Supporting Information

Unraveling Reversible DNA Cross-links with a Biological Machine

Shane R. Byrne & Steven E. Rokita*
Chemistry Biology Interface Graduate Program and Department of Chemistry
Johns Hopkins University, 3400 N. Charles St.
Baltimore, MD 21218 United States

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General materials. T4 polynucleotide kinase, NiCo21(DE3) competent cells, dNTPs, ddNTPs, Klenow (exo-) polymerase, ϕ29 polymerase, Human apurinic/apyrimidinic endonuclease 1 (APE1) and uracil-DNA glycosylase (UDG) were purchased from New England Biolabs. 9-Aminoacridine was prepared as published previously.[1] 5'-ATP-agarose resin (catalog number A2767) and dTTP (sodium salt solution, PCR grade) were purchased from Sigma-Aldrich. P6 Micro Bio-Spin columns and Bradford reagent were purchased from Bio-Rad. PD-10 spin columns for desalting were purchased from GE Healthcare. HisPur™ Ni-NTA resin was purchased from ThermoFisher Scientific. Ultracentrifugal filters with a 10,000 MW cutoff (Amicon) were purchased from Millipore.

Expression and purification of T7gp4. Procedures described below are adapted from a published protocol[2] using the plasmid pET19-56-Kan for expressing a fusion of His_{10} and the 56 kDa fragment of T7gp4 described previously.[3] The plasmid was transformed into *Escherichia coli* NiCo21(DE3) chemically competent cells in the presence of kanamycin (50 µg/mL). Cultures were grown in LB media (1 L) and kanamycin (50 µg/mL) at 37 ºC with vigorous shaking (220 rpm) until OD_{600} reached ~ 1. Expression of T7gp4 was then induced with isopropyl-β-D-thio-galactopyranoside (1 mM) at 37 ºC for 3 h with vigorous shaking (220 rpm). Cells were harvested by centrifugation (5000 rpm, 15 min, 4 ºC), flash frozen in liquid N_2 and stored at -80 ºC until use.

Frozen cell pellets were thawed on ice and resuspended in 50 mM potassium phosphate pH 8, 500 mM NaCl and 10 mM imidazole (20 mL, loading buffer). Cells were lysed by treatment with lysozyme (100 µg/mL) on ice for 1 h followed by three rounds of flash freezing in liquid nitrogen and thawing on ice. Cell debris was removed by centrifugation (35,000 g, 1 h, 4 ºC) and the resulting supernatant was applied by gravity to a column containing 1 mL of HisPur™ nickel-nitroloacetic acid (Ni-NTA) resin. The column was washed with loading buffer (30 mL) followed by loading buffer supplemented to 100 mM imidazole (20 mL). The protein was then eluted with Ni-NTA elution buffer (loading buffer with imidazole supplemented to 500 mM). The fractions containing T7gp4 were desalted with a PD-10 spin column (1000 g, 2 min, 4 ºC) equilibrated with ATP-agarose loading buffer (20 mM potassium phosphate pH 6.8, 0.5 mM DTT, 10% glycerol, 500 mM KCl, and 10 mM $MgCl_2$).

The desalted protein was applied by gravity to a column containing ATP-agarose resin (0.5 mL) and washed with ATP-agarose loading buffer (10 mL). The column was then washed with 20 mM potassium phosphate pH 6.8, 0.5 mM DTT, 20 mM EDTA, 10% glycerol, and 500 mM KCl until no proteins eluted. T7gp4 was then released by switching to the loading buffer supplemented with an additional 500 mM KCl. The isolated T7gp4 was concentrated with an Amicon Ultra Centrifugal Filter (MW cut-off of 10,000 kDa), washed with water (3 x 500 µL) and stored in 20 mM potassium phosphate pH 7.5 with 50% glycerol.

