**Escherichia coli** DinB (DNA polymerase IV) possesses an enzyme architecture resulting in specialized lesion bypass functionality and the potential for creating −1 frameshifts in homopolymeric nucleotide runs. We have previously shown that the mutagenic potential of DinB is regulated by the DNA damage response protein UmuD$_2$. In the current study, we employ a pre-steady-state fluorescence approach to gain a mechanistic understanding of DinB regulation by UmuD$_2$. Our results suggest that DinB, like its mammalian and archaeal orthologs, uses a template slippage mechanism to create single base deletions on homopolymeric runs. With 2-aminopurine as a fluorescent reporter in the DNA substrate, the template slippage reaction results in a prechemistry fluorescence change that is inhibited by UmuD$_2$. We propose a model in which DNA templates containing homopolymeric nucleotide runs, when bound to DinB, are in an equilibrium between non-slipped and slipped conformations. UmuD$_2$, when bound to DinB, displaces the equilibrium in favor of the non-slipped conformation, thereby preventing frameshifting and potentially enhancing DinB activity on non-slipped substrates.

DNA polymerases of the Y family catalyze replication on damaged DNA templates, thereby providing cells with a mechanism to tolerate DNA damage by a process called translesion DNA synthesis (TLS) (1). However, there is a potential mutagenic cost to TLS that is due to the intrinsic architecture of Y family polymerases. Although Y family and replicative polymerases share a similar “right-handed” fold, those in the Y family, which lack proofreading capability, make minimal contacts with substrate DNA and dNTP, resulting in higher error rates (1). Therefore, Y family polymerases are regulated to prevent inappropriate access to DNA replication intermediates and thus sustain genomic integrity (2).

Y family polymerases belonging to the DinB class are found in all domains of life and include *Escherichia coli* DinB (polymerase IV), *Sulfolobus solfataricus* Dpo4, *Sulfolobus acidocaldarius* Dbh, and, in eukaryotes, DNA polymerase κ (Pol κ) (3). These enzymes are capable of copying over certain dG lesions, including 7,8-dihydro-8-oxo-2'-deoxyguanosine and N$^{2}$-furfuryl-dG, and make few base substitution errors while doing so (4–8). In addition, DinB orthologs are also necessary for the final extension steps to complete TLS (9, 10). DinB and its orthologs produce single-base deletions at high rates (~10$^{-2}$ to 10$^{-3}$) on repetitive DNA sequences both in vitro and in vivo (11–18). The sequence specificity for single-base deletion formation by DinB and its archaeal orthologs (Dpo4 and Dbh) is remarkably similar, with all family members having elevated −1 frameshift potential on homopolymer sequences flanked by a 5′ G (13, 17, 18). It seems likely that some feature of the architecture of DinB orthologs that enables them to bypass N$^{2}$-dG lesions, namely an open active site and lack of proofreading, results in single-base deletions via a common mechanism on similar repetitive sequences.

Single-base deletions have been proposed to occur by three different mechanisms, illustrated in Fig. 1. Misinsertion misalignment occurs when an incorrect nucleotide enters the active site of the polymerase (step 1) and is incorporated at the primer terminus, creating a base substitution error (step 2) (19, 20). A subsequent DNA rearrangement results in the newly added terminal nucleotide of the primer pairing with the complementary base 5′ to the templating base (+1-position), forcing the templating (0-position) base to become unpaired in an “extrahelical” structure (step 3). In the template slippage model proposed by Streisinger (Streisinger slippage) (21), the polymerase can potentially form two enzyme-DNA binary conformations that are presumably in equilibrium. The non-slipped conformation has all of the bases properly base-paired (top structure). The slipped preinsertion binary conformation (bottom) occurs when the primer and template strands misalign, resulting in an unpaired base (step 1). However, the equilibrium can be displaced in favor of the slipped conformation by binding of a dNTP complementary to the next templating base to form a slipped ternary complex (step 2). The slipped preinsertion binary and preinsertion ternary intermediates do not require phosphodiester bond formation for stability because the terminal base of the primer is correctly paired with a base on...
the template strand. The primer of the slipped ternary complex is then extended by phosphodiester bond formation (step 3). In the dNTP-stabilized misalignment mechanism (22–24), an incoming nucleotide pairs with a base 5’ to the templating base (+1), forcing the templating base (0) to become extrahelical (step 1). The extrahelical base is then stabilized by phosphodiester bond formation (step 2). Although all the mechanisms outlined above have a similar end point, each pathway results in different intermediates that can be distinguished by fluorescence methods (25, 26).

