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DinB Upregulation Is the Sole Role of the SOS Response in Stress-Induced Mutagenesis in *Escherichia coli*

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ABSTRACT

Stress-induced mutagenesis is a collection of mechanisms observed in bacterial, yeast, and human cells in which adverse conditions provoke mutagenesis, often under the control of stress responses. Control of mutagenesis by stress responses may accelerate evolution specifically when cells are maladapted to their environments, *i.e.*, are stressed. It is therefore important to understand how stress responses increase mutagenesis. In the *Escherichia coli* Lac assay, stress-induced point mutagenesis requires induction of at least two stress responses: the RpoS-controlled general/starvation stress response and the SOS DNA-damage response, both of which upregulate DinB error-prone DNA polymerase, among other genes required for Lac mutagenesis. We show that upregulation of DinB is the only aspect of the SOS response needed for stress-induced mutagenesis. We constructed two *dinB*(o^c) (operator-constitutive) mutants. Both produce SOS-induced levels of DinB constitutively. We find that both *dinB*(o^c) alleles fully suppress the phenotype of constitutively SOS-"off" *lexA*(Ind^-) mutant cells, restoring normal levels of stress-induced mutagenesis. Thus, *dinB* is the only SOS gene required at induced levels for stress-induced point mutagenesis. Furthermore, although spontaneous SOS induction has been observed to occur in only a small fraction of cells, upregulation of *dinB* by the *dinB*(o^c) alleles in all cells does not promote a further increase in mutagenesis, implying that SOS induction of DinB, although necessary, is insufficient to differentiate cells into a hypermutable condition.

GENOMIC stability and mutation rates are tightly regulated features of all organisms. Understanding how cells regulate mutation rates has important implications for evolution, cancer progression and chemotherapy resistance, aging, and acquisition of antibiotic resistance and evasion of the immune system by pathogens, all processes driven by mutagenesis and all of which occur during stress.

Stress-induced mutagenesis refers to a group of related phenomena in which cells poorly adapted to their environment (*i.e.*, stressed) increase mutation rates as part of a regulated stress response (reviewed by GALHARDO *et al.* 2007). Abundant examples, particularly in microorganisms, show the induction of specific pathways of mutagenesis in response to stresses. The types of genetic alteration induced by stress include base substitutions, small deletions and insertions, gross chromosomal rearrangements and copy-number variations, and movement of mobile elements. These various pathways require the functions of different sets of genes and proteins. Thus, there appear to be multiple molecular mechanisms of stress-inducible mutagenesis that operate in different organisms, cell types, and growth-inhibiting stress conditions.

However, a common theme in the many mechanisms of stress-inducible mutagenesis described to date is the requirement for the function of one or more cellular stress responses. Starvation stress-induced mutagenesis in *Bacillus subtilis* requires the *comK* regulatory gene that controls the stress response that in turn allows competence for natural transformation in response to starvation (SUNG and YASBIN 2002). The RpoS-controlled general or starvation stress response is required for starvation-induced excisions of phage Mu in *Escherichia coli* (GOMEZ-GOMEZ *et al.* 1997), for base-substitution mutagenesis in aging *E. coli* colonies (BJEDOV *et al.* 2003), for starvation-induced point mutations (SAUMAA *et al.* 2002) and transpositions (ILVES *et al.* 2001) in *Pseudomonas putida*, and for starvation-induced gene amplification (LOMBARDO *et al.* 2004) and frameshift

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mutagenesis (Layton and Foster 2003; Lombardo *et al.* 2004) in the *E. coli* Lac assay, described in more detail below. The SOS DNA-damage stress response is required for the stress-induced frameshift mutagenesis in the *E. coli* Lac assay discussed below, for *E. coli* mutagenesis in aging colonies (Taddei *et al.* 1997), for ciprofloxacin (antibiotic)-induced resistance mutagenesis (Cirz *et al.* 2005), and for mutagenesis conferring resistance to bile salts in Salmonella (Prieto *et al.* 2006). The stringent response to amino-acid starvation is required for a transcription-associated mutagenesis in *E. coli* that targets stringent-response-controlled genes (Wright *et al.* 1999) and for amino-acid-starvation-induced mutagenesis in *B. subtilis* (Rudner *et al.* 1999). Two different stress responses to hypoxia in human cancer cells also increase mutagenesis. One does so by specific downregulation of mismatch-repair genes (Mihaylova *et al.* 2003; Koshiji *et al.* 2005; Bindra and Glazer 2007). The other is postulated to promote genome rearrangement by its demonstrated downregulation of RAD51 and BRCA1 functions required for high-fidelity repair of DNA double-strand breaks (DSBs) (Bindra *et al.* 2004). These stress responses exert temporal control or restriction of mutagenesis, which favors genomic stability when cells and organisms are well adapted to their environments (*i.e.*, not stressed) and increases mutagenesis, potentially accelerating evolution, specifically during stress when cells are maladapted to their environments. Except for the human examples, the ways by which the stress responses upregulate mutagenesis are mostly not understood. We focus here on how a stress response controls mutagenesis in an *E. coli* model system.

Stress-induced mutagenesis is perhaps best understood in the *E. coli* model system. A widely used assay system uses a +1 frameshift allele of a *lacIZ* fusion gene located in the F′128 plasmid in cells with a deletion of the chromosomal *lac* genes (Cairns and Foster 1991). When these cells are plated on lactose minimal medium, a few Lac^+ revertant colonies are observed. Many of these arise from spontaneous generation-dependent mutations that occur during growth of the culture. Prolonged incubation of these plates results in the continuous accumulation of additional Lac^+ revertants, which arise through two mechanisms, both different from the mechanisms that produce the generation-dependent mutants (reviewed by Galhardo *et al.* 2007).

First, within the first few days, most of the Lac^+ colonies are "point mutants" that possess a compensatory −1 frameshift mutation in the *lacIZ* gene (Foster and Trimarchi 1994; Rosenberg *et al.* 1994). Cells that carry these mutations also carry increased numbers of secondary unselected mutations in other genomic regions, whereas most Lac^- cells starved on the same plates do not, indicating that a subpopulation of the cells undergoes genomewide hypermutation (Torkelson *et al.* 1997; Rosche and Foster 1999; Godoy *et al.* 2000). Therefore, a subset of the starved cells experiences increased mutagenesis when compared with the majority of the cells. Hereafter we refer to this subpopulation as "hypermutable." This hypermutable cell subpopulation (HMS) appears to be important to the formation of most or all of the Lac^+ stress-induced mutants (Gonzales *et al.* 2008). The hypermutable state is transient, ceasing after growth impairment is ended and growth resumes (Longerich *et al.* 1995; Torkelson *et al.* 1997; Rosenberg *et al.* 1998; Rosche and Foster 1999; Godoy *et al.* 2000).

Second, longer incubation also results in the formation of a significant proportion of *lac*-amplified colonies in which the leaky *lacIZ* allele is amplified to 20–50 tandem copies, which produce sufficient enzyme activity to allow growth on lactose (Hastings *et al.* 2000; Powell and Wartell 2001; Kugelberg *et al.* 2006; Slack *et al.* 2006). In summary, *E. coli* cells may either increase point-mutation rates or undergo extensive genomic rearrangement in response to a growth-limiting environment.

Both of these processes require induction of the general or starvation stress response controlled by RpoS (Lombardo *et al.* 2004). Point mutagenesis, but not amplification, also requires induction of the SOS DNA-damage stress response (Cairns and Foster 1991; McKenzie *et al.* 2000, 2001). In this article, we focus on the role of the SOS response in the mechanism of stress-induced point mutagenesis. See Hastings (2007) for a review of the mechanisms of stress-induced amplification and genome rearrangement.

The molecular mechanism of point mutagenesis in the Lac system is now considerably well understood. It entails a switch from the normally high-fidelity DNA synthesis associated with recombination-dependent double-strand-break repair to an error-prone synthesis specifically under stress (Ponder *et al.* 2005). Several genetic requirements are known for stress-induced point mutagenesis, including DNA-recombination functions (Harris *et al.* 1994, 1996; Foster *et al.* 1996; He *et al.* 2006) in addition to the genes required for induction of the SOS DNA-damage response (Cairns and Foster 1991; McKenzie *et al.* 2000) and the σ^S (RpoS) general/starvation stress-response (Layton and Foster 2003; Lombardo *et al.* 2004) regulons, and the *dinB* gene encoding DNA polymerase (Pol) IV (McKenzie *et al.* 2001).

DinB is the founding member of the most widespread subfamily of Y-family specialized DNA polymerases, with orthologs in bacteria, archaea, and eukaryotes, including humans (reviewed by Nohmi 2006). DinB/Pol IV can perform high-fidelity translesion DNA synthesis (TLS) across a number of different DNA lesion substrates (Jarosz *et al.* 2006; Bjedov *et al.* 2007; Yuan *et al.* 2008). However, this enzyme shows a significant error rate when copying undamaged DNA templates (Kobayashi *et al.* 2002). Some mutations in DinB can abolish its TLS activity, without interfering with the

mutator phenotype caused by overexpression of DinB, suggesting that mutagenesis and TLS are independent activities of Pol IV (GODOY *et al.* 2007). Eighty-five percent of the stress-induced Lac$^+$ point mutations generated in the nongrowing cells arise in a DinB-dependent manner (MCKENZIE *et al.* 2001).

The *dinB* gene is under the control of the SOS response, which upregulates its transcription 10-fold (KIM *et al.* 2001). Additionally, the alternative σ (transcription) factor σ^S (RpoS), which is responsible for the general stress response, upregulates *dinB* expression transcriptionally by 2- to 3-fold upon entry into stationary phase (LAYTON and FOSTER 2003). Proteins such as Ppk (STUMPF and FOSTER 2005) and the chaperones GroEL (LAYTON and FOSTER 2005), RecA, and UmuD (GODOY *et al.* 2007) all seem to modulate DinB activity. An interesting *in vivo* role of DinB is SOS untargeted mutagenesis of phage λ (KIM *et al.* 1997). In it, −1 frameshift mutations in runs of G's are generated, similarly to the predominant mutations detected in the *lac* gene during stress-induced mutagenesis (FOSTER and TRIMARCHI 1994; ROSENBERG *et al.* 1994). On the other hand, DinB has no effect on the spontaneous mutation rate in growing cells (MCKENZIE *et al.* 2001, 2003; KUBAN *et al.* 2004; WOLFF *et al.* 2004). DinB is implicated as the DNA polymerase that, only during the stress responses, makes DSB-repair-associated errors that become stress-induced point mutations (PONDER *et al.* 2005).

The role of the SOS response in controlling mutagenesis in the Lac assay is a complex issue because several SOS-controlled genes are required for the process. *dinB*, *recA*, *ruvA*, and *ruvB* are all required for mutagenesis (CAIRNS and FOSTER 1991; HARRIS *et al.* 1994, 1996; FOSTER *et al.* 1996; MCKENZIE *et al.* 2001; HE *et al.* 2006) and are all upregulated by SOS (COURCELLE *et al.* 2001). Also, the F-encoded *psiB* gene exerts a negative effect on mutagenesis in SOS-derepressed cells (MCKENZIE *et al.* 2000) and is thought to inhibit SOS induction and RecA (reviewed by COX 2007). We sought to determine whether the requirement for induction of the SOS response in stress-induced mutagenesis reflects a need for upregulation solely of *dinB* or whether any other gene(s) is required at SOS-induced levels. We present evidence below that indicates, first, that DinB is the only SOS-controlled gene required at induced levels for efficient stress-induced point mutagenesis and, second, that, although SOS-induced levels of DinB are required, they are not sufficient to differentiate cells into a hypermutable condition.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media: The bacterial strains used in this work are shown in Table 1. *dinB*(o^c) alleles were constructed as described below. Other strains were constructed using P1-mediated transduction as described (MILLER 1992). The antibiotics used were as follows: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; tetracycline, 10 μg/ml; kanamycin, 30 μg/ml; and rifampicin, 40 μg/ml. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was used at 40 μg/mL. M9 minimal medium (MILLER 1992) was supplemented with 10 μg/ml of vitamin B1 and either 0.1% glycerol or 0.1% lactose. Luria–Bertani–Herskowitz (LBH) medium was used as described by TORKELSON *et al.* (1997).

