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UmuD and RecA Directly Modulate the Mutagenic Potential of the Y Family DNA Polymerase DinB

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SUMMARY

DinB is the only translesion Y family DNA polymerase conserved among bacteria, archaea, and eukaryotes. DinB and its orthologs possess a specialized lesion bypass function but also display potentially deleterious −1 frameshift mutagenic phenotypes when overproduced. We show that the DNA damage-inducible proteins $UmuD_2$ and RecA act in concert to modulate this mutagenic activity. Structural modeling suggests that the relatively open active site of DinB is enclosed by interaction with these proteins, thereby preventing the template bulging responsible for −1 frameshift mutagenesis. Intriguingly, residues that define the $UmuD_2$-interacting surface on DinB statistically covary throughout evolution, suggesting a driving force for the maintenance of a regulatory protein-protein interaction at this site. Together, these observations indicate that proteins like RecA and $UmuD_2$ may be responsible for managing the mutagenic potential of DinB orthologs throughout evolution.

INTRODUCTION

Decades after their discovery, the *dinB* (Kenyon and Walker, 1980) and *umuDC* (Elledge and Walker, 1983; Kato and Shinoura, 1977) genes of *Escherichia coli* were shown to encode specialized Y family DNA polymerases, DNA Pol IV (Wagner et al., 1999) and Pol V (Reuven et al., 1999; Tang et al., 1999) respectively, that catalyze the insertion of deoxyribonucleoside triphosphates (dNTPs) opposite potentially lethal replication blocking lesions in a process termed translesion synthesis (TLS) (Goodman, 2002; Ohmori et al., 2001). TLS can proceed with a range of fidelities (Goodman, 2002), but in all cases Y family polymerases replicate undamaged DNA with a reduced fidelity relative to the enzymes that replicate the majority of the genome (Friedberg et al., 2002). Therefore, Y family polymerases must be excluded from improper access to replication intermediates to maintain genomic integrity (Sutton and Walker, 2001a).

Both *dinB* and *umuDC* are regulated transcriptionally by the SOS regulatory network (Friedberg et al., 2006), their expression being induced when LexA undergoes facilitated autocleavage upon interaction with the RecA::ssDNA nucleoprotein filament formed after DNA damage. The subsequent control of UmuC function is remarkably complex and involves the gene products of the cotranscribed *umuD* gene. $UmuD_2$ undergoes an SOS-mediated posttranslational modification when it too is subject to facilitated autodigestion upon interaction with RecA::ssDNA nucleoprotein filaments to yield $UmuD'_2$ (Burckhardt et al., 1988; Nohmi et al., 1988). The conversion of $UmuD_2$ to $UmuD'_2$ activates UmuC polymerase function (Reuven et al., 1999; Tang et al., 1999), and RecA::ssDNA filaments are required in *trans* for $UmuD'_2$C-catalyzed TLS (Schlacher et al., 2006). In addition, RecF, RecO, and RecR cooperate to alleviate the inhibition of $UmuD'_2$C-mediated TLS that is brought about by DNA Pol III (Fujii et al., 2006). Finally, $UmuD'_2$C must interact with the β processivity clamp of DNA Pol III to function in vivo (Becherel et al., 2002; Sutton et al., 2001).

DinB is the only Y family DNA polymerase conserved among all domains of life (Ohmori et al., 2001), and under conditions of DNA damage it is the most abundant DNA polymerase in *E. coli* (Kim et al., 2001). Despite the remarkable evolutionary conservation of DinB, the details of its biochemical regulation are comparatively unknown (Fuchs et al., 2004). We recently showed that an important function of DinB and its orthologs is to carry out highly proficient and accurate TLS past a particular class of N^2-deoxyguanosine adducts (Jarosz et al., 2006). On the surface, these observations seem incompatible with DinB's role in the elevated frequency of −1 frameshift mutations observed during λ untargeted mutagenesis (Brotcorne-Lannoye and Maenhaut-Michel, 1986) and adaptive mutagenesis (McKenzie et al., 2001; Tompkins et al., 2003) or with the −1 frameshift mutator effect caused by DinB overproduction (Kim et al., 1997).

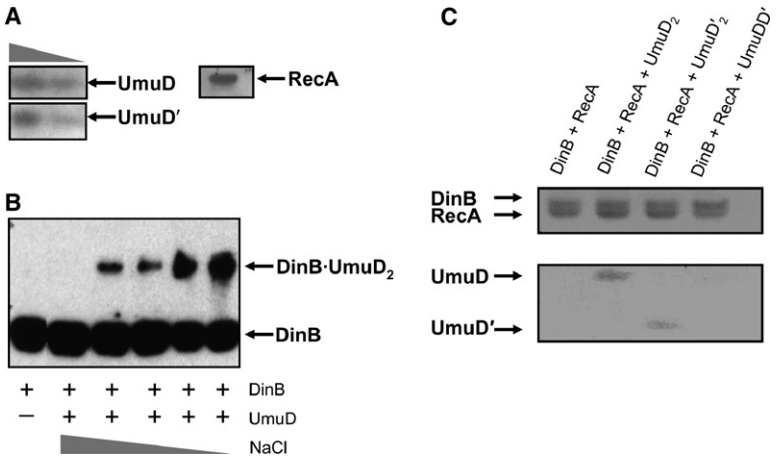


Figure 1. DinB Interacts Directly with UmuD$_2$ and RecA

(A) Far-western blot demonstrates that UmuD directly interacts with ^{32}P-labeled (His)$_6$-HMK-DinB. Either 50 or 100 pmol of UmuD or UmuD′ were separated by 12% SDS-PAGE and transferred to a PDVF membrane. The HMK-DinB protein probe was radioactively labeled and incubated with the membrane for 15 min, after which the membrane was exposed to film.

(B) Crosslinking experiment suggests that DinB interacts with the UmuD$_2$ homodimer. One hundred picomoles of DinB and UmuD$_2$ were mixed in a 10 μl volume in 50 mM HEPES (pH 7.5), 25–500 mM NaCl, and 1 mM DTT and incubated for 10 min at 25°C.

(C) DinB forms stable binary and ternary complexes with RecA and UmuD$_2$. DinB(His)$_6$ pulls down RecA alone and in combination with UmuD and UmuD′ on a Ni^{2+} affinity resin. UmuDD′ cannot be observed to similarly interact.

Previous studies of DinB and its orthologs, as well as of other Y family DNA polymerases, have focused on the pivotal role of processivity clamps in regulation of TLS and mutagenesis (Becherel et al., 2002; Bergoglio et al., 2002; Beuning et al., 2006a; Bunting et al., 2003; Frampton et al., 2006; Lenne-Samuel et al., 2002; Ogi et al., 2005). However, given DinB's documented potential to cause deleterious −1 frameshift mutations (Kim et al., 2001; Nohmi, 2006), we thought it possible that DinB might be subject to control beyond transcriptional induction and interaction with the processivity clamp. We therefore searched for additional regulatory factors that might manage this potentially problematic function. Remarkably, we found that UmuD, UmuD′, and RecA, previously known only to regulate UmuC function, regulate both the activity and mutagenic properties of DinB via protein-protein interactions that enclose its active site. Our findings suggest that mechanistic features of this regulation may be maintained in eukaryotes consistent with a common pattern of regulation for these DNA polymerases through evolution.