DinB family members have been proposed to use all three mechanisms for creating single-base deletions, although only one, template slippage, requires a homopolymeric nucleotide run. In a study of Pol κ fidelity, Ohashi et al. (12) demonstrated that the enzyme generated single-base deletions at high rates (~10⁻³). On repetitive sequences, Pol κ generated deletions at a rate that was 2–3-fold higher than on non-repetitive sequences, consistent with the use of template slippage. Ohashi et al. (12) also concluded, based on the high deletion rate on non-repetitive sequences, that Pol κ additionally generates single base deletions either by misinsertion or dNTP-stabilized misalignment. Subsequent biochemical data obtained by Wolfe et al. (27) demonstrated that Pol κ efficiently extends mispaired termini by a primer-template misalignment, supporting a misinsertion misalignment mechanism. Moreover, Wolfe et al. (27) were unable to observe chemical incorporation by Pol κ on substrates that can only be extended by dNTP-stabilized misalignment. An archaean family member, Dbh, creates pyrimidine deletions as much as 50% of the time on the sequence 5’-G-Py-Py; a template slippage mechanism is implicated by stopped-flow fluorescence experiments, which demonstrate the unstacking of a nucleotide on the template strand used by other DinB family members. In the current study, we use a pre-steady-state fluorescence approach to demonstrate that E. coli DinB does indeed use a template slippage mechanism when making single-base deletions (17, 26). However, the authors reported that deletion formation required a run of at least 3 identical bases (26), an observation that is more consistent with template slippage used by other DinB family members. In the current study, we use a pre-steady-state fluorescence approach to demonstrate that E. coli DinB does indeed use a template slippage mechanism when making single-base deletions on substrates that contain homopolymeric nucleotide runs.

We had previously shown that RecA and UmuD₂ prevent DinB-mediated 1 frameshift mutagenesis both in vivo and in vitro and have suggested that mechanistic principles of this regulation may be conserved throughout evolution (29). RecA and UmuD, like DinB, are transcriptionally up-regulated as part of the SOS response after DNA damage (for a review, see Ref. 30), and these proteins form a DinB-RecA-UmuD₂ complex (29). We have previously described an in silico model for the DinB-RecA-UmuD₂ complex in which the accessory proteins constrain the active site of DinB, preventing rearrangements necessary for the formation of deletion intermediates (29). In support of this model, we report that the addition of UmuD₂ and RecA to our DinB reactions results in the inhibition of a non-covalent step within the template slippage pathway, consistent with the accessory proteins preventing the formation of slipped DNA intermediates on homopolymeric nucleotide runs.

**EXPERIMENTAL PROCEDURES**

*Materials—* Native DinB was purified as described previously (6), except that the purified protein was stored in 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol. Native UmuD was purified as described previously (31). RecA protein and T4-polynucleotide kinase were purchased from New England Biolabs. Synthetic oligonucleotides were purchased from the W. M. Keck Biotechnology Resource Laboratory (Yale Medical School) and were purified as described previously (13). Substrate sequences are provided in supplemental Table S1. Ultrapure dNTPs were purchased from GE Healthcare. [γ⁻³²P]ATP was purchased from PerkinElmer Life Sciences.

*Time-based Fluorescence Emission Assay—* Reactions containing 2-amino-purine (2-AP) substrates were excited at 310 nm, and emission was monitored at 360 nm at 22 °C using a Photon Technology International scanning spectrophotometer. Emission of 1 μM annealed primer-template DNA, 6–15 μM DinB (as indicated) in DinB reaction buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 4 mM MgCl₂) was monitored for 50 s (65-μl total volume), and extension was then initiated by adding 2 μl of dNTP (3 mM final), and emission was monitored for an additional ≥100 s. When indicated, RecA and UmuD were added to the reaction in a DinB/RecA/UmuD₂ ratio of 1:2:10.

*Stopped-flow Fluorescence—* Stopped-flow experiments were performed at 22 °C on an Applied Photophysics SX.18MV spectrophotometer. To measure dNTP incorporation, one drive syringe contained DNA and proteins in DinB reaction buffer, and the other contained dNTP and MgCl₂ in DinB reaction buffer. Nucleotide incorporation was initiated by rapid mixing of the contents of each syringe (80 μl each). The final concentrations of components were 200 nM substrate DNA, 3 μM DinB, 6 μM RecA and 60 μM UmuD₂, 2 mM dNTP, and 2 mM additional MgCl₂. The excitation wavelength for 2-AP substrates was 310 nm, and fluorescence emission was detected using a 345-nm long pass filter. Three individual traces of at least 10 s for each reaction were averaged and fitted to exponential equations using SigmaPlot (Systat Software) to determine a reaction rate. Representative fittings are shown in supplemental Fig. S1.

