LETTERS

A single amino acid governs enhanced activity of DinB DNA polymerases on damaged templates

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Translesion synthesis (TLS) by Y-family DNA polymerases is a chief mechanism of DNA damage tolerance[1]. Such TLS can be accurate or error-prone, as it is for bypass of a cyclobutane pyrimidine dimer by DNA polymerase η (XP-V or Rad30) or bypass of a (6-4) TT photoproduct by DNA polymerase V ($UmuD'_2C$), respectively[2,3]. Although DinB is the only Y-family DNA polymerase conserved among all domains of life, the biological rationale for this striking conservation has remained enigmatic[4]. Here we report that the *Escherichia coli dinB* gene is required for resistance to some DNA-damaging agents that form adducts at the N^2-position of deoxyguanosine (dG). We show that DinB (DNA polymerase IV) catalyses accurate TLS over one such N^2-dG adduct (N^2-furfuryl-dG), and that DinB and its mammalian orthologue, DNA polymerase κ, insert deoxycytidine (dC) opposite N^2-furfuryl-dG with 10–15-fold greater catalytic proficiency than opposite undamaged dG. We also show that mutating a single amino acid, the 'steric gate' residue of DinB (Phe13 → Val) and that of its archaeal homologue Dbh (Phe12 → Ala), separates the abilities of these enzymes to perform TLS over N^2-dG adducts from their abilities to replicate an undamaged template. We propose that DinB and its orthologues are specialized to catalyse relatively accurate TLS over some N^2-dG adducts that are ubiquitous in nature, that lesion bypass occurs more efficiently than synthesis on undamaged DNA, and that this specificity may be achieved at least in part through a lesion-induced conformational change.

Although DinB is strongly upregulated as part of the SOS DNA damage response and $dinB^+$ function has been implicated in untargeted mutagenesis of λ phage, adaptive mutagenesis and −1 frameshift mutagenesis when $dinB^+$ is overexpressed in exponential phase[5–9], these phenotypes seem inadequate to account for the strong conservation of the DinB subfamily of DNA polymerases during evolution. We therefore exposed an *E. coli* strain bearing a precise deletion of the *dinB* gene to various DNA-damaging agents to gain insights into DinB function *in vivo*. The Δ*dinB* strain shows a marked sensitivity to nitrofurazone (NFZ; Supplementary Fig. S1a) that can be complemented *in trans* by $dinB^+$ under its native promoter on a low copy-number plasmid (see Fig. 3a). The killing curve of a Δ*umuC* strain is indistinguishable from that of wild type (Fig. S1a), indicating that DinB is responsible for most TLS over potentially lethal NFZ-induced adducts. The Δ*dinB* mutant also shows increased sensitivity to killing by 4-nitroquinoline-1-oxide (4-NQO; Supplementary Fig. S1b and see Fig. 3b), but in this case TLS by $UmuD'_2C$ makes a contribution to survival in a $dinB^+$ background (Supplementary Fig. S1b). Deletion of *polB*, which encodes DNA polymerase II (pol II) and is also induced by the SOS response[10], does not increase sensitivity to either agent (data not shown).