RESULTS

DinB Interacts with Numerous Cellular Factors

To identify proteins that might regulate DinB function, we covalently coupled purified recombinant DinB to an affinity resin (see Figure S1A available online). Interacting proteins from lysates of constitutively SOS-induced *E. coli* were eluted and separated by SDS-PAGE. Those that bound in a DinB-dependent fashion included the chaperones GroEL and DnaK and also the ribosomal protein L3 as well as lesser amounts of other ribosomal proteins (Figure S1B). The presence of the chaperones was anticipated because GroEL has been shown to regulate the function of both DinB (Layton and Foster, 2005) and UmuD′$_2$C (Donnelly and Walker, 1989) and DnaK regulates UmuC levels in vivo (Grudniak et al., 2005). The ribosomal proteins L3 and others seem less likely to be bona fide regulators of DinB function although this remains a formal possibility.

However, we also identified UmuD, UmuD′, and RecA as DinB-interacting proteins. This was a complete surprise because, despite their intensively studied, highly nuanced roles in regulating UmuC function (Friedberg et al., 2006; Jarosz et al., 2007; Nohmi, 2006), none of these factors had previously been implicated in regulating DinB function aside from the indirect role of RecA in mediating DinB induction via the SOS regulatory network. Intriguingly, the levels of UmuD in vivo (180 molecules in non-SOS-induced cells; 2400 molecules in SOS-induced cells) parallel those of DinB (250 molecules in non-SOS-induced cells; 2500 molecules in SOS-induced cells) and greatly exceed what is required to interact with UmuC (17 molecules in non-SOS-induced cells; 200 molecules in SOS-induced cells) (Kim et al., 1997; Woodgate and Ennis, 1991). We therefore investigated the ability of these proteins to affect DinB function in vivo and in vitro.

DinB Forms a Stable Interaction with UmuD$_2$ and RecA

To ascertain whether the interactions we observed between DinB, RecA, UmuD, and UmuD′ were direct, we first performed a far-western blot in which we probed membranes containing UmuD, UmuD′, and RecA with DinB. Each of the interactions appears to be direct in nature both in this experiment (Figure 1A) and in similar far-western blots performed on eluates from the DinB affinity column (data not shown). To analyze the stoichiometry of the DinB•UmuD interaction, we crosslinked DinB and UmuD with formaldehyde and analyzed the products by immunoblot using an antibody against DinB. The crosslinked species corresponds to the molecular weight of a DinB•UmuD$_2$ complex and the reaction appears to be inhibited by high concentrations of NaCl (Figure 1B), suggesting that the interface may partly involve ionic or polar interactions. The propensity of RecA to multimerize

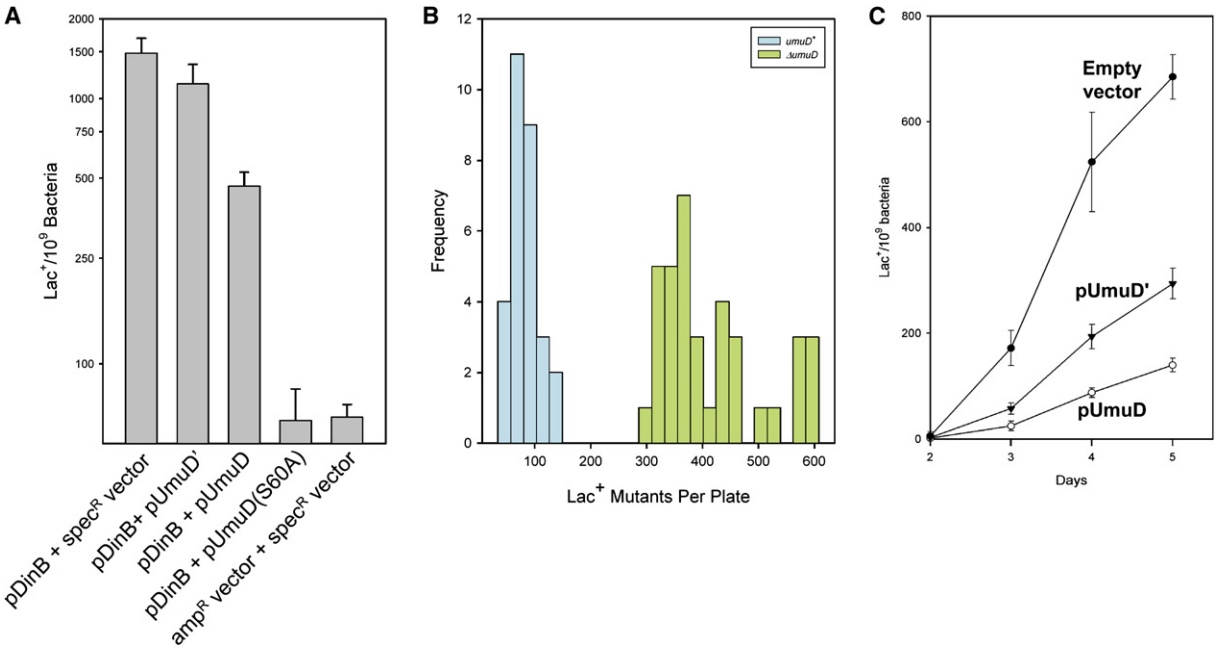


Figure 2. UmuD$_2$ Regulates the −1 Frameshift Activity of DinB In Vivo

(A) Lac$^+$ reversion assay of the frameshift allele in CC108 demonstrates that UmuD$_2$ modulates DinB's −1 frameshift function.

(B) Deletion of *umuD*$^+$ results in a significant increase in −1 frameshift mutagenesis. A histogram of Lac$^+$ mutants obtained from *umuD*$^+$ and *ΔumuD* strains reveals that even the modest chromosomally encoded levels of UmuD$_2$ have a significant impact on −1 frameshift mutagenesis. Experiments were performed as indicated in the Experimental Procedures. y axis shows the frequency of plates with the number of Lac$^+$ mutants indicated in the bins on the x axis.

(C) UmuD$_2$ also affects the number of Lac$^+$ revertants in an adaptive mutagenesis experiment. FC40 with empty vector (closed circles); FC40 with pUmuD′ (closed triangles); FC40 with pUmuD (open circles). Error bars represent one standard deviation.

(McGrew and Knight, 2003) made it difficult to establish the stoichiometry of the DinB•RecA interaction.

To test whether DinB, UmuD$_2$ and/or UmuD′$_2$, and RecA form a stable ternary complex in solution, a DinB variant with a hexahistidine affinity tag at its C terminus was incubated with RecA—both alone and in combination with UmuD$_2$, UmuD′$_2$, and the heterodimeric species UmuDD′ (Battista et al., 1990). Complexes that formed with DinB were isolated using Ni^{2+} affinity resin. Using physiologically relevant concentrations (2.5 μM) of each protein, we observed the formation of a stable stoichiometric complex between DinB and RecA (Figure 1C); fluorescence anisotropy does not reveal such an association (Schlacher et al., 2005). Moreover, it appears that DinB's interaction with RecA does not preclude association with UmuD or UmuD′. Unexpectedly, we did not observe an interaction between DinB and the UmuDD′ heterodimer alone (data not shown) or in combination with RecA (Figure 1C) in this experiment. Taken together, these data indicate that DinB, RecA, and UmuD$_2$ (and to a lesser extent UmuD′$_2$), can form ternary complexes under physiological conditions.

UmuD Suppresses DinB-Dependent Mutagenic Phenomena In Vivo

Because the cellular levels of UmuD are similar to those of DinB and are much higher than those of UmuC, we wondered whether the −1 frameshift mutator effect associated with overexpression of DinB might be a consequence of the number of molecules of DinB exceeding those of UmuD in the cell. Interestingly, that appears to be the case as we found that co-overproduction of UmuD eliminates much of the −1 frameshift mutagenesis caused by DinB overproduction (Figure 2A). Co-overproduction of a noncleavable UmuD variant, UmuD(S60A), completely eliminates DinB-dependent frameshift mutagenesis (Figure 2A), indicating that full-length UmuD is sufficient for maximal inhibition. These plasmids result in a 3- to 4-fold increase in UmuD levels over those encoded by the chromosomal *umuD* gene as determined by immunoblot (Figure S2).