*Chemical Quench Reactions—* Primer oligonucleotides were 5’-end labeled with [γ⁻³²P]ATP by T4-polynucleotide kinase and annealed to template oligonucleotide by heating to 95 °C for 1 min, followed by annealing at 37 °C for 45 min. Rapid quench-flow (KinTek Corp., model QRF-3) and manual quench kinetic measurements were performed under single-turnover conditions at 22 °C with final concentrations of 10 nM annealed primertemplate DNA and 1 μM DinB in DinB reaction buffer. When indicated, 2 μM RecA and 20 μM UmuD final concentrations were added to the reaction. Reactions were initiated with dNTP solution (final concentration 1 mM dNTP in DinB reaction buffer with an additional 1 mM MgCl₂). Reactions were quenched with 5 μl of quench buffer (80% formam-
DinB Template Slippage Is Inhibited by UmuD₂

We therefore designed several DNA substrates (supplemental Table S1; substrates shown in abbreviated form in Figs. 2–5) with the 2-AP reporter either as the templating base (0-position; substrates I-3C, I-4C, and III-C) or base-paired with the primer terminus (−1-position; substrate II-G) within nucleotide runs of varying lengths to deduce the DNA stacking environment of these positions. With 2-AP at the 0-position, we expect that the template slippage pathway will result in a prechemistry fluorescence decrease when 2-AP slips backward and base-pairs with the terminal nucleotide of the primer. In contrast, the dNTP-stabilized misalignment mechanism will give a fluorescence increase as the 2-AP becomes unstacked from its neighbors. With 2-AP at the −1-position, opposite the primer terminus, the template slippage pathway predicts a prechemistry fluorescence increase as the 2-AP loses its base-pairing partner, whereas dNTP-stabilized misalignment should result in little or no fluorescence change. The fluorescence changes associated with the misinsertion-misalignment pathway (Fig. 1) are likely to be similar to those predicted for dNTP-stabilized misalignment, but consideration of reaction rates, as described below, clearly rules out this pathway.

DinB Uses a Template Slippage Mechanism on Homopolymeric Nucleotide Runs—In preparation for our studies on the effects of UmuD and RecA, we characterized the action of DinB on substrates containing 2-AP within homopolymeric nucleotide runs. We used stopped-flow fluorescence to observe the fluorescence changes that take place when a DinB-DNA binary complex is mixed with a dNTP complementary to the base 5′ to the templating position (base-skipping reaction; red traces) (Fig. 2). The fluorescence changes observed were those predicted by the template slippage mechanism: a decrease with 2-AP at the 0-position (duplexes I-3C and I-4C; Fig. 2, A and B) and an increase with 2-AP at the −1-position (duplex II-G; Fig. 2D). Fitting of the stopped-flow fluorescence traces for the base-skipping reactions gave similar rates (≈45 s⁻¹; Table 1) of the initial fluorescence change for all of these substrates, suggesting that these rates are limited by the same process and are not influenced significantly by the length of the homopolymeric run involved in the slippage process. Moreover, the initial fluorescence change must correspond to a prechemistry step because its rate (≈45 s⁻¹) is 1000-fold faster than the corresponding dNTP incorporation rate (≈3 × 10⁻² s⁻¹) measured by chemical quench. The existence of an early non-covalent step was corroborated by stopped-flow experiments using substrates I-3C(dd), I-4C(dd), and II-G(dd), which are analogous to...
the parental substrates except that the primer strand has a 2',3'-dideoxynucleotide and thus cannot form a phosphodiester bond with the incoming nucleotide. When mixed with the dNTP appropriate for the base-skipping reaction, all three dideoxy-terminated substrates resulted in fluorescence changes whose amplitudes and rates were similar to the major change observed with the corresponding deoxy-terminated substrates (Fig. 3, A–C, and Table 1). Thus, base-skipping reactions utilizing deoxy- and dideoxy-terminated substrates containing homopolymeric nucleotide runs result in rapid fluorescence changes consistent with the extrusion of a base within the duplex region prior to chemistry.

Variants of these DNA substrates, in which the +1 base is not complementary to the added dNTP (duplexes I-4G and II-C; supplemental Table S1), gave no fluorescence changes on the time scale tested (Fig. 2, C and E, blue traces), demonstrating that the fluorescence changes observed for the base-skipping reactions require complementarity between the incoming nucleotide and the +1 base. The I-4G and II-C duplexes were also used to measure, by chemical quench, the rates of the misinsertion events (2-AP dGTP and A dCTP, respectively) that would be the initiating events in the misinsertion-misalignment mechanism for base skipping. The misinsertion reactions were ~10-fold slower than the base-skipping reactions templated by a dNTP complementary to the +1 base (Table 1); compare, for example, the chemical incorporation rate of II-G base skipping ((6.2 ± 0.1) × 10⁻²) and II-C misinsertion control ((5.0 ± 1.9) × 10⁻³). This indicates that the majority of the base-skipping mutations catalyzed by DinB on nucleotide runs are not likely to be initiated by a misinsertion event, and thus the misinsertion-misalignment pathway (Fig. 1) makes, at best, a minimal contribution.

The fluorescence changes were recorded after mixing a binary complex of DinB bound to the indicated substrate with either the dNTP required for base-skipping, error-free incorporation or misinsertion. For each trace, the relevant DNA sequence at the primer terminus is shown along with the appropriately colored incoming dNTP (Ap, 2-AP; a crossed out arrow indicates the absence of incorporation; full sequences of the DNA substrates are given in supplemental Table S1). A and B, for substrates I-3C and I-4C with 2-AP as the templating base, a rapid fluorescence decrease is observed for both the base-skipping (red) and error-free (green) reactions compared with the buffer control (black), consistent with template slippage. D, for substrate II-G (2AP (−1)) the base-skipping reaction (red) results in a rapid fluorescence increase, which, like substrates I-3C and I-4C, is consistent with template slippage. The error-free reaction (green) resulted in a decrease. C and E, no fluorescence changes are observed for the misinsertion control reactions (blue traces) using substrates I-4G and II-C when compared with a buffer control. The data were plotted on a logarithmic time scale so as to show early fluorescence changes. See Table 1 and supplemental Table S2 for the rate data obtained from these experiments.