The plasmids used in this study are listed in Table 2. Plasmids containing P_{dinB}*lacZ* fusions used in β-galactosidase assays for gene expression analysis were constructed by amplification of the *dinB* promoter (from bases −432 to −2 of *dinB*) with primers 5′-TCGGCTGAATTCTGTTCGA CTCGCTCGATAAT-3′ and 5′-CGGTACAAGCTTGCTCACCT CTCAACACTGGT-3′ and by cloning into the pFZY plasmid (KOOP *et al.* 1987) using the *Eco*RI and *Hin*dIII sites introduced in the primers. The *dinB* promoter was amplified from strain SMR4562 and cloned into pFZY to generate plasmid pPdinB and amplified from strains SMR10308 and SMR10309 to generate the plasmids pPdinBOC1 and pPdinBOC2, respectively.

Construction of the *dinB*(o^c) alleles and strains bearing them: We created each of two mutations predicted from previous work on other SOS genes (FRIEDBERG *et al.* 2005) to inactivate the predicted LexA-binding site in *dinB* (Figure 1A). The Lac assay strains carry two copies of *dinB*, one in the chromosome and one in F′128 (discussed in the RESULTS). The constructions required several steps as below. Primer sequences are given in the supporting information, File S1.

First, we linked the *cat* selectable marker with *dinB*. We chose to put a selectable marker in the *lafU* (formerly known as *mbhA*) gene, which is present immediately upstream from the 5′-end of *dinB*. The FRT*cat*FRT cassette was amplified from pKD3 (DATSENKO and WANNER 2000) using primers CatupdinB-F and CatupdinB-R. The product was used to obtain SMR4562 recombinants containing the *lafU*::FRT*cat*FRT insertion (allele Δ*lafU2*::FRT*cat*FRT), using short-homology recombination as described (DATSENKO and WANNER 2000). One recombinant containing the Δ*lafU2*::FRT*cat*FRT in the F′ plasmid was selected. This strain (SMR10292) was used to amplify the Δ*lafU2*::FRT*cat*FRT-*dinB*$^+$ region by PCR using primers CatupdinB-F and dinBcatnock-R. This product was used as a template for PCR-mediated site-directed mutagenesis, altering the *dinB* promoter.

Next we constructed a Δ*lafU-dinB* deletion strain to be used as a recipient for allelic replacement with the site-directed *dinB*-mutant genes linked to Δ*lafU2*::FRT*cat*FRT. We created a FC36-derivative containing a deletion encompassing the 3′ half of Δ*lafU* and the whole *dinB* gene using primers kandinBchrom-F and DinBRCAT to amplify FRTKanFRT from pKD13 (DATSENKO and WANNER 2000). The products were used for short-homology recombination in the FC36 background, creating strain SMR10299. A similar deletion in the same region in the F′ plasmid was created by short-homology recombination in SMR4562, using FRT*cat*FRT amplified from pKD3 with primers CatupdinB-F and DinBRCAT. Location of the deletion in the F′ plasmid was confirmed by the ability to conjugate the *cat* gene conferring chloramphenicol resistance. The *cat* gene was removed by FLP-mediated site-specific recombination using the pCP20 plasmid (DATSENKO and WANNER 2000). The resulting F′128 Δ*lafU-dinB*::FRT [allele Δ(*lafU-dinB*) *2096*(::FRT)] was mated into strain SMR10299, creating strain SMR10303 [SMR4562 Δ(*lafU-dinB*) *2097*(::FRT-KanFRT) [F′ Δ(*lafU-dinB*) *2096*(::FRT)]]. This strain was used as a recipient for allelic replacement using the site-directed *dinB* mutants produced by PCR with the Δ*lafU2*::FRT*cat*FRT-*dinB* fragment as a template. The sequence of the promoter and coding sequence of the *dinB* gene from the Kan^R Cam^R recombinants was determined by PCR and DNA sequencing to

TABLE 1

Bacterial strains used in this study

Name	Relevant genotype	Reference or source
FC29	Δ(*lac-proB*)XIII *ara thi* [F′ Δ(*lacI-lacZ*)]	Cairns and Foster (1991)
FC40	Δ(*lac-proB*)XIII *ara thi* Rif^R [F′ *lacI33*Ω*lacZ proAB*$^+$]	Cairns and Foster (1991)
FC231	FC40 *lexA3*(Ind$^-$)	Cairns and Foster (1991)
SMR868	FC40 *lexA3*(Ind$^-$)	McKenzie *et al.* (2000)
SMR4562	Identical to FC40, independent construction	McKenzie *et al.* (2000)
SMR5400	SMR4562 *sulA211 lexA51*(Def) Δ*psiB*::*cat*	McKenzie *et al.* (2000)
SMR9436	SMR4562 Δ*ruvC*::FRTKanFRT	Magner *et al.* (2007)
SMR5889	SMR4562 Δ*dinB50*::FRT [F′ Δ*dinB50*::FRT]	McKenzie *et al.* (2001)
SMR10292	SMR4562 [F′ *lafU2*::FRT*cat*FRT]	This study
SMR10299	FC36 Δ(*lafU-dinB*)*2097*(::FRTKanFRT)	This study
SMR10303	SMR4562 Δ(*lafU-dinB*)*2097*(::FRTKanFRT) [F′ Δ(*lafU-dinB*)*2096*(::FRT)]	This study
SMR10304	SMR4562 Δ(*lafU-dinB*)*2097*(::FRTKanFRT) [F′ *lafU2*::FRT*cat*FRT *dinBo-21*(o^c)]	This study
SMR10306	SMR4562 Δ(*lafU-dinB*)*2097*(::FRTKanFRT) [F′ *lafU2*::FRT*cat*FRT *dinBo*-22(o^c)]	This study
SMR10308	SMR4562 [F′ *lafU2*::FRT*cat*FRT *dinBo-21*(o^c)]	SMR4562 × P1(SMR10304)
SMR10309	SMR4562 [F′ *lafU2*::FRT*cat*FRT *dinBo*-22(o^c)]	SMR4562 × P1(SMR10306)
SMR10310	SMR868 [F′ *lafU2*::FRT*cat*FRT *dinBo-21*(o^c)]	SMR868 × P1(SMR10304)
SMR10311	SMR868 [F′ *lafU2*::FRT*cat*FRT *dinBo*-22(o^c)]	SMR868 × P1(SMR10306)
SMR10314	SMR868 [F′ *lafU2*::FRT*cat*FRT]	SMR868 × P1(SMR10292)
SMR10760	FC231 [F′ *lafU2*::FRT*cat*FRT]	FC231 × P1(SMR10292)
SMR10761	FC231 [F′ *lafU2*::FRT*cat*FRT *dinBo-21*(o^c)]	FC231 × P1(SMR10304)
SMR10762	FC231 [F′ *lafU2*::FRT*cat*FRT *dinBo*-22(o^c)]	FC231 × P1(SMR10306)
SMR10766	SMR4562 Δ*ruvC*::FRTKanFRT [F′ *lafU2*::FRT*cat*FRT]	SMR10292 × P1(SMR9436)
SMR10767	FC231 Δ*ruvC*::FRTKanFRT [F′ *lafU2*::FRT*cat*FRT *dinBo-21*(o^c)]	SMR10761× P1(SMR9436)
SMR10768	FC231 Δ*ruvC*::FRTKanFRT [F′ *lafU2*::FRT*cat*FRT]	SMR10760 × P1(SMR9436)
SMR10838	SMR4562 [pPdinB]	This study
SMR10839	SMR4562 [pPdinBOC1]	This study
SMR10840	SMR4562 [pPdinBOC2]	This study
SMR10841	SMR5400 [pPdinB]	This study
SMR10842	SMR5400 [pPdinBOC1]	This study
SMR10843	SMR5400 [pPdinBOC2]	This study
SMR11023	SMR4562 [F′ *lafU2*::FRT*cat*FRT Δ*yafNOP*::FRTKanFRT]	This study
SMR11024	SMR4562 [F′ *lafU2*::FRT*cat*FRT Δ*yafNOP*::FRTKanFRT *dinBo-21*(o^c)]	This study
SMR11026	FC231 [F′ *lafU2*::FRT*cat*FRT Δ*yafNOP*::FRTKanFRT]	FC231 × P1(SMR11023)
SMR11027	FC231 [F′ *lafU2*::FRT*cat*FRT Δ*yafNOP*::FRTKanFRT *dinBo-21*(o^c)]	FC231 × P1(SMR11024)

ensure that the desired mutation was introduced and that no other mutation in *dinB* was generated inadvertently by PCR. One recombinant containing the *dinBo-21*(o^c) mutation (SMR10304) and one containing the *dinBo-22*(o^c) mutation (SMR10306) were chosen. Those strains were used as P1 donors of Δ*lafU2*::FRT*cat*FRT *dinBo-21*(o^c) and Δ*lafU2*::FRT*cat*FRT *dinBo-22*(o^c), respectively, to transduce the *dinB*(o^c) alleles to all the genetic backgrounds of interest, including SMR4562 and strains FC231 and SMR868 carrying *lexA3*(Ind$^-$).

Deletion of the *yafNOP* genes in the *dinB* operon was performed using short-homology recombination (Datsenko and Wanner 2000) as follows. Strains SMR10292 [SMR4562 (F′ Δ*lafU2*::FRT*cat*FRT)] and SMR10308 [SMR4562 (F′ Δ*lafU2*::FRT*cat*FRT *dinBo-21*(o^c))] were used as recipients for deletion by transformation with a DNA fragment amplified from pKD13 with primers yafNwL and yafPwR. Homologous incorporation of this DNA fragment, which contains the FRTKanFRT marker, results in a deletion of the *yafNOP* genes. Kan^R recombinants were selected, and location of the marker

TABLE 2

Plasmids used in this study

Name	Description and source
pFZY	Low-copy plasmid with multicloning site abutting a promoterless *lacZ* (Koop *et al.* 1987)
pPdinB	Bases −432 to −2 of *dinB* from strain SMR4562 cloned into pFZY, producing a P_{dinB}*lacZ* fusion
pPdinBOC1	Bases −432 to −2 of *dinB* from strain SMR10308 cloned into pFZY, producing a $P_{dinBo\text{-}21(o^c)}$*lacZ* fusion
pPdinBOC2	Bases −432 to −2 of *dinB* from strain SMR10309 cloned into pFZY, producing a $P_{dinBo\text{-}22(o^c)}$*lacZ* fusion

in the F′ episome was confirmed both by ability to transfer the resistance during mating and by cotransduction of Kan^R and Cam^R (present in the linked *lafU2*::FRT*cat*FRT in both strains). The strains resulting from deletion of *yafNOP* from the episomes of SMR10292 and SMR10308 were named SMR11023 and SMR11024, respectively. Both strains were used respectively as P1 donors to transfer the *lafU2*::FRT*cat*FRT Δ*yafNOP*::FRTKanFRT linkage and the *lafU2*::FRT*cat*FRT *dinBo-21*(o^c) Δ*yafNOP*::FRT:KanFRT linkage into the FC231 background, creating strains SMR11026 and SMR11027.

β-Galactosidase assays: β-Galactosidase assays were performed to determine the relative expression of *lacZ* under the control of the different versions of the *dinB* promoter cloned into the low-copy plasmid pFZY (Koop *et al.* 1987). Cells were grown in LBH medium until mid-log phase, and the levels of β-galactosidase were determined in samples of the cultures as described (Miller 1992).

DinB Western blots: For DinB detection on Western blots, stationary-phase cultures grown from single colonies in 5ml of M9 B1 glycerol medium for 48 hr were harvested, and cells were suspended in sample loading/lysis buffer (62.5 mM Tris, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 0.5% β-mercaptoethanol), correcting for the OD_{600} of the terminal culture. For 1 ml of a culture at OD_{600} of 2 (measured at $OD_{600} \leq 1$ with diluted samples), 100 μl of sample loading buffer was used. Twenty microliters of each sample was separated by electrophoresis on a SDS polyacrylamide gel (12.5%). Proteins were transferred to a Hybond-LFP PVDF membrane (Amersham Biosciences), and the membrane was probed with a polyclonal DinB rabbit antiserum (Kim *et al.* 2001). A goat anti-rabbit secondary antibody conjugated to the Cy5 fluorescent dye (Amersham Biosciences) was used for detection of DinB, using the Typhoon scanner (Amersham Biosciences).