Strikingly, we observed that a precise deletion of the chromosomal *umuD* gene also results in an increased frequency of −1 frameshift mutagenesis. Indeed, deletion of *umuD* (Table S1) leads to a considerable and unexpected increase in the frequency of −1 frameshift mutagenesis from 8.0 (±2.5) × 10^{-8} (*umuD*$^+$) to 41.5 (±9.3) × 10^{-8} (*ΔumuD*) ($p = 3.96 \times 10^{-24}$) (Figure 2B). Fluctuation analysis (Foster, 2006) reveals a similar increase in estimated mutation rates between the two strains. The so-calculated mutation rates (with 95% confidence limits in parentheses) are 1.7 × 10^{-8} (1.3 × 10^{-8}; 1.9 × 10^{-8}) for *umuD*$^+$ and 7.1 × 10^{-8} (5.7 × 10^{-8}; 8.2 × 10^{-8}). These results emphasize the ability of even the modest levels of

chromosomally encoded UmuD to modulate DinB's mutagenic function. Interestingly, the mutants do not appear to conform either to a classical Luria-Delbruck distribution or to others that we tested, an observation that may be of biological significance.

We then investigated the possibility that the *umuD* gene products might similarly modulate the phenomenon of adaptive mutagenesis in the widely studied *E. coli* strain FC40 (Cairns and Foster, 1991), which is dependent on DinB-promoted −1 frameshifts (McKenzie et al., 2001; Tompkins et al., 2003). Under the conditions required to observe such mutagenesis, DinB levels are elevated by ~2- to 4-fold (Kim et al., 2001; Layton and Foster, 2003). Remarkably, overproduction of UmuD or UmuD′ strikingly reduced adaptive mutagenesis (Figure 2C). The 5-fold reduction in the frequency of adaptive mutagenesis caused by UmuD overexpression is equivalent to the decrease caused by *dinB* inactivation (McKenzie et al., 2001; Tompkins et al., 2003). These observations suggest that the *umuD* gene products are able to modulate the −1 frameshift activity of DinB both in exponential phase and also under the conditions of an adaptive mutagenesis experiment.

Initially, we were surprised that deletion of *umuD* has no effect on *dinB*$^+$-dependent NFZ resistance, but this point is discussed further below. Although *ΔrecA* strains do exhibit sensitivity to this agent (data not shown), the multitudinous physiological roles of RecA (Friedberg et al., 2006; McGrew and Knight, 2003) complicate the interpretation of this result.

To investigate whether DinB reciprocally affects UmuD$_2$ function, we examined the effect of DinB overproduction on UV-induced mutagenesis, a phenomenon that is critically dependent on *umuD*$^+$ as well as on *umuC*$^+$ (Elledge and Walker, 1983; Kato and Shinoura, 1977). Expression of *dinB*$^+$ from a low-copy-number plasmid suppressed UV-induced mutagenesis by ~7-fold (Table S2). It is a formal possibility that this effect reflects competition between DinB and UmuD′$_2$C for the primer terminus, but it may also be related to DinB-dependent inhibition of RecA-mediated UmuD$_2$ autocleavage (Figure 5E), consistent with DinB and UmuD$_2$ interacting in vivo.

Identification of the Molecular Interface between DinB and UmuD$_2$

Our observation that UmuD suppresses the −1 frameshift activity of DinB in vivo was especially intriguing in light of structural studies of archaeal DinB homologs, which have been shown to possess remarkably open active sites (Ling et al., 2001; Silvian et al., 2001). We therefore analyzed the interaction between DinB and UmuD$_2$ using cellulose filter peptide arrays (Frank, 2002). The membranes were probed with either DinB or UmuD$_2$ (Figure S3), and interacting peptides were identified and mapped onto structural models of DinB (Jarosz et al., 2006) or UmuD$_2$ (Beuning et al., 2006c; Sutton et al., 2002). Interestingly, the UmuD$_2$-interacting peptides on DinB localize to a single face of the protein (Figure 3A). Further, the presence of an extended interacting surface on DinB suggests that its interaction with UmuD$_2$ is qualitatively different from its interaction with the β processivity clamp, which depends on a highly conserved peptide motif (Bunting et al., 2003; Dalrymple et al., 2001). Most intriguingly, the interaction interface suggests that UmuD$_2$ may suppress mutagenesis by helping to enclose the strikingly open active site of DinB, thereby preventing the DNA template bulging necessary for −1 frameshift mutagenesis (Ling et al., 2001; Potapova et al., 2002).

The DinB-interacting interface forms a somewhat less contiguous surface when mapped onto a specific UmuD$_2$ model we had originally proposed (Sutton et al., 2002). However, the DinB binding interface forms a more extensive surface when mapped onto one of four isoenergetic models of UmuD$_2$ (Beuning et al., 2006c), in which its N terminus is raised to reveal an interacting surface across the side of the protein (Figure 3B). These observations suggest that our previous model of UmuD$_2$ may represent only one of several biologically relevant UmuD$_2$ conformers (Figure S4).

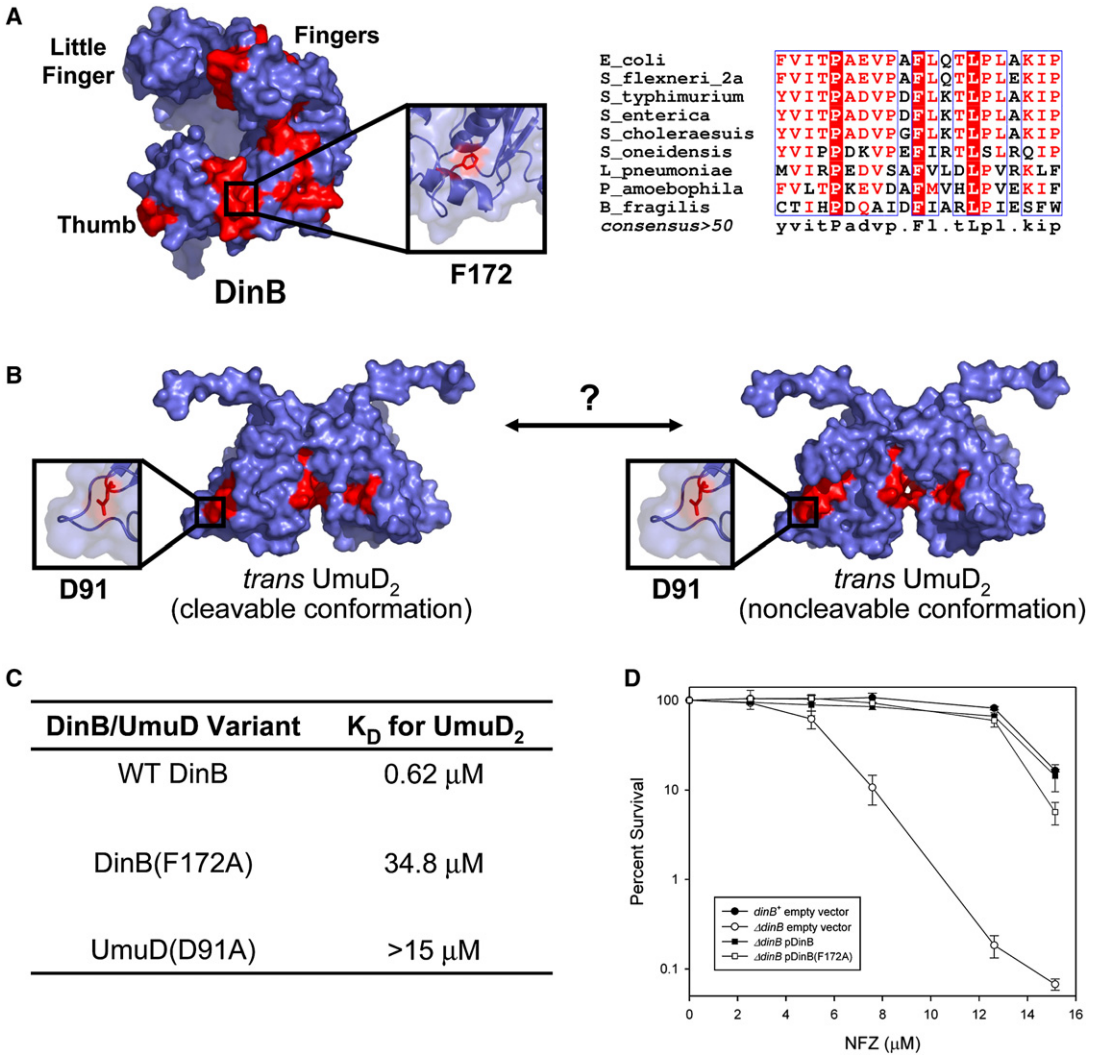
In an effort to design a DinB variant that is unable to interact with UmuD$_2$, we identified a strongly interacting group of peptides from the DinB peptide array and examined conservation of this region in numerous *umuD*-containing organisms. Three residues, P166, F172, and L176, were strikingly conserved, and we determined the effect of changing each residue to an alanine (Figure 3A). Although the mutant proteins DinB(P166A) and DinB (L176A) were insoluble (data not shown), we were able to express and purify DinB(F172A) in soluble form (Figure S5). Moreover, we found that the *dinB* allele encoding DinB(F172A) complements the NFZ sensitivity of a *ΔdinB* strain (Figure 3D), indicating that this mutant is proficient for TLS in vivo.