FIGURE 2. Stopped-flow fluorescence traces of substrates I and II suggest that DinB creates single-base deletions via a template slippage pathway. The fluorescence changes were recorded after mixing a binary complex of DinB bound to the indicated substrate with either the dNTP required for base-skipping, error-free incorporation or misinsertion. For each trace, the relevant DNA sequence at the primer terminus is shown along with the appropriately colored incoming dNTP (Ap, 2-AP; a crossed out arrow indicates the absence of incorporation; full sequences of the DNA substrates are given in supplemental Table S1). A and B, for substrates I-3C and I-4C with 2-AP as the templating base, a rapid fluorescence decrease is observed for both the base-skipping (red) and error-free (green) reactions compared with the buffer control (black), consistent with template slippage. D, for substrate II-G (2AP (−1)) the base-skipping reaction (red) results in a rapid fluorescence increase, which, like substrates I-3C and I-4C, is consistent with template slippage. The error-free reaction (green) resulted in a decrease. C and E, no fluorescence changes are observed for the misinsertion control reactions (blue traces) using substrates I-4G and II-C when compared with a buffer control. The data were plotted on a logarithmic time scale so as to show early fluorescence changes. See Table 1 and supplemental Table S2 for the rate data obtained from these experiments.
DinB Template Slippage Is Inhibited by UmuD₂

Error-free (non-slipped) incorporation of a correctly paired dNTP onto substrates I-3C, I-4C, and II-G was associated with a rapid fluorescence decrease of ~120 s⁻¹ (Fig. 2, A, B, and D (green traces) and Table 1). The similarity of the rates of the fluorescence changes in the base-skipping (~45 s⁻¹) and normal incorporation (~120 s⁻¹) reactions suggests that they may correspond to analogous molecular processes. Interestingly, the DinB error-free reactions resulted in biphasic curves where the second fluorescent change varied considerably in amplitude (range 0.05 to 0.25 V) but had a rate similar to the chemical incorporation rate (~2 s⁻¹) (supplemental Table S2). The second fluorescence change was not observed in reactions using dideoxy-terminated substrates, suggesting that it may indeed correspond to the rate-limiting step for chemistry. The same conclusion was reached in a similar pre-steady-state fluorescence study of Dbh (25) and is consistent with studies of other DinB orthologs (12, 27, 33, 34) However, the template slippage mechanism is at odds with an earlier fluorescence study of DinB by Tippin et al. (26), which concluded that DinB uses a dNTP-stabilized misalignment mechanism. This disagreement is particularly puzzling because substrates I-3C and I-4C have the same sequence around the primer terminus as that used by Tippin et al. (26). To rule out the possibility that the mechanism of the base-skipping reaction might be influenced by DNA sequence remote from the primer terminus, we carried out a time-based fluorescence emission assay (Fig. 4) using substrate III-C, which has 2-AP at the templating position (0-position) and is identical to the substrate used in an earlier DinB study (17). The base-line fluorescence signal of III-C and purified DinB was established, and then the reaction was started by adding dGTP (base skipping) or dTTP (error-free control). In both cases, we observed a fluorescence decrease, consistent with our previous conclusion that DinB uses a template slippage mechanism (Fig. 4A). By contrast, the earlier study had reported a

| DNA Substrate | Primer Terminus | Reaction Type | dNTP | Fluorescence Change | Chemistry |
|---------------|----------------|--------------|------|---------------------|-----------|
|               |                |              |      |                     |           |
| I-3C          | CTTTT          | Base-skip    | dGTP | -                   | 41        |
| I-3C(dd)      | GAAAXC⁻        | Error-free   | dTTP | -                   | 110       |
| I-4C          | CTTTT          | Base-skip    | dGTP | -                   | 47        |
| I-4C(dd)      | GAAAXC⁻        | Error-free   | dTTP | -                   | 140       |
| I-4G          | CTTTT          | Misinsertion | dGTP | N.D.                | 2.8 x 10⁻³ |
| II-G          | CT             | Base-skip    | dCTP | +                   | 50        |
| II-G(dd)      | GXAG⁻          | Error-free   | dTTP | -                   | 120       |
| II-C          | CT             | Misinsertion | dCTP | N.D.                | 5.0 x 10⁻³ |
| III-C*        | CTTTTT         | Base-Skip    | dGTP | -                   | 1.5 x 10⁻² |
| III-G         | CTTTT          | Misinsertion | dGTP | N.D.                | 1.8 x 10⁻³ |

*The relevant sequence surrounding the primer terminus is shown. See supplemental Table S1 for complete substrate sequences.

