Supporting Information

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SI Materials and Methods

Plasmid Construction. Details of plasmids used for triplex-forming oligonucleotide (TFO) assays and for the generation of substrates containing single lesions are given in Table S1.

Plasmid pSRTB1 (1) contains a TFO binding site located between +39 and +60 downstream of the T7A1 promoter. Transcription can be stalled at +21 by the inclusion of 3′ dUTP in transcription reactions that lack UTP. Plasmids pSRTB8, pSRTB9, and pSRTBlacO are derivatives of plasmid pSRTB1 in which additional spacer DNA has been inserted between the stall site and the TFO binding site. These plasmids were constructed in a series of steps, as follows. Plasmid pSRTB2 was derived from pSRTB1 (1) by site-directed mutagenesis to introduce a unique BbvCI site downstream of the stall site at +21. pSRTB4 was derived from pSRTB2 by cutting with BbvCI and inserting annealed oligos that created a HindIII site downstream of the BbvCI site. pSRTB7 was derived from pSRTB4 by cutting with BbvCI and inserting annealed oligos. pSRTB8 was derived from pSRTB7 by cutting with BbvCI and HindIII and inserting annealed oligos that increase the distance between the stall site and the TFO site to 100 bp. pSRTB9 was derived from pSRTB8 by cutting with BsrGI and Hind III and inserting a 522-bp BsrGI-HindIII fragment containing part of the firefly luciferase gene from plasmid pGL2 (Promega). This increased the distance between the stall site and the TFO site to 581 bp. pSRTBlacO was derived from pSRTB7 by cutting with HindIII and inserting annealed oligos that introduce a *lac01* operator sequence centered at +66 and increase the distance between the stall site and the TFO site to 79 bp.

Plasmids pSRTB8B3 and pSRTB8B5 are derivatives of pSRTB8, and they contain cassettes of three or five BbvCI recognition sites, respectively, between the T7A1 promoter and the TFO binding site. They were derived from pSRTB8 by cutting with BbvCI and inserting annealed oligos. pSRTB8B3ops is a derivative of pSRTB8B3 into which the *ops* (operon polarity suppressor) pause site from the *rfaQ* gene has been inserted between the T7A1 promoter and the BbvCI cassette, so that RNA polymerase (RNAP) will pause at +46. Its design was based on pSRTB8B3, with the insertion of a short region containing the *ops* pause sequence from the *rfaQ* gene (5′-GGCGGTAGCGTGCTTTTTTC-3′) flanked by NcoI and XhoI restriction sites upstream of the three BbvCI sites. The required sequence was generated by gene synthesis (Eurofins MWG) and used to replace the promoter and reporter region in pSRTB8B3.

Plasmid pHWL1-T7A1-2lacO is a derivative of pHWL1-T7A1-2 (2), into which a *lacO* sequence has been inserted centered at +73. It contains an ∼170-bp repair analysis cassette, flanked by BsrGI and SphI sites, downstream of the T7A1 promoter and *lacO* sequence. It was derived from pHWL1-T7A1-2 (2) by linearizing the plasmid at the unique BsrGI centered at +39.5 with respect to the T7A1 transcription start site and inserting annealed oligos 5′-GTACGCAAGCTTATCCTCTATAGCAGAATTGTGAGCGCTCACAATTCT-3′ and 5′-GTACAGAATTGTGAGCGCTCACAATTCTGCTATAGAGGATAAGCTTGC-3′.

Plasmid pHWL1-2 (2) is a derivative of pHWL1-T7A1-2 in which the T7A1 promoter has been removed and the BsrGI-SphI analysis cassette has been shortened.

Creation of DNA Substrates Containing Single Lesions. Plasmid substrates containing single lesions were prepared as described by Luzzietti et al. (3). Briefly, plasmid pSRTB8B3, pSRTB8B5, or pSRTB8B3ops was nicked on the template strand using Nb. BbvCI (New England BioLabs) or on the nontemplate strand with Nt. BbvCI (New England BioLabs). The nicked substrates were annealed with a 20-fold molar excess of lesion-containing oligonucleotides complementary to the unnicked strand (Tables S2 and S3). The reactions were incubated at 80 °C for 2 min, and the temperature was then reduced to 20 °C at 1 °C per min. Excess oligonucleotides were removed using a PCR clean-up column (Qiagen), and the annealed substrates were ligated using T4 DNA ligase. Successfully ligated closed-circular plasmids were purified by extraction from an agarose gel. Oligonucleotides containing biotinylated deoxythymidine (bio-dT) were obtained from Eurofins MWG, and the oligonucleotide containing a cyclopyrimidine dimer (CPD) was obtained from Eurogentec.