A reciprocal approach was used to generate a UmuD$_2$ mutant that might be impaired with respect to its ability to interact with DinB. The variant UmuD(D91A) is soluble and purifies as wild-type UmuD. It is also proficient for facilitated autoproteolysis (Figure S6).

We determined that the dissociation constant between DinB and UmuD$_2$ is 0.62 μM using fluorescence spectroscopy (Figure 3C; Figure S7). Provocatively, the cellular concentration of UmuD$_2$ rises from ~0.15 μM under non-SOS-induced conditions to ~2 μM under conditions of SOS induction (Kim et al., 2001), indicating that DinB and UmuD$_2$ are capable of interaction within the range of physiologically relevant concentrations. Furthermore, the DinB(F172A) and UmuD(D91A)$_2$ proteins were each greatly impaired with respect to their ability to bind their partners (Figure 3C), indicating that the interfaces we identified by peptide array mapping are functionally relevant.

Mutation of the Interface between DinB and UmuD$_2$ Impairs Function In Vivo

To determine whether the physical interaction between DinB and UmuD$_2$ we observed and analyzed in vitro is important for modulation of DinB-dependent frameshift



DinB/UmuD Variant	K_D for UmuD$_2$
WT DinB	0.62 μM
DinB(F172A)	34.8 μM
UmuD(D91A)	>15 μM

Figure 3. Molecular Characterization of the Interaction between DinB and UmuD$_2$

(A) Peptide array mapping of the UmuD binding interface on DinB reveals a surface composed of the thumb and finger domains of the polymerase. Several hydrophobic residues in the most strongly interacting peptide are conserved among DinB orthologs from organisms containing *umuD*.

(B) Peptide array mapping of the DinB binding interface on UmuD$_2$ reveals a discontinuous interface on a structural model of *trans*-UmuD$_2$ that is enlarged in an alternative isoenergetic *trans*-UmuD$_2$ conformer.

(C) Alanine mutants of DinB F172 or UmuD D91 result in a weakened interaction determined by fluorescence spectroscopy.

(D) A low-copy-number plasmid encoding DinB(F172A) (pYG768-F172A; open squares) is fully able to rescue the NFZ sensitivity of a *ΔdinB E. coli* strain bearing the pWSK29 empty vector (open circles) to the levels of an isogenic *dinB*$^+$ strain (closed circles), just as a plasmid encoding wild-type DinB (pYG768; closed squares). Error bars represent one standard deviation determined from three independent experiments.

mutagenesis in vivo, we examined whether the −1 frameshifts produced by DinB(F172A) could be inhibited by UmuD. Overproduction of DinB(F172A) results in an increase in −1 frameshift mutagenesis by ~6-fold (Figure 4A). The precise reasons for the difference between this value and that obtained from wild-type DinB overproduction, which could include reduced −1 frameshift activity or affinity for frameshift intermediates in vivo, are unclear. However, the increase in −1 frameshift mutagenesis observed from DinB(F172A) overproduction is remarkably statistically significant ($p = 4 \times 10^{-15}$). Co-overproduction of UmuD or UmuD′ does not substantially reduce the −1 frameshift mutation frequency due to DinB(F172A) overproduction (Figure 4A). These data suggest that a direct interaction of UmuD$_2$ or UmuD′$_2$ at the interface we have identified on DinB is important for modulation of −1 frameshift mutagenesis in vivo.

Reciprocally we examined whether UmuD(D91A) could suppress the −1 frameshift mutagenesis promoted by overproduction of wild-type DinB. Consistent with the weakened affinity of UmuD(D91A) for DinB, its overproduction only modestly suppresses −1 frameshift

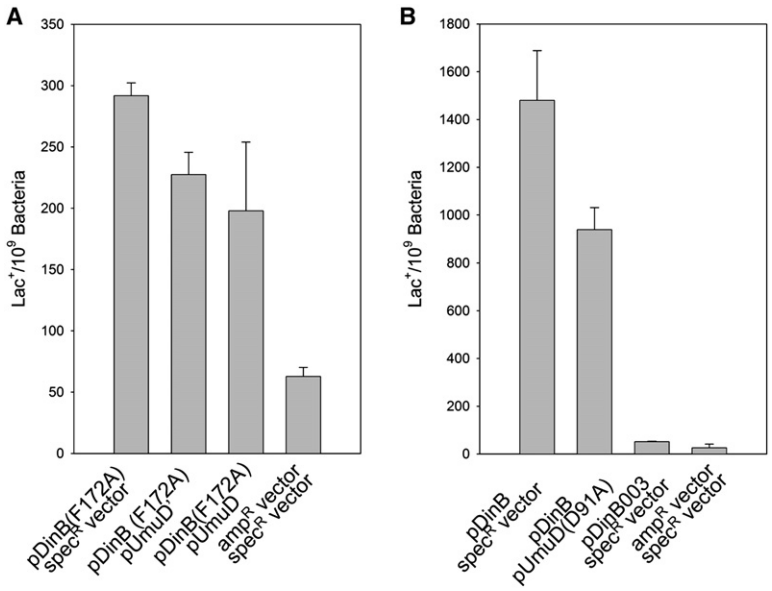


Figure 4. Single Amino Acid Changes on the Interface between DinB and UmuD Perturb Regulation of −1 Frameshift Activity

(A) The DinB(F172A) variant has a lower affinity for UmuD$_2$ and is not as responsive as wild-type DinB to regulation by UmuD$_2$.