Stress-induced mutagenesis assays: Stress-induced *lac* reversion assays were performed as described (Harris *et al.* 1996) with four independent cultures of each strain. The proportion of Lac$^+$ point mutants and *lac*-amplified colonies was determined by plating cells from 20 colonies of each culture for each day in which Lac$^+$ colonies were counted (days 2–5) on LBH rifampicin X-gal plates. This allows the distinction between Lac$^+$ point mutants (solid-blue colonies) and *lac*-amplified cells, given the *lac*-unstable sectoring-colony phenotype diagnostic of *lac* amplification (Hastings *et al.* 2000).

Determination of the mutation sequences in the *lac* gene: Lac$^+$ point mutants from experiment day 5 were identified as described above and purified on LBH plates containing rifampicin and X-Gal. A 300-nucleotide region spanning the *lac* +1 allele was amplified by PCR using primers lacIL2 (5′-AGGCTATTCTGGTGGCCGGA-3′ and lacD2 (5′-GCCTCTTC GCTATTACGCCAGCT-3′). DNA sequencing was performed by Seqwright (Houston) using primer, lacU (5′-ATATCCCG CCGTTAACCACC-3′).

RESULTS

Construction and characterization of the *dinB*(o^c) alleles: To test the hypothesis that *dinB* might be the sole SOS gene required at induced levels for stress-induced point mutagenesis, we constructed *dinB* mutants in which the transcriptional repression by LexA, the repressor controlling the expression of the SOS regulon, is alleviated. This was achieved by site-directed mutagenesis of the *dinB* promoter, altering the binding site of the LexA repressor. These are used (see below) to express *dinB* at SOS-induced levels in strains in which the rest of the SOS genes are repressed. The sequences of the operator-constitutive *dinB*(o^c) mutations that were constructed are shown in Figure 1A.

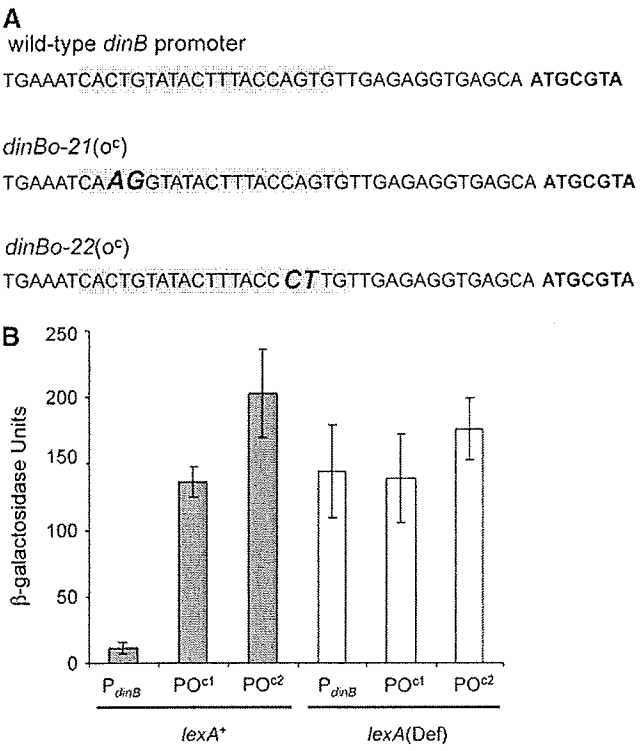

Figure 1.—Construction and characterization of two *dinB*(o^c) alleles. (A) Location of the operator-constitutive mutations in the *dinB* promoter. The SOS operator (from Fernandez De Henestrosa *et al.* 2000) is shaded, and the mutations introduced in each of the alleles are in boldface and italic type. The beginning of the *dinB* ORF is shown in boldface type. (B) Activity of the *dinB* promoter in transcriptional fusions with *lacZ*, measured in both wild-type (SMR4562) and its LexA-defective (null), *lexA51*(Def), derivative strain SMR5400, in which SOS is constitutively highly induced. The strains from left to right are SMR10838, SMR10839, SMR10840, SMR10841, SMR10842, and SMR10843. P_{dinB} indicates the wild-type *dinB* promoter present in plasmid pPdinB, PO^{c1} indicates the *dinBo-21*(o^c) promoter contained in plasmid pPdinBOC1, and PO^{c2} indicates the *dinBo-22*(o^c) promoter contained in plasmid pPdinBOC2. Mean ± 1 standard error of the mean (SEM) for three independent determinations.

To test whether these mutations behave as *bona fide* operator-constitutive alleles, we fused the *dinB* promoter regions from the two *dinB*(o^c) alleles to *lacZ* and measured the levels of β-galactosidase expression from these P_{dinB}*lacZ* fusions carried in a low-copy plasmid (Figure 1B). Introduction of these plasmids into wild-type cells resulted in ~10-fold higher *lacZ* expression from both $P_{dinB(o^c)}$*lacZ* fusions when compared with wild-type P_{dinB}. This is in agreement with previous estimates of transcriptional induction of *dinB* during the SOS response

(COURCELLE *et al.* 2001; KIM *et al.* 2001). *lexA51*(Def) cells have no functional LexA repressor and show constitutive SOS expression (MOUNT 1977). We find that *lacZ* expression is increased in a *lexA51*(Def) strain when driven by the wild-type *dinB* promoter, but see no significant increase with the *dinB*(o^c) promoters, showing that levels of *dinB* transcription similar to that achieved by true SOS derepression are achieved by the *dinB*(o^c) mutations. The *lexA51*(Def) strain SMR5400 also carries a mutation in the *sulA* gene, which allows survival under constitutive SOS induction (MOUNT 1977), and a mutation in the F-encoded *psiB* gene, which has been shown to exert a negative effect on stress-induced mutagenesis (MCKENZIE *et al.* 2000) probably by affecting SOS induction (reviewed by COX 2007).

In the Lac-assay strains such as FC40 and SMR4562, *dinB* is present both in the chromosome and in the F′128, at which locus it is more highly expressed (KIM *et al.* 2001). Introduction of both *dinB*(o^c) alleles into the episomal *dinB* locus results in about five- to six-fold increased DinB-protein levels in stationary-phase cells compared with an otherwise isogenic SMR4562 derivative in both wild-type and *lexA3*(Ind$^-$) backgrounds (Figure 2). This indicates that both *dinB*(o^c) alleles are functional *in vivo*, conferring an increased basal *dinB* expression. Furthermore, both alleles confer levels of expression similar to those observed in *lexA51*(Def) cells (Figure 2), at least in the growth conditions used by us in the stress-induced mutagenesis experiments (cells grown for 48 hr in M9 B1 glycerol minimal medium). It was noted before (KIM *et al.* 2001) that expression of *dinB* in the F′128 plasmid is higher than that from the chromosomal *dinB*. Our finding that both *dinB*(o^c) alleles, when present only in the episome, increase DinB to levels similar to that observed in the *lexA51*(Def) strain (in which both the episomal and the chromosomal copy are constitutively highly expressed), also implies that the episomal expression is more pronounced than the chromosomal expression. To facilitate further strain construction and genetic analysis, we carried out the subsequent experiments in cells bearing a single *dinB*(o^c) allele in the F′128 plasmid.

***dinB*(o^c) mutations restore stress-induced point mutagenesis in SOS-off strains:** Because DinB is a key player in stress-induced mutagenesis, we wanted to examine whether *dinB* is the only gene required at SOS-induced levels for stress-induced point mutagenesis in the Lac assay. The SOS response is induced when DNA damage is sensed in the form of single-strand DNA (reviewed by FRIEDBERG *et al.* 2005). RecA binds the single-strand DNA and becomes active as a co-protease that facilitates cleavage of the LexA repressor, resulting in upregulation of the SOS genes, including *dinB*. To determine whether *dinB* upregulation constitutes the sole role of the SOS response in stress-induced point mutagenesis, we tested the effect of the *dinB*(o^c) alleles on *lac* reversion in both wild-type and *lexA3*(Ind$^-$) backgrounds. The *lexA3*(Ind$^-$) mutation creates an uncleavable LexA/SOS repressor such that derepression of the SOS response genes during an SOS response is prevented (MOUNT *et al.* 1972). Previously, this allele was shown to cause reduced stress-induced point mutagenesis in the Lac assay (CAIRNS and FOSTER 1991; MCKENZIE *et al.* 2000, 2001), indicating that one or more SOS-controlled genes are needed at induced levels for efficient stress-induced mutagenesis. Representative results from single experiments with each of the two *dinB*(o^c) alleles constructed are shown in Figure 3, A and B, and quantification of the stress-induced point mutagenesis rates from multiple experiments is shown in Figure 3C. Strikingly, either allele provides a complete suppression of the phenotype of the *lexA3*(Ind$^-$) strain. These results show that the reduced stress-induced mutagenesis in a *lexA3*(Ind$^-$) strain is caused specifically by the failure to upregulate *dinB*, and not any other gene in the LexA/SOS regulon. This finding places DinB as the central SOS-regulated protein in stress-induced mutagenesis and indicates that upregulation of other SOS genes such as *recA*, *ruvA*, and *ruvB* beyond their constitutive levels of expression is irrelevant.

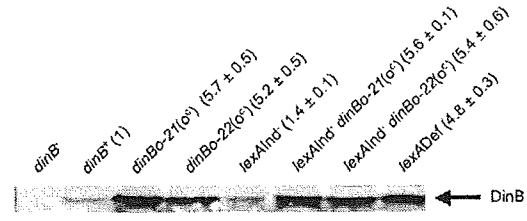

FIGURE 2.—DinB Western blots. Stationary-phase cells grown in M9 B1 glycerol medium were harvested and analyzed using a rabbit polyclonal DinB antiserum as described (MATERIALS AND METHODS). Values shown represent the average DinB protein levels relative to wild type determined in three independent experiments ± SEM. Similar results were obtained with Western blots performed with a DinB monoclonal antibody. Strains are the following: *dinB*, SMR5889; *dinB*$^+$, SMR10292; *dinBo-21*(o^c), SMR10308; *dinBo-22*(o^c), SMR10309; *lexA3*(Ind$^-$), SMR10760; *lexA3*(Ind$^-$) *dinBo-21*(o^c), SMR10761; *lexA3*(Ind$^-$) *dinBo-22*(o^c), SMR10762; and *lexA*(Def), SMR5400.

SOS-induced levels of DinB are not sufficient to increase stress-induced point mutagenesis: We note that providing SOS-induced levels of DinB to all cells, with the *dinB*(o^c) mutations, did not stimulate stress-induced mutagenesis above wild-type levels in the *lexA3*(Ind$^-$) strain (Figure 3 and legend), even though normally SOS is expected to be induced spontaneously in only ~1% of cells (PENNINGTON and ROSENBERG 2007). Neither did DinB overproduction increase mutagenesis in the wild-type genetic background (Figure 3 and legend). These results indicate that DinB upregulation by the SOS response, although required, is not sufficient to differentiate the mutating subpopulation; *dinB*(o^c) appears not to make all cells in the population