Before forming stable N^2-dG adducts *in vivo*, nitrofurans such as NFZ must be reduced and acetylated[11]. Likewise, at least half of the adducts that 4-NQO produces are N^2-dG adducts[11,12]. To address whether the NFZ resistance of a $dinB^+$ strain arises from N^2-dG lesion bypass, wild-type DinB was expressed and purified from *E. coli* (Supplementary Fig. S2), and oligonucleotide substrates were constructed that contained a site-specific N^2-furfuryl-dG (Supplementary Fig. S3a), a structural analogue of the principal N^2-dG adduct formed by NFZ. Whereas *E. coli* DNA pol I is strongly blocked by this lesion (Fig. 1a), DinB has markedly different properties. In the presence of all four deoxyribonucleotide triphosphates, DinB shows an increased catalytic proficiency on the N^2-furfuryl-dG template relative to an undamaged template (Fig. 1b). Standing-start[13] experiments (see Supplementary Fig. S4) indicate that DinB is 15-fold more proficient at adding dC opposite N^2-furfuryl-dG than opposite undamaged dG (Fig. 1c). DNA pol κ, the mammalian DinB orthologue, is also considerably more proficient at adding dC opposite N^2-furfuryl-dG than undamaged dG (Fig. 1d), indicating that this striking specificity has been conserved in eukaryotes. Furthermore, DinB bypass of N^2-furfuryl-dG is not only proficient, but also accurate (Fig. 1e). This is achieved in part from a preference for correct dC insertion and in part from a preference for elongating from dC correctly paired with N^2-furfuryl-dG (Table 1).

These observations suggest that a physiological role of DinB and its orthologues is to catalyse accurate TLS over some N^2-dG adducts. This hypothesis received strong support from our construction of a *dinB* mutant that almost eliminates the ability of DinB to perform this type of TLS without impairing its ability to replicate undamaged DNA. We designed the *dinB* mutant after constructing a homology model of DinB encountering an N^2-furfuryl-dG lesion based on the structure of *Sulfolobus solfataricus* Dpo4[14,15] (Supplementary Fig. S5a–d). We noted a pocket in the enzyme next to the template base that could potentially accommodate the N^2-furfuryl-dG adduct, bringing it into proximity with Phe 13. This residue corresponds to Phe 12 of the *S. acidocaldarius* DinB homologue (Dbh), the 'steric gate' that prevents the improper incorporation of ribonucleotide substrates by that enzyme[16]. Speculating that an active site rearrangement involving the N^2-furfuryl-dG adduct, the Phe 13 steric gate residue and the incoming nucleotide might favour catalysis, we mutated the planar hydrophobic Phe 13 steric gate to a sterically different but still hydrophobic valine residue.

Primer extension assays using DinB(F13V), which purified to homogeneity indistinguishably from wild-type DinB (Supplementary Fig. S6), showed that DinB(F13V) is almost unable to carry out TLS over N^2-furfuryl-dG, although its activity on undamaged DNA is largely unaffected (Table 1 and Fig. 2a). The F13V mutation has a modest effect on the ability of DinB to discriminate against ribonucleotides, increasing the frequency of their misincorporation from $<10^{-5}$ (limit of detection) to $\sim10^{-3}$. Because the steric gates of all

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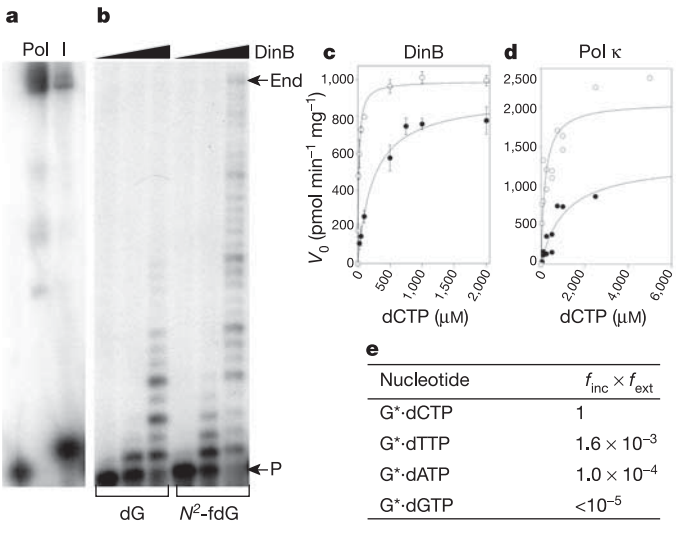
DinB orthologues are phenylalanine or tyrosine residues, we considered whether the corresponding mutation in these enzymes would likewise separate their TLS activities from their ability to replicate undamaged templates. We therefore assayed the archaeal DinB orthologue Dbh and its steric gate mutant Dbh(F12A)[16] on N^2-furfuryl-dG and undamaged templates. Whereas wild-type Dbh replicates both templates with comparable efficiencies at 37 °C, the F12A derivative shows disproportionately reduced activity on the damaged template (Fig. 2b, c).

