Role of *Escherichia coli* DNA Polymerase IV in In Vivo Replication Fidelity

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We have investigated whether DNA polymerase IV (Pol IV; the *dinB* gene product) contributes to the error rate of chromosomal DNA replication in *Escherichia coli*. We compared mutation frequencies in mismatch repair-defective strains that were either *dinB* positive or *dinB* deficient, using a series of mutational markers, including *lac* targets in both orientations on the chromosome. Virtually no contribution of Pol IV to the chromosomal mutation rate was observed. On the other hand, a significant effect of *dinB* was observed for reversion of a *lac* allele when the *lac* gene resided on an F'(*pro-lac*) episome.

Several mechanisms control the fidelity of the DNA replication process. These include correct base selection by the DNA polymerase, removal of base insertion errors by 3'-exonucleolytic proofreading, and correction by DNA mismatch repair (29). In *Escherichia coli*, base selection and proofreading are performed by the DNA polymerase III (Pol III) holoenzyme, the enzyme that replicates the bacterial chromosome. It is generally considered a highly accurate enzyme (29). Mismatch repair is performed by the *mutHLS* mismatch repair system (17). In combination, these three processes yield an error rate of 10^{-9} to 10^{-11} error per base pair replicated per cell division (6, 29).

In addition to Pol III, *E. coli* possesses four other DNA polymerases, Pol I, Pol II, Pol IV, and Pol V, whose precise functions are still being defined. Pol IV and Pol V belong to the recently described Y family of DNA polymerases (H. Ohmori, E. C. Friedberg, R. P. P. Fuchs, M. F. Goodman, F. Hanaoka, D. Hinkle, T. A. Kunkel, C. W. Lawrence, Z. Livneh, T. Nohmi, L. Prakash, S. Prakash, T. Todo, G. C. Walker, Z. Wang, and R. Woodgate, Letter, *Mol. Cell* **8**:7-8, 2001). Pol IV and Pol V are generally considered low-fidelity DNA polymerases in view of their lack of proofreading activity and their high in vitro error rates (14, 37). Both enzymes are inducible as part of the SOS regulon, and under SOS-induced conditions they function in lesion bypass and mutagenesis (3, 14, 21, 22, 28, 33, 37, 39).

In contrast to Pol V, which is tightly controlled and essentially not present outside of SOS-induced conditions, Pol IV has a relatively high basal expression level (21), an estimated 250 molecules of Pol IV per cell (20) compared to about 30 molecules of Pol III and much less of the complete Pol III holoenzyme (25). It has been proposed (14) that in certain instances, such as at blocked DNA replication forks, Pol IV might gain access to the replication fork and perform remedial

DNA synthesis. Thus, its abundance and potential access to the replication point raise the question of whether this low-fidelity enzyme may contribute to the replication error rate and spontaneous mutagenesis. Consistent with this potential role, overexpression of Pol IV was shown to result in a mutator phenotype (20, 40). Another instance where Pol IV has been implicated is mutagenesis in nondividing cells (adaptive or stationary-phase mutagenesis) (9). Adaptive mutations are routinely assessed through reversion of the *lacI33 Ω lacZ* frameshift allele residing on an F' episome (9). Loss of Pol IV significantly diminishes these mutations, implicating Pol IV in their production (9, 26, 38).

In this report we examine the possible role of Pol IV in contributing to the chromosomal mutation rate in dividing cells. We did this by carefully analyzing the level and specificity of spontaneous mutation in mismatch repair-defective *mutL* strains with or without Pol IV present. Mismatch repair-defective strains are most suitable for this purpose, as their mutation rates most directly reflect the error rate of ongoing DNA replication. We used a variety of mutational detection systems, including a system developed previously in our laboratory that permits measurement of reversion of a series of *lacZ* alleles residing in two orientations in the *E. coli* chromosome with respect to the direction of replication (7), causing *lac* sequences to be replicated either as a leading or a lagging strand during chromosomal replication. Thus, in addition to assessing the general role of Pol IV by comparing *din⁺* to *dinB* strains, it may be possible to assess whether Pol IV might work differentially in one of the two strands, specifically. To inactivate Pol IV, we used the Δ *dinB::kan* allele, which also includes a partial deletion of the *yafN* gene (21).