Figure S1. Unwinding a model replication fork with T7gp4. (A) OD1:5'-[^{32}P]-OD2 (10 nM) containing extended poly(T) sequences in 40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM potassium glutamate and 1 mM dTTP was incubated with the indicated concentration of T7gp4 (monomer) for 10 min at 37 ºC. Reaction was quenched with EDTA (40 mM) and loading dye containing bromophenol blue and xylene cyanol in 30% glycerol. (B) Products were separated by native polyacrylamide gel electrophoresis (PAGE, 10%) and quantified by phosphorimagery (% relative to total radiolabel). As electrophoretic standards, lane ss contains only 5'-[^{32}P]-OD2 and lane ds contains the annealed duplex OD1:5'-[^{32}P]-OD2.

Figure S2 Distribution of adducts formed by bisQMP after T7gp4 treatment. OD1:5'-[^{32}P]-OD2 was cross-linked and purified by standard conditions except the bisQMP concentration was lowered to 50 µM and reaction time was limited to 30 min for single-hit conditions. (A) The resulting mixture of OD1:5'-[^{32}P]-OD2 (10 nM) with and without cross-linking in 40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM potassium glutamate and 1 mM dTTP was incubated in the alternate absence and presence of T7gp4 (55 nM monomer) for the indicated time at 37 ºC. Reaction was quenched with EDTA (40 mM), lyophilized and then dissolved in 10% piperidine (30 µL). The resulting solution was heated for 30 min at 90 ºC and lyophilized again. To remove residual piperidine, samples were dissolved in water and lyophilized in three consecutive repetitions. (B) Products were separated by denaturing PAGE (PAGE, 20%). As electrophoretic standards, lane ss contains 5'-[^{32}P]-OD2 after piperidine treatment. Lane A+G contains 5'-[^{32}P]-OD2 after incubation with 5% formic acid (30 min, 37 ºC) and subsequent piperidine treatment. Lane xl was generated by piperidine treatment of OD1:5'-[^{32}P]-OD2 after incubation with bisQMP as described above.

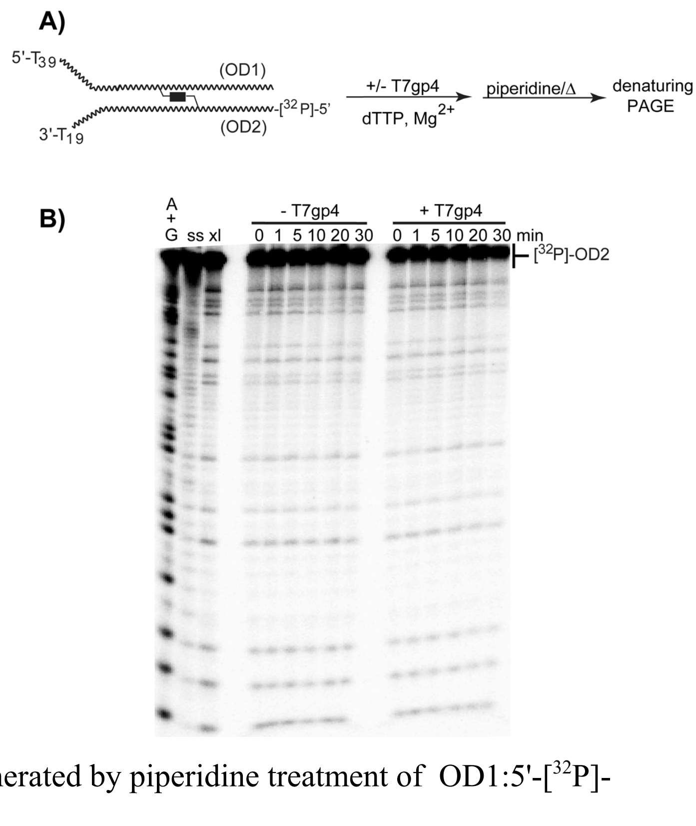
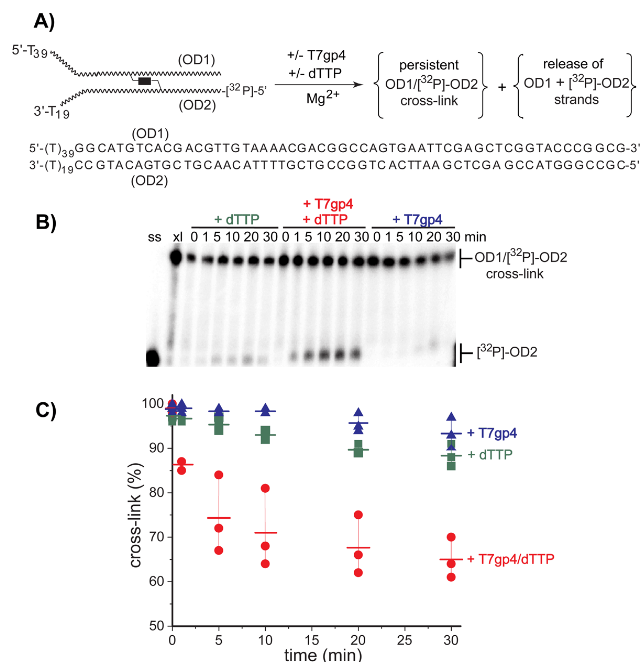