(B) The UmuD(D91A) variant has a lower affinity for DinB and does not regulate −1 frameshift activity as well as wild-type UmuD. Error bars represent one standard deviation.

mutagenesis (Figure 4B). Moreover, even significant overproduction of DinB(F172A) was insufficient to impair UV-induced mutagenesis (Table S2). These observations suggest that a direct interaction between DinB and UmuD$_2$ is crucial for the ability of each protein to modulate the function of the other in vivo.

UmuD$_2$ Inhibits DinB-Dependent −1 Frameshift Activity In Vitro

To gain more detailed insights into the mechanisms governing modulation of DinB function by UmuD in vivo, we reconstituted DinB-dependent −1 frameshift activity in vitro with a substrate containing a G:G mispair that can be extended with either (1) dGTP to generate a full-length product or (2) dATP to generate a −1 frameshift product that is one nucleotide shorter than the template (Figure 5A) (Kobayashi et al., 2002; Wagner et al., 1999). DinB is unable to extend from this mispair using dGTP under our experimental conditions, and although it can act on this substrate with modest efficiency using dATP, addition of UmuD$_2$ alone did not alter its −1 frameshift activity (data not shown). However, we then discovered that, when RecA is added in a stoichiometric ratio with DinB, the addition of UmuD$_2$ nearly completely inhibits the reaction (Figure 5B). In striking contrast, addition of UmuD$_2$ and RecA results in an ~20-fold enhancement of the ability of DinB to extend from a correctly paired terminus in the same sequence context (Figure 5B). These observations indicate that UmuD$_2$ and RecA act in concert to modulate DinB function in a highly sophisticated manner, promoting its ability to extend a properly paired primer terminus while suppressing its ability to extend a mismatched terminus.

Our discovery that RecA is required for UmuD$_2$ to modulate DinB mutagenic function in vitro led us to examine whether *recA*$^+$ is similarly required for UmuD-dependent suppression of −1 frameshift mutagenesis in vivo. It is, as co-overproduction of UmuD or UmuD(S60A) has little effect on the frequency of DinB-dependent −1 frameshift mutagenesis in a *ΔrecA* strain (Figure 5C). Intriguingly, the frequency of −1 frameshift mutagenesis caused by DinB overproduction is also modestly elevated in a *ΔrecA* strain. Taken together, these findings provide strong evidence that RecA is required for UmuD$_2$-dependent modulation of DinB function.

In vitro, UmuD$_2$ reduced the maximal −1 frameshift activity of wild-type DinB by one half at a concentration of 840 nM, while a concentration of 3.6 μM is needed to cause an equivalent effect on DinB(F172A) (Figure 5D). Relative to wild-type UmuD$_2$, UmuD(D91A)$_2$ also shows a marked 10-fold decrease in its ability to inhibit DinB-dependent −1 frameshift activity in vitro. Additionally, we observed that DinB is able to inhibit the RecA-mediated autocleavage of UmuD$_2$ in vitro, and moreover that the DinB(F172A) variant was unable to do so efficiently (Figure 5E). All of these data underscore the notion that a physical interaction between DinB and UmuD$_2$ exists under physiological conditions and is required for UmuD$_2$-dependent modulation of DinB function.

A TLS-Deficient DinB Variant Is Proficient for −1 Frameshift Function

It has been suggested that the −1 frameshift mutator signature of DinB is a direct consequence of structural features that enable it to act as a TLS DNA polymerase (Ling et al., 2001; Potapova et al., 2002). To ascertain whether DinB's −1 frameshift mutator activity is separable from its function in TLS, we examined the DinB(F13V) variant, which is able to catalyze DNA synthesis on undamaged DNA but is virtually unable to perform TLS on certain adducted templates (Jarosz et al., 2006). We found that, when overexpressed, this mutant is able to

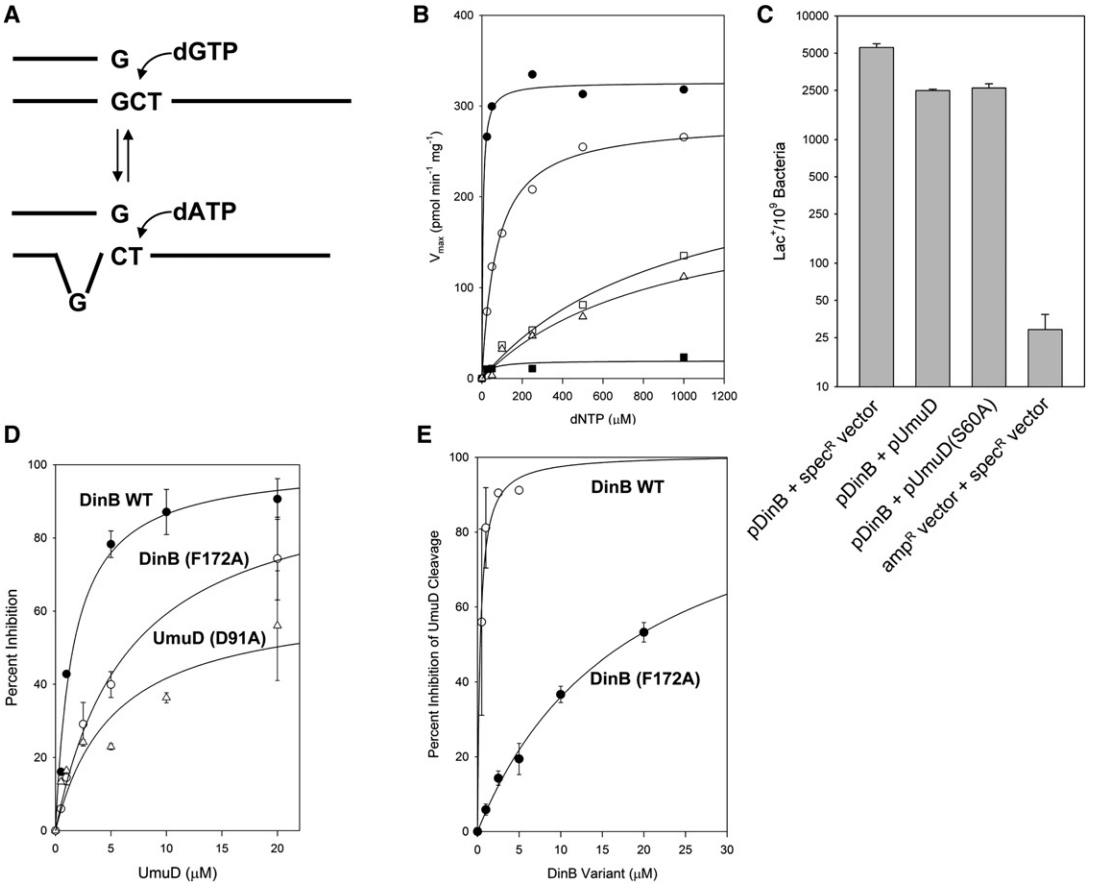


Figure 5. UmuD$_2$ and RecA Directly Modulate DinB −1 Frameshift Function

(A) Schematic of a mismatched DNA substrate that can be extended either by dGTP to generate a full-length product or by dATP, thereby forming a dNTP-stabilized misalignment and generating a −1 frameshift product.

(B) Plot of reaction velocity versus dNTP substrate concentration for DinB in combination with RecA and UmuD$_2$. Extension of the GG mismatch with dATP by DinB in combination with stoichiometric quantities of RecA (open squares) is detectable but weaker than extension of a GC base pair in the same sequence context (open circles). The addition of saturating UmuD$_2$ (10 μM) profoundly inhibits DinB activity on a GG mismatch (closed squares) but stimulates DinB activity on a GC (closed circles) by more than 20-fold. Data for DinB alone (without RecA or UmuD$_2$) on the GG mismatch substrate are shown in open triangles.