To determine whether the F13V mutation specifically eliminates N^2-dG lesion bypass without affecting other properties of DinB, we examined bypass of two other well-studied lesions, (+)-*trans-anti*-benzo[a]pyrene-N^2-dG (N^2-B[a]P-dG; Supplementary Fig. S3b) and a tetrahydrofuran abasic site analogue[17,18] (Supplementary Fig. S3c). Although DinB-catalysed bypass of the N^2-B[a]P-dG lesion is inefficient[17] as compared with bypass of N^2-furfuryl-dG, the F13V mutation similarly eliminates its ability to perform this type of TLS (Fig. 2d). Furthermore, like the wild-type enzyme[18], DinB(F13V) is unable to bypass a tetrahydrofuran abasic site analogue efficiently (Supplementary Fig. S7), indicating that the F13V mutation has not relaxed the specificity of DinB *in vitro*. Although it is possible that the F13V mutation also affects DinB bypass of some other lesion, these data indicate that it specifically eliminates bypass of N^2-dG lesions.

To establish whether N^2-dG lesion bypass is required for *dinB*-dependent resistance to NFZ and 4-NQO, we examined the ability of a low-copy number plasmid carrying the *dinB(F13V)* allele under its own promoter to complement a Δ*dinB* strain for NFZ and 4-NQO resistance (Fig. 3a, b). Although the mutant protein is expressed from this plasmid *in vivo* (data not shown), pdinB(F13V) is unable to complement NFZ or 4-NQO resistance, which is consistent with the notion that an N^2-dG adduct is responsible for NFZ lethality. Furthermore, pdinB(F13V) exacerbates the sensitivity of the Δ*dinB* strain to these agents, to an even greater degree than a plasmid encoding a catalytically inactive DinB(D103N) mutant protein (pdinB003)[19] (Fig. 3a, b), suggesting that it is interfering with some cellular process that can otherwise contribute modestly to NFZ resistance. The plasmid-borne *dinB(F13V)* allele does not affect viability of the *dinB*$^+$ strain, but it has a dominant negative effect on survival after treatment with either NFZ or 4-NQO (Supplementary Fig. S8). We conclude that this dominance is largely due to an impairment of TLS rather than ribonucleotide misincorporation into DNA by DinB(F13V) because dominance is still observed in an *rnhB* mutant[20] (Supplementary Fig. S8) and the mutant enzyme still favours dNTP incorporation *in vitro* (Table 1). Taken together, our data indicate that the aromatic steric gate residue of DinB is required for TLS over N^2-dG adducts both *in vivo* and *in vitro*.



e

Nucleotide	$f_{inc} \times f_{ext}$
G*·dCTP	1
G*·dTTP	1.6×10^{-3}
G*·dATP	1.0×10^{-4}
G*·dGTP	$<10^{-5}$

Figure 1 | Bypass of N^2-furfuryl-dG. **a**, Primer (lane 1) extension products of *E. coli* pol I (5 nM) on undamaged dG (lane 2, 95.3% extension) and N^2-furfuryl-dG-damaged templates (lane 3, 8.2% extension; see Supplementary Information). **b**, Running-start primer (P) extension reactions with 1, 10 or 50 nM DinB protein and 250 μM dNTPs. Lanes 1–3, undamaged dG template (0.03, 2.2 and 81.8% extension, respectively); lanes 4–6, N^2-furfuryl-dG-damaged template (0.05, 65.5 and 91.1% extension, respectively). **c**, Plot of initial reaction velocity versus initial concentration of dCTP in standing-start assays on undamaged dG (filled circles) and N^2-furfuryl-dG-damaged templates (open circles). Error bars represent 1 s.d. determined from three reactions. **d**, As **c**, but with the mammalian DinB orthologue pol κ. **e**, Fidelity of DinB bypass of N^2-furfuryl-dG measured by using standing-start incorporation and extension assays[13]. Error of these measurements is ~20%.