Figure S3. Unwinding DNA and release of a reversible interstrand cross-link. (A) A replication fork model (OD1:5'-[^{32}P]-OD2, 10 nM) containing an interstrand cross-link formed by bisQMP and a radiolabel on the excluded strand was incubated in 40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 50 mM potassium glutamate with the alternative and combined presence of T7gp4 (55 nM monomer) and dTTP (1 mM). (B) After incubation at 37 ºC for the indicated time, reaction was quenched by addition of EDTA (40 mM) and analyzed by denaturing polyacrylamide gel electrophoresis (PAGE, 10%). As electrophoretic standards, lane ss contains only 5'-[^{32}P]-OD2 and lane xl contains OD1:5'-[^{32}P]-OD2 cross-linked by bisQMP. (C) The remaining cross-link (% based on total radiolabel) was determined by phosphoimagery in three replicates and their average values are indicated by the cross-bars.



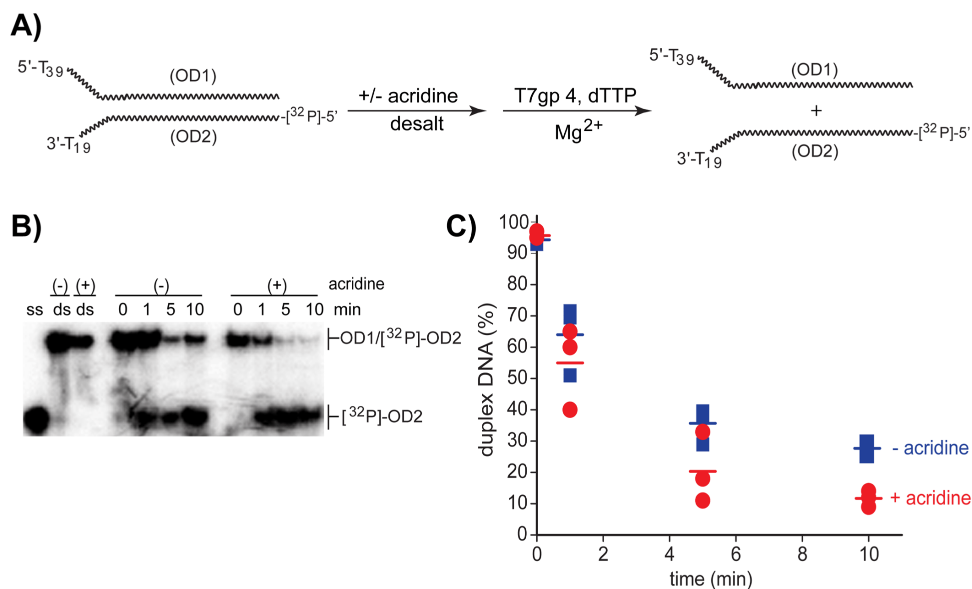


Figure S4. Unwinding a model replication fork in the presence of 9-aminoacridine. (A) OD1:5'-[^{32}P]-OD2 (10 nM) containing a radiolabel on the excluded strand in 40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM potassium glutamate and 1 mM dTTP was incubated with T7gp4 (55 nM monomer) for the indicated time at 37 ºC. Alternatively, OD1:5'-[^{32}P]-OD2 in 10 mM 2-(N-morpholino)ethanesulfonate (MES) pH 7 and 50 mM NaF was incubated for 2 h with 250 µM 9-aminoacridine under ambient conditions. Low molecular weight species were then removed using a P6 micro-spin column. The isolated DNA was treated with T7gp4 under conditions described above. Unwinding was quenched with EDTA (40 mM) and loading dye containing bromophenol blue and xylene cyanol in 30% glycerol. (B) Products were separated by native polyacrylamide gel electrophoresis (PAGE, 10%) and quantified by phosphorimagery (% relative to total radiolabel). As electrophoretic standards, lane ss contains only 5'-[^{32}P]-OD2. Lanes ds contain the annealed duplex OD1:5'-[^{32}P]-OD2 in the presence and absence of 9-aminoacridine as indicated. (C) The remaining duplex (% based on total radiolabel) was determined by phosphoimagery in three replicates and their average values are indicated by the cross-bars.