Patch Synthesis Assay. Patch synthesis assays to monitor nucleotide excision repair (NER) and transcription-coupled NER (TCR) were performed essentially as described by Manelyte et al. (2). Unless stated otherwise, all reactions contained 1× repair buffer [40 mM Hepes, 100 mM KCl, 8 mM $MgCl_2$, 4% (vol/vol) glycerol, 5 mM DTT, 100 μg/mL BSA] supplemented with 2 mM ATP, 100 μg/mL NADH, 50 μM dGTP, 50 μM dCTP, 50 μM dTTP, 10 μM dATP, 5 μCi [α^{32}P] dATP, 1.25 U/mL *Escherichia coli* DNA polymerase I (New England BioLabs), and 24 U/mL *E. coli* DNA ligase (New England BioLabs). Where indicated, the following additional components were added: 250 nM Mfd, 100 nM Lac repressor, "NER repair mix" (final concentration of 8 nM UvrA, 100 nM UvrB, 70 nM UvrC, and 5 nM UvrD), "Free transcription mix" (for transcription without stalling; final concentration of 12.5 nM RNAP, 200 μM GTP, 200 μM CTP, and 200 μM UTP), and "Stalled RNAP mix" [for transcription stalling at +21; final concentration of 12.5 nM RNAP, 200 μM GTP, 200 μM CTP, 100 μM adenylyl (3′-5′) uridine (ApU), and 500 μM 3′ dUTP]. ApU is necessary to allow RNAP to initiate transcription from the T7A1 promoter in the absence of UTP, and 3′ dUTP prevents RNAP extending the transcript beyond 21 nt. All reactions were initiated by the addition of 1.5 nM closed-circular substrate DNA plus 1.5 nM supercoiled control DNA [pHWL1-2 irradiated with 30 J/m^2 of 254-nm UV light as described by Manelyte et al. (2): The analysis cassette in this plasmid is not transcribed and acts as an internal global NER control in each reaction]. Reactions were incubated at 37 °C for 20 min and were stopped by phenol/chloroform extraction and ethanol precipitation. For experiments using single-lesion templates (Figs. 1, 2, and 5), the DNA pellets were resuspended in 10 μL 1× New England Biolabs buffer 2 + 100 μg/mL BSA and incubated at 37 °C for 45 min with 2.5 units each of HindIII, BmtI, BsrGI, and SphI. Digestion of the substrates based on pSRTB8B3 or pSRTB8B5 with HindIII and BmtI releases a DNA fragment encompassing the site of damage/repair in which the template strand is 62 nt long and the nontemplate strand is 54 nt long. Digestion of the pHWL1-2 control DNA with BsrGI and SphI releases a nontranscribed DNA fragment in which the strands are 141 and 133 nt long. Digests were stopped by the addition of an equal volume of formamide stop buffer and were incubated at 95 °C for 3 min. Samples were run on denaturing acrylamide gels and analyzed as described for in vitro transcription reactions. For quantification, the amount of radioactivity incorporated into bands of interest was normalized to the amount incorporated into the 133-nt band of the UV-irradiated pHWL1-2 internal control.

The DNA substrate used for patch synthesis assays in which the effect of a protein roadblock on TCR was analyzed (Fig. 3 and Fig. S3) was 1.5 nM supercoiled pHWL1-T7A1-2lacO plasmid DNA that had been irradiated with 30 J/m^2 of 254-nm UV light as described by Manelyte et al. (2). UV-irradiated supercoiled pHWL1-2

DNA (1.5 nM) was also present to act as an internal nontranscribed control. These assays were conducted as described above, with the following exceptions: RNAP concentration was 8 nM, GTP and CTP concentration in reactions with stalled RNAP was 10 μM, UvrA was untagged [purified as described by Manelyte et al. (2)], KCl concentration was 50 mM, and Hind III and BmtI were omitted from the restriction digests following patch synthesis. Digestion of pHWL1-T7A1-2lacO with BsrGI and SphI releases a DNA fragment in which the template strand is 165 nt long and the nontemplate strand is 173 nt long.

The fluence of UV light used to generate randomly distributed CPD lesions in the pHWL1-2 control DNA and the pHWL1-T7A1-2lacO substrate is expected to produce an average of one to two lesions per plasmid [1 J/m^2 of 254 nm UV light produces ~2.4 CPDs per 10^8 Da of DNA (4). Plasmids pHWL1-T7A1-2lacO and pHWL1-2 have molecular masses of 2.5 MDa and 2.4 MDa, respectively (660 Da/bp), and so are expected to contain an average of 1.8 CPDs per plasmid after exposure to 30 J/m^2 of 254-nm UV light]. The majority of these UV-irradiated substrate molecules will therefore not contain lesions within the reporter cassette in which patch synthesis is measured. Consequently, the absolute level of repair detected in these substrates is lower than that detected in the single-lesion templates, in which nearly all of the substrate molecules contain a lesion within the region analyzed (Fig. S1).