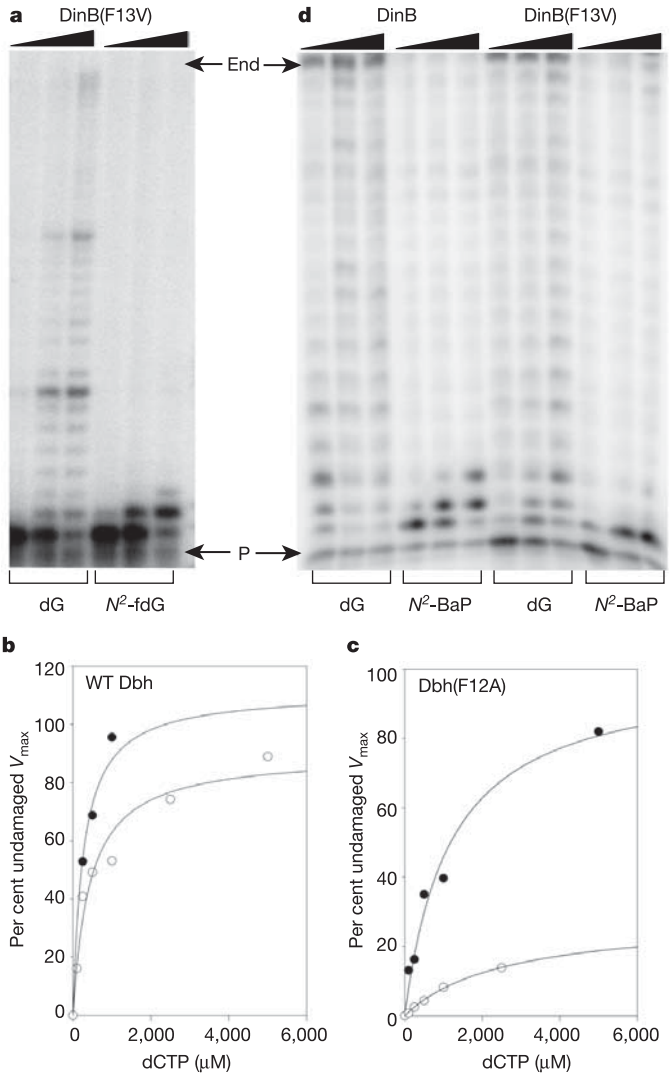


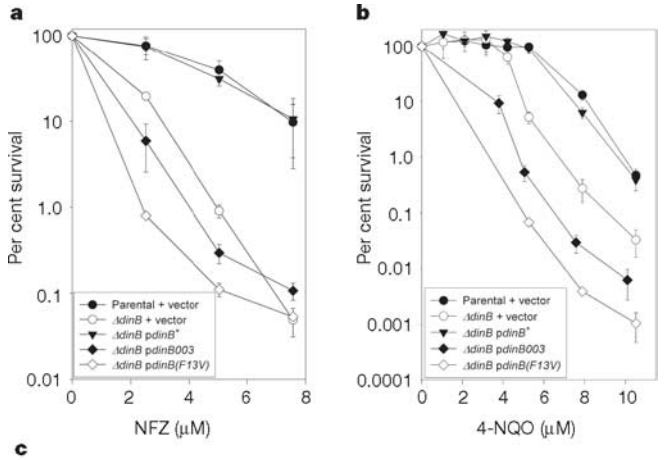
Figure 2 | A single mutation in DinB or its archaeal orthologue Dbh separates their TLS and DNA polymerase activities. **a**, Running start primer extension reactions using 1, 10 and 50 nM DinB(F13V) on undamaged dG (lanes 1–3, 0, 79.1 and 86.8% extension, respectively) and N^2-furfuryl-dG-damaged templates (lanes 4–6, 0, 7.5 and 16.3% extension, respectively). DinB(F13V) retains DNA polymerase activity but is compromised for TLS. **b**, Plot of relative initial velocity versus initial dCTP concentration for Dbh on undamaged dG (filled circles) and N^2-furfuryl-dG (open circles). **c**, As **b** but for Dbh(F12A); activity is disproportionately reduced on the N^2-furfuryl-dG-damaged template. **d**, Running-start primer (P) extension assays with 1, 10 and 50 nM DinB or DinB(F13V) on undamaged and N^2-B[a]P-dG-damaged templates. Lanes 1–12 show 29.8, 62, 88.7, 1.3, 17.8, 32.4, 11.5, 35.5, 53.8, 0, 0 and 0.2% extension, respectively.