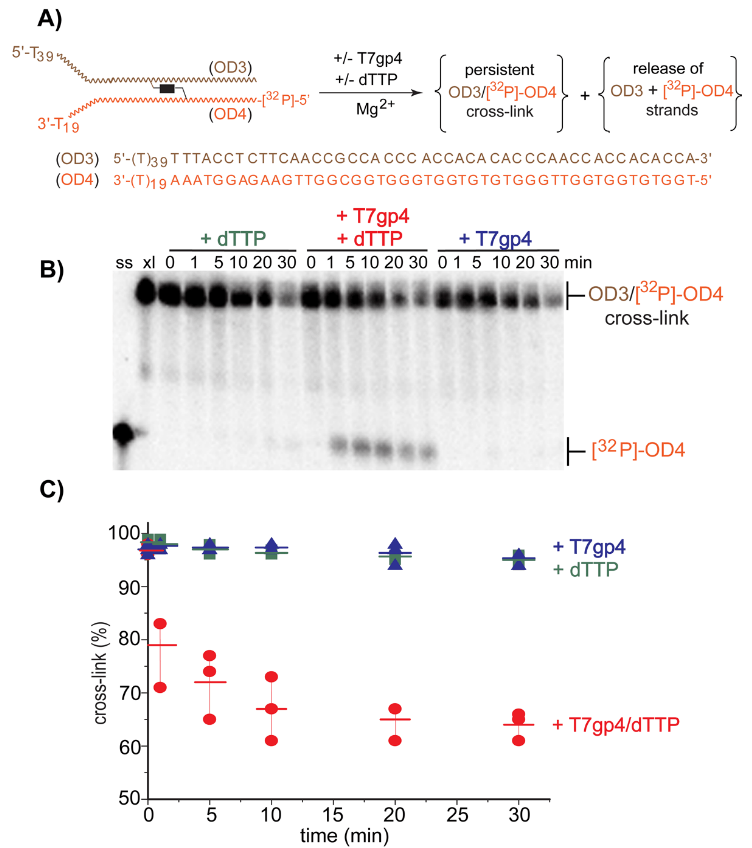


Figure S5. Unwinding DNA and release of a reversible interstrand cross-link from a second model of a replication fork. (A) OD3:5'-[^{32}P]-OD4 (10 nM) containing an interstrand cross-link formed by bisQMP and a radiolabel on the excluded strand was incubated in 40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 50 mM potassium glutamate with the alternative and combined presence of T7gp4 (55 nM monomer) and dTTP (1 mM). (B) After incubation at 37 ºC for the indicated time, reaction was quenched by addition of EDTA (40 mM) and analyzed by denaturing polyacrylamide gel electrophoresis (PAGE, 10%). As electrophoretic standards, lane ss contains only 5'-[^{32}P]-OD4 and lane xl contains OD3:5'-[^{32}P]-OD4 cross-linked by bisQMP. (C) The remaining cross-link (% based on total radiolabel) was determined by phosphoimagery in three replicates and their average values are indicated by the cross-bars.