TFO-Displacement Assays. TFO-displacement assays (Fig. 4) were conducted essentially as described by Smith et al. (1). A TFO labeled with ^{32}P was bound to a specific site on supercoiled plasmid templates. Unless stated otherwise, transcription complexes were stalled at +21 by incubating 5 nM triplex-containing DNA with 20 nM RNAP holoenzyme and 10 μM ATP, 10 μM GTP, 10 μM CTP, 10 μM 3′ dUTP, and 100 μM ApU for 15 min at 20 °C in TR buffer [50 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT]. TFO displacement was initiated by the addition of 250 nM Mfd plus 2 mM dATP, and reactions were incubated at 20 °C. Samples were quenched by the addition of 1/4 volume of GSMB buffer [15% glycerol, 3% (wt/vol) SDS, 250 mM MOPS (pH 5.5), 0.4 mg/mL bromophenol blue], run on acidic agarose gels (1), and analyzed using a phosphorimager and ImageQuant software (GE Healthcare Life Sciences). Experiments analyzing the effect of Lac repressor were done in 1× repair buffer with 50 mM KCl, and Lac repressor was added with the RNAP at a final concentration of 100 nM where indicated.

Electrophoretic Mobility Shift Assay. Electrophoretic mobility shift assays were used to confirm the presence of bio-dT lesions in plasmid substrates. Reactions contained either 10 nM uncut, closed-circular substrates (Fig. S1*A*) or 30 nM substrates digested with EcoRI and Hind III (Fig. S1*B*). Streptavidin (Sigma–Aldrich) was added at 10-fold molar excess to both biotin-modified and unmodified substrates in TE buffer [10 mM Tris·HCl (pH 8), 1 mM EDTA] for 30 min at room temperature. The reactions were stopped by the addition of 1/6 volume STEB [100 mM Tris·HCl (pH 8), 100 mM EDTA, 40% (wt/vol) sucrose, 0.4 mg/mL bromophenol blue]. These were analyzed by electrophoresis on a 1% agarose gel when in the closed-circular form (Fig. S1*A*) or a by 6% (wt/vol) acrylamide/1× Tris-borate-EDTA buffer native gel when digested to linear fragments (Fig. S1*B*). Control reactions without the addition of streptavidin were run on each gel. The DNA bands and streptavidin–DNA complexes were detected using ethidium bromide staining.

NER Incision Assay. The ability of a bio-dT lesion to act as a substrate for the bacterial NER proteins was tested using an agarose gel-based DNA incision assay (Fig. S1 *C*, *E*, and *F*). This assay monitors the conversion of covalently closed-circular DNA into nicked DNA by the action of UvrA, UvrB, and UvrC proteins at DNA lesions and separates the two forms on the basis of their differing mobility on agarose gels run in the presence of ethidium bromide (5). The assay therefore detects lesions located anywhere within the plasmid, and not only within the small reporter cassette that is analyzed in the patch synthesis assay. Template DNA at a concentration of 2.5 nM was mixed with 100 nM UvrA, 100 nM UvrB, and 200 nM UvrC in 1× repair buffer. These mixtures were incubated at 37 °C for 5 min, and reactions were then initiated by the addition of 1 mM ATP. The reactions were incubated at 37 °C for the indicated time intervals. Aliquots were removed, the reaction was stopped by adding 1/2 volume STEB, and enzymes were inactivated by heating at 67 °C for 20 min. Samples were analyzed by electrophoresis on 1% agarose gels containing 0.5 μg/mL ethidium bromide. Nicked and intact DNA species were detected by ethidium bromide staining. Quantification was performed using a GelDoc-It imaging system (UVP) and ImageQuant software. The intensity values for the closed-circular DNA bands were multiplied by a correction factor of 2 [to correct for the topologically induced differences in the capacity of relaxed covalently closed-circular DNA and nicked circular DNA to bind ethidium bromide (5)] before the percentage of DNA in the nicked form was calculated.

In the control reactions shown in Fig. S1*C*, the Uvr proteins were replaced with storage buffer [10 mM Tris·HCl (pH 8), 1 mM EDTA, 2 mM DTT, 200 mM KCl, 50% (vol/vol) glycerol] or with 5 units of T4 endonuclease V (T4EV), which nicks the DNA at CPD sites (no ATP was added to reactions containing T4EV). To provide a positive control for the experiment shown in Fig. S1*C*, B5-control-template strand plasmid DNA that had been irradiated with 30 J/m^2 of 254-nm UV light to generate randomly located CPDs was assayed alongside the biotinylated substrates.