Table 1 | Kinetic parameters for N^2-furfuryl-dG bypass, mismatch extension and rNTP insertion

Substrate†	Enzyme	V_{max} (pmol min^{-1} mg^{-1})	K_m (μM)	V_{max}/K_m (pmol min^{-1} mg^{-1} M^{-1})‡	f_{inc}
dNTP insertion					
G·dCTP	WT DinB	910.3	240.1	3.8×10^6	1.0
G*·dCTP	WT DinB	992.8	16.1	6.2×10^7	16.3
G*·dTTP	WT DinB	155.5	36.7	4.2×10^6	1.1
G*·dATP	WT DinB	81.6	20.2	4.0×10^6	1.1
G*·dGTP	WT DinB	Undetectable	-	-	-
G·dCTP	DinB(F13V)	1461.0	117.7	1.2×10^7	1.0
G*·dCTP	DinB(F13V)	300.8	227.1	1.3×10^6	0.1
Mismatch extension					
G*C	WT DinB	807.7	2.4	3.4×10^8	1.0
G*T	WT DinB	101.2	14.2	8.2×10^6	2.4×10^{-2}
G*A	WT DinB	46.4	86.2	5.4×10^5	1.6×10^{-3}
rCTP insertion					
G·rCTP	WT DinB	Undetectable	-	-	-
G*·rCTP	WT DinB	28.3	932.0	3.0×10^4	N/A
G·rCTP	DinB(F13V)	23.7	325.0	7.3×10^4	N/A
G*·rCTP	DinB(F13V)	8.1	293.0	2.8×10^4	N/A
Template parameters					
Undamaged dG	WT DinB	274.0	13.8 ± 1.7	2.0×10^7	1.0
N^2-furfuryl-dG	WT DinB	1,040.9	20.5 ± 5.2	5.1×10^7	2.6

†G* represents N^2-furfuryl-dG; the s.e.m. is ~20%.
‡V_{max} and K_m for the DNA substrates reveal preferential activity on N^2-furfuryl-dG.

Does DinB prevent mutagenesis caused by NFZ or 4-NQO *in vivo*, or does it promote mutagenesis, a behaviour frequently attributed to Y-family DNA polymerases? Loss of *dinB*$^+$ does not alter the frequency of NFZ-induced rifampicin-resistant (Rif^r) mutations (mean ± 1 s.d., $29.6 \pm 9.7 \times 10^{-9}$ for *dinB*$^+$ versus $31.4 \pm 11.8 \times 10^{-9}$ for Δ*dinB*). Given the markedly greater ability of a *dinB*$^+$ strain to survive NFZ treatment as compared with a Δ*dinB* strain, this indicates that DinB is not a mutagenic polymerase when bypassing NFZ-induced lesions that are lethal in its absence. Loss of *dinB*$^+$ function results in an increase in the frequency of Rif^r mutants upon 4-NQO treatment ($48.1 \pm 1.7 \times 10^{-9}$ for *dinB*$^+$ versus $453 \pm 181 \times 10^{-9}$ for Δ*dinB*). This implies that DinB carries out accurate TLS over a class of lethal 4-NQO-induced lesions that are bypassed in a more error-prone fashion in its absence, most likely by $UmuD'_2C$ (ref. 21). These data indicate that the *dinB* gene product bypasses the lethal lesions generated by NFZ and 4-NQO with unexpectedly high fidelity *in vivo*, thus resembling the behaviour of DNA pol η when bypassing cyclobutane pyrimidine dimers[2,22].