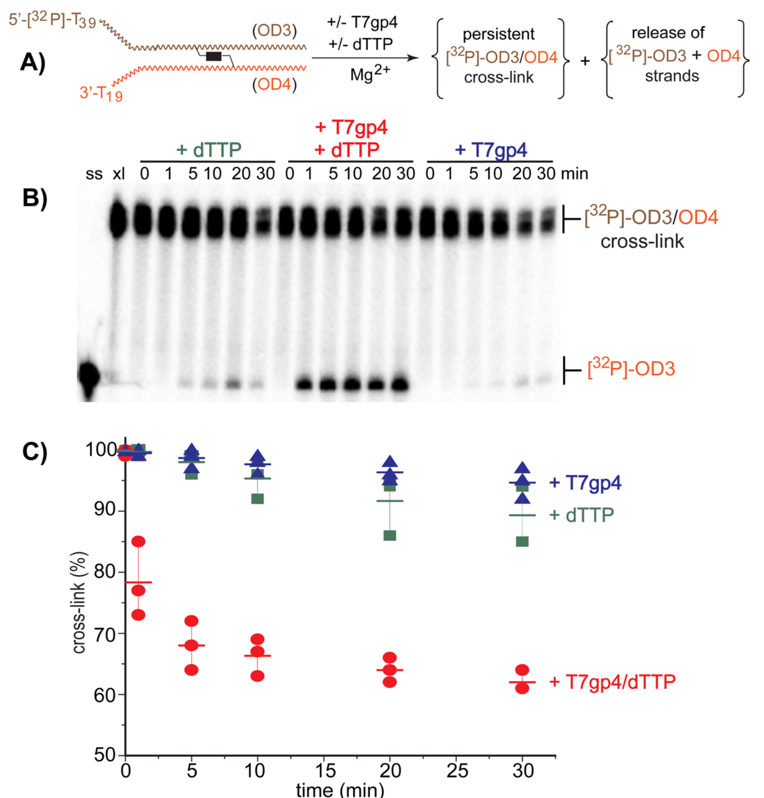


Figure S6. Monitoring unwinding of DNA and release of a reversible interstrand cross-link when the translocated strand is radiolabeled. (A) 5'-[^{32}P]-OD3:OD4 (10 nM) containing an interstrand cross-link formed by bisQMP was incubated in 40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 50 mM potassium glutamate with the alternative and combined presence of T7gp4 (55 nM monomer) and dTTP (1 mM). (B) After incubation at 37 ºC for the indicated time, reaction was quenched by addition of EDTA (40 mM) and analyzed by denaturing polyacrylamide gel electrophoresis (PAGE, 10%). As electrophoretic standards, lane ss contains only 5'-[^{32}P]-OD3 and lane xl contains 5'-[^{32}P]-OD3:OD4 cross-linked by bisQMP. (C) The remaining cross-link (% based on total radiolabel) was determined by phosphoimagery in three replicates and their average values are indicated by the cross-bars.