** Increase (+) or decrease (−) in fluorescence signal following dNTP addition. The absence of an amplitude change is indicated by N.D. (none detected).

† Rates were determined by fitting the stopped-flow fluorescence data to exponential equations. See "Experimental Procedures" for methods and supplemental Fig. S1 for representative fittings. For data that fitted to >1 exponential, the rate of the largest amplitude fluorescence change is reported here. The full data are reported in supplemental Table S2.

(/dd), a dideoxy-terminated substrate.

The base-skip reaction of substrates III-C and III-G was analyzed by time-based emission measurement in the fluorometer. The rate of the fluorescence decrease for the III-C reaction was fast, consistent with the stopped-flow rates reported for the other substrates.

The misinsertion reactions for substrates I-4G and III-G were initiated with dGTP, whereas the reaction for substrate II-C was initiated with dCTP.

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**TABLE 1**
Rates of chemical incorporation and fluorescence changes catalyzed by DinB

| DNA Substrate | Primer Terminus | Reaction Type | dNTP | Fluorescence Change | Chemistry |
|---------------|----------------|--------------|------|---------------------|-----------|
| I-3C          | CTTTT          | Base-skip    | dGTP | 41                  | 9.4 x 10⁻³ |
| I-3C(dd)      | GAAAXC⁻        | Error-free   | dTTP | 110                 |           |
| I-4C          | CTTTT          | Base-skip    | dGTP | 23                  | 2.3 x 10⁻² |
| I-4C(dd)      | GAAAXC⁻        | Error-free   | dTTP | 31                  |           |
| I-4G          | CTTTT          | Misinsertion | dGTP | N.D.                | 2.8 x 10⁻³ |
| II-G          | CT             | Base-skip    | dCTP | 50                  | 6.2 x 10⁻² |
| II-G(dd)      | GXAG⁻          | Error-free   | dTTP | 120                 | 2.3       |
| II-C          | CT             | Misinsertion | dCTP | N.D.                | 5.0 x 10⁻³ |
| III-C*        | CTTTTT         | Base-Skip    | dGTP | -                   | 1.5 x 10⁻² |
| III-G         | CTTTT          | Misinsertion | dGTP | N.D.                | 1.8 x 10⁻³ |

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The same conclusion was reached in a similar pre-steady-state fluorescence study of Dbh (25) and is consistent with studies of other DinB orthologs (12, 27, 28, 33, 34) However, the template slippage mechanism is at odds with an earlier fluorescence study of DinB by Tippin et al. (26), which concluded that DinB uses a dNTP-stabilized misalignment mechanism. This disagreement is particularly puzzling because substrates I-3C and I-4C have the same sequence around the primer terminus as that used by Tippin et al. (26). To rule out the possibility that the mechanism of the base-skipping reaction might be influenced by DNA sequence remote from the primer terminus, we carried out a time-based fluorescence emission assay (Fig. 4) using substrate III-C, which has 2-AP at the templating position (0-position) and is identical to the substrate used in an earlier DinB study (17). The base-line fluorescence signal of III-C and purified DinB was established, and then the reaction was started by adding dGTP (base skipping) or dTTP (error-free control). In both cases, we observed a fluorescence decrease, consistent with our previous conclusion that DinB uses a template slippage mechanism (Fig. 4A). By contrast, the earlier study had reported a
A Non-covalent Step in the DinB-dependent Template Slippage

fluorescence increase for the base-skipping reaction (17). However, consistent with this earlier study, we did not observe a fluorescence change for the misinsertion misalignment control reaction using substrate III-G (Fig. 4B). Although the disagreement between previous DinB reports (17, 26) and the data presented here is puzzling, our observations and conclusion that DinB uses a template slippage mechanism on substrates with homopolymeric nucleotide runs are entirely consistent with studies of other DinB orthologs (12, 25, 27, 28, 33, 34).

A Non-covalent Step in the DinB-dependent Template Slippage Pathway Is Inhibited by Saturating Quantities of UmuD<sub>2</sub>