T4EV Incision Assay. The introduction of single CPD lesions into templates was detected by means of an agarose gel-based DNA incision assay using T4EV (Fig. S1*D*). Five-nanomolar DNA was treated with 5 units of T4EV in TE buffer. The reaction was incubated at 37 °C for 45 min and then stopped by addition of 1/6 volume of STEB. Samples were analyzed by electrophoresis on a 1% agarose gel containing 0.5 μg/mL ethidium bromide. Nicked and intact DNA species were detected by ethidium bromide staining. Control templates containing no inserted CPDs underwent the same treatment and were run on the same gel.

1. Smith AJ, Szczelkun MD, Savery NJ (2007) Controlling the motor activity of a transcription-repair coupling factor: Autoinhibition and the role of RNA polymerase. *Nucleic Acids Res* 35(6):1802–1811.
2. Manelyte L, Kim Y-IT, Smith AJ, Smith RM, Savery NJ (2010) Regulation and rate enhancement during transcription-coupled DNA repair. *Mol Cell* 40(5):714–724.
3. Luzzietti N, Knappe S, Richter I, Seidel R (2012) Nicking enzyme-based internal labeling of DNA at multiple loci. *Nat Protoc* 7(4):643–653.
4. Mitchell DL, Jen J, Cleaver JE (1992) Sequence specificity of cyclobutane pyrimidine dimers in DNA treated with solar (ultraviolet B) radiation. *Nucleic Acids Res* 20(2): 225–229.
5. Lloyd RS, Haidle CW, Robberson DL (1978) Bleomycin-specific fragmentation of double-stranded DNA. *Biochemistry* 17(10):1890–1896.