Figure S7. Representative data for Figure 2, supplementing conditions after initial unwinding of DNA containing a reversible interstrand cross-link. (A) OD3:5'-[^{32}P]-OD4 (10 nM) containing a cross-link formed by bisQMP and a radiolabel on the excluded strand was incubated at 37 ºC in 40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 50 mM potassium glutamate with T7gp4 (55 nM monomer) and dTTP (1 mM) for 30 min before supplementing with an additional aliquot of either T7gp4 (55 nM monomer) or dTTP (1 mM). Incubations were continued at 37 ºC for the indicated times and then quenched with EDTA (40 mM). (B) Reaction products were identified after separation by denaturing PAGE (10%). The control entitled spontaneous hydrolysis contained no T7gp4 or dTTP throughout the incubations and that entitled no supplement contained the standard concentrations of T7gp4 and dTTP in the initial incubation but no additional T7gp4 or dTTP in the subsequent incubation.

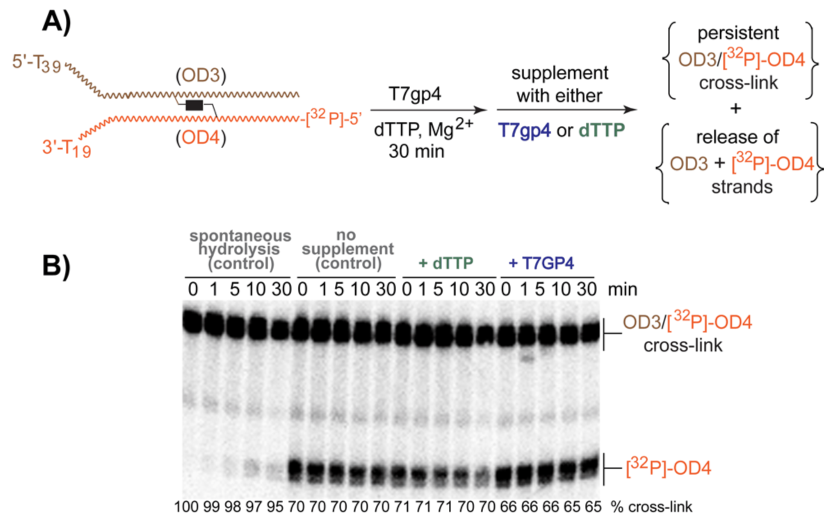


Figure S8. Unwinding DNA containing a reversible interstrand cross-link with excess T7gp4 for an extended incubation and further supplements of dTTP and T7gp4. (A) OD3:5'-[^{32}P]-OD4 (10 nM) containing a cross-link formed by bisQMP and a radiolabel on the excluded strand was incubated at 37 ºC in 40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 50 mM potassium glutamate with excess T7gp4 (500 nM monomer) and dTTP (1 mM) for 0 - 30 min. After 30 min, equivalent samples were then supplemented with an additional aliquot of only dTTP (1 mM) or both T7gp4 (500 nM monomer) and dTTP (1 mM). These latter samples were incubated at 37 ºC for an additional 30 min (60 min total) before quenching with EDTA (40 mM). (B) Reaction products were identified after separation by denaturing PAGE (10%).