We have hypothesized that UmuD and RecA suppress the base-skipping reaction of DinB by enclosing the open active site of DinB to prevent template bulging (29). To test this hypothesis, we added RecA and UmuD<sub>2</sub> to our stopped-flow and time-based fluorescence assays, predicting an inhibition of the early non-covalent fluorescence change. In the complex purified in our earlier study, DinB-RecA-UmuD<sub>2</sub> were in a 1:1:1 ratio (29); here we used saturating quantities of UmuD<sub>2</sub> with and without RecA to ensure that each DinB molecule was bound. The stopped-flow fluorescence traces obtained using DinB, DinB-UmuD<sub>2</sub> (complex in 1:1:1 ratio), and DinB-RecA-UmuD<sub>2</sub> (complex in a 1:2:1 ratio) indicate that UmuD<sub>2</sub> does indeed inhibit the prechemistry fluorescence increase in the DinB base-skipping reaction. Unlike the experiments with DinB alone in which we observed a very rapid fluorescence change (Fig. 5A), we did not observe any fluorescence emission changes for DinB-UmuD<sub>2</sub> (Fig. 5B) and DinB-RecA-UmuD<sub>2</sub> (Fig. 5C) during the first 10 s of the reaction on substrate II-G. The absence of a rapid change in fluorescence is consistent with our hypothesis that UmuD<sub>2</sub> encloses the DinB active site, preventing the formation of slipped intermediates within nucleotide runs. The general nature of this conclusion is further supported by the absence of rapid fluorescence changes during base-skipping reactions on substrates I-3C and I-4C (supplemental Fig. S2). To test if UmuD<sub>2</sub> inhibits these conformational changes for extended periods of time, we monitored fluorescence emission for 100 s using a fluorometer. On substrate II-G, base skipping in a reaction with DinB alone resulted in the expected rapid fluorescence increase followed by a slower fluorescence increase with a rate of 3.0 × 10<sup>-2</sup> s<sup>-1</sup> (Fig. 5D). Saturating quantities of RecA and UmuD<sub>2</sub> eliminated the immediate fluorescence increase, consistent with our hypothesis and stopped-flow results (Fig. 5E). However, a slow fluorescence increase that does not reach completion within 150 s was detected. Chemical quench assays confirm the inhibition of the base-skipping reaction by RecA and UmuD<sub>2</sub>, demonstrating a decrease in the rate (~8-fold) of covalent nucleotide incor-
DinB complex chemical incorporation and fluorescence change rates on substrate II-G

| Protein(s)         | Reaction type | dNTP     | Fluorescence change | Chemistry | Rate | Amplitude |
|--------------------|---------------|----------|---------------------|-----------|------|-----------|
|                    |               |          | Rate                |           |      | 1 nM s^-1 |
| DinB               | Base-skip     | dCTP     | +                   |           | 50   | 7.2       |
|                    | Error-free    | dTTP     | -                   |           | 120  | 9.4       |
| DinB-UmuD$_2$      | Base-skip     | dCTP     | ND                  | ND        | 3.8  | 8.0 x 10^-3 |
|                    | Error-free    | dTTP     | ND                  | ND        | 9.4  | 0.76      |

a Increase (+) or decrease (−) in fluorescence signal following dNTP addition. The absence of an amplitude change is indicated by ND (none detected).

b Rates were determined by fitting the stopped-flow fluorescence data to exponential equations. See “Experimental Procedures” for methods and supplemental Fig. S1 for representative fittings. For data that fitted to 1 exponential, the rate of the largest amplitude fluorescence change is reported here. The full data are reported in supplemental Table S2.

c Amount of the 10 nM DNA primers extended by DinB after 5 min.

DinB Template Slippage Is Inhibited by UmuD$_2$

A significant decrease in fluorescence change was observed for the error-free reaction in the presence of UmuD$_2$. The absence of fluorescence change indicates that the reaction was completely error-free. The presence of UmuD$_2$ did, however, eliminate the rapid prechemistry fluorescence decrease in the error-free reaction, whereas a slower fluorescence change with a rate similar to chemistry was observed for substrate II-G.
DISCUSSION

**DinB and Its Orthologs Use a Template Slippage Mechanism on Substrates Containing Homopolymeric Nucleotide Runs**—Our stopped-flow fluorescence experiments with a series of DNA substrates containing a 2-AP reporter demonstrated that DinB creates single-base deletions within homopolymeric nucleotide runs via a template slippage mechanism. The 2-AP reporter positioned either at the 0- or −1-position within a run resulted in a rapid prechemistry fluorescence change, interpreted as a template slippage isomerization that causes a base within the duplex region to adopt an extrahelical, or unstacked, conformation. In the template slippage model (Fig. 1), the misalignment should occur prior to dNTP chemical incorporation, consistent with the observed rate of the fluorescence change. The clearest evidence supporting the template slippage model during the base-skipping reaction is provided by substrate II-G, having 2-AP at the −1-position, where we observe a fluorescence increase consistent with 2-AP being unstacked from its 5′ and 3′ neighbors in an extrahelical conformation.