c

Plasmid	Spontaneous	NFZ
Vector	11.7 ± 4.5	29.6 ± 9.7
pdinB+	62.9 ± 22.3	37.4 ± 17.5
pdinB(F13V)	17.1 ± 6.6	57.9 ± 33.7
pdinB003	15.2 ± 7.8	10.6 ± 7.5

Figure 3 | Importance of DinB F13 residue *in vivo*. **a**, Unlike pdinB$^+$, pdinB(F13V) is unable to restore NFZ resistance in the Δ*dinB* strain but instead exacerbates the sensitivity, like pdinB003, which encodes a catalytically inactive DinB(D103N) protein. Error bars indicate 1 s.d. determined from three experiments. **b**, pdinB(F13V) and pdinB003 also exacerbate the sensitivity of the Δ*dinB* strain to 4-NQO. **c**, Spontaneous and induced mutation frequencies per 10^9 bacteria to Rif^r.

The *in vitro* data discussed above indicate that the F13V mutation almost eliminates the ability of DinB to bypass N^2-furfuryl-dG and does not relax its specificity with respect to the lesions that it can bypass, but does DinB(F13V) replicate undamaged DNA with reduced fidelity? Using a set of Δ*dinB* strains carrying various plasmid-borne *dinB* alleles (Fig. 3a, b), we examined the frequencies of spontaneous and NFZ-induced mutation to Rif^r. We observed no increase in the frequency of spontaneous or NFZ-induced Rif^r mutations between the *dinB*$^+$ and *dinB(F13V)* alleles (Fig. 3c), indicating that the F13V mutation does not result in DinB(F13V) becoming a mutator polymerase. We also compared the effect of *dinB(F13V)* on spontaneous mutation to that of *dinB*$^+$ using derivatives of the strain CC102 (ref. 23). This strain carries a *lacZ* allele that reverts by a GC to AT transition, the most frequent DinB error that we detected *in vitro*. Here again, we detected no increase in Lac$^+$ reversion between the *dinB(F13V)* derivative ($11 \pm 5 \times 10^{-9}$) and that of the *dinB*$^+$ strain ($8 \pm 5 \times 10^{-9}$), indicating that the F13V mutation does not decrease the fidelity of DinB.

The highly conserved steric gate residue, Phe 13, clearly has a crucial role in bypass of N^2-dG adducts by DinB, but further work will be required to establish whether it participates in an N^2-dG lesion-induced conformational change that permits preferential replication of this type of damaged DNA template. Nevertheless, some of our observations are consistent with such a lesion-induced conformational change (Table 1) including, first, the ability of wild-type DinB to incorporate detectably low levels of rNTPs only when acting on the N^2-furfuryl-dG bearing template; second, no detectable increase in rNTP incorporation by DinB(F13V) on the N^2-furfuryl-dG bearing template relative to an undamaged control; and last, a lower apparent Michaelis constant (K_m) for dCTP when DinB is bound to an N^2-furfuryl-dG standing-start template rather than to the corresponding dG template, coupled with a higher apparent maximal enzyme-catalysed reaction velocity (V_{max}) for the damaged primer or template itself.