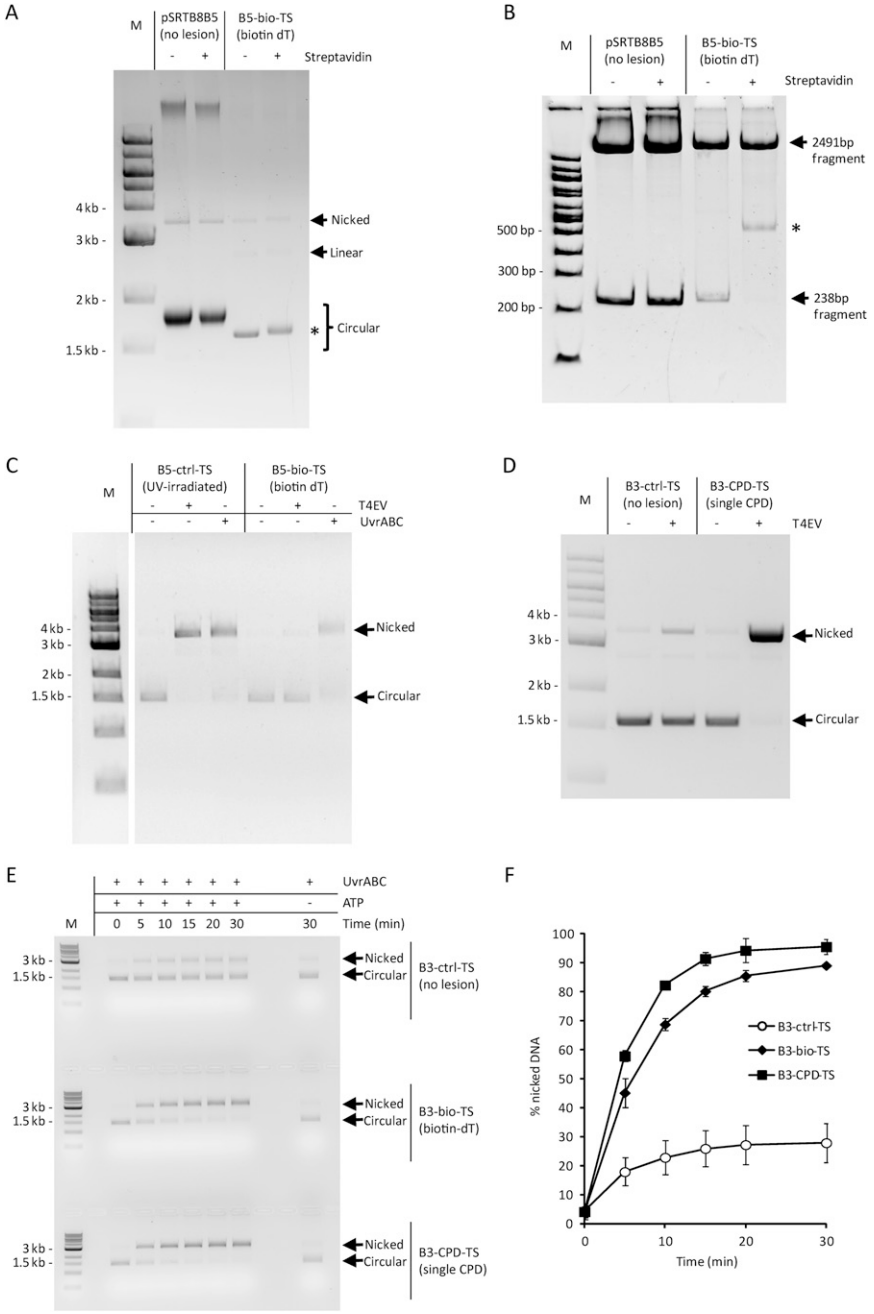

Fig. S1. Analysis of single-lesion substrates. (*A*) Analysis of bio-dT substrate by electrophoretic mobility shift assay (EMSA). An EMSA gel shows binding of streptavidin to a purified substrate containing an annealed and ligated bio-dT oligonucleotide (B5-bio-TS). Unmodified supercoiled pSRTB8B5 (the starting material for the creation of the biotinylated template) was used as a control. The single-lesion template was predominantly in a closed-circular form (circular). A small shift in mobility in the presence of streptavidin (*) indicated the presence of bio-dT. (*B*) Analysis of bio-dT substrate by EMSA. An EMSA gel shows binding of streptavidin to B5-bio-TS substrate that had been digested with EcoRI and HindIII to release a 238-bp fragment carrying the site of modification. The decreased mobility of this band in the presence of streptavidin (*) confirmed the presence of bio-dT. (*C*) Analysis of bio-dT substrate by a NER incision assay. B5-bio-TS and a UV-irradiated control were incubated with UvrA, UvrB, and UvrC or with T4EV (which nicks DNA at CPDs) for 45 min, and conversion of closed-circular DNA to nicked DNA was then detected by gel electrophoresis. Very little of the B5-bio-TS was nicked by T4EV, indicating that the substrate was largely free of UV-induced lesions. B5-bio-TS was nicked by the Uvr proteins, indicating that bio-dT is a substrate for the bacterial NER proteins. The UV-irradiated control DNA was nicked efficiently by both enzymes. (*D*) Analysis of single CPD substrate by T4EV incision assay. Substrate DNA prepared by insertion of a CPD-containing oligonucleotide (B3-CPD-TS) or an unmodified control oligonucleotide (B3-ctrl-TS) was incubated with T4EV. Little of the control substrate was nicked, indicating that the plasmid backbone was largely free of UV-induced lesions. Almost all of the B3-CPD-TS substrate was nicked by T4EV, indicating that the single CPD lesion had been incorporated. (*E*) NER incision time course comparing incision of bio-dT and CPD. Substrate DNAs prepared by insertion of an unmodified control oligonucleotide (B3-ctrl-TS), a bio-dT containing oligonucleotide (B3-bio-TS), or a CPD-containing oligonucleotide (B3-CPD-TS) were incubated with UvrA, UvrB, and UvrC for the times indicated, and conversion of closed-circular DNA to nicked DNA was then detected by gel electrophoresis. Control reactions containing the repair proteins but lacking ATP were incubated for 30 min. Little of the control substrate was nicked, confirming that the majority of the substrate plasmids contained no preexisting lesions. The substrates containing single bio-dT or CPD lesions were both nicked efficiently by UvrABC in an ATP-dependent fashion, confirming that bio-dT is a substrate for the bacterial NER proteins. (*F*) Quantification of the NER incision time course

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experiment shown in *E*. The intensity of the closed-circular and nicked DNA bands was quantified, and a twofold correction factor was applied to the closed-circular DNA to account for differences in ethidium bromide binding capacity. The amount of nicked DNA at each time point is shown as a percentage of the total DNA in the lane. Data are the average of three independent experiments and are shown with SD. Incision of the substrate containing a bio-dT lesion was only slightly slower than incision of the substrate containing a single CPD, suggesting that the two lesions are recognized with similar efficiency by the Uvr proteins.