Mutational spectra of DinB orthologs suggest a preference to create single-base deletions within homopolymeric runs adjacent to a 5′ G (12, 13, 17, 18). The presence of the 5′ G at the end of the homopolymeric run may be related to the ability of these enzymes to efficiently and accurately bypass N2-dG lesions during TLS (4–8), and the similar sequence specificity argues for a common mechanism for creating deletion mutations (i.e. template slippage). A fluorescence study of Dbh demonstrated a prechemistry step that could correspond to the formation of a slipped DNA intermediate during a base-skipping reaction (25). Moreover, DNA structures analogous to template slippage intermediates have been observed in co-crystals of Dbh-DNA complexes with an extrahelical base (28) and Dpo4 bound to damaged DNA (33, 34). Both Dpo4 bound to a substrate containing an abasic site (Ab-2a structure) (33) and to a benzo[a]pyrene-deoxyguanosine DNA substrate (34) have the unpaired DNA lesion in an extrahelical conformation stabilized by a correct base pair at the primer terminus, analogous to the intermediates we have proposed for the base-skipping pathway (Figs. 2–4). The mammalian ortholog Pol κ, however, creates single-base deletions at high rates on both non-repetitive and repetitive sequences by different mechanisms. The ability to create single-base deletions on non-repetitive sequences results from its ability to efficiently extend from substrates that have formed via a primer-template realignment as a consequence of a dNTP misinsertion event (i.e. misinsertion misalignment) (27). The ability of Pol κ to use a template slippage mechanism is inferred from the enzyme’s inability to use a dNTP-stabilized misalignment in vitro (27) and elevated frameshift rates on repetitive versus non-repetitive sequences (12). Thus, Pol κ-mediated mutations created via different mechanisms suggest that DinB orthologs may make single-base deletions by various pathways in a sequence context-dependent manner. However, it seems likely that members of the DinB family of DNA polymerases all use a template slippage mechanism on repetitive sequences to create single-base deletions.

Contrary to the results reported here, an earlier study by Tippin et al. (26) concluded that DinB creates single nucleotide deletions via a dNTP-stabilized misalignment mechanism. Using substrates similar to our I-3C and I-4C, with 2-AP at the 0-position adjacent to a run of A residues, Tippin et al. (26) observed a fluorescence increase, the opposite of the fluorescence change we report here. Compared with the fluorescence changes we observed for the base-skipping reaction catalyzed by DinB, the fluorescence change reported by Tippin et al. (26) was less robust, required a deoxy (not dideoxy) primer terminus, and had a slower rate, similar to that of chemical incorporation in a base-skipping situation. Tippin et al. (26) also reported a requirement for a homopolymeric run of >2 identical bases; this last observation is a classic characteristic of a template slippage reaction and therefore argues against their assignment of a dNTP-stabilized misalignment mechanism (21–24). In the same study, Tippin et al. (26) described base-skipping reactions of polymerase μ that, like our DinB data, display all of the hallmarks of a template slippage pathway. Adding to the puzzling nature of the discrepancy are the remarkably similar rates of dNTP error-free and base-skipping incorporation by DinB reported here and by Tippin et al. (26). Moreover, the DinB production methods used by us (6) and by Goodman and colleagues (35) both produce enzymes capable of bypassing benzo[a]pyrene adducts with similar efficiencies.

**UmuD2 Prevents Single-base Deletions by Inhibiting the Formation of a Template Slippage Intermediate**—In the DinB-catalyzed template slippage reaction, a DNA rearrangement must occur. Because the 2-AP probe is sensitive to nucleotide base-stacking interactions (32), our stopped-flow fluorescence experiments monitor this DNA rearrangement and provide mechanistic insights into our DinB-RecA-UmuD2 model (29). We argued above that the major fluorescence change observed for the DinB base-skipping reaction corresponds to DNA template-primer misalignment. This non-covalent fluorescence change (i.e. template slippage) is blocked by DinB-UmuD2 or DinB-RecA-UmuD2, with the result that we do not observe the prechemistry fluorescence change seen for DinB within the first 10 s of the reaction (Fig. 5). We had previously shown that RecA was required to inhibit DinB extension from slipped primer termini and speculated that RecA may aid in targeting DinB-UmuD2 to the primer terminus (29). However, the high DNA and protein concentrations used in this assay may reduce the requirement for RecA. In the presence of UmuD2 and RecA, a base-skipping reaction catalyzed by DinB results in a slow fluorescence increase observed on a longer time scale similar to the nucleotide incorporation rate (Fig. 5 and Table 2). An absolute block to the template slippage pathway is unlikely because these complexes would be expected to be dynamic in solution, allowing UmuD2 molecules to freely release from DinB, allowing slow, but detectable, catalysis to occur over several minutes. Moreover, the ~0.1 mM UmuD2 concentration used in our assays may alter its protein folding. UmuD2 is an intrinsically disordered protein displaying a CD spectrum characteristic of a random coil at low concentrations (~5 μM) and a spectrum indicating the presence of a β-sheet at high concentrations (~1 mM) (31). This suggests multiple protein concentration-dependent conformations, only a subset of which may be competent to bind and inhibit DinB catalysis. These multiple protein-dependent
DinB Template Slippage Is Inhibited by UmuD₂

**FIGURE 6. Proposed explanation for the modulation of DinB activity by UmuD₂.** In the absence of SOS induction (left), DinB (gray box) is bound by UmuD₂ with full-length arms, which favors binding of the DNA substrate in the non-slipped conformation, thereby preventing base skipping and possibly enhancing error-free incorporation. Upon DNA damage (right), the SOS response is induced, and the arms of UmuD₂ are cleaved by the RecA nucleoprotein filament to form UmuD₁. UmuD₁ is unable to bind DinB and influence the equilibrium between slipped and non-slipped conformations of DNA, thus allowing the formation of single-base deletions via template slippage.