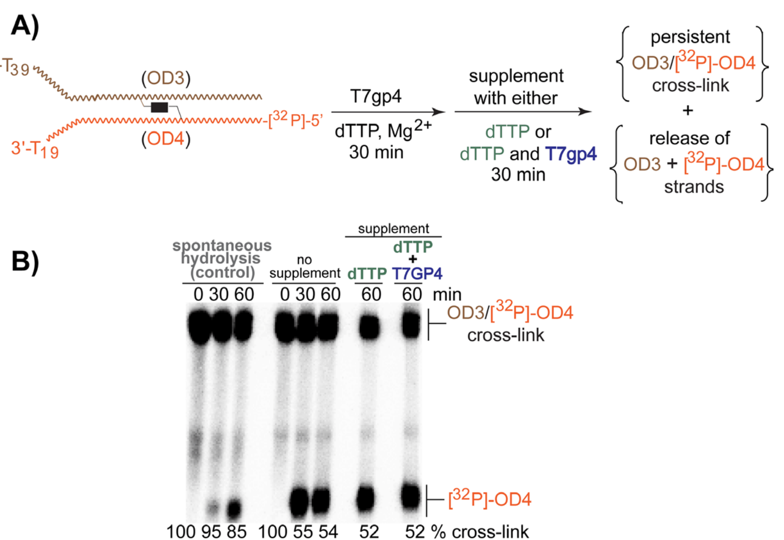


Figure S9. Data for Figure 3, challenging T7gp4 with an irreversible cross-link. (A) The replication fork model (OD3:5'-[^{32}P]-OD4) containing a cross-link formed by mechlorethamine and a radiolabel on the excluded strand was treated in the alternative and combined presence of T7gp4 and dTTP as described in Figure S4. (B) After incubation at 37 ºC for the indicated time, reaction was quenched by addition of EDTA (40 mM) and analyzed by denaturing PAGE (10%). As electrophoretic standards, lane ss contains only 5'-[^{32}P]-OD4 and lane xl contains OD3:5'-[^{32}P]-OD4 cross-linked by mechlorethamine.

Figure S10. Dissociation of T7gp4 from duplex DNA containing a reversible cross-link. OD3:OD4 (10 nM) containing reversible cross-links formed by bisQMP in 40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM potassium glutamate and T7gp4 (55 nM monomer concentration) was incubated at 37 ºC for 10 min in the presence and absence of dTTP (1 mM). The remaining free T7gp4 was then quantified by its ability to unwind a radiolabeled equivalent of the cross-linked OD3:OD4 that was added subsequently. dTTP (1 mM) was also added to the samples previously lacking dTTP. The unwinding assay was maintained at 37 ºC and quenched at the indicated times with EDTA (40 mM). (B) Products were separated by 10% denaturing PAGE and visualized by phosphorimagery. (C) Experiments were performed in triplicate and the remaining cross-linked DNA (%) is reported relative to total radiolabel. Their average values are indicated by the cross-bars.

Figure S11. Unwinding DNA and release of nucleobase adducts from the excluded strand. (A) A model replication fork (OD3:5'-[^{32}P]-OD4, 10 nM) containing reversible adducts formed by monoQMP was incubated in the alternative and combined presence of T7gp4 (55 nM monomer) and dTTP (1 mM). (B) After incubation at 37 ºC for the indicated time, reaction was quenched by addition of EDTA (40 mM) and analyzed by denaturing PAGE (10%). As electrophoretic standards, lane ss contains only 5'-[^{32}P]-OD4 and lane alk contains OD4 after treatment with monoQMP. (C) The remaining cross-link (% based on total radiolabel) was measured by phosphoimagery in three replicates and their average values are indicated by the cross-bars. The low resolution of this gel relative to that of Figure 4 may be attributed to the high guanine content of OD4.