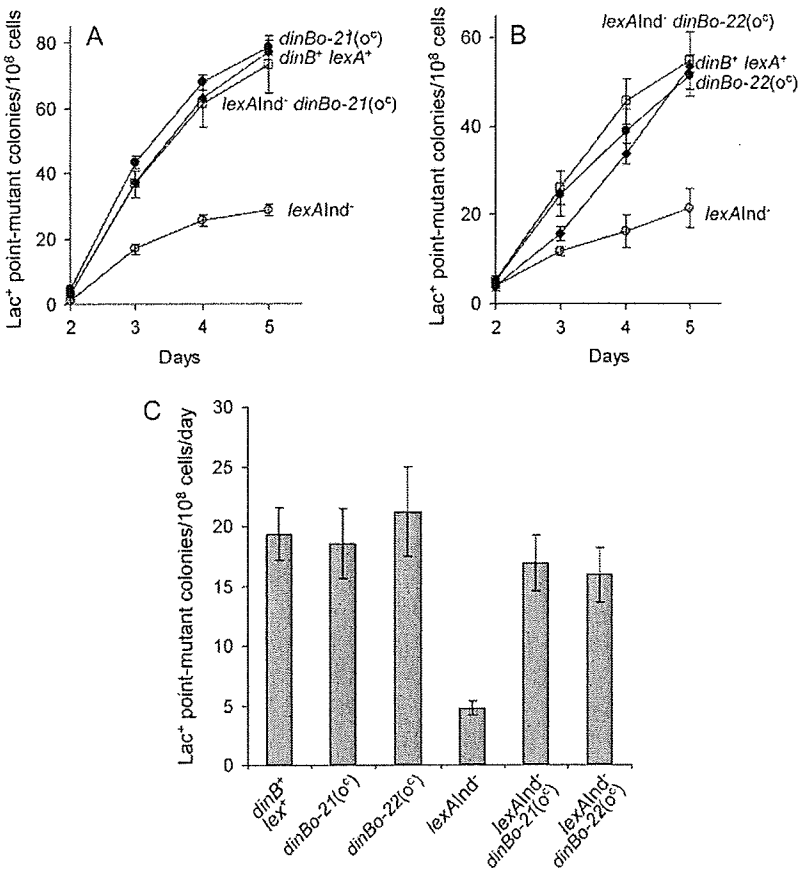

FIGURE 3.—Two *dinB* operator-constitutive alleles restore stress-induced Lac point mutagenesis to SOS-off *lexA*(Ind$^-$) cells. (A) Effect of the *dinBo-21*(o^c) allele in stress-induced mutagenesis: a representative experiment. (B) Effect of the *dinBo-22*(o^c) allele in stress-induced mutagenesis: a representative experiment. Note that, for both alleles, the stress-induced point mutagenesis-defective phenotype of *lexA3*(Ind$^-$) cells is fully suppressed; however, overproduction of DinB with these alleles does not stimulate mutagenesis above wild-type levels. Data represent means ± SEM for four cultures. Strains are the following: *dinB*$^+$ *lex*$^+$, SMR10292 (solid diamonds in A and B); *lexA*(Ind$^-$), SMR10760 (open circles in A and B); *dinBo-21*(o^c), SMR10308 (solid circles in A); *dinBo-22*(o^c), SMR10309 (solid circles in B); *lexA* (Ind$^-$) *dinBo-21*(o^c), SMR10761 (open squares in A); and *lexA*(Ind$^-$) *dinBo-22*(o^c), SMR10672 (open squares in B). (C) Quantification of stress-induced point-mutation rates from six independent experiments, each with all genotypes done in parallel. Strains were as above except that three experiments were performed in the *lexA3*(Ind$^-$) strains listed above, whereas an additional three experiments were performed, with similar results, in an independently constructed, identical set of *lexA3*(Ind$^-$) strains: *lexA*(Ind$^-$), SMR10314; *lexA*(Ind$^-$) *dinBo-21*(o^c), SMR10310; and *lexA*(Ind$^-$) *dinBo-22*(o^c), SMR10311. Rates represent the increase of Lac$^+$ point mutant colonies per day observed between days 3 and 5 of each experiment. Means ± 1 SEM are shown. *P*-values were obtained for pairwise comparisons by the nonparametric Mann–Whitney rank-sum test using the SYSTAT 11 statistics software by SYSTAT software and are as follows. The mutation rate of *dinB*$^+$ is not different from those of *dinBo-21*(o^c) ($P = 0.699$), *dinBo-22*(o^c) ($P = 0.699$), *lexA*(Ind$^-$) *dinBo-21*(o^c) ($P = 0.818$), or *lexA*(Ind$^-$) *dinBo-22*(o^c) ($P = 0.18$), but is significantly different from the rate of *lexA*(Ind$^-$) ($P = 0.002$), and the *lexA*(Ind$^-$) rate differs from those of *lexA*(Ind$^-$) *dinBo-21*(o^c) ($P = 0.002$) and *lexA*(Ind$^-$) *dinBo-22*(o^c) ($P = 0.002$).

mutable. This might reflect either of two possible realities. First, in principle, it could be possible that during stress-induced mutagenesis conditions all cells are SOS-induced such that providing an operator-constitutive *dinB* does not provide any more DinB protein than the population of cells already has, and so does not increase mutagenesis further. This is unlikely (discussed below). Second, and more likely, it could be that during stress-induced mutagenesis only a small fraction of cells is SOS induced, as is the case for growing cells in which $\sim 10^{-2}$ are (PENNINGTON and ROSENBERG 2007), but that in this cell subpopulation some other condition must be met to allow mutagenesis. For example, it is likely that possession of a DNA double-strand break at which the mutagenic repair occurs (PONDER *et al.* 2005) is also required such that DinB upregulation alone is not sufficient.

SOS induction of other genes in the *dinB* operon is irrelevant for stress-induced mutagenesis: *dinB* is part of a four-gene operon including *dinB*, *yafN*, *yafO*, and *yafP* (MCKENZIE *et al.* 2003). The functions of the three *yaf* genes are unknown. The whole operon, including the three genes downstream of *dinB*, is induced as part of the SOS response (COURCELLE *et al.* 2001). Thus, in the experiments described above, all three *yaf* genes were also upregulated by the operator-constitutive mutations in the *dinB* promoter. We show that the restoration of mutability to SOS-off *lexA*(Ind$^-$) cells conferred by the *dinB*(o^c) mutations was not conferred by increased *yafNOP* expression, only by increased *dinB* expression, because it also occurred in strains carrying a deletion of the *yafNOP* genes in *cis* with (downstream of) the *dinBo-21*(o^c) mutation in F′128 (Figure 4). Although intact *yaf* genes are present in the chromosome of this strain, they will be repressed by the *lexA*(Ind$^-$)-encoded uncleavable LexA/SOS repressor, such that only DinB is produced at SOS-induced levels in this strain. Therefore, *dinB* is indeed the only gene of the SOS regulon that is required at SOS-induced levels for stress-induced mutagenesis in the Lac assay. These experiments do not rule out a role for the *yaf* genes (expressed at uninduced levels) in mutagenesis, a topic that will be addressed in a future publication (L. SINGLETARY, J. GIBSON, E. TANNER, G. J. MCKENZIE, P. L. LEE and S. M. ROSENBERG, unpublished data).

The mechanism of stress-induced *lac* reversion in *dinB*(o^c) cells is similar to that in wild-type cells: The

results obtained show that the *dinB*(o^c) alleles are able to rescue completely the mutagenesis-defective phenotype of *lexA3*(Ind$^-$) (SOS-off) cells in stress-induced mutagenesis (Figure 3). This could result from restoration of the same stress-induced mutagenesis pathway and mechanism that operates in wild-type cells. Alternatively, it was possible that constitutive expression of *dinB* might activate a different mutagenesis mechanism that coincidentally yielded similar mutant frequencies in the course of several days. We provide two lines of support for the first possibility that the normal pathway and mechanism of stress-induced point mutagenesis was restored to *lexA3*(Ind$^-$) (SOS-off) cells by the *dinB*(o^c) mutations.

First, we find that the Lac-reversion-mutation sequences in *lexA3*(Ind$^-$) *dinB*(o^c) cells are indistinguishable from the characteristic point-mutation sequences seen normally in stress-induced point mutagenesis (in *lexA*$^+$ *dinB*$^+$ cells) (Figure 5A). The mutations are dominated by −1 deletions at mononucleotide repeats that occur preferentially in the same preferred hotspot sequences as observed in *lexA*$^+$ *dinB*$^+$ cells. This characteristic mutation sequence spectrum is highly specific and different from, for example, spontaneous reversions of this *lac* allele during growth, which are more heterogeneous, including −1 deletions not at mononucleotide repeats and larger frameshift-reverting additions and deletions in about half the mutations (Foster and Trimarchi 1994; Rosenberg *et al.* 1994). These results imply that mutations occur via a similar or the same mechanism in both genetic backgrounds, supporting the idea that the rescue of the *lexA3*(Ind$^-$) phenotype by the *dinB*(o^c) alleles restored the same mutagenesis mechanism that normally operates in *lexA*$^+$ *dinB*$^+$ cells.

Second, a hallmark of stress-induced mutagenesis in the Lac assay is its requirement for homologous-recombination, double-strand-break-repair functions, including *recA*, *recB*, and *ruvAB*, and *ruvC* (Harris *et al.* 1994, 1996; Foster *et al.* 1996; He *et al.* 2006), because the mutagenesis results from error-prone double-strand-break-repair events (Ponder *et al.* 2005). Similarly, we find that deletion of *ruvC* reduces stress-induced mutagenesis in *lexA3*(Ind$^-$) (SOS-off) cells carrying a *dinB*(o^c) allele (Figure 5B). Thus the mutagenesis restored to *lexA3*(Ind$^-$) cells by the *dinB*(o^c) mutation requires *ruvC*. This supports the conclusion that a similar or the same recombination-dependent mutagenesis pathway is operating in *lexA3*(Ind$^-$) *dinB*(o^c) cells as is normal in cells wild-type for *lexA* and *dinB*.

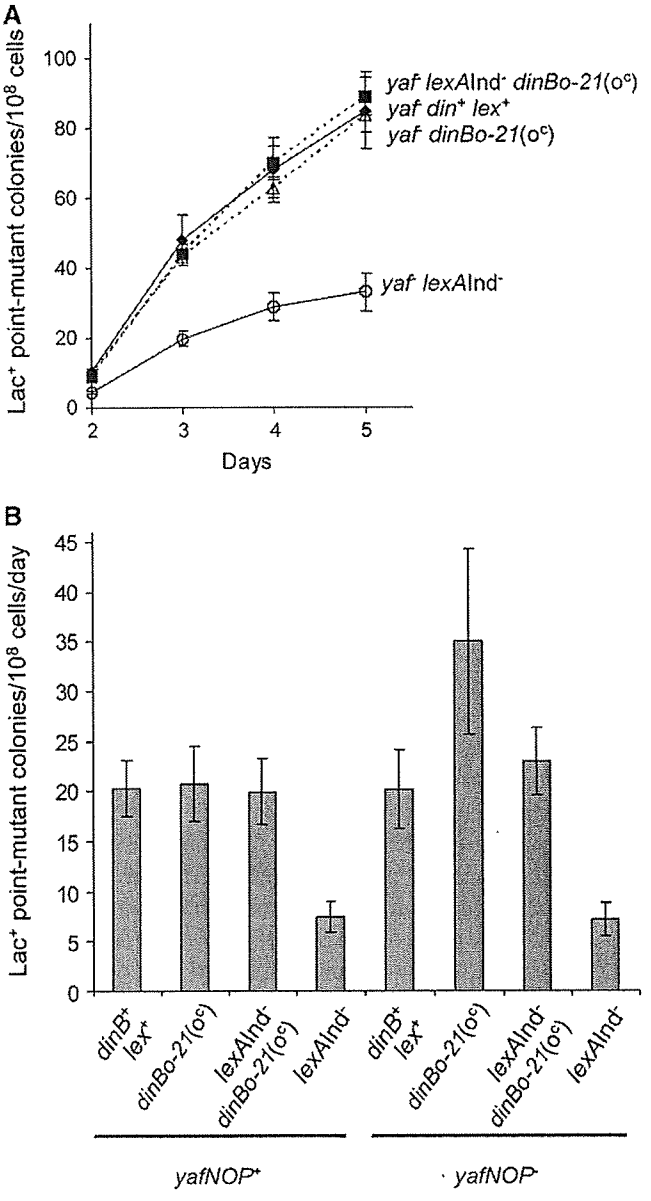

FIGURE 4.—Stress-induced mutagenesis proficiency in SOS-off *lexA3*(Ind$^-$) cells with a *dinB*(o^c) mutation does not require SOS induction of the *yafNOP* genes. (A) Representative experiment showing that deletion of the *yafNOP* genes in *cis* with *dinBo-21*(o^c) does not affect the ability of this promoter mutation to rescue the phenotype of SOS-off *lexA3*(Ind$^-$) cells. (B) Quantification of stress-induced point-mutation rates (calculated as in Figure 3) from four independent experiments. Means ± SEM are shown. *P*-values (calculated as in Figure 3) are as follows. For the *yaf*$^+$ background, the *dinB*$^+$ rate is not different from the rates observed with *dinBo-21*(o^c) (P = 0.886) or *lexA*(Ind$^-$) *dinBo-21*(o^c) (P = 0.686) but differs from that of *lexA*(Ind$^-$) (P = 0.029), and the rate of *lexA*(Ind$^-$) differs from that of *lexA*(Ind$^-$) *dinBo-21*(o^c) (P = 0.029). Similarly, for the *yaf*$^-$ background, the *dinB*$^+$ rate is not significantly different from the rates of *dinBo-21*(o^c) (P = 0.114) or *lexA*(Ind$^-$) *dinBo-21*(o^c) (P = 0.886) but differs from the *lexA*(Ind$^-$) rate (P = 0.029), and the *lexA*(Ind$^-$) rate differs from that of *lexA*(Ind$^-$) *dinBo-21*(o^c) (P = 0.029). Strains are the following: *dinB*$^+$, SMR10292; *lexA*(Ind$^-$), SMR10760; *dinBo-21*(o^c), SMR10308; *lexA*(Ind$^-$) *dinBo-21*(o^c), SMR10761; *yaf*$^-$ *dinB*$^+$ *lexA*$^+$, SMR11023 (solid diamonds); *yaf*$^-$ *dinBo-21*(o^c), SMR11024 (open triangles); *yaf*$^-$ *lexA*(Ind$^-$), SMR11026 (open circles); *yaf*$^-$ *lexA*(Ind$^-$) *dinBo-21*(o^c), SMR11027 (solid squares).