DinB may have a role as a mutator polymerase under certain conditions of biological stress or in some sequence contexts[7,19]. Because other amino acids can act as steric gates in other DNA

polymerases[24], however, the evolutionary conservation of this aromatic steric gate residue with a crucial role in TLS suggests that N^2-dG adduct bypass is an important and physiologically relevant property of the DinB subfamily of Y-family DNA polymerases. Present at levels comparable to those of 8-oxo-G *in vivo*, N^2-dG adducts are formed from byproducts of diverse cellular processes, including lipid peroxidation[25]. Furthermore, there is evidence that these minor groove adducts may be recalcitrant to excision repair[26]. In mammalian cells, the N^2-dG adduct of the carcinogen acetylaminofluorine persists even though it is the least common dG isomer formed[27]. Finally, our results emphasize that Y-family DNA polymerases, although perhaps relatively error-prone under some conditions[7,8,10,19], can also be specialized for proficient and accurate replication of a particular class of damaged DNA.

METHODS

Sensitivity and mutation frequency determination. Details of strain and plasmid construction are included as Supplementary Information. From single colonies, strains were grown for 16 h to saturation, diluted 1:1,000 into LB medium and then grown to 5×10^9 c.f.u. per ml. From this freshly saturated culture, dilutions were plated on LB agar containing ampicillin and 0–15 μM NFZ or 4-NQO. Stock solutions of NFZ and 4-NQO were freshly prepared in *N*,*N*-dimethyl formamide. About ten of these colonies were suspended in M9 salts and deposited on plates containing rifampicin ($100\ \mu g\ ml^{-1}$) to determine the number of Rif^r mutants. This number was corrected for the number of viable cells in each colony. We measured GC to AT transitions using a Δ*dinB* derivative of the CC102 strain[23].

Template synthesis and construction. The N^2-furfuryl-dG adduct was made by a postsynthetic derivatization approach[28], described in detail in the Supplementary Information. MALDI-TOF mass spectrometry of the purified oligonucleotide gave a mass of 4,986.26 (4,986.27 calculated) for the single, negatively charged moiety (Supplementary Fig. S9). The 16-nucleotide lesion-bearing oligomer was ligated to 5′-GGTTACTCAGATCAGGCCTGC-3′ at the 5′ end and 5′-GGCTGCAGCTGTACTATCATATGC-3′ at the 3′ end by standard protocols, and gel-purified to remove the ligation scaffolds 5′-AGGTCTTCG-CAGGCCTGA-3′ and 5′-CAGCTGCAGCCGGACGCC-3′. The benzo[a]pyrene lesion is in the sequence context 5′-GACTACGTACTGTCACATXCACACGC-TATCTGGCCAGATCCGC-3′.

Primer extension assays. Details of the protein purification procedure are given in the Supplementary Information. Assays were performed and quantified by using either standing or running start primers of the sequences 5′-GCATATGA-TAGTACAGCTGCAGCCGGACGCC-3′ or 5′-GCATATGATAGTACAGCTG-CAGCCGGACGC-3′, respectively, for all templates except the N^2-B[a]P-dG-bearing substrate[13]. For that substrate, the primer 5′-GCGGATCTGGCCAGA-TAGCGTGT-3′ (running) was used. In brief, assays were conducted in a 10-μl volume containing 1, 10 or 50 nM DinB, 10 nM primer and template, either 250 μM dNTPs or 0–2,000 μM dCTP, 50 mM HEPES (pH 7.5), 100 mM KCl, 7.5 mM $MgCl_2$, 5% glycerol and 0.1% bovine serum albumin. Reactions were initiated with dNTPs and quenched after incubation for 15 min (or as noted in the figure legends) at 37 °C. The per cent extension is defined as the percentage of primers that are extended past the lesion. The same conditions were used to assay Dbh and pol κ, except that the enzyme concentration was 10 nM and 2 nM, respectively. Products were analysed on a 12% denaturing polyacrylamide gel and quantified on a phosphorimager (Molecular Dynamics).

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