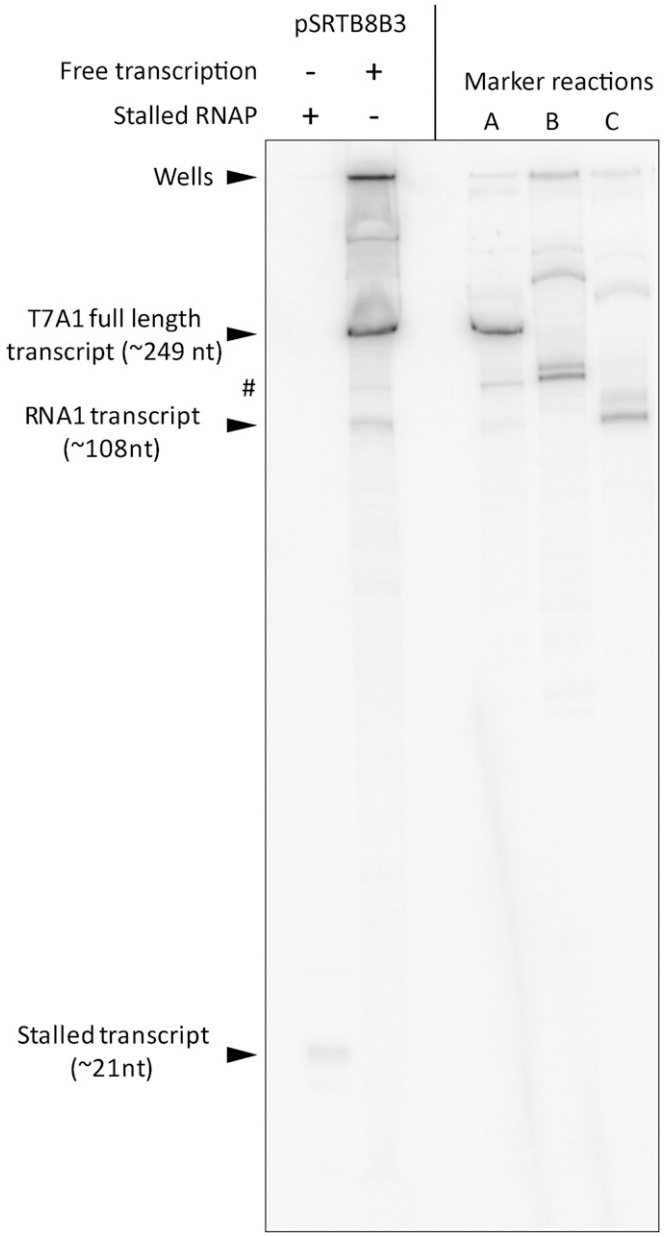

Fig. S2. Effect of 3′ dUTP on in vitro transcription. Supercoiled pSRTB8B3 plasmid DNA was incubated at 37 °C for 20 min with 12.5 nM RNAP, 10 μM GTP, 2.5 μCi [α^{32}P] GTP, 200 μM CTP, 2 mM ATP, and either 100 μM ApU plus 500 μM 3′ dUTP (Stalled RNAP) or 200 μM UTP (Free transcription). The in vitro-transcribed RNA was analyzed on a denaturing 15% (wt/vol) acrylamide gel. The T7A1 full-length transcript ends at a λ*oop* terminator 249 bp downstream of the transcription start site. The RNA I transcript is encoded as part of the ColE1 replication origin of pSR plasmids. The 21-nt product generated by 3′ dUTP-induced stalling and a product caused by pausing or termination within the A/T-rich TFO binding site (labeled #) are indicated. Markers were generated by multiround transcription in the presence of all four NTPs on purified linear fragments of pSRTB8B3 that contained only the T7A1 transcription unit. Transcripts produced from an EcoRI-BamHI fragment encoding a 249-nt transcript (lane A), an EcoRI-ClaI fragment encoding a 160-nt run-off transcript (lane B), and an EcoRI-HindIII fragment encoding a 114-nt run-off transcript (lane C) are shown.

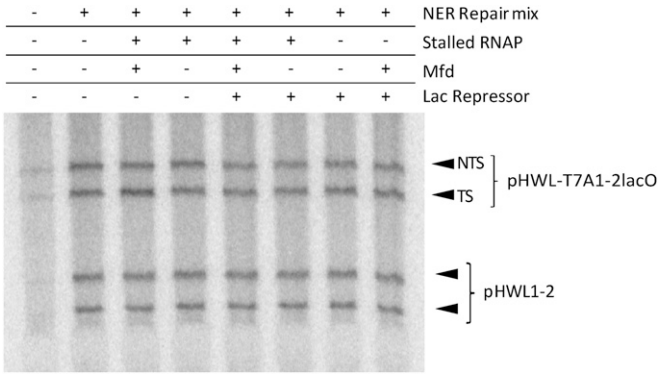

Fig. S3. Effect of a protein roadblock on TCR at a distance. A patch synthesis assay monitored repair of randomly located UV-induced lesions in the TS and nontemplate strand (NTS) of a transcribed reporter cassette in plasmid pHWL1-T7A1-2 lacO. Reactions also contained UV-irradiated plasmid pHWL1-2 as a nontranscribed control. All reactions contained DNA polymerase, DNA ligase, ATP, NADH, and the radiolabeled dNTP mixture required for patch synthesis. NER Repair mix indicates that UvrA, UvrB, UvrC, and UvrD were added, and Stalled RNAP indicates that RNAP was added with GTP, CTP, ApU, and 3′ dUTP. Mfd and Lac repressor protein were added where indicated.