DISCUSSION

How stress responses confer temporal regulation of mutagenesis: The coupling of mutagenesis programs to

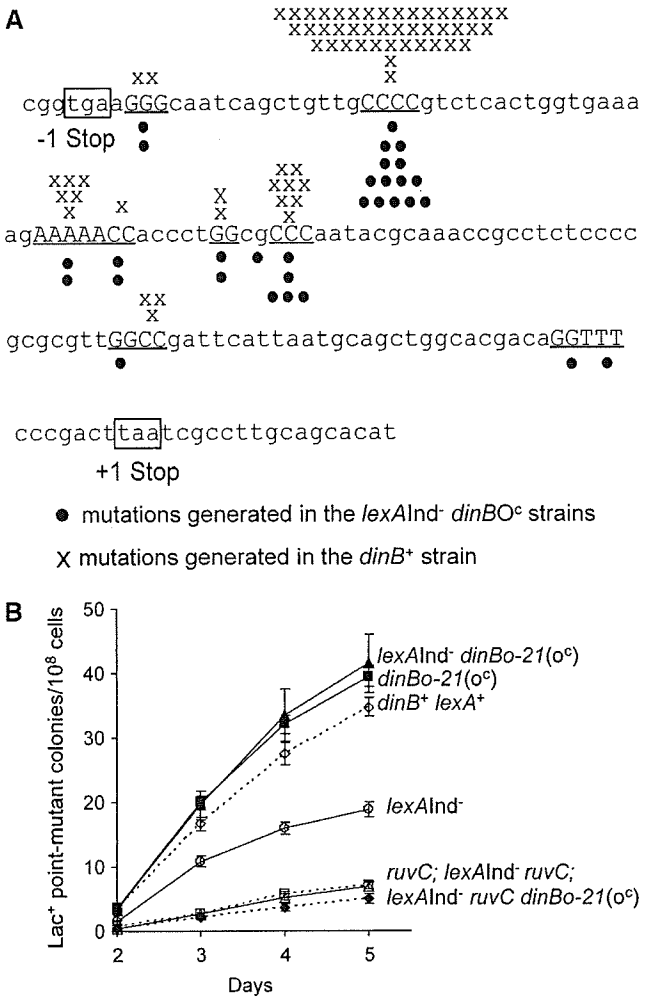

FIGURE 5.—Stress-induced mutagenesis in SOS-off *lexA3* (Ind$^-$) cells with *dinB*(o^c) alleles occurs via a mechanism similar to normal stress-induced mutagenesis in the Lac assay. (A) Sequences of Lac$^+$ mutations are the same in *lexA3*(Ind$^-$) *dinB*(o^c) strains and in *lexA*$^+$ *dinB*$^+$ cells. Both sets of reversion mutations are nearly all −1 deletions in mononucleotide repeats. The positions of the −1 deletions observed in the *lexA3* (Ind$^-$) *dinBo-21*(o^c) and *lexA3*(Ind$^-$) *dinBo-22*(o^c) strains (SMR10761 and SMR10762) are shown as circles, and the position of the −1 deletions observed in *lexA*$^+$ *dinB*$^+$ cells (data from FOSTER and TRIMARCHI 1994 and ROSENBERG *et al.* 1994) are marked as X's. The region shown is part of the *lacIZ* fusion gene present in these strains. Compensatory frameshift mutations in a 130-nt region between the two out-of-frame stop codons (boxed) can restore gene function. (B) Stress-induced mutagenesis promoted by the *dinBo-21*(o^c) allele in the *lexA3*(Ind$^-$) background requires RuvC. A representative experiment performed with four independent cultures of each strain is shown. Means ± SEM. This result was repeated twice. Strains are the following: *dinB*$^+$ *lex*$^+$, SMR10292 (open diamonds); *dinBo-21*(o^c), SMR10308 (solid squares); *lexA*(Ind$^-$) *dinBo-21*(o^c), SMR10761 (solid triangles); *lexA*(Ind$^-$), SMR10760 (open circles); *ruvC*, SMR10766 (open triangles); *lexA*(Ind$^-$) *ruvC*, SMR10768 (open squares); and *lexA*(Ind$^-$) *ruvC dinBo-21*(o^c), SMR10767 (closed diamonds).

cellular stress responses observed in bacterial and eukaryotic cells (reviewed in the Introduction and by GALHARDO *et al.* 2007) provides a temporal regulation of mutagenesis, limiting mutagenesis to times of stress. This may potentially accelerate genetic change, and thus the ability to evolve, specifically when cells and organisms are maladapted to their environments, *i.e.*, are stressed. Here we demonstrate that, in the case of the *E. coli* Lac assay, the requirement for the SOS stress response can be deconvoluted to the need for induction of one specific gene, *dinB*. A number of other stress responses have been shown to upregulate mutagenesis, such as the RpoS response in *E. coli*, Salmonella, and Pseudomonas; the stringent response in *E. coli* and *B. subtilis*; the competence response of *B. subtilis*; and two human responses to hypoxic stress (see Introduction). All of these modulate the expression of tens to hundreds of different genes. It is not yet known whether any other of these stress responses can be narrowed down to relevant effects on one or a few genes.

Roles of SOS in other stress-induced mutagenesis mechanisms: The SOS response is a major upregulator of mutagenesis during stress conditions but may not function identically in each case. For example, the SOS response is required for phage λ untargeted mutagenesis (ICHIKAWA-RYO and KONDO 1975), stress-induced point mutagenesis in *E. coli* in the Lac assay (MCKENZIE *et al.* 2000), ciprofloxacin (antibiotic)-resistance mutagenesis induced by exposure to ciprofloxacin (CIRZ *et al.* 2005), mutagenesis in aging colonies in a laboratory *E. coli* strain (TADDEI *et al.* 1995), and bile-resistance mutagenesis in Salmonella (PRIETO *et al.* 2006). Although DinB is required for λ untargeted mutagenesis (KIM *et al.* 1997) and for Salmonella bile-resistance mutagenesis (PRIETO *et al.* 2006), it is not yet known whether the SOS requirement in either case is based on DinB upregulation. In the ciprofloxacin-induced mutagenesis, the SOS-controlled DNA polymerases DinB, Pol II, and Pol V are all required, as are the double-strand-break-repair genes, including SOS-regulated *recA*, *ruvA*, and *ruvB* (CIRZ *et al.* 2005). Part of this mutation pathway's requirement for SOS is likely to be for production of DNA Pol V (CIRZ *et al.* 2005) because this polymerase is required for mutagenesis and is produced virtually not at all without an SOS response (reviewed by NOHMI 2006). It is not yet known whether upregulation of any, all, or none of the other two SOS-controlled DNA polymerases additionally account for the requirement for an SOS response for ciprofloxacin-induced mutagenesis. Conversely, in the Lac assay, we measure frameshift reversion, which DinB promotes but Pols II and V do not, whereas the ciprofloxacin-induced mutations are base substitutions, which all three SOS polymerases promote (CIRZ and ROMESBERG 2007). CIRZ and ROMESBERG (2007) have pointed out that the mutagenesis pathway in the Lac system might be

identical to that in ciprofloxacin-resistance mutagenesis and might also require Pols II and V if base substitution mutations were assayed. In a different assay system, mutagenesis in aging colonies required an SOS response, did not require DinB, and required Pol I instead (Taddei *et al.* 1997). Therefore, in the mutagenesis mechanism operating during that stress, the SOS requirement must be for some other function. Thus, although the SOS response is required for multiple examples of stress-inducible mutagenesis, its means of promoting mutagenesis in at least some of these different stress circumstances is different.

SOS induction of DinB is necessary but not sufficient for stress-induced mutability and for differentiation of a hypermutable cell subpopulation: Several lines of evidence indicate that although SOS induction of DinB is necessary, it is not sufficient for creating the transient mutable state in which most Lac reversions occur (Gonzales *et al.* 2008). Rather, the evidence also supports a model in which at least three events must occur: (1) a double-strand break and its repair, (2) SOS induction, and (3) induction of the RpoS stationary-phase and general stress response. The concerted induction of these stress responses in cells bearing double-strand DNA ends (DSEs) is proposed to differentiate the hypermutable subpopulation, as depicted in Figure 6A.

The first evidence for this model comes from experiments in which DNA double-strand breaks were delivered to the DNA by expression of the I-*Sce*I double-strand endonuclease *in vivo* (Ponder *et al.* 2005). DinB-dependent, stress-induced mutagenesis was stimulated >1000-fold near the DSBs, but only weakly (3-fold) in a another DNA molecule (with no DSB) in the same cell. SOS is induced robustly by I-*Sce*I-induced DSBs (Pennington and Rosenberg 2007). Therefore, these results indicate that the SOS-mediated DinB upregulation caused by I-*Sce*I-mediated DNA cleavage was not sufficient for mutagenesis. A DSB was also required locally. The mutations appear to occur in acts of error-prone DSB repair (Ponder *et al.* 2005). Figure 6B shows a model for such mutagenic double-strand-break repair occurring in stressed cells.

Second, RpoS is required for stress-induced Lac reversion (Layton and Foster 2003; Lombardo *et al.* 2004). Moreover, in the study using I-*Sce*I-induced DSBs (Ponder *et al.* 2005), the DinB-dependent DSB-associated mutagenesis was provoked only in cells that either were in stationary phase or were expressing the RpoS stationary-phase and general stress-response transcriptional activator inappropriately during log phase. Again, because the SOS response is induced efficiently by I-*Sce*I-mediated double-strand breakage (Pennington and Rosenberg 2007), this implies that repair of a DSB under the influence of the SOS response is also not sufficient for DinB-dependent mutagenesis; RpoS must also be induced. Additionally, although some kinds of