(C) *recA*$^+$ is required for UmuD-dependent inhibition of DinB-promoted −1 frameshift mutagenesis in vivo. Overproduction of DinB promotes −1 frameshift mutagenesis in a *ΔrecA* background, but the co-overproduction of UmuD$_2$ or a noncleavable UmuD(S60A) variant has little effect on mutation frequency.

(D) Plot of percent frameshift inhibition versus UmuD variant concentration. The frameshift activity of DinB is efficiently inhibited by UmuD$_2$ (closed circles), but the frameshift activity of DinB(F172A) is more inert to UmuD$_2$ suppression (open circles). The UmuD(D91A) variant is also very inefficient at inhibiting the −1 frameshift activity of wild-type DinB (open triangles).

(E) DinB efficiently inhibits UmuD$_2$ autocleavage in vitro. Wild-type DinB (open circles) inhibits UmuD$_2$ autocleavage far better than DinB(F172A) (closed circles). Error bars represent one standard deviation.

promote −1 frameshift mutagenesis in vivo (Figure 6A), indicating that DinB's −1 frameshift mutator activity can be genetically separated from its ability to carry out proficient and accurate TLS over certain N^2-dG adducts.

Curiously, the mutation frequency induced by overexpression of DinB(F13V) is consistently greater than that produced by overexpression of wild-type DinB (Figure 2A) even though the levels of each protein are comparable in vivo (Figure S8). This observation suggests either that DinB(F13V) has an increased −1 frameshift mutator activity or that some other mechanism is responsible for this phenomenon in vivo. Although the first explanation is formally possible, the −1 frameshift activity of DinB(F13V) in vitro is slightly reduced (~3-fold) relative to wild-type DinB. This observation suggests that DinB(F13V) promotes increased mutagenesis by virtue of either its recruitment to, or association with, frameshift intermediates. Curiously, we were unable to observe UmuD$_2$-dependent inhibition of DinB(F13V) −1 frameshift activity either in vivo or in vitro (Figures 6A and 6B). These

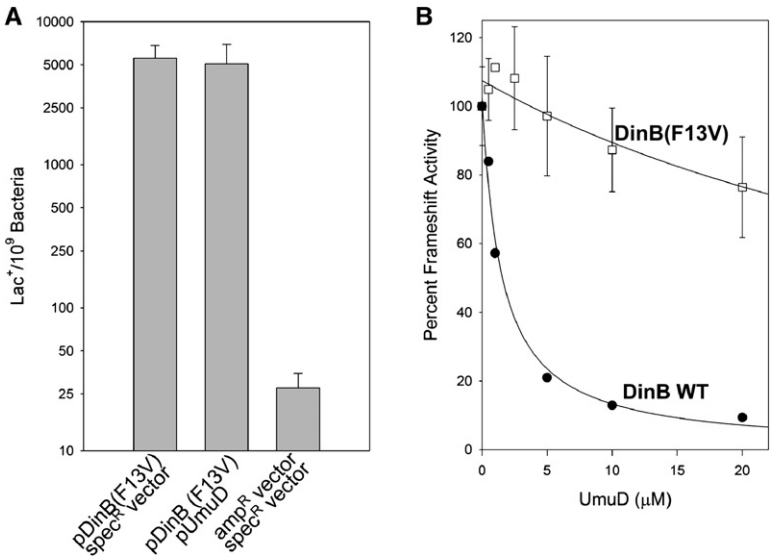


Figure 6. A TLS-Deficient Variant of DinB Is Proficient for −1 Frameshift Function

(A) The DinB(F13V) variant can promote −1 frameshift mutagenesis but is not controlled by co-overproduction of UmuD.

(B) The −1 frameshift activity of DinB(F13V) is poorly inhibited by UmuD$_2$ in vitro. Plot of frameshift activity versus UmuD concentration indicates that DinB(F13V) (open squares) retains much of its frameshift activity at concentrations of UmuD that inhibit virtually all DinB frameshift activity (closed circles). All reactions contain RecA in stoichiometric ratios with DinB. Error bars represent one standard deviation.

observations may be at least in part due to the fact that DinB(F13V) has a reduced affinity for UmuD$_2$ relative to the wild-type enzyme (Figure S9).

RecA and UmuD$_2$ May Modulate DinB Function by Restricting Its Open Active Site

In an effort to explain how UmuD$_2$ suppresses the intrinsic −1 frameshift mutator activity of DinB in a RecA-dependent manner, we generated a model of a ternary complex among DinB, RecA, and UmuD$_2$. The structure of RecA (Story et al., 1992) and models of DinB (Jarosz et al., 2006) and UmuD$_2$ (Beuning et al., 2006c) were docked using several constraints. First, UmuD$_2$ was optimally positioned on DinB using our peptide array data (Figures 3A and 3B; Figure S3). We then used distance constraints between RecA and UmuD$_2$ from published monocysteine crosslinking studies (Lee and Walker, 1996) to orient RecA relative to UmuD$_2$. Finally, we analyzed the RecA binding interface on DinB with an additional peptide array experiment (Figure S10). Together, these data were used to generate the working model shown in Figures 7A and 7B, which suggests that RecA and UmuD$_2$ act in concert to enclose the relatively open active site of DinB, perhaps thereby preventing the template bulging necessary for −1 frameshift mutagenesis. It is also provocative that in our model RecA is positioned appropriately to interact with the end of a RecA-nucleoprotein filament, suggesting that this interaction may also play a pivotal role in targeting DinB to RecA-coated substrates, a concept that has been previously proposed for UmuD′$_2$C (Bailone et al., 1991; Frank et al., 1993).

DISCUSSION

In this article, we have used several in vitro methods to demonstrate unanticipated direct interactions among DinB, UmuD$_2$, UmuD′$_2$, and RecA (Figures 1A–1C) and to present evidence that these interactions are physiologically relevant. Indeed, we have been able to isolate a ternary complex of these three proteins that is stable on the minute timescale (Figure 1C). The dissociation constant between DinB and UmuD$_2$ is 620 nM, a concentration that is squarely within the physiological range of UmuD$_2$ concentrations (0.35 μM uninduced; 4.5 μM SOS induced). The estimated K_D between DinB and RecA is ~1 μM, and the RecA concentration under normal conditions is 2.5 μM. The cellular levels of all of these proteins rise at least 10-fold upon SOS induction (Kim et al., 2001; Salles and Paoletti, 1983; Woodgate and Ennis, 1991). Therefore, many molecules of DinB are likely to exist as at least a binary and perhaps ternary complex under physiological conditions. Furthermore, these findings provide a potential rationale for the observation that the physiological levels of the *umuD* gene products greatly exceed those of UmuC.