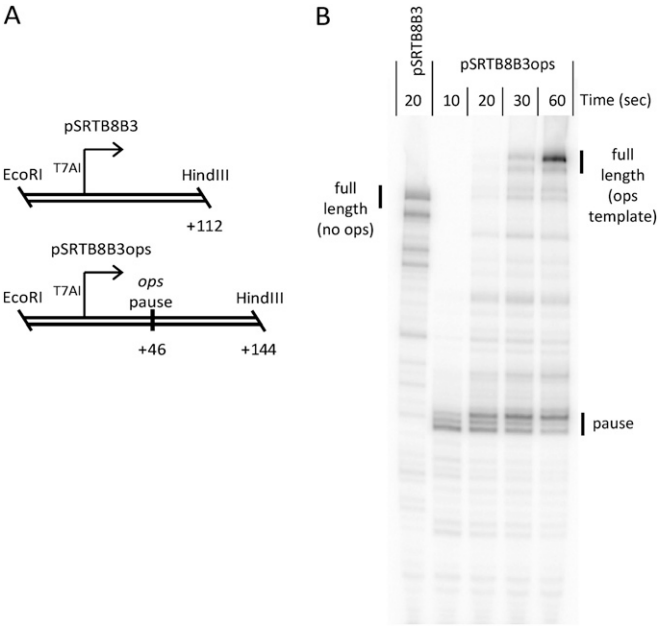

Fig. S4. Effect of the *rfaQ ops* pause site on in vitro transcription. (*A*) Substrates used in these assays are shown. The reactions used gel-purified linear EcoRI-HindIII fragments that contained only the T7A1 transcription unit and generated run-off transcripts. (*B*) Indicated linear templates were incubated with 20 nM RNAP; 200 μM CTP, ATP, and UTP; 10 μM GTP; and 2.5 μCi [α^{32}P] GTP at 37 °C for the indicated times, and the in vitro-transcribed RNA was analyzed on a denaturing 12% (wt/vol) acrylamide gel.

Table S1. Summary of plasmids used for single-lesion and TFO assays

Plasmid	Sequence of nontemplate strand from +1 of T7A1 promoter (all 5′ to 3′)
pSRTB1	ATCGAGAGGG ACACGGCGAA TAGCCATCGA TCAAGCTG*TT CTTTTCTTTT CTTCTTCTTT* G
pSRTB2	ATCGAGAGGG ACACGGCGAA TAG**CCTCAGC** TCAAGCTG*TT CTTTTCTTTT CTTCTTCTTT* G
pSRTB4	ATCGAGAGGG ACACGGCGAA TAG**CCTCAGC** AGAAGCTTGG CATCAGCTCA AGCTG*TTCTT TTCTTTTCTT CTTCTTT*G
pSRTB7	ATCGAGAGGG ACACGGCGAA TAGCCTCATA GCAATGATGG T**CCTCAGC**AA GCTTGGCATC AGCTCAAGCT G*TTCTTTTCT TTTCTTCTTC TTT*G
pSRTB8	ATCGAGAGGG ACACGGCGAA TAGCCTCATA GCAATGATGG T**CCTCAGC**TT GTGTACAATT G**CCTCAGC**GA TCCATCGCTC AGACTAGAGC GATTCAGCAA GCTTGGCATC AGCTCAAGCT G*TTCTTTTCT TTTCTTCTTC TTT*G
pSRTB9	ATCGAGAGGG ACACGGCGAA TAGCCTCATA GCAATGATGG T**CCTCAGC**TT GTGTACATCG ACTGAAATCC CTGGTAATCC GTTTTAGAAT CCATGATAAT AATTTTCTGG ATTATTGGTA ATTTTTTTTG CACGTTCAAA ATTTTTTGCA ACCCCTTTTT GGAAACAAAC ACTACGGTAG GCTGCGAAAT GTTCATACTG TTGAGCAATT CACGTTCATT ATAAATGTCG TTCGCGGGCG CAACTGCAAC TCCGATAAAT AACGCGCCCA ACACCGGCAT AAAGAATTGA AGAGAGTTTT CACTGCATAC GACGATTCTG TGATTTGTAT TCAGCCCATA TCGTTTCATA GCTTCTGCCA ACCGAACGGA CATTTCGAAG TATTCCGCGT ACGTGATGTT CACCTCGATA TGTGCATCTG TAAAAGCAAT TGTTCCAGGA ACCAGGGCGT ATCTCTTCAT AGCCTTATGC AGTTGCTCTC CAGCGGTTCC ATCCTCTAGA GGATAGAATG GCGCCGGGCC TTTCTTTATG TTTTTGGCGT CTTCCATGGT AGCTTCCTTA GCTCCTGAAA ATCTCGTCGA AGCTTGGCAT CAGCTCAAGC TG*TTCTTTTC TTTTCTTCTT CTTT*G
pSRTBlacO	ATCGAGAGGG ACACGGCGAA TAGCCTCATA GCAATGATGG T**CCTCAGC**AA GCTT***GAATTG TGAGCGGATA ACAATTTC***AG CTTGGCATCA GCTCAAGCTG *TTCTTTTCTT TTCTTCTTCT TT*G
pSRTB8B3	ATCGAGAGGG ACACGGCGAA TAGCCTCATA GCAATGATGG T**CCTCAGC**TC AGCTAG**CCTC AGC**CTACAAT CA**CCTCAGC**G ATCCATCGCT CAGACTAGAG CGATTCAGCA AGCTTGGCAT CAGCTCAAGC TG*TTCTTTTC TTTTCTTCTT CTTT*G
pSRTB8B5	ATCGAGAGGG ACACGGCGAA TAGCCTCATA GCAATGATGG T**CCTCAGC**TC ATGTCAT**CCT CAGC**ACACTT GAC**CCTCAGC** TCAGCTAG**CC TCAGC**CTACA ATCA**CCTCAG C**GATCCATCG CTCAGACTAG AGCGATTCAG CAAGCTTGGC ATCAGCTCAA GCTG*TTCTTT TCTTTTCTTC TTCTTT*G
pSRTB8B3ops	ATCGAGAGGG ACACGGCGAA TAGCCTCATC CATGGGGCGG TAGCGTGCTT TTTTCCTCGA GAGCAATGAT GGT**CCTCAGC** TCAGCTAG**CC TCAGC**CTACA ATCA**CCTCAG C**GATCCATCG CTCAGACTAG AGCGATTCAG CAAGCTTGGC ATCAGCTCAA GCTG*TTCTTT TCTTTTCTTC TTCTTT*G