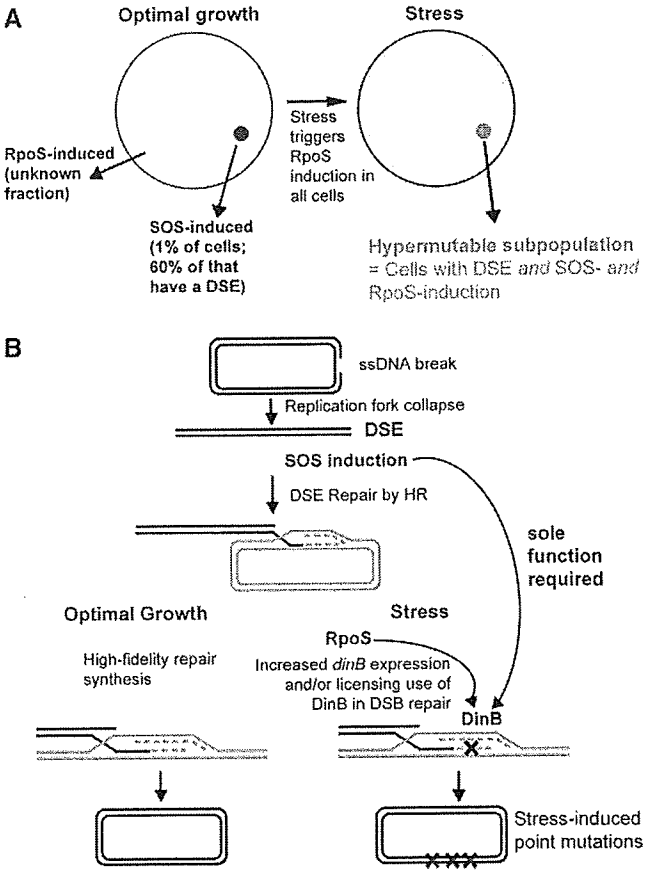

Figure 6.—Models for the role of SOS induction and DinB in differentiation of a hypermutable cell subpopulation and double-strand-break-repair-associated mutagenesis during stress. (A) Venn-diagram model of cell subpopulations that overlap to produce a transiently hypermutable cell subpopulation. In this model (modified from Galhardo *et al.* 2007), differentiation of a HMS (green) is proposed to occur when three conditions are met: (1) induction of the SOS response in cells bearing (2) a double-strand break or a DSE (blue) and (3) induction of the RpoS regulon (yellow) by suboptimal growth conditions. During stress, all cells induce RpoS and, we suggest, the fraction of cells bearing both a DSE and SOS induction [$\sim 6 \times 10^{-3}$ of growing cells (Pennington and Rosenberg 2007)] might remain roughly constant. In this model, the *dinB*(o^c) mutations, which upregulate DinB in all cells, would not increase HMS size because the DSEs are not available in all cells. (B) Mutagenic double-strand-break repair during stress. Ponder *et al.* (2005) showed that repair of a double-strand break is a high-fidelity, nonmutagenic process in unstressed cells (left) but switches to a mutagenic mode during stress under the control of the RpoS general stress response (right). This process requires a DSE and its repair; induction of the SOS response (McKenzie *et al.* 2000), which we show here, is solely to provide DinB upregulation, and induction of the RpoS regulon (Ponder *et al.* 2005). Some as yet-unknown function regulated by the RpoS response licenses the use of DinB in those conditions. This function could be the documented increase in DinB expression (Layton and Foster 2003) or the induction of another regulatory factor, or a combination of both. ssDNA, single-strand DNA; HR, homologous recombination; X's, DNA polymerase errors/mutations; parallel lines, double-strand DNA; dashed lines, newly synthesized DNA strands.

DNA damage can induce RpoS (Merrikh *et al.* 2009), if it occurs in these experimental conditions, damage induction of RpoS appears not to be sufficient for mutagenesis; another RpoS-inducing input must contribute.

Finally, in growing cells, only ~1% of cells are SOS induced spontaneously (Pennington and Rosenberg 2007), and in this study we observed that making every cell in the population experience SOS-induced levels of DinB production, using the *dinB*(o^c) alleles, did not increase stress-induced mutagenesis in otherwise wild-type cells (Figure 3). This could mean either of two things:

1. Unlike growing cells, in stationary phase, all of the cells are already SOS induced, and so the *dinB*(o^c) alleles do not change the number of cells expressing DinB or the DinB levels in most cells. This possibility is unlikely, given the large increase in DinB levels that we observed with the *dinB*(o^c) alleles measured in stationary-phase cells (Figure 2).
2. More plausibly, the data imply that, even though the *dinB*(o^c) alleles confer SOS-induced levels of DinB to all cells, this is not sufficient for mutagenesis. These data support the model in which a DNA double-strand break and its repair, an RpoS response, and an SOS response (shown to act solely via DinB production) are all required for stress-induced point mutagenesis (Figure 6, A and B). We note that if all cells had become hypermutable when DinB was overproduced in all cells, mutation rate would have been higher than normal because the hypermutable cells would no longer be a small subpopulation. This was not observed (Figures 3 and 4).

Role of SOS/DinB in a hypermutable cell subpopulation: We previously suggested a model for the origin of the hypermutable cell subpopulation that appears to underlie most double-strand-break-repair-associated stress-induced point mutagenesis (Gonzales *et al.* 2008) on the basis of three requirements for stress-induced mutability discussed above: a genomic DSB/DSE (and its repair), an SOS response, and an RpoS response (Galhardo *et al.* 2007). The simultaneous occurrence of these three events is proposed to differentiate the hypermutating cells. It is unknown what fraction of the cells in a stationary population experience an SOS response, but ~1% of the cells in log-phase cultures display spontaneous SOS induction, ~60% of those (~6×10^{-3}) due to a spontaneous DSB/DSE (Pennington and Rosenberg 2007). When these cells enter the stationary phase, RpoS is likely to be induced in all of them (Hengge-Aronis 2002). Thus, if the numbers for growing cells hold, then the ~6×10^{-3} of cells with a DSB and an SOS response would become the HMS when RpoS induction occurred in the whole population in stationary phase (Figure 6A). We can now refine this model to note that the sole component of the SOS response required would be DinB upregulation.

The additional requirement for RpoS—to license the use of DinB in error-prone DSB/DSE repair (shown by Ponder *et al.* 2005)—could be based either solely on RpoS upregulation of DinB or on RpoS-controlled expression of other factors that permit DinB use (Figure 6B). The SOS and RpoS responses increase DinB expression ~10-fold and 2- to 3-fold, respectively (Kim *et al.* 2001; Layton and Foster 2003). The identities of potential DinB-licensing factors in the RpoS regulon are not yet known. This control would provide a restriction of the mutagenesis to periods of stress, and only to those few cells with a DSB/DSE. The restriction of mutagenesis to a cell subpopulation may allow clonal populations to hedge their bets during adaptation to changing environments, both conserving the original genome sequence, which is well adapted to the previous environment and useful if resources become available again suddenly, and simultaneously exploring the new adaptive landscape in the subpopulation.

Regulation of DinB mutator activity: In many other assay systems in which DinB-dependent mutagenesis has been observed, stress responses other than, or in addition to, SOS are required. In Salmonella bile-induced resistance mutagenesis, which is DinB dependent, the SOS and RpoS responses are required (J. Casadesus, personal communication, and Prieto *et al.* 2006). In *B. subtilis* starvation-associated mutagenesis, the ComK competence stress response is required for the DinB-dependent mutagenesis (Sung and Yasbin 2002). In *E. coli*, β-lactam antibiotics induce *dinB* transcription independently of SOS (Perez-Capilla *et al.* 2005). *P. putida* DinB-dependent, stress-induced mutagenesis requires RpoS (Saumaa *et al.* 2002). It is not known whether any stress response other than SOS is required for DinB-dependent, ciprofloxacin-induced resistance mutagenesis (Cirz *et al.* 2005). Thus, it is plausible that DinB-dependent mutagenesis might usually require more than one stress-response input to occur. Although effects of DinB in SOS mutagenesis of *E. coli* (Kuban *et al.* 2006) have been observed, it is not known whether the DinB-dependent mutations may have arisen in cells also induced for RpoS or another stress response simultaneously.

What factors modulate DinB mutator activity? As a translesion DNA polymerase that inserts bases opposite several otherwise replication-blocking DNA adducts, DinB performs this role mostly in a high-fidelity fashion (Jarosz *et al.* 2006; Bjedov *et al.* 2007; Godoy *et al.* 2007). This, and the existence of *dinB* mutations that separate translesion from mutagenic functions (Godoy *et al.* 2007), imply that mutator activity occurs during synthesis that is not part of translesion synthesis (although it does occur during DSB repair; Ponder *et al.* 2005). Alternatively, DinB mutator activity could be taking place at sites of yet-unidentified DNA lesions.

A recent study implicated the SOS-induced UmuD protein as a candidate to inhibit DinB mutagenic

potential during SOS induction (Godoy *et al.* 2007). UmuD is produced virtually only during an SOS response (Courcelle *et al.* 2001). Consequently, UmuD is not expected to be present in *lexA*(Ind$^-$) *dinB*(o^c) cells, but stress-induced mutation rates were similar to those in wild-type cells (Figures 3 and 4). Therefore, UmuD appears not to inhibit DinB in its role in stress-induced point mutagenesis. Other levels of control are likely to exist.

One case of strongly DinB-dependent mutagenesis thought to occur with only one stress-response input in physiological conditions is SOS-mediated untargeted mutagenesis of phage λ (Kim *et al.* 1997). Under those conditions, mutagenesis of the phage DNA is heavily dependent on DinB, presumably relying on the physiological levels of *dinB* expression achieved *in vivo* during SOS induction. It is interesting to note that extensive double-strand-end-initiated recombination between the multiple copies of the phage DNA is expected to occur during a lytic cycle (Thaler and Stahl 1988). Those might be the sites of mutagenic action of DinB. Nevertheless, it is not known whether other factors, λ or host encoded, might also play a role in that process. Another example is the recent finding of increased DinB-dependent mutagenesis in cells lacking the ClpXP protease (Al Mamun and Humayun 2009). RpoS does not seem to be involved in the observed mutagenesis. However, the lack of a major protease is likely to have many pleiotropic effects, and there remains the possibility that other responses, which foster DinB activity, are triggered in those cells.

Evolution of stress-induced mutagenesis pathways: The occurrence of many different molecular mechanisms of stress-inducible mutagenesis (reviewed in the Introduction and by Galhardo *et al.* 2007), and even of mechanisms by which a single stress response such as SOS promotes mutagenesis under different stress circumstances, suggests that these mutagenesis programs have evolved many times at least somewhat independently. This is also suggested by a survey of starvation-stress-inducible mutability in 787 *E. coli* natural isolates (Bjedov *et al.* 2003). Those authors found that whereas >80% of the natural isolates displayed stress-inducible mutator activity, the ability to do so correlated well with ecological niche and poorly with strain phylogeny, suggesting multiple recent evolutions of the stress-inducible mutagenesis pathways. We have suggested that stress-inducible mutagenesis mechanisms are both somewhat varied and recently acquired because they confer a benefit to cells that is under periodic (alternating positive and negative) second-order selection (Galhardo *et al.* 2007). That is, these pathways are useful and selected in changing environments in which adaptation is promoted by mutability and responsiveness and superfluous and perhaps costly in static environments. Despite the variability and potential multiple origins, the basic themes of the regulation of mutability temporally by stress responses, and potentially spatially in genomes (Galhardo *et al.* 2007), are widespread and appear to be potentially important evolutionary strategies.

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DinB Upregulation Is the Sole Role of the SOS Response in Stress-Induced Mutagenesis in *Escherichia coli*

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FILE S1

Primers used in this study

yafNwL:
5'TGTATATTCTGGTGTGCATTATTATGAGGGTATCACTGTATGCATCGAATTATTCCGGGGATCCGTCGACC3'

yafPwR
5'ATACCAGGCGGGCGTTATTTTCATTGCAAGCTGGATTTAATGTTGCGGTTTTGTAGGCTGGAGCTGCTTC3'

CatupdinB-F:
5'GAAGCGAATCTGGAGATGGAGATTGTTCCCCAGGGATTACGCGTGGTGTAGGCTGGAGCTGCTTC3'

CatupdinB-R:
5'CGCGCTGCCGCGTTCAAACATATTGCGGTTCTGGTCGTCTTTAATCATATGAATATCCTCCTTAG3'

dinBcatnock-R: 5' GATACCCTCATAATAATGC 3'

kandinBchrom-F:
5'CGCCACCGAGCTTGGTGAGCTGGCAACCAGTATCAACACCATTGCGTGTAGGCTGGAGCTGCTTC3'

DinBRCAT:
5'GTGATACCCTCATAATAATGCACACCAGAATATACATAATAGTATCATATGAATATCCTCCTTA 3'

dinBOC1F: 5' CCCTGAAATCAAGGTATACTTTAC 3'

dinBOC1R: 5' GTAAAGTATACCTTGATTTCAGGG 3'

dinBOC2F: 5' GTATACTTTACCCTTGTTGAGAGGT 3'

dinBOC2R: 5' ACCTCTCAACAAGGGTAAAGTATAC 3'

For site directed mutagenesis, each of these two complementary primer pairs (last four primers listed) were used to amplify *dinB* fragments with the desired mutations in the borders. PCR amplification with external primers and both fragments as templates was used to generate full length products.

