Supporting Information

Cohen et al. 10.1073/pnas.1005203107

SI Material and Methods

Bacterial Strains and Plasmids. The strains and plasmids used in this study are listed in Table S3 and were constructed using standard molecular biology techniques. Plasmids were maintained with ampicillin (100 μg/mL) when necessary.

DNA Damage Sensitivity Assays. Independent overnight E. coli cultures grown in LB medium were diluted in M9 minimal salts and plated on LB agar containing NFZ, 4-NQO, or MMS. For UV survival assays, cells were plated on LB agar and then irradiated with UV light (0–40 J/m²) by using a G15T8 UV lamp (GE) at 254 nm, then incubated in the dark. A concentrated stock solution of NFZ or 4-NQO was first made in N,N-dimethylformamide, stored at −20 °C, and diluted appropriately for each experiment. Percent survival was determined relative to growth in the absence of DNA-damaging agent.

Microarray Analysis. Cultures were grown in Luria–Bertani (LB) medium at 30 °C to exponential phase. RNA samples were prepared from three independent cultures of AB1157 (nusA”) or AB1157 nusA11 (SEC164) using a Qiagen RNeasy extraction kit according to the manufacturer’s directions, and RNA samples were treated with DNA-free (Ambion) to remove residual DNA according to the manufacturer’s instructions. Microarray data collection and analysis were performed as described in refs. 1 and 2. Microarray *.CEL files were combined with *.CEL files from arrays that comprise the M3D compendium (1) [http://m3d.bu.edu (E. coli v3_Build_3)] and RMA-normalized (3) with RMAexpress. Each gene’s SD of expression, σ, was calculated across the entire compendium and used to construct the z-scale difference between that gene’s normalized expression in the nusA11 strain versus the nusA+ control:

\[ \Delta z_{exp} = \frac{X_{exp} - X_{ref}}{\sigma} \]

This allowed us to measure each gene’s change in expression for a given experiment in units of SD, a form of the z-test. For each set of strains in the experiment set, we converted z scores to p values and chose significantly up- and down-regulated genes by selecting those with a q value of <0.05 (false discovery rate) (4).

Protein Purification. NusA and NusA11 proteins were purified from BL21(DE3) pLysS cells containing a His6-NusA overexpression plasmid (pNusA or pNusA11) (Table S3) by a two-step purification protocol using Ni-NTA and monoQ affinity chromatography. For purification of E. coli RNAP BL21(DE3) pLysS cells overexpressing [θ(θ’,θ′′)θθ]αω from pRL4545, a derivative of pRL4930 (5). Cells were lysed by sonication and RNAP purified by polyethyleneimine precipitation followed by ammonium sulfate precipitation, Ni-NTA, and Heparin affinity chromatography.

In Vitro Transcription. DNA and RNA oligonucleotides used to generate transcription substrates are listed in Table S3 and were purified by denaturing PAGE before use. The 14-mer oligonucleotide containing the N2-furfuryl-dG adduct was synthesized and purified as described in ref. 6. The nucleic acid templates for in vitro transcription were assembled in reconstitution buffer (RB) [20 mM Tris HCl (pH 8.0), 40 mM KCl, 5 mM MgCl₂] by heating (NT)DNA and (T)DNA containing a 9-ntd noncomplementary region (bubble) and RNA primer to 75 °C and cooled slowly to 25 °C. Oligonucleotide 6897 (1 μM) is used for (NT)DNA. Oligonucleotides 6897, 6883, and 6896 are used (1 μM each) to generate full-length undamaged strand; 6881, 6883, and 6896 are used to generate 1-ntd gap template; 6897, 1-dG, and 6896 are used to generate damaged template; 6897 and 6896 are used to generate 14-ntd gap template. Template strand was ligated with 2,000 units of T4 DNA ligase (NEB) and 1 mM ATP. Reconstitution of TECs was performed by incubating core E. coli RNAP (2.5 μm) with the nucleic acid scaffold in RB for 10 min at room temperature. At 37 °C TECs were diluted in RB to contain 50 nM TECs before adding 10 μM UTP and 10 μCi [α-32P]GTP to label and extend the RNA to position G12. Next, ATP, UTP, and GTP (10 μM each) were added to allow RNAP to elongate to G27. Addition of CTP (10 μM) allows for transcription to continue to the end of the template in the case of full-length, undamaged substrates. Samples were removed at predetermined times, quenched with an equal volume 2× loading dye [8 M urea, 50 nM EDTA, 90 mM Tris-borate buffer (pH 8.3), 0.02% bromophenol blue, 0.02% xylene cyanol], and analyzed by denaturing 20% polyacrylamide gel electrophoresis. The gel was exposed to a PhosphorImager screen and analyzed using ImageQuant software (GE Healthcare). To map the position of the transcript generated on the N2-f-dG template, a C28 marker was made by adding 50 μM dCTP instead of CTP to a reaction with the full-length, undamaged template. Scaffolds to test transcription when N2-f-dG is present on the nontranscribed strand are described in Fig. S2 and Table S3. Ligation and reaction conditions were as above.