Forward mutagenesis to rifampin and nalidixic acid resistance and reversion of the *trpE9777* frameshift allele. We first tested a series of chromosomal markers by using strain NR13145 [*ara thi* Δ (*pro-lac*) *mutL::Tn10 trpE9777*] and its Δ *dinB::kan* derivative, strain NR13146. The strains are derivatives of KA796 (32) into which the *trpE9777* (30, 34), *mutL::Tn10* (31), and Δ *dinB::kan* (21) alleles were introduced by P1 transduction. The strains allow measurement of *trpE9777*

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FIG. 1. Effect of *dinB* deletion on frequencies of mutants (per 10^8 cells) of mismatch repair-defective *mutL* strains. Light gray bars, *dinB* positive; dark gray bars, *dinB* deficient. The numbers above each bar indicate the average frequencies of mutants calculated for 30 independent cultures. This experiment was performed a total of four times with similar results. In no case was a significant difference for the *dinB*-to-*dinB*⁺ comparison found ($P > 0.5$). Error bars represent standard errors of the means.

frameshift reversion as well as forward mutagenesis to rifampin resistance (Rif^R) and nalidixic acid resistance (Nal^R). The *trpE9777* allele contains an extra A · T base pair added to a run of five A · T base pairs (M. J. Bronson and C. Yanofsky, Letter, J. Mol. Biol. **88**:913-915, 1974), and reversion occurs by loss of the extra pair (34). The data shown in Fig. 1 indicate that there were no significant differences in mutability between the *dinB*-positive and *dinB*-deficient strains for this marker. Likewise, no effects of *dinB* were observed for the Rif^R or Nal^R forward-mutagenesis systems, which allow scoring of base substitution mutagenesis at multiple sites in the *rpoB* or *gyrA* gene, respectively (11, 41). These experiments were performed multiple times, and no significant differences between *dinB* and *dinB*⁺ strains were observed in any of them (see Fig. 1).

Chromosomal *lac* system for assaying leading- versus lagging-strand mutagenesis. Our laboratory has developed a system that allows comparison of the mutabilities of the *lacZ* gene when it is present in the two opposing orientations on the bacterial chromosome. Inversion of the target gene places *lac* sequences that were previously copied by leading-strand replication into the lagging strand and vice versa. Thus, a comparison of the mutabilities of the same target sequence in the two orientations can reveal differential mutabilities, if any, during leading- or lagging-strand replication. The two orientations of the *lac* operon with respect to the direction of replication through the target have been arbitrarily designated right (R) and left (L) (7). Using this system, it was previously deduced (7) that base substitutions likely occur more frequently during leading-strand replication. Likewise, the frequencies of frameshift mutations are not equal on the two DNA strands (12), although the complexity of frameshift mutagenesis (1) does not permit a ready strand assignment in this case (12). These sets of strains with *lac* alleles in the two orientations provide a potentially discriminating tool to assay the influence of Pol IV on chromosomal replication fidelity.

The strains used have been described previously (7, 12, 13). They are *mutL*::Tn5 derivatives of MC4100 (Δlac) carrying a series of defined *lac* alleles (4, 5) in the λ attachment site and having either a *dinB*⁺ or $\Delta dinB::kan$ genotype. For the *lac* alleles, we used four base substitution markers and four frame-

shift markers. The base substitution markers (5) were two transitions (G · C → A · T and A · T → G · C) and two transversions (G · C → T · A and A · T → T · A). The frameshift markers (4) were two (+1) frameshifts [addition of G · C to a (G · C)₆ run or A · T to an (A · T)₆ run] and two (−1) frameshifts [loss of G · C from (G · C)₆ or A · T from (A · T)₇].