We find that DinB-dependent −1 frameshift mutagenesis can be suppressed by co-overproduction of UmuD (Figures 2A and 2B), and elevated by absence of *umuD*$^+$ on the chromosome (Figure 2B). Moreover, a noncleavable UmuD variant completely suppresses −1 frameshift mutagenesis, indicating that only the function of full-length UmuD is required to control DinB's mutator potential (Figure 2A). Reciprocally, the DinB-binding-deficient UmuD(D91A) variant (Figure 3C), although perfectly proficient for RecA-mediated autoproteolysis (Figure S6), does not efficiently reduce DinB-dependent −1 frameshift mutagenesis (Figure 4B). Intriguingly, when mapped onto a structural model of UmuD$_2$, the residue D91 in UmuD does not form a contiguous interface with the other residues identified as part of the DinB-binding interface, as it is occluded by the curled N terminus of UmuD (Figure 3B). However, recent computational studies have suggested that isoenergetic conformations of UmuD$_2$

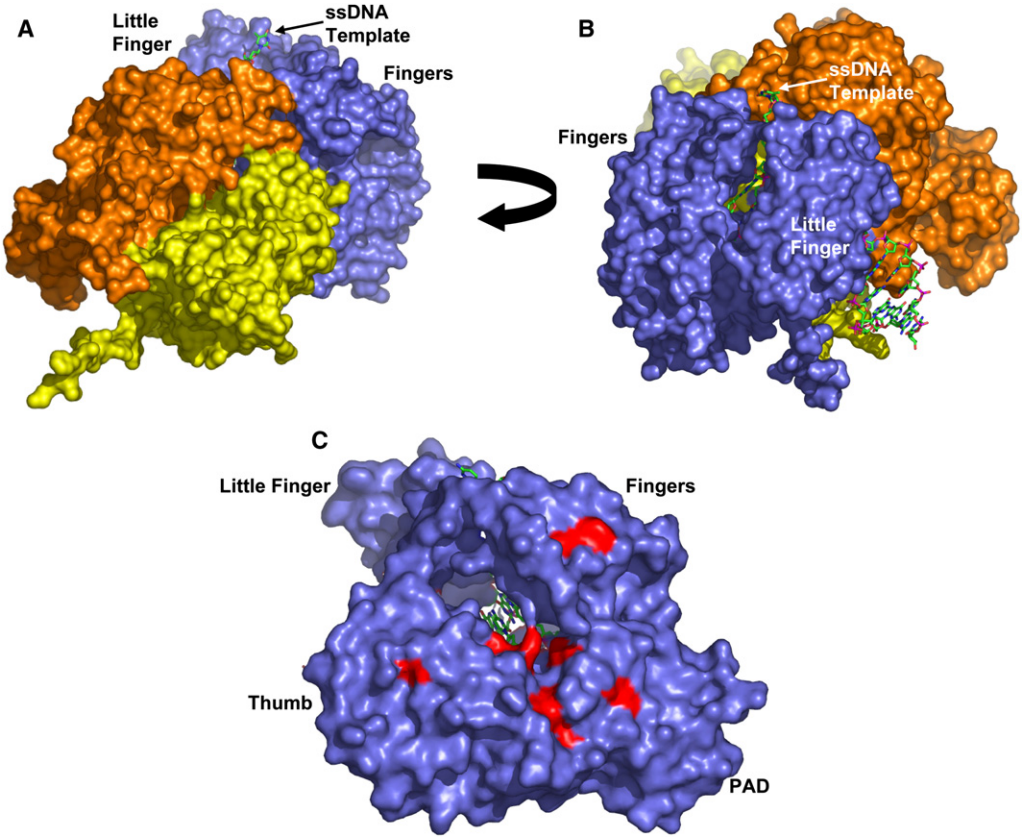


Figure 7. RecA and UmuD$_2$ May Enclose the Open Active Site of DinB

(A and B) In silico modeling of a ternary complex of the proteins. The surface representation of DinB is shown in blue, UmuD$_2$ in yellow, and RecA in orange. The DNA is relatively enclosed in the complex.

(C) Statistical covariance of DinB/pol κ residues across evolution. Residues that display statistical covariance with the UmuD$_2$ binding interface on *E. coli* DinB define an interface in a similar position on pol κ (shown in red), suggesting a possible rationale for the maintenance of this interface as a site of regulatory protein-protein interactions.

can be formed in which its N terminus is raised (Beuning et al., 2006c), thereby potentially favoring interaction with DinB but also rendering it incompetent for autoproteolysis (Figure S4). Such conformational flexibility is consistent with the recent discovery that UmuD$_2$ and UmuD′$_2$ are intrinsically disordered proteins (Simon et al., 2007). Our observation that DinB inhibits UmuD$_2$ autoproteolysis in vitro and UV-induced mutagenesis in vivo is also consistent with this notion. Overproduction of UmuD, and to a lesser extent UmuD′, also inhibits adaptive mutagenesis (Figure 2C), which occurs via a −1 frameshift event (Cairns and Foster, 1991). Irrespective of the precise molecular mechanisms of adaptive mutagenesis, it is clear that the *umuD* gene products can play a role in modulating it.

Our efforts to reconstitute the UmuD$_2$ modulation of DinB frameshift activity in vitro using a mismatched substrate (Kobayashi et al., 2002; Wagner et al., 1999) revealed that RecA needs to be present in stoichiometric quantities with DinB for this to occur (Figure 5B). Consistent with these observations, we found that *recA*$^+$ is required for the suppression of DinB-dependent −1 frameshift mutator activity in vivo, as the co-overproduction of UmuD$_2$ does not suppress this mutagenesis in a *recA* strain (Figure 5C). A noncleavable UmuD(S60A) variant is fully proficient for suppression of −1 frameshift mutagenesis (Figure 2A), indicating that RecA's role in this phenomenon is distinct from its function in promoting UmuD autocleavage.

Strikingly, addition of RecA and UmuD$_2$ to an assay in which DinB replicates a template with a properly paired terminus results in a remarkable increase in DinB catalytic proficiency (Figure 5B; open and closed circles). This observation suggests that RecA and UmuD$_2$ may modulate DinB function on diverse DNA substrates.

Taken together, these discoveries significantly expand our view of how the fidelity of Y family DNA polymerases is regulated in response to DNA damage or environmental stress. Under normal conditions, cellular levels of UmuD$_2$ are relatively low but comparable to those of DinB. Upon SOS induction, UmuD$_2$ predominates for 30–40 min, a phase during which accurate repair and damage tolerance mechanisms operate (Sutton and Walker, 2001b). The

subsequent phase, in which UmuD′$_2$ predominates, is when potentially mutagenic TLS by UmuD′$_2$C takes place. Our results suggest that in uninduced cells, as well as during the first phase of SOS induction, UmuD$_2$ and RecA would act in concert to restrict the −1 frameshift mutagenic potential of DinB by closing in its active site and simultaneously stimulating its activity on templates with properly paired termini. This control would then be relaxed during the second, more mutagenic phase of SOS induction. Our results further suggest that DinB would be mutagenic under conditions of chronic SOS induction.

We propose, as have others (Ling et al., 2001; Potapova et al., 2002), that the propensity of DinB and its orthologs for −1 frameshift mutagenesis may arise as a result of their unique active sites that are specialized for TLS function. DinB overproduction, whether synthetic or apparently natural as during adaptive mutagenesis, renders the cell vulnerable to the −1 frameshift mutator potential of DinB, which likely results at least in part from exceeding the capacity of its UmuD$_2$ manager. Similarly, we observe a significant increase in −1 frameshift mutagenesis when the *umuD*$^+$ gene is deleted (Figure 2B). Unexpectedly, this regulation cannot be fully explained by a simple analysis of the relative levels of DinB and UmuD$_2$. The CC108 episome contains an additional copy of *dinB*$^+$ that expresses ~750 molecules of DinB in addition to the 250 molecules expressed from the chromosomal *dinB*$^+$ gene (Kim et al., 2001). In a *ΔumuD* CC108 strain (0 molecules UmuD$_2$; 1000 molecules DinB) we observe a 5-fold increase in −1 frameshift mutagenesis relative to a *umuD*$^+$ CC108 strain (90 molecules UmuD$_2$; 1000 molecules DinB). If all molecules of DinB participate equally in −1 frameshift mutagenesis under these conditions, this experiment should result in at most a 1.1-fold effect. Under the conditions of overproduction used in this study (Figure 2A), the number of molecules of DinB (Kim et al., 2001) likewise exceeds those of UmuD. These observations suggest that other limiting cellular factors may be required for DinB to promote −1 frameshift mutagenesis and moreover that these factors may also interact with UmuD$_2$.