Far-Western Blotting. An equivalent number of BL21 cells expressing UvrA from pMP47 or containing the empty vector (pET11) were lysed by boiling in SDS-loading dye and lysates were separated by SDS/PAGE (4–12%), transferred to a poly(vinylidene difluoride) membrane, and probed with purified recombinant NusA (2 μM final concentration). Anti-NusA Western blotting was then performed as described in ref. 7. Monoclonal anti-NusA antibody was obtained from Neoclon.

RNA Polymerase Mutant Screen. Mutagenized libraries of pRL706 (8) transformed into AB1157 were grown in LB medium supplemented with ampicillin induced with 1 mM IPTG. Under induced conditions, it has been estimated that ~85%–90% of cellular RNAPs have incorporated the plasmid-encoded His-tagged subunit (8). Cultures were diluted in M9 minimal salts and 10-fold dilutions stamped onto LB agar containing ampicillin (100 μg/mL; Molecular Probes). Plates were incubated at 37 °C and scored for NFZ sensitivity or resistance the next day. All isolates were isolated and repeated for confirmation. Plasmids from confirmed clones were isolated and sequenced. Of ~800 mutants screened, 6 NFZR mutants and 23 NFZS mutants were isolated.

Live-Cell Microscopy. Live-cell microscopy was performed as described in refs. 9 and 10. Aliquots of cells were stained with the membrane dye FM-64 (240 ng/mL) and 1 μM MitoTracker. Plates were then placed on a pad of 4% low melt agarose in a solution of M9 minimal salts and covered with a coverslip. The following Chroma filter sets were used: 41002b (TRITC) for FM-64 and 41012 for GFP. Images were acquired using a Nikon E800 microscope with a charge-coupled device camera (Hamamatsu model C4742-95) and OpenLab software (Improvision). Images were colorized in OpenLab and then transferred to Photoshop (Adobe) for figure assembly.


**Fig. S1.** (A and B) Sensitivity of *nusA11* mutant strains to NFZ (μM) (A) and 4-NQO (μM) (B) can be complemented in trans at 30 °C in AB1157. pBR322 is used as an empty vector and pNAG2010 is *pnuA*+, and are described in Table S2. SD determined from at least three independent cultures. (C) Sensitivity of Δ*nusA* strains to NFZ, UV, and MMS can be complemented in trans. Growth of 10-fold serial dilutions, labeled to the left, of designated strains is depicted in photograp. *nusA*+ represents MDS42 and Δ*nusA* represents MDS42 Δ*nusA*. −, empty vector (pBR322); +, *pnuA*+ (pNAG2010).
Fig. S2. (A) $N^2$-f-dG on the nontranscribed strand does not block transcription. Schematic of experimental design: three oligonucleotides, one containing the $N^2$-f-dG lesion or an undamaged proxy, are ligated together to generate the nontranscribed strand (NT) using the transcribed strand (T) as a scaffold. “X” indicates a site of $N^2$-furfuryl-dG lesion or proxy dG. A 9-ntd noncomplementary region between the (T)DNA and (NT)DNA allows for the annealing of an RNA primer (shown in red) to initiate transcription. Oligonucleotides 6920 (black), 6883 or f-dG (green), and 6930 (blue) are used to generate the nontranscribed strand. For each nucleic acid scaffold, undamaged or $N^2$-f-dG (in the NT strand), purified RNAP, ATP, and [$\alpha$-32P]GTP are added to allow for radiolabel incorporation into the RNA transcript and extend to G11 (first lane). The addition of excess cold ATP, UTP, and GTP extends the transcription elongation complex (TEC) to U18 position, 5 nucleotides before the $N^2$-f-dG lesion (second lane). Addition of CTP allows for the visualization of the run-off transcript (third lane; band labeled RO). All reactions represent 5-min time points after addition of nucleotide. (B) For nucleic acid templates labeled undamaged and $N^2$-f-dG (transcribed) in vitro transcription reactions were carried out as in Fig. 3. The first lane represents the migration of the transcript generated in the presence of radiolabeled GTP, UTP, and limiting ATP. The second lane represents the migration of the transcript generated when excess GTP, UTP, and ATP are added generating a G27 transcript marker. The third lane represents the migration of the transcript generated after the addition of CTP, allowing RNAP to transcribe through the template generating the complete transcript. All samples in this figure were removed 1 min after addition of nucleotide(s). The migration of the run-off transcript generated using the full-length/undamaged template when all NTPs are added is labeled RO. The final lane represents the migration of a C28 marker generated by the addition of dCTP to a reaction using the full-length template. These results demonstrate that the $N^2$-f-dG lesion stalls transcription four nucleotides before the site of the lesion.
In vitro transcription reactions were performed as in Fig. 3, except samples were removed 1, 5, and 10 min after addition of CTP. For each template, the first three lanes are the same as those shown in Fig. 3B, with the last of these three lanes representing the product generated 1 min after addition of CTP, noted as 1' above. An empty lane separates the 1' time point and the products generated 5 (5') and 10 (10') minutes after addition of CTP, as labeled above. These results demonstrate that even with prolonged incubation time, *E. coli* RNAP cannot bypass a template strand gap of 14-ntd or the $\text{N}^2\text{-f-dG}$ lesion. Nonspecific higher migrating bands are observed in reactions using 14-ntd and $\text{N}^2\text{-f-dG}$ templates as these bands are observed before all nucleotides are added to the reaction.

