pol IV during Stress-Induced Mutagenesis**

The data presented imply that *E. coli* DNA polymerases I, II and III compete with DNA Pol IV during double-strand-break (DSB)-repair-associated stress-induced mutagenesis. First, we have ruled out models in which the absence of Pols I and II, or an altered function allele of Pol III, affect mutagenesis indirectly by affecting the level of SOS-induction and thus dinB expression (Figure 1A,C,G, Table 1). These models had been compelling given that the mutations affecting Pol I [50] and Pol III [46] do demonstrably affect SOS induction, which is required for stress-induced mutagenesis by virtue of it upregulation of Pol IV [37,38].

Second, we ruled out possible SOS-independent effects of the relevant mutations in Pols I, II, and III on DinB protein levels (Figure 2). Third, we excluded the possibilities that Pol I or Pol III altered Pol IV-dependent mutation rates by proofreading and correcting Pol IV-generated errors (Figure 1D,H, Table 1). Fourth, whereas it seemed likely previously that DNA Pol III might be required for the Pol IV-dependent stress-induced mutagenesis [35,36,44,45], our data exclude this possibility. We found that a Pol III mutant protein that reduces DinB-dependent stress-induced mutagenesis does not do this because Pol III itself makes or facilitates the DNA polymerase errors that become the mutations (Figure 1H, Table 1), but rather Pol III appears to act by excluding Pol IV. The data support models in which the mutation rate in this assay is a direct result of (is modulated by) DNA polymerases I, II and III competing with the lower-fidelity Pol IV.

**Multiple DNA Polymerases in Double-Strand-Break Repair During Stress**

Previous work indicates that the sites of mutagenesis at which the DNA polymerases compete are sites of DSB repair via homologous recombination (HR). The mutagenesis requires HR-DSB repair proteins [32,33,34], and a DSB in the same DNA molecule in which the mutations occur [31]. DSBs made in the same molecule as *lac* stimulated DSB-repair-protein-, RpoS-, SOS- and DinB-dependent mutagenesis 6000-fold, whereas DSBs made in a different molecule in the same cell increased mutagenesis only 3-fold, strongly supporting error-prone DSB-repair models for the mutagenesis. DSB repair was high-fidelity and non-mutagenic in unstressed cells, but switched to a mutagenic mode using Pol IV either when cells were stressed and expressed both the RpoS general-stress response and the SOS DNA-damage response (which is activated in essentially all acts of DSB repair [46]) or if the RpoS response was expressed artificially in unstressed cells [31]. Those data revealed an RpoS-controlled switch from high-fidelity to mutagenic DSB repair, using Pol IV, under stress. Although other models have been considered (see [26,42]), this basic interpretation of the data above has not, to our knowledge, been called into question and we are not aware of alternative interpretations. The data presented here imply that all of the DNA polymerases are present at the sites of DSB repair synthesis during stress.

The implication that all five DNA polymerases are present in acts of DSB repair under stress does not mean that all make the errors that become stress-induced mutations. First, of the five DNA polymerases, Pol IV is by far the most robust producer of -1 deletion errors [54,55] as would produce frameshift reversions in Lac system [56,57]. Second, the data presented here support models in which, at least for frameshift reversion, Pols I, II, and III compete with and dampen the mutagenic effect of Pol IV. Conversely, in DSB-repair-protein-dependent base-substitution mutagenesis conferring ciprofloxacin [39] or β-lactam resistance [40], Pols II, IV and V were all required, or Pol IV was required and Pol V partially required, respectively. Thus, the job of base-substitution mutagenesis appears to be shared. It may be that some DNA polymerases make errors that others extend at the mispaired primer terminus, protecting them from proof-reading functions of more processive polymerases [39]. Others of the five DNA polymerases might dampen mutagenesis simply by being present and competing with the mutagenic DNA polymerases, and influencing the dynamics of DNA polymerase choice thereby.