The data in Fig. 2 show that, as before (7, 12, 13), significantly different frequencies of mutants were observed when the effect of orientation (R versus L) on all four base substitution alleles and three of the four frameshift alleles in the *dinB*⁺ control strains was tested. However, introduction of the *dinB* allele did not significantly affect the level of any of the observed mutations, regardless of the gene orientation (with one minor exception described below). The lack of differences is statistically significant (see legend to Fig. 2) and was observed in numerous repeated experiments (4 to 10 repeats for each *lac* allele). In the case of the −1G frameshift event only, a small but repeatable difference was observed between the *dinB*⁺ and *dinB* strains. In each repeated experiment with this allele, we observed a very moderate (~20%) but consistent decrease in the mutability of the *dinB* derivative carrying the L-oriented *lac* operon. No difference was observed for the corresponding R-oriented strain. This suggests that for this *lacZ* allele, Pol IV may play a minor, strand-specific role.

Effects of *dinB* in F'(*pro-lac*) strains. Lac reversion in non-growing cells (stationary or adaptive mutagenesis) has been shown to be, to a large extent, *dinB* dependent (9, 26, 35, 38). These experiments have generally used a particular frameshift allele, *lacI33*Ω*lacZ*, residing on an F'(*pro-lac*) episome. Among other differences, such as those in growth and selection conditions, these experiments differ from ours in that they are generally conducted at higher basal Pol IV levels. The F'(*pro-lac*) episome used (F'128) also contains a copy of the *dinB* gene. Moreover, multiple copies of F' are often present in the cell (10, 15). Thus, these experiments are generally conducted under conditions of enhanced Pol IV levels (21).

To complement our chromosomal experiments, we also conducted an experiment with a set of strains containing F'(*pro-lac*). We assayed for two chromosomal markers (Rif^R and *trpE9777* reversion) and one F'(*pro-lac*)-contained marker, the *lacZ* gene from strain CC104, which permits measurement of G · C → T · A transversions (5). The strains used were as described above for Fig. 1, except that the relevant F' (either *dinB* positive or with the $\Delta dinB::kan$ deletion) was introduced by conjugation. The data in Table 1 show the results obtained for four different *dinB* configurations: fully positive (gene present on both chromosome and F'), semideficient (gene absent on either F' or the chromosome), and fully deficient. The data show that the frequencies for the two chromosomal markers are unaffected or are little affected by the *dinB* copy number, whereas the episomal G · C → T · A marker is significantly affected. In four independent experiments, the average reductions due to the *dinB* defect were 1.9-fold (loss of the F' copy), 1.3-fold (loss of the chromosomal copy), and 3.0-fold (loss of both copies). This result indicates that, in this system, Pol IV contributes significantly (~70%) to the production of G · C → T · A transversions. The mutants on the Lac plates were counted after about 40 to 45 h of incubation, when Lac⁺ revertants first appeared. Thus, even though the *lac* G · C → T · A transversion allele has been shown to produce (modest

TABLE 1. Mutant frequencies in F'(*pro-lac*) strains with deletion of chromosomal and/or episomal *dinB* genes

Strain ^a	<i>dinB</i> genotype ^b		Mutation frequency (mean $\pm$ SEM) ^c		
	Chromosome	F'	Rif ^r	Trp ⁺	Lac ⁺
NR13153	+	+	440 $\pm$ 38	283 $\pm$ 18	7.3 $\pm$ 0.5
NR13155	+	—	415 $\pm$ 48	253 $\pm$ 35	3.5 $\pm$ 0.5
NR13157	—	+	485 $\pm$ 30	260 $\pm$ 20	5.3 $\pm$ 0.8
NR13159	—	—	425 $\pm$ 40	243 $\pm$ 25	1.6 $\pm$ 0.3

^a The strains, which were created as described in the text, were mismatch repair defective (*mutL::Tn10*).

^b +, gene present; —, gene absent.

^c Presented are frequencies per 10⁸ cells of Rif^r, reversion of the *trpE9777* frameshift, and reversion of the episomal *lacZ* G · C → T · A transversion. The numbers are the averages for 16 independent cultures.

amounts of) “adaptive” mutants upon prolonged incubation on the plate (16, 23), the observed mutants must be considered preexisting (growth dependent). Similar results were obtained in this experiment when the Δ *dinB::kan* allele was replaced by the nonpolar Δ *dinB::zeo* allele (2; data not shown).