Sequences shown are of the template strand, from the first transcribed base of the T7A1 promoter to the end of the TFO binding site. BbvCI sites are indicated by bold text, BmtI sites by single-underlined text, HindIII sites by double-underlined text, TFO binding sites by italic text, *lacO* by bold italic text, and the *rfaQ ops* pause site by shaded text. All plasmids are identical outside the sequence shown.

Table S2. Details of single-lesion substrates created by ligating oligonucleotides into plasmid DNA containing a single-strand gap

Substrate	Nicking enzyme	Plasmid	Oligo	Summary
B3-ctrl-TS	Nb. BbvCI	pSRTB8B3	31-TS	Control: no lesion
B3-bio-TS	Nb. BbvCI	pSRTB8B3	31bio-TS	Bio-dT at +68, TS
B3-CPD-TS	Nb. BbvCI	pSRTB8B3	31CPD-TS	TT CPD at +68/+69, TS
B5-ctrl-TS	Nb. BbvCI	pSRTB8B5	63-TS	Control: no lesion
B5-bio-TS	Nb. BbvCI	pSRTB8B5	63bio-TS	Bio-dT at +100, TS
B5-bio-NTS	Nt. BbvCI	pSRTB8B5	63bio-NTS	Bio-dT at +102, NTS
B3ops-bio-TS	Nb. BbvCI	pSRTB8B3ops	31bio-TS	Bio-dT at +100, TS; *ops* pause at +46

ctrl, control; NTS, nontemplate strand; TS, template strand.

Table S3. Sequences of the oligonucleotides used to create the single-lesion substrates listed in Table S2

Oligo	Oligo sequence (5′ to 3′)
31-TS	TGAGGTGATTGTAGGCTGAGGCTAGCTGAGC
31bio-TS	TGAGGTGATXGTAGGCTGAGGCTAGCTGAGC (X = bio-dT)
31CPD-TS	TGAGGTGAXXGTAGGCTGAGGCTAGCTGAGC (X = TT CPD)
31bio-NTS	TCAGCTCAGCTACCTCAGCCTACAAXCACC (X = bio-dT)
63-TS	TGAGGTGATTGTAGGCTGAGGCTAGCTGAGCTGAGGGTCAAGTGTGCTGAGGATGACATGAGC
63bio-TS	TGAGGTGATXGTAGGCTGAGGCTAGCTGAGCTGAGGGTCAAGTGTGCTGAGGATGACATGAGC (X = bio-dT)
63bio-NTS	TCAGCTCATGTCATCCTCAGCACACTTGACCCTCAGCTCAGCTAGCCTCAGCCTACAAXCACC (X = bio-dT)