Our discovery that UmuD$_2$ and RecA appear to modulate the mutagenic potential of DinB by enclosing its open active site may have implications for the control of DinB orthologs in other organisms. Overproduction of mammalian pol κ has similarly deleterious mutagenic consequences to DinB overproduction (Bavoux et al., 2005), despite the fact that its active site appears to be comparatively closed (Lone et al., 2007). A comparison of the pol κ structure with our working model of the DinB•UmuD$_2$•RecA complex suggests that part of the pol κ structure may play a role equivalent to RecA, but that its function might be further regulated by a partner protein interacting in a manner analogous to UmuD interacting with DinB.

Although the UmuD protein is only conserved among certain bacteria, we wondered whether the UmuD-binding interface on DinB might be maintained throughout evolution to interact either with a highly diverged UmuD or with a different but functionally equivalent partner protein. Despite the fact that residues on DinB that participate in this interface are not strongly conserved, we considered whether they would exhibit statistical covariance through evolution. We therefore assembled an alignment of numerous DinB and pol κ sequences from all domains of life and examined which positions showed statistically significant covariance (Afonnikov and Kolchanov, 2004) with at least two of three residues that comprise the UmuD$_2$-interacting interface of DinB. Strikingly, these residues define an interface on pol κ that is similar to the one we identified experimentally on DinB (Figure 7C). These observations suggest that there may be an evolutionary driving force for the maintenance of this interface, perhaps as a site for regulatory protein-protein interactions.

We had anticipated that deletion of either *umuD* or *recA* would affect DinB TLS function and were therefore initially surprised that deletion of *umuD* had no effect on *dinB*$^+$-dependent resistance to NFZ in a wild-type *E. coli* strain. Deletion of *recA* dramatically increased sensitivity to NFZ but, because of the central role of RecA in coordinating numerous aspects of the DNA damage response (Friedberg et al., 2006), we were unable to infer that this results from a loss of DinB-mediated TLS. This led us to wonder whether the −1 frameshift mutator activity of DinB is genetically separable from its ability to proficiently and accurately bypass certain N^2-dG adducts. We have previously characterized a separation of function mutant of DinB's steric gate residue, DinB(F13V), which is active as a conventional DNA polymerase but is virtually unable to catalyze TLS on N^2-dG adducted templates (Jarosz et al., 2006). In vivo, DinB(F13V) is exceptionally proficient at promoting −1 frameshift mutagenesis, although its levels are comparable to wild-type by immunoblot (Figure S8), indicating that certain mechanistic attributes of DinB that are required for its proficient and accurate N^2-dG lesion-bypass ability are not required for its −1 frameshift mutator activity (Figure 6A). This observation is consistent with the fact that the DinB(F172A) variant can confer NFZ resistance to a *ΔdinB* strain, despite the fact that it is somewhat compromised with respect to its −1 frameshift mutator activity.

Taken together our results indicate that DinB plays at least two separable roles in nature, one in which it promotes survival by accurately bypassing a common class of N^2-dG adducts and another in which it promotes mutagenesis under stressful conditions. The additional levels of DinB regulation we have described suggest ways in which the balance of these two diverse roles could be tuned to the physiological conditions being experienced by the organism.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification

Native DinB, UmuD, UmuD′, and RecA, and their variants, were purified as described previously (Beuning et al., 2006b; Jarosz et al., 2006; Konola et al., 1995), but 50 mM HEPES (pH 7.2) was used as

a buffering agent. Plasmids expressing DinB(F172A) and UmuD(D91A) were constructed from pDFJ1 and pSG5 (Beuning et al., 2006b) using a QuikChange kit (Stratagene). Both DinB(F172A) and UmuD(D91A) behaved as wild-type DinB and UmuD during purification. A plasmid encoding (His)$_6$HMK-DinB (Chen and Hai, 1994) was constructed in pET16B using standard cloning procedures. (His)$_6$HMK-DinB was purified using Ni^{2+}-NTA affinity resin (QIAGEN) following the manufacturer's instructions.

Affinity Chromatography

The details of the procedure are described in the Supplemental Data accompanying this article online.

Crosslinking and Binding Measurements

Crosslinking reactions were initiated with formaldehyde as described (Sutton et al., 1999) and allowed to proceed for 10 min before quenching with SDS-PAGE loading buffer containing 5% β-mercaptoethanol. Fluorescence spectroscopy was performed as described (Beuning et al., 2006b).

Far-Western Assays and Peptide Array Experiments

Far-western blots, as well as kinase labeling of purified (His)$_6$HMK-DinB with [γ-^{32}P]ATP by heart muscle kinase (Sigma), were performed according to standard procedures as previously described (Sutton et al., 1999). Cellulose filter peptide arrays were synthesized with overlapping 12-mer peptides offset by two residues (MIT CCR Core Facility). The arrays were probed with 100 nM UmuD$_2$ or DinB, and washed and developed as described (Niehbuhr and Wehland, 1997). Control arrays were performed using DinB or UmuD$_2$ antibodies alone.

Mutagenesis Assays

DinB-dependent −1 frameshift mutagenesis experiments were performed as previously described (Kim et al., 1997), except that IPTG was not added to the media. Ampicillin (100 μg/ml) and spectinomycin (60 μg/ml) were used as necessary for plasmid maintenance. Adaptive mutagenesis was performed as previously described (Godoy et al., 2000). A table of the strains and plasmids used in this study is provided as Supplemental Data (Table S1).

DinB and UmuD$_2$ Activity Assays

DinB was assayed as described previously (Jarosz et al., 2006), except 50 nM enzyme and 10 nM primer/template was used. The oligonucleotides 5′-ATCCTAGTCCAGGCTGCTGACAACTCGGGAACGTGCTAC ATGAAT-3′, 5′-ATTCATGTAGCAGCGTTCCC-3′, and 5′-ATTCATGT AGCAGCGTTCCG-3′ were designed based on those used previously (Wagner et al., 1999). Reactions were initiated with the appropriate dNTP, quenched after 20 min, and separated on a 16% denaturing polyacrylamide gel, which was quantified using a Typhoon phosphorimager (GE Healthcare). UmuD$_2$ autocleavage reactions were performed as described (Beuning et al., 2006b).

Molecular Modeling and Statistical Covariance

A model of the DinB•UmuD$_2$ complex was constructed using the application 3D dock (http://www.bmm.icnet.uk/docking) based on the following constraints: E168 < 6 Å from either UmuD chain, and L176, P177, K180, and F172 < 8 Å from D91 of either UmuD chain. The UmuD$_2$•RecA model was made using the following constraints: RecA S117 < 7 Å from either UmuD chain (Lee and Walker, 1996); UmuD residues L101, R102, V34, and S81 are 6–12 Å from RecA; and UmuD residue E11 is 6–25 Å from RecA. Resulting complexes were filtered based on the RecA residues T243 and R244 < 10 Å from DinB to generate a model of the DinB•RecA•UmuD$_2$ ternary complex. We performed the same procedure by docking DinB to the UmuD$_2$•RecA models using the constraints described above and obtained similar results. Statistical covariance was performed by aligning 84 DinB and pol κ sequences from diverse organisms using ClustalW and analyzing significant pairwise correlation of alignment positions with the CRASP algorithm (Afonnikov and Kolchanov, 2004).

Supplemental Data

Supplemental Data include ten figures and two tables and can be found with this article online at http://www.molecule.org/cgi/content/full/28/6/1058/DC1/.

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