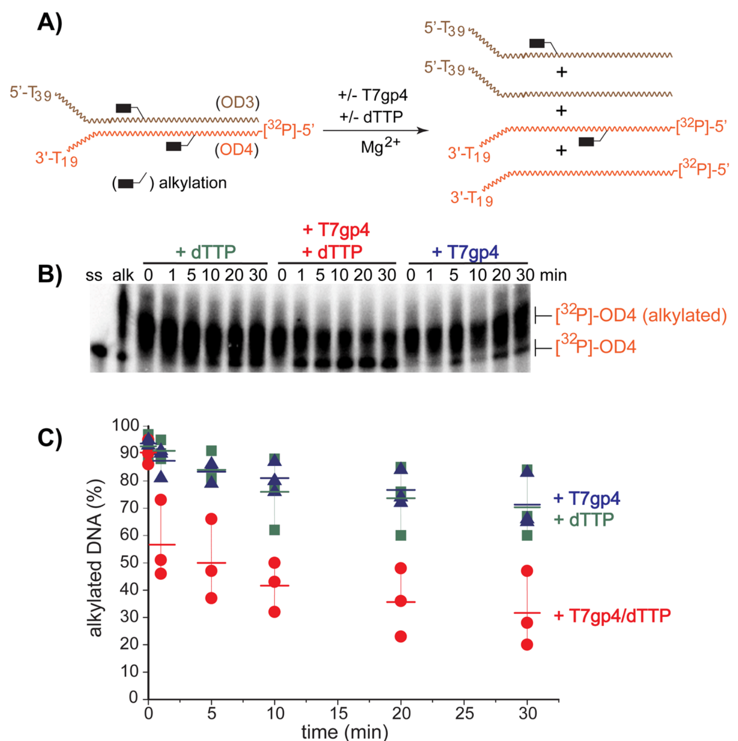


Figure S12. Release of a reversible cross-link by T7gp4 during unwinding of the excluded strand. (A) The duplex OD3:5'-[^{32}P]-OD4 (10 nM) containing reversible cross-links formed by bisQMP was incubated in the alternative and combined presence of T7gp4 (55 nM monomer) and dTTP (1 mM). (B) After incubation at 37 ºC for the indicated time, reaction was quenched by addition of EDTA (40 mM) and analyzed by denaturing PAGE (10%). As electrophoretic standards, lane ss contains only 5'-[^{32}P]-OD4. Lane alk contains OD4 after treatment with monoQMP and lane xl contains OD3:5'-[^{32}P]-OD4 cross-linked by bisQMP.

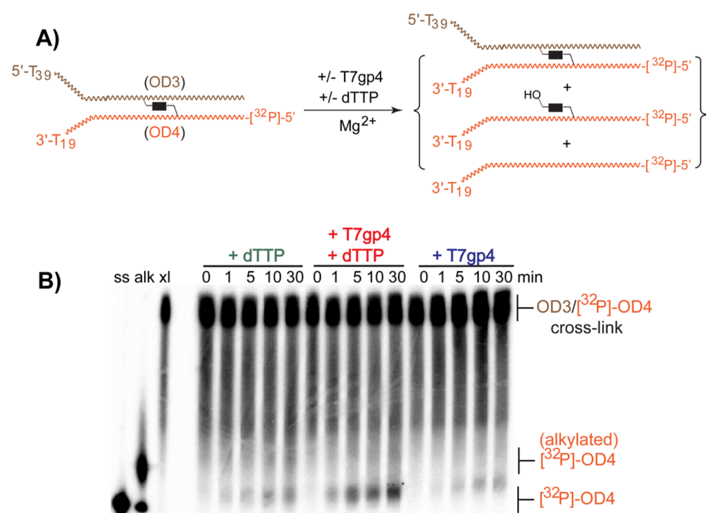


Figure S13. Dissociation versus deglycosylation of guanine N7 adducts. (A) OD3:5'-[^{32}P]-OD4 (10 nM) containing reversible adducts formed by monoQMP (50 μM) was incubated in the alternative absence and presence of T7gp4 (55 nM monomer) and dTTP (1 mM). After the indicated time, these samples were divided equally into two aliquots and frozen. One fraction was lyophilized and then dissolved in 10% piperidine (30 μL). The resulting solution was heated for 30 min at 90 ºC and lyophilized again. To remove residual piperidine, samples were dissolved in water and lyophilized in three consecutive repetitions. The second fraction was precipitated with NaOAc (0.3 M, pH 5.2) and cold ethanol (100 μL) and resuspended in 50 mM KOAc, 1 mM DTT and 20 mM Tris-OAc pH 7.9. Reaction was initiated by addition of APE1 (3 U). Samples were incubated at 37 ºC for 1 h and then quenched by heating at 65 ºC for 20 min. (B) Samples were combined with formamide loading dye and separated by denaturing PAGE (20%). An analog of OD4 containing a U at position 24 was treated with UDG (3 U) for 10 min at 37 ºC, before treatment with APE1 (lane "U"). The duplex lacking monoQMP treatment was also subject to heat and piperidine treatment (lane “no QM”).

References

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