Addition of purified NusA or NusA11 to in vitro transcription reactions of full-length/undamaged template (A), 14-ntd gapped template (B), or $\text{N}^2\text{-f-dG}$ containing template (C). Lanes labeled “−” were performed identically as those done in Fig. 3B. The first lanes represent products formed in the presence of radiolabeled GTP, UTP, and limiting ATP, the addition of excess GTP, UTP, and ATP to lane two and the addition of CTP to lane 3. Lanes labeled +NusA represent reactions performed in the presence of 100 nM purified NusA or NusA11 for lanes labeled +NusA11.
Fig. S5. (A) Percent survival of rpoB mutants expressed in AB1157 to 12.5 μM NFZ at 30 °C. A higher dose of NFZ is used compared with experiments done at 37 °C to observe sensitivity of strains expressing rpoB+. In this and all graphs in this figure, error bars represent the SD determined from at least three independent cultures. (B) Sensitivity of rpoB mutants expressed in a ΔdinB background to 10 μM NFZ at 37 °C. (C) Residues of rpoB D185 (blue), V287 (orange), and D320 (yellow) mapped on to the structure of T. thermophilus RNAP elongation complex (2O5I). β catalytic subunit shown in pale green, β' in pink, DNA in green, and RNA in red. V287 and D320 are located in a lineage-specific sequence insertion in the lobe domain of E. coli RNAP, and V287 is not conserved between E. coli and T. thermophilus. The residue highlighted in orange is the residue of T. thermophilus that is positioned closest to V287 of E. coli. The crystal structure predicts that when RNAP stalls at the −4 position relative to the N²-f-dG lesion in the transcribed strand, the N²-f-dG adduct would be located in the minor groove of the dsDNA ahead of the transcription bubble.
Fig. S6. SOS induction and RecA-GFP foci of exponentially growing cells. (A–C) SOS induction of exponentially growing cells. Representative micrographs of wild-type (AB1157) (SEC677) (A), lexA(Def) (SEC678) (B), and nusA11 (SEC679) (C) cells during exponential growth (OD$_{600}$ ∼ 0.3). These analyses demonstrate that ∼0.2% of wild-type cells are induced for the SOS response (n = 602) compared with 100% of lexA(Def) (n = 44) and 0.8% of nusA11 (n = 453) cells. Cell outlines (red) were visualized with the vital membrane stain FM4-64, and SOS induction was monitored from PsulA-GFP fusion (green). (D) Cell length distributions of stationary-phase wild type/AB1157 (blue) and nusA11 (orange) show that nusA11 strains are elongated compared with wild type. (E) Cell length distributions of exponentially growing wild-type/AB1157 (blue) and nusA11 (orange) cells. (F–I) RecA-GFP foci in exponentially growing cells: Representative micrographs of AB1157 (F) and nusA11 (H) strains during exponential growth (OD$_{600}$ ∼ 0.3), and distribution of RecA-GFP foci in nusA+ (G) and nusA11 (I) cells. Cell outlines (red) were visualized with the vital membrane stain FM4-64, and RecA-GFP foci are shown in green. All experiments in this figure were performed at the permissive temperature, 30 °C.

Other Supporting Information Files

Table S1 (DOC)
Table S2 (DOC)
Table S3 (DOC)