Conclusions. The present results indicate that a lack of functional Pol IV does not significantly lower mutation frequencies in growing mismatch repair-deficient *E. coli* cells. Therefore, we conclude that Pol IV does not contribute significantly to the normal chromosomal error rate. This does not mean that Pol IV does not participate in chromosomal activities; it means only that this involvement, if any, is generally error free compared to other error-producing mechanisms. Presumably, the access of Pol IV to the replication fork is carefully controlled, permitting only limited DNA synthesis by this error-prone enzyme.

Our experiments did show a small (~20%) but consistent role for Pol IV in the reversion of the –1G frameshift *lacZ* allele, but only when the *lacZ* gene was in the L orientation (Fig. 2). This result suggests that Pol IV does contribute to errors in this configuration. Due to the complexity of frameshift mutagenesis (1), whether this role is an involvement in leading- or lagging-strand replication cannot be readily deduced. However, overexpression of Pol V preferentially increased base substitution mutagenesis in the lagging strand, presumably because of easier access (24). Preliminary data suggest that the same may be true for the mutator effect resulting from Pol IV overexpression (unpublished data). Overall, based on multiple repeats of the experiments shown in Fig. 2, we estimate that the 20% effect observed for the case of the –1G *lacZ* allele represents a general upper limit for the contribution of Pol IV in any of the other chromosomal tests reported here.

In contrast to the case with the chromosomal mutations, our data indicate that Pol IV can play a significant role in mutation production on the F'(*pro-lac*) episome, even in growing cells

(Table 1). Presumably, one factor contributing to this mutation production is the increased *dinB* gene dosage in this experimental setting, as the F' carries an additional copy of the gene and multiple F' copies may be present. Overproduction of Pol IV has been demonstrated to lead to a mutator phenotype (20, 41). Interestingly, and consistent with the data of Kim et al. (21), who noted that the episomal *dinB* copy produced more Pol IV protein than the chromosomal copy, we noticed that the deletion of *dinB* from the episome decreased the frequency of mutants more than the deletion of *dinB* from the chromosome (1.3- versus 1.9-fold; average result of four experiments).

Interestingly, increased *dinB* copy number did not significantly increase the frequency of chromosomal Rif^r mutations or *trpE9777* frameshift reversions (Table 1). This finding suggests that location of the target gene on the F' itself might also be necessary for enhanced mutagenesis. A rigorous demonstration of this will require comparison of the *lacZ* target genes on the chromosome and F' under the various *dinB* gene dosage conditions. In the meantime, one might speculate that the same principles that underlie the production of growth-independent mutations (which are to a large extent *dinB* dependent) are also relevant to the growth-dependent mutations observed here. The duplications and amplifications of episomal *lac* sequences that have been shown to be prerequisites for the production of mutations under nongrowing conditions (19) are, in fact, produced during the preceding growth phase (19). These amplified sequences may include both *lac* and *dinB* (19, 35). Therefore, even in growing cells, conditions may be favorable for preferential mutagenesis of episomal *lac* sequences in a *dinB*-dependent manner. Although experiments reported here used the Δ (*dinB-yafN*) allele, for which polar effects on the *yaf* genes have been suggested to contribute to certain mutational end points (26, 27), we obtained identical results with the nonpolar Δ *dinB::zeo* allele (2; data not shown). Thus, we conclude that the mutator effect of Pol IV on F'(*pro-lac*) is due to Pol IV and not to other members of the *yaf* operon.