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The Steric Gate Amino Acid Tyrosine 112 Is Required for Efficient Mismatched-Primer Extension by Human DNA Polymerase κ†

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ABSTRACT: Human DNA is continuously damaged by exogenous and endogenous genotoxic insults. To counteract DNA damage and ensure the completion of DNA replication, cells possess specialized DNA polymerases (Pols) that bypass a variety of DNA lesions. Human DNA polymerase κ (hPolκ) is a member of the Y-family of DNA Pols and a direct counterpart of DinB in *Escherichia coli*. hPolκ is characterized by its ability to bypass several DNA adducts [e.g., benzo[*a*]pyrene diolepoxide-N^2-deoxyguanine (BPDE-N^2-dG) and thymine glycol] and efficiently extend primers with mismatches at the termini. hPolκ is structurally distinct from *E. coli* DinB in that it possesses an ~100-amino acid extension at the N-terminus. Here, we report that tyrosine 112 (Y112), the steric gate amino acid of hPolκ, which distinguishes dNTPs from rNTPs by sensing the 2′-hydroxy group of incoming nucleotides, plays a crucial role in extension reactions with mismatched primer termini. When Y112 was replaced with alanine, the amino acid change severely reduced the catalytic constant, i.e., k_{cat}, of the extending mismatched primers and lowered the efficiency, i.e., k_{cat}/K_m, of this process by ~400-fold compared with that of the wild-type enzyme. In contrast, the amino acid replacement did not reduce the insertion efficiency of dCMP opposite BPDE-N^2-dG in template DNA, nor did it affect the ability of hPolκ to bind strongly to template-primer DNA with BPDE-N^2-dG/dCMP. We conclude that the steric gate of hPolκ is a major fidelity factor that regulates extension reactions from mismatched primer termini.

The human genome is continuously exposed to a variety of genotoxic agents such as polycyclic aromatic hydrocarbons, ultraviolet light, and reactive oxygen species (*1*). To counteract genotoxic insults, cells possess a number of defense strategies that enable them to complete chromosome replication and maintain the integrity of the genome. One of these strategies is translesion DNA synthesis (TLS)[1] accomplished by specialized DNA polymerases (Pols) (*2*, *3*). These Pols can bypass a variety of DNA lesions, which would otherwise block DNA replication, to ensure the continuity of chromosome replication. Human cells possess more than 14 Pols, and approximately half of them are involved in TLS across DNA lesions, repair of DNA damage, or both (*4*, *5*). In particular, Y-family Pols play major roles in the damage tolerance process by carrying out error-free TLS, although in some cases they mediate error-prone TLS, which can result in mutagenesis, carcinogenesis, and genetic diversity (*6*–*8*).

Of the four human Y-family Pols, i.e., Polκ, -η, and -ι and REV1, Polκ is unique in that its orthologues are present not only in Eukarya but also in bacteria and Archaea (*9*–*11*). The *Escherichia coli* orthologue is DinB or Pol IV, which bypasses several DNA lesions, such as N^2-guanine adducts of benzo[*a*] pyrene diolepoxide (BPDE-N^2-dG), in an error-free manner (*12*, *13*). It is also active in spontaneous mutagenesis in λ phage and in stationary-phase *E. coli* cells (*14*–*17*). Like DinB, hPolκ bypasses BPDE-N^2-dG in an error-free manner by incorporating dCMP opposite the lesion (*18*–*23*) and correctly mediates bypass DNA synthesis across other lesions, e.g., thymine glycol, at least in vitro (*24*). hPolκ is also distinct from other human Y-family Pols or even from *E. coli* DinB in that it efficiently extends

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[1]Abbreviations: Pol, DNA polymerase; hPolκ, human DNA polymerase κ; BPDE-N^2-dG, benzo[*a*]pyrene diolepoxide-N^2-deoxyguanine; Y112, tyrosine 112; TLS, translesion DNA synthesis; rNTP, ribonucleotide triphosphates; F12, phenylalanine 12; PCR, polymerase chain reaction; 8-OH-dG, 8-hydroxyguanine; O^6-MedG, O^6-methylguanine; O^4-MedT, O^4-methylthymine; MOE, molecular operating environment; K_d, equilibrium dissociation constant; HNE-dG, *trans*-4-hydroxy-2-nonenal-dG.

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primers with terminal mismatches (*25*). hPolκ extends mismatched primers with a frequency of $10^{-1}-10^{-2}$, which is more than 10 times greater than that seen with hPolη or *E. coli* DinB. Thus, hPolκ may play a role in TLS in vivo as an extender of mismatched primers generated by other Pols (*2*). Structural analysis of the catalytic core of hPolκ suggests that the N-terminus, approximately 75 amino acids that are absent in bacterial and archaeal counterparts (Figure 1 of the Supporting Information), forms an "N-clasp" domain that enhances the binding of hPolκ to DNA (*26*). Despite distinct biochemical and structural characteristics, the amino acids in the vicinity of the terminal base in primers are similar to those of other family members. Therefore, the exact mechanism by which hPolκ mediates efficient mismatch extension reactions remains unresolved.

In this study, we examined the role of tyrosine 112 (Y112), the steric gate amino acid of hPolκ, in TLS and extension reactions from mismatched primer termini. The steric gate amino acid is the residue that distinguishes dNTPs from rNTPs by sensing the 2′-hydroxy group of rNTPs (*27*, *28*). We have previously demonstrated that the steric gate of the Archaea orthologue of hPolκ, i.e., phenylalanine 12 (F12) of *Sulfolobus acidocaldarium* (*Sac*) Pol Y1 (also known as DBH), plays a role in fidelity during DNA synthesis by regulating the efficiency with which oxidized dNTPs are incorporated into DNA (*29*). Both F13, the steric gate amino acid of *E. coli* DinB, and F12 of DBH were also shown recently to play important roles in TLS across N^2-deoxyguanine−DNA adducts (*12*). Here, we report that the steric gate amino acid of hPolκ is critical for mismatch extension reactions. When Y112 is replaced with alanine (A), the catalytic efficiency of extension from mismatched DNA substrates decreases more than 400-fold. Unlike in the bacterial and archaeal orthologues, the steric gate is unimportant in regulating correct counterbase insertion opposite N^2-deoxyguanine−DNA adducts. We suggest that the steric gate amino acid might have evolved into a major fidelity factor regulating mismatch extension reactions when the ancestral *E. coli* DinB- and archaeal DBH-type proteins developed the extended N-terminal domain. We propose a possible mechanism by which hPolκ extends mismatched primer termini depending on the steric gate.

EXPERIMENTAL PROCEDURES

More detailed experimental protocols are available in the Supporting Information.

Construction of the hPolκ Overexpression Vector. The hPolκ gene (NCBI GenBank POLK entry accession number XM_003930.2) was amplified by polymerase chain reaction (PCR) from the Clontech human testis large insert cDNA library. The amplified fragments were digested with *Nco*I and *Bam*HI, and the resulting fragment was ligated into similarly digested vector pYG8582, which is the same as pET-16b (Novagen) but has the translational DB (downstream box) enhancer (*30*). The resulting plasmid pYG8583 carries the coding sequence for N-terminal 10-His-tagged full-length hPolκ. To construct a C-terminally truncated hPolκ expression vector, a synthetic linker was ligated between the *Xba*I and *Bam*HI sites of pYG8582. This construct was then digested with *Xba*I and *Avr*II, and after dephosphorylation of both ends, the digested plasmid was ligated to the *Xba*I fragment of pYG8583, which carried the N-terminal portion of the hPolκ coding sequence. The resulting construct, overexpressed C-terminally truncated 10-His-tagged hPolκ$_{1-559}$, was named pYG8591. We refer to it as hPolκ throughout this study.

Construction of Mutant hPolκ Overexpression Vectors. The Y112A mutant of hPolκ was made from pYG8591 by site-directed mutagenesis using PCR. An amino acid change of Y112 to valine (V) was introduced by the same method. Overexpression vectors for Y112A and Y112V were named pYG331 and pYG332, respectively.

Overexpression and Purification. To express the wild-type and mutant proteins, plasmids pYG8591, pYG331, and pYG332 were transformed into Rosetta competent cells (Novagen), and expression was induced by adding IPTG. The harvested cells were resuspended in BugBuster lysis buffer (Novagen), and soluble proteins were collected by centrifugation. hPolκ and the mutant proteins were purified by binding to BD TALON Superflow resin (BD Biosciences) and eluted in accordance with the manual provided by BD Biosciences. The eluted proteins were further purified by gel filtration, followed by ion exchange chromatography (HiTrap Heparin HP, GE Healthcare) using an FPLC system (AKTAexplorer 10S, GE Healthcare). The purified proteins were stored at −80 °C.

Primer Extension Assay. Standard polymerase reactions (10 μL) were performed in 40 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 10 mM DTT, 0.1 mg/mL BSA, 60 mM KCl, 2.5% glycerol, and 250 μM dNTPs. To this reaction mixture were added annealed oligonucleotides (100 nM), consisting of primers (14mers, 18mers, or 19mers) and template 5′-GC GCGCTTCTGGCCAATXGCAGAATTCCTAGGGAAG-3′ (36mer), where X represents lesions, i.e., BPDE-N^2-dG, 8-hydroxyguanine (8-OH-dG), O^6-methylguanine (O^6-MedG), O^4-methylthymine (O^4-MedT), or thymine glycol, at position 19. If no lesions were present, X represents dG. The 36mer templates containing the lesions were synthesized by Japan BioServices Co. (Saitama, Japan), except for BPDE-N^2-dG. The template DNA bearing BPDE-N^2-dG, i.e., (−)-*trans-anti*-benzo[*a*]pyrene diol epoxide adduct at N^2-dG, at position 19 of the 36mer DNA was synthesized as reported previously (*31*). All other oligonucleotides were synthesized by BEX Corp. (Tokyo, Japan) and double purified by high-performance liquid chromatography. The primers were labeled with Cy3 at the 5′-terminus; the exact sequences are shown in Table 1 of the Supporting Information and in the top part of Figures 1, 2, and 4. Wild-type hPolκ, Y112A, or Y112V was added at a concentration of 40, 10, or 5 nM, respectively, in the experiments shown in Figures 1, 2, and 4, followed by incubation for 15 min (Figures 1 and 2) or 20 min (Figure 4) at 37 °C. When ribonucleotide incorporation was assayed, rNTP instead of dNTP was included in the reaction mixtures at concentrations of 0, 50, 100, 250, or 500 μM. After the reactions were terminated, the products were resolved by electrophoresis on a 15% polyacrylamide gel and visualized with the Molecular Imager FX (Bio-Rad). The band intensities were quantified with Quantity One (Bio-Rad).

Steady-State Kinetic Analyses. The constituents of the reaction mixtures were the same as those in the primer extension assay except that only one dNTP was included and the protein concentrations were 5 nM. The reactions were initiated by adding dNTP. Both the concentration of dNTP and the incubation time were varied depending on the DNA substrate (matched or mismatched primer termini with or without lesions in the template). The products were resolved by electrophoresis on a 15% polyacrylamide gel, and the band intensities were quantified as described in the primer extension assay. Less than 20% of the

primers were extended in the steady-state kinetic analyses, ensuring single-hit kinetics. For each DNA substrate, the rate of incorporation was plotted as a function of dNTP concentration, and the V_{max} and K_m values were determined by Enzyme Kinetics Module 1.1 of SigmaPlot 2001 software (SPSS Inc., Chicago, IL). k_{cat} was calculated by dividing V_{max} by the enzyme concentration. All values are means ± standard errors from three experiments.

DNA Binding BIAcore Assay. The DNA binding assay was performed using the BIAcore3000 instrument (Biacore). The oligonucleotides that were used were basically the same as those used in the primer extension assay, except that primers were biotinylated at their 5′-termini to enable immobilization. The equilibrium dissociation constants (K_d) were calculated from the kinetic traces using BIAevaluation version 4.0 (Biacore) and employing local fitting according to the "1:1 binding with drifting baseline" predefined model.

Molecular Modeling. To visualize the relative position between Y112 or A112 and mismatched primer termini, active sites of wild-type hPolκ and its derivative Y112A complexed with a G:T mismatch and dATP were modeled on the basis of the reported crystallographic structure [Protein Data Bank entry 2OH2 (*16*)] using the 2007.09 version of the Molecular Operating Environment (MOE) (Chemical Computing Group Inc., Montreal, QC). Sequences of the template and primer were replaced with those which were applied to the experiments presented here, i.e., 3′-TCCTTAAGACGGTAA-5′ and 5′-AGGAATTCTGCT-3′, where G and T represent the mismatched template and primer bases, respectively. The incoming dTTP in the original structure was replaced with dATP, and Y112 was replaced with alanine in the model of Y112A. After all replacements, the total potential energy of the complex was minimized under the conditions where all coordinate sets of hPolκ were fixed.