Conformations may also contribute to the fast low amplitude changes seen prior to the major amplitude change during the DinB-UmuD₂ error-free reaction (supplemental Table S2). The fast low amplitude change may represent only a fraction of the UmuD₂ molecules initially being in a conformation that enables DinB binding, thus allowing the formation of the non-covalent intermediate to occur in a subset of the substrate population.

Our previous suggestion that polymerase-bound DNA substrates containing homopolymeric nucleotide runs would be in equilibrium between slipped and non-slipped conformations (25) is supported by a Dbh-DNA binary complex co-crystal in which the DNA contained an extraheiral base (28). A recent study of polymerase λ suggests that this model may potentially be applicable to all polymerases. Deletion and insertion errors of polymerase λ occur through the ability of this polymerase to bind the template in multiple conformations relative to the primer strand during catalytic cycling (36). Moreover, those authors argued that, because all DNA polymerases make single-base deletions, make and break non-covalent contacts with substrate DNA, and alter the positioning of residues and substrates within their active sites, repositioning of the template strand relative to the primer during catalytic cycling offers a general mechanism by which all DNA polymerases could create deletion and insertion errors (36).

Based on the results reported above and the potential for polymerases to bind a substrate in multiple conformations, we propose that UmuD₂ displaces the DinB-DNA binary equilibrium in favor of the non-slipped DNA conformation (Fig. 6). In the presence of DinB alone, binding of dNTP corresponding to the base-skip reaction will displace the equilibrium toward the slipped conformation; conversely, binding of dNTP complementary to the templating base (error-free incorporation) will favor the non-slipped conformation. The prechemistry fluorescence changes in Fig. 5A are entirely consistent with these predictions. In the presence of UmuD₁, however, the rapid fluorescence changes for the error-free (decrease) and base-skipping (increase) reactions are not observed, consistent with the equilibrium strongly favoring the non-slipped conformation in the binary complex and the slipped conformation being inhibited during the formation of an insertion ternary complex. Thus, we propose that in vivo in the absence of DNA damage (non-SOS conditions), when the number of DinB (250 molecules) and UmuD (180 molecules) are similar and these proteins are capable of interacting ($K_d = 0.62 \mu M$) (15, 29, 37), UmuD₂ prevents template bulging by constraining the open DinB active site and thereby biasing the DNA substrate equilibrium toward the non-slipped conformation and limiting the reaction to error-free dNTP incorporation. The biasing of the equilibrium toward the non-slipped conformation not only provides a mechanism to prevent single-base deletions by UmuD₂ but also provides an explanation for the enhancement of DinB activity in the presence of UmuD₂ seen in steady-state reactions when protein and nucleotide levels mimic physiological conditions more closely than the saturating DinB and dNTP concentrations used here (29). Under SOS conditions, the N-terminal 24 amino acids of UmuD are cleaved by the DNA-RecA nucleoprotein filament to form UmuD’ (30). Estimation of the DinB and UmuD₂ dissociation constant ($K_d > 20 \mu M$)⁶ suggests an inability of these proteins to form a stable complex in vivo, consistent with our previous observation that UmuD₂ is unable to inhibit single-base deletions in vivo on homopolymeric nucleotide runs as measured by the Cairns and Foster adaptive mutagenesis system and the Cupples and Miller CC108 Lac⁺ reversion assays (29, 38, 39). Therefore, in our proposed model under SOS conditions, the DNA substrate equilibrium is not significantly affected by UmuD₂ allowing the formation of single-base deletions on homopolymeric nucleotide runs via template slippage. These regulatory mechanisms enable DinB to act as a relatively accurate DNA polymerase under non-stressed conditions while allowing it to transform into a more flexible and mutagenic DNA polymerase after SOS induction.

**Evolutionary Perspective**—Several residues along the predicted DinB-UmuD₂-interacting surface show statistical covariance across evolution, suggesting that the ability of DinB and its orthologs to create single-base deletions may be regulated by comparable protein contacts (29). We speculate that the N-clasp domain of Pol κ may play a functionally similar role to UmuD in regulating polymerase activity. The N-clasp is a unique protein domain that encircles the DNA substrate while interacting with the thumb, finger, and little finger domains partially enclosing the active site. The partial closing of the active site by an intrinsic domain provides a potential explanation for the absence of some frameshift hot spots in the full-length Pol κ mutational spectra that were observed for DinB in the same forward mutation assay (12, 17). Analogous to the

⁶ S. M. Simon and G. C. Walker, unpublished observation.
UmuD₂ (inactive for TLS) to UmuD’₂ (active for TLS) cleavage, the N-clasp acts like a switch between inactive and active enzyme modes, thereby modulating Pol κ activity in a situation-dependent manner (12, 40, 41). Moreover, the N-clasp could directly affect Pol κ dNTP incorporation because the very N terminus reaches over the fingers domain into the active site (42). Although speculative, the positioning of the N-clasp and its ability to modulate Pol κ activity suggest commonalities in the regulation of polymerase activity by the N-clasp and UmuD on their respective DinB orthologs.

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