The question of the role of Pol IV in replication fidelity has been addressed before. Strauss et al. (36), using *dnaE* mutator strains containing an impaired Pol III, noted that about 75% of mutations in this background were *dinB* dependent, based on measurements of either *lac* reversion on F' or chromosomal Rif^r mutations. Interestingly, they also noted a corresponding 1.5- to 3-fold *dinB*-dependent reduction in the *dnaE*⁺ control strain, implying a role for Pol IV in creating these mutations. However, the effect of the Δ *dinB::kan* allele in the control strain was limited to the mismatch repair-proficient background. No effect was observed in the mismatch repair-deficient *mutS* background, in which replication errors are more directly viewed. Experiments in our laboratory with *mut*⁺ derivatives of the strains shown in Fig. 2 have not revealed any effect of *dinB* when chromosomal Rif^r mutations or chromo-

FIG. 2. Frequencies of base substitution or frameshift mutants (Lac⁺ revertants per 10⁸ cells) of *dinB*⁺ and *dinB* pairs of strains containing the *lac* operon in two orientations. The strains were mismatch repair defective (*mutL*). The base substitution or frameshift measured in each case is indicated above the panel. Light gray bars indicate frequencies of mutants of the *dinB*⁺ strains; dark gray bars indicate frequencies of mutants of the *dinB*-deficient strains. L and R indicate the transcription orientation of the *lac* operon (7). The numbers above the bars are the average frequencies of mutants calculated for 30 independent cultures, and error bars represent standard errors of the means. For all of these experiments, the observed differences in *lac* reversion frequency between *dinB*⁺ and *dinB* pairs of strains are not statistically significant (*P* > 0.5), with the exception of a small (20%) effect for the –1G allele in the L orientation (*P* < 0.05).

somal G · C→T · A or -1G *lac* reversions are being scored (data not shown). This discrepancy remains to be explored.

McKenzie et al. (26) demonstrated the lack of an effect of the *dinB10* allele on the mutability of a series of chromosomal markers. These experiments were performed in the mismatch repair-proficient background, and their results are therefore not directly comparable to those of our experiments, which focus on the production of replication errors. Nevertheless, the absence of an effect in both studies is consistent with the lack of a role for Pol IV in chromosomal replication fidelity. With regard to the effect of *dinB* on episomal genes, the experiments of McKenzie et al. indicated a lack of effect of *dinB10* on the *lacI33ΩlacZ* allele (26), a result that appears to contradict our results with the episomal *lac* G · C→T · A transversion (Table 1). However, a reasonable explanation may be that DNA mismatch repair is efficient in removing most or all of the Pol IV-mediated frameshift revertants of *lacI33ΩlacZ*, masking the effect of Pol IV on replication errors. Efficient correction of the *lacI33ΩlacZ* revertants has been demonstrated (8, 9, 18). In a second study, McKenzie et al. (27) made a distinction between the nonpolar *dinB* alleles, such as *dinB10*, and the polar alleles, such as Δ *dinB::kan*, which exerts a polar effect on the distal members of the *dinB-yafNOP* operon. An effect (approximately twofold) was observed on *lacI33ΩlacZ* reversion with the polar Δ *dinB::kan* allele but not with the nonpolar alleles (27), leading the authors to conclude that the distal members of the operon might be involved. In our study, we observed an effect on the episomal *lacZ* G · C→T · A marker with both the polar Δ *dinB::kan* allele (Table 1) and the nonpolar Δ *dinB::zeo* allele, suggesting that only Pol IV is involved. These differences may reflect both the action of mismatch repair, which may dramatically influence the extent to which replication errors can be observed, and any mechanistic differences in the genesis of *lacI33ΩlacZ* frameshifts and *lacZ* G · C→T · A transversions. This issue requires further study.

In summary, no evidence has been found for a significant contribution of Pol IV to chromosomal mutagenesis in growing cells, although episomal mutagenesis on F'(*pro-lac*) may be enhanced by Pol IV action.

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ADDENDUM IN PROOF

Recently, Wolff et al. (E. Wolff, M. Kim, K. Hu, H. Yang, and J. H. Miller, *J. Bacteriol.* **186**:2900–2905, 2004), based on an analysis of the spectral fingerprint of DNA polymerase IV-induced mutations, also concluded that Pol IV does not contribute significantly to mutations occurring during exponential growth.

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