RESULTS

Y112 Is the Steric Gate Amino Acid of hPolκ. By aligning the amino acid sequences of various Y-family Pols, we postulated that Y112 of hPolκ was the steric gate amino acid. To explore this possibility, Y112 was substituted with A or V; the resulting proteins and the wild-type enzyme (Figure 1 of the Supporting Information) were subjected to primer extension assays with rNTPs and dNTPs. The truncated form of hPolκ consisting of the 559 N-terminal residues is far more stable than the full-length protein and was therefore used throughout the study. In the presence of four rNTPs, the Y112A and Y112V proteins efficiently incorporated rNTPs and extended the primer, while the wild-type enzyme did not (Figure 1). In the presence of dNTPs, all three proteins incorporated dNTPs and extended the primers. These results clearly indicate that Y112 functions as the steric gate of hPolκ.

Translesion Activity of hPolκ Y112A and Y112V. To examine whether the steric gate residue of hPolκ is required for TLS across lesions, we performed in vitro primer extension assays using Y112A, Y112V, and the wild-type enzyme (Figure 2). The DNA lesions we analyzed were BPDE-N^2-dG, 8-OH-dG, O^6-MedG, O^4-MedT, and thymine glycol. hPolκ more efficiently bypasses the (−)-*trans-anti*-BPDE-N^2-dG adduct than the (+)-*trans-anti*-BPDE-N^2-dG adduct (*32*). To examine the effects of the amino acid substitution of Y112 on the bypass activities carefully, we employed the (−)-*trans-anti*-BPDE-N^2-dG

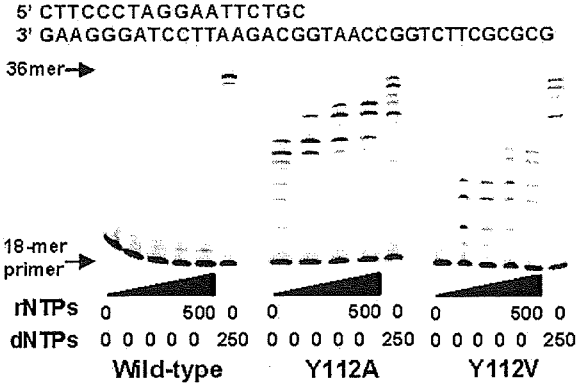

FIGURE 1: Incorporation of rNTPs by wild-type and mutant hPolκ proteins was analyzed by a primer extension assay using Cy3-labeled 18mer primers annealed to 36mer templates (i.e., 18/36G in Table 1 of the Supporting Information). The primer-template DNA (0.1 μM) was incubated with 40 nM wild type, Y112A, or Y112V in the presence of rNTPs for 15 min at 37 °C. Concentrations of rNTP were increased up to 500 μM. dNTP at a concentration of 250 μM was added as the control. The samples were resolved by 15% denaturing polyacrylamide gel electrophoresis and analyzed as described in Experimental Procedures.

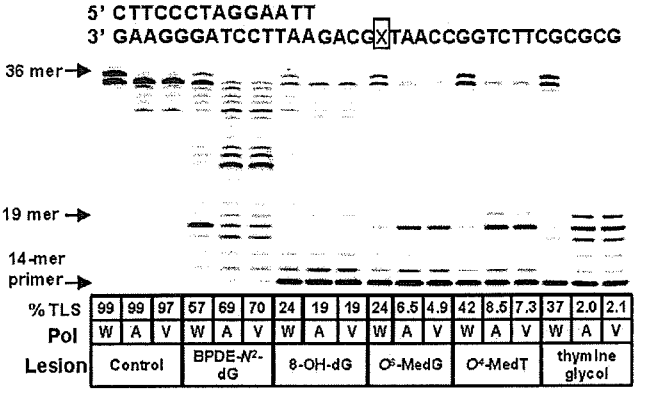

FIGURE 2: Effect of substituting A or V for Y112 on TLS across several lesions in DNA. The Cy3-labeled 14mer primer was annealed to each of six different 36mer templates containing an unmodified dG, dT, or lesions at position 19 (indicated as a boxed X). Reactions were allowed to proceed for 15 min at 37 °C with 100 nM primer-template and 10 nM hPolκ protein. W, A, and V represent wild-type hPolκ, Y112A, and Y112V, respectively. % TLS indicates the percentage of the amount of primers opposite and beyond the lesion relative to the total amount of the primer.

adduct in this study, although (+)-*trans-anti*-BPDE-N^2-dG is the major adduct of benzo[*a*]pyrene (*32*). Both Y112A and Y112V exhibited primer extension activities across BPDE-N^2-dG in DNA comparable to those of the wild-type enzyme. Rather, the mutant proteins displayed substantially reduced activities in inserting a base opposite other types of lesions except for 8-OH-dG compared to the wild-type enzyme. In particular, Y112A and Y112V exhibited a severely reduced ability to deal with thymine glycol in DNA. To gain insight into the roles of the steric gate amino acid in TLS in a quantitative manner, we conducted steady-state kinetic analyses of insertion and extension steps across BPDE-N^2-dG and thymine glycol in DNA (Table 1). The Y112A incorporation efficiency, i.e., k_{cat}/K_m, for dCMP opposite template BPDE-N^2-dG was 8-fold greater than that of the wild-type enzyme. In contrast, the amino acid change decreased the efficiency of incorporation of dCMP opposite undamaged dG by 90%. In the extension step, replacement of Y112 with

Table 1: Steady-State Kinetic Parameters for Incorporation and Extension across BPDE-N^2-dG and Thymine Glycol in DNA by the Wild Type and Y112A[a]

		Incorporation						
		k_{cat} (min^{-1})		K_m (μM)		k_{cat}/K_m (μM^{-1} min^{-1})		
template	dNTP	WT[b]	Y112A	WT	Y112A	WT	Y112A	Y112A/WT
G	dCTP	7.9 ± 2.0	8.2 ± 1.5	6.7 ± 2.6	60 ± 22	1.2	1.4×10^{-1}	1/8.6
BPG[c]		0.5 ± 0.088	1.6 ± 0.30	1900 ± 560	750 ± 340	2.7×10^{-4}	2.1×10^{-3}	7.8/1
T	dATP	13 ± 2.2	13 ± 2.0	3.4 ± 1.4	46 ± 15	3.8	2.9×10^{-1}	1/13
ThGl[d]		5.6 ± 0.97	0.20 ± 0.060	190 ± 74	2300 ± 1100	2.9×10^{-2}	8.4×10^{-5}	1/350
		Extension						
		k_{cat} (min^{-1})		K_m (μM)		k_{cat}/K_m (μM^{-1} min^{-1})		
base pair at th 3′-primer termini (primer-template)		WT[b]	Y112A	WT	Y112A	WT	Y112A	Y112A/WT
G/C		18 ± 1.1	15 ± 0.73	3.5 ± 0.40	65 ± 6.1	5.2	2.3×10^{-1}	1/23
BPG/C		11 ± 0.69	9.3 ± 1.7	44 ± 7.7	910 ± 380	0.25	1.0×10^{-2}	1/24
T/A		15 ± 0.72	18 ± 1.4	1.4 ± 0.16	17 ± 3.1	11	1.1	1/11
ThGl/A		4.4 ± 0.18	0.2 ± 0.026	240 ± 21	810 ± 240	1.8×10^{-2}	2.5×10^{-4}	1/75

[a] Incorporation reactions were analyzed by determining the steady-state kinetic parameters for incorporation of dCMP opposite template guanine (G) or BPDE-N^2-dG (BPG) and for incorporation of dAMP opposite template thymine (T) or thymine glycol (ThGl). Exact sequences of primer-template DNA, i.e., 18/36G or 18/36T, are described in Table 1 of the Supporting Information. The primer-template (100 nM) and the proteins (5 nM) were incubated at 37 °C. Extension reactions were analyzed by determining the steady-state kinetic parameters for incorporation of dAMP opposite template thymine (T) adjacent to G/C, BPG/C, T/A, or TG/A at the primer-template termini. Exact sequences of primer-template DNA, i.e., 19C/36G or 19A/36T, are described in Table 1 of the Supporting Information. The primer-template (100 nM) and the proteins (5 nM) were incubated at 37 °C. [b] WT, wild-type hPolκ. [c] BPG, BPDE-N^2-dG. [d] ThGl, thymine glycol.

A decreased the k_{cat}/K_m of dAMP incorporation opposite template dT adjacent to either BPDE-N^2-dG or dG by 95%. Therefore, the overall TLS across BPDE-N^2-dG was only moderately compromised by the amino acid change. For TLS across thymine glycol, however, the amino acid change severely reduced both the insertion and extension efficiency. The insertion and extension efficiencies of Y112A were only ~0.3 and ~1%, respectively, compared to that of the wild-type enzyme. From these results, we concluded that the steric gate amino acid of hPolκ is nonessential for TLS at least across (−)-*trans*-*anti*-BPDE-N^2-dG in DNA. Instead, Y112 may play roles in incorporation steps opposite lesions other than N^2-dG adducts, such as thymine glycol, and in extension steps from primer termini.

Strong Binding of hPolκ to Primer-Template DNA with BPDE-N^2-dG/dCMP. Although the amino acid replacement of Y112 with A or V did not severely reduce TLS efficiency across BPDE-N^2-dG in DNA, it might still modulate binding to primer-template DNA. To test this possibility, we examined the physical interactions between hPolκ and primer-template DNA with or without lesions using surface plasmon resonance and calculated the equilibrium dissociation constants (K_d) (Figure 3 and Table 2 of the Supporting Information). Wild-type hPolκ bound to primer-template DNA with BPDE-N^2-dG ~3-fold more strongly than it bound to DNA without the lesion. Strong binding depended on the length of the primer and on the dNMP opposite the lesion. Specifically, strong binding was observed when the length of the primer was the same as the length of the template between the 3′-end and the lesion, i.e., a 19mer primer. Alternatively, the length of the primer could be one or two base pairs longer than the length of template, i.e., a 20mer or 21mer primer. No strong binding was observed when the primer was shorter than 19 bp or longer than 21 bp. Additionally, for strong binding, the dNMP opposite the lesion had to be correct (dCMP); strong binding was not observed when the 19mer primers had dGMP, dTMP, or dAMP at the 3′-terminus. No strong binding was observed in primer-template DNA with other lesions, such as thymine glycol, or in primer-template DNA with mismatched termini (data not shown). Importantly, replacing the steric gate amino acid with A or V did not enhance or weaken binding to primer-template with BPDE-N^2-dG lesions (Figure 2 and Table 3 of the Supporting Information), nor did they modulate the binding affinity for control DNA. These results suggest that the steric gate amino acid may play roles in TLS other than modulating the ability of hPolκ to bind to damaged or undamaged DNA.

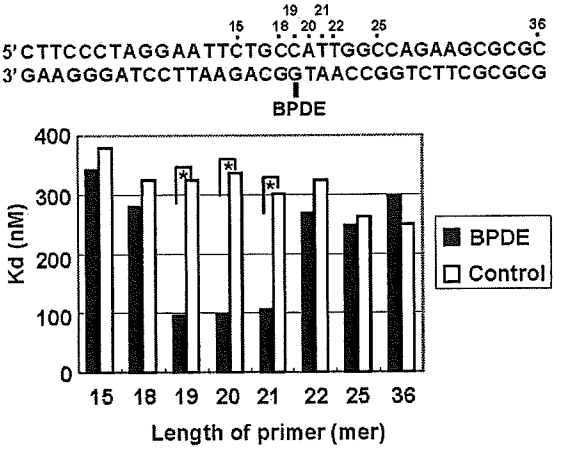

FIGURE 3: Affinity of hPolκ for primer-template DNA with a BPDE-N^2-dG adduct. Primers of different lengths, i.e., 15mers, 18mers, 19mers, 20mers, 21mers, 22mers, 25mers, or 36mers, were annealed with template DNA (36mer) with BPDE-N^2-dG at position 19. As a control, template DNA with an undamaged dG at position 19 was annealed to the various primers. The affinity between protein and DNA was measured with a BIAcore 3000, and the K_d was calculated with BIAevaluation version 4.0. The heights of bars represent the average K_d values of three experiments. An asterisk indicates $P < 0.001$ (t-test).