**Model**

A model for the mechanism by which DNA polymerase competition could modulate HR-DSB-repair-associated stress-induced mutagenesis is illustrated in Figure 3. In this model, in growing cells, Pols I, II and III prevent Pol IV-mediated synthesis, keeping mutation rates low (left side of Figure 3). Under stress, Pol IV levels are upregulated about 10-fold by the SOS response [3,58] and ~2-fold by the RpoS-controlled general or stationary-phase/starvation stress response [29]. The upregulation of Pol IV is the only contribution of the SOS response to stress-induced frameshift mutagenesis [38], whereas it is unknown whether RpoS induction ushers Pol IV into acts of DSB-repair synthesis solely by increasing Pol IV levels (mass action) or by upregulating factors that might assist Pol IV. Either way, in the model, when upregulated by SOS and upregulated/assisted by RpoS induction under stress, Pol IV may then compete more successfully with Pols I, II, and III causing increased mutagenesis under stress (right side of Figure 3). An antimutator allele of Pol III (*dnaE913*) decreases Pol IV-dependent mutagenesis [35,36,44,45] and Figure 1G. Our
data suggest that the dnaE915 mutant of Pol III* is better able to exclude Pol IV from the site of DNA synthesis during stress. It is likely that wild-type Pol III also competes with Pol IV effectively during DSB repair because DSB-repair synthesis in growing cells is strongly Pol III-dependent [59].

In summary, the evidence presented suggests that all five DNA polymerases are available during stress-induced mutagenesis, and that DNA polymerases I, II, and III compete with Pol IV at the primer terminus. The mechanism(s) by which various DNA polymerases win the competition under various circumstances are important biologically for determining whether cells will survive replication-blocking lesions and/or will experience mutagenesis. These mechanisms are likely to be tightly regulated by means specific to the circumstance and stress experienced.

Materials and Methods

Bacterial Strains and Media

E. coli strains (Table 2) were constructed using standard bacteriophage P1-mediated transduction techniques [60]. Relevant genotypes were confirmed by sensitivities to UV light, antibiotic resistances, and/or PCR amplification, followed by restriction digestion or DNA sequencing. Antibiotics were used in the following concentrations (μg/ml): chloramphenicol, 25; kanamycin, 30; rifampicin, 100; spectinomycin, 50; streptomycin, 50; tetracycline, 10. All M9 media [60] also contained 10 μg/ml vitamin B1 and either 0.1% glycerol or 0.1% lactose. Luria-Bertani-Herskowitz (LBH) medium was described previously [61].

Stress-induced mutagenesis assays

The Lac assay was performed as described [34], except that in some experiments the cultures were grown at 32°C for 3 days, instead of 37°C for 2 days, before plating on M9 lactose medium. This produces much higher mutation rates as seen in Figure 1E, F and I, but does not change the relative mutability between different strains [31]. All experiments presented had less than 2-fold net population change during days 1–3 post-plating (monitored per [34]). All strains within an individual experiment were treated similarly with respect to culture temperature and length of culture time. In the Lac assay, Lac+ colonies result either from –1 bp deletions that compensate for the +1 bp insertion in the lacZ fusion gene [56,57], or from tandem amplification of the leaky lac allele to 20–50 copies [62]. Because by day 5 of an experiment, amplification accounts for only a few percent of the Lac+ colonies [62], we have not corrected the numbers to subtract amplification. Therefore, the data presented show total Lac+
Western analyses of Pol IV protein levels

48-hour 5 ml M9 glycerol cultures were normalized to OD600 of 1.0, and 1 ml of each pelleted and resuspended in 0.1 ml of Laemmli buffer [63] and boiled for 5 minutes. 15 μl from each sample were separated by discontinuous SDS-PAGE on a 14% Tris-glycine gel, and transferred to 0.2 micron Hybond ECL PVDF film in 1x Dunn’s modified carbonate buffer (10 mM NaHCO3, 3 mM Na2CO3, pH 9.5) on 20% methanol at 100 volts for 2 hrs at 4°C [64]. The membrane was incubated in blocking buffer (PBS-T [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, 0.1% Tween-20], 5% milk) for 1 hr, washed twice in PBS-T, treated with primary antibody [65], diluted 1:2000 in blocking buffer, for 1 hr, washed twice in PBS-T, then treated with secondary antibody (Goat anti-rabbit IgG-Cy5, GE healthcare), diluted 1:2000 in blocking buffer for one hour with agitation. The membrane was washed four times in PBS-T, three times in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4), and dried at 37°C for one hour. Cy5 fluorescence was detected on a Typhoon 9410 scanner (PMT set to 400).

Western blot densitometry was performed using Adobe Photoshop.

Supporting Information

Table S1 Details of stress-induction-mutation experiments summarized in Table 1. Found at: doi:10.1371/journal.pone.0010862.s001 (0.19 MB DOC)

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Author Contributions

Conceived and designed the experiments: PJH MNH RSH SMR. Performed the experiments: PJH MNH PCT NCF AS RLF MPR RSH. Analyzed the data: PJH MNH RSH SMR. Contributed reagents/materials/analysis tools: PJH MNH PCT AS RSH. Wrote the paper: PJH MNH SMR.

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