The Topodynamics of Incision of UV-irradiated Covalently Closed DNA by the Escherichia coli Uvr(A)BC Endonuclease*

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The Escherichia coli Uvr(A)BC endonuclease (Uvr-(A)BC) initiates nucleotide excision repair of a large variety of DNA damages. The damage recognition and incision steps by the Uvr(A)BC is a complex process utilizing an ATP-dependent DNA helix-tracking activity associated with the UvrA2B1 complex. The latter activity leads to the generation of highly positively supercoiled DNA in the presence of E. coli topoisomerase I in vitro. Such highly positively supercoiled DNA, containing ultraviolet irradiation-induced photoproducts (uvDNA), is resistant to the incision by Uvr(A)BC, whereas the negatively supercoiled and relaxed forms of the uvDNA are effectively incised. The E. coli gyrase can contribute to the above reaction by abolishing the accumulation of highly positively supercoiled uvDNA thereby restoring Uvr(A)BC-catalyzed incision. Eukaryotic (calf thymus) topoisomerase I is able to substitute for gyrase in restoring this Uvr(A)BC-mediated incision reaction. The inability of Uvr(A)BC to incise highly positively supercoiled uvDNA results from the failure of the formation of UvrAB-dependent obligatory intermediates associated with the DNA conformational change. In contrast to Uvr(A)BC, the Micrococcus luteus UV endonuclease efficiently incises uvDNA regardless of its topological state. The in vitro topodynamic system proposed in this study may provide a simple model for studying a topological aspect of nucleotide excision repair and its interaction with other DNA topology-related processes in E. coli.

The Uvr(A)BC endonuclease from Escherichia coli consists of an ensemble of the uvrA, uvrB, and uvrC gene products. They act in a sequential manner to produce dual incision of damaged DNA strand at the fourth (fifth) phosphodiester bond 3’ and the eighth phosphodiester bond 5’ to the damaged site, respectively (1–3). The NER† process is completed in vitro by the subsequent action of DNA helicase II (UvrD), DNA polymerase I, dNTPs, and ligase (4, 5). The Uvr(A)BC is able to act on a very broad spectrum of DNA damage (1–3, 6). In the damage recognition and incision steps, an intricate ATP hydrolysis-coupled process is employed rather than direct damage binding-incision utilized by most of the damage-specific repair enzymes (1–3, 6). In particular, a DNA helix-tracking activity of UvrA2B1 complex is engaged in search and/or preprepping of damage by Uvr(A)BC (1, 7). Such DNA helix-tracking activity generates domains of positive and negative supercoils ahead and behind moving complex, respectively (7, 8). The differential accumulation of either positive or negative supercoils, which can be achieved in the presence of “anchoring” entities (9, 10), could potentially affect the tracking activity of the UvrA2B1 complex and, hence, influence the DNA repair process. In the cell, the topoisomerase activities are involved in preventing such accumulation and maintaining the proper level of DNA superhelicity (8, 11–13). It was found that topo I and gyrase mutations as well as gyrase inhibitors (novobiocin and nalidixic acid) increase sensitivity of E. coli cells to the killing effects of UV irradiation, affect to different degrees the amount of UV-stimulated repair synthesis, and inhibit recovery of UV-irradiated nonreplicative λ phage (14–18). These observations give a hint of the existence of the topological aspect of NER in vitro. However, the effect of damaged DNA topological state on and involvement of topoisomerase in NER in E. coli have received so far very limited biochemical characterization (19–22). In this report, we addressed this question using the in vitro system including Uvr proteins and topoisomerases. This topodynamic (23, 24) system allows for the differential regulation of the uvDNA topological state. It was found that highly positively supercoiled uvDNA generated by UvrA2B1 helix-tracking activity in the presence of only E. coli topo I is not incised by Uvr(A)BC, whereas it is efficiently incised by Micrococcus luteus UV endo. The addition to this reaction mixture of enzymatic activities removing positive supercoils (E. coli gyrase or eukaryotic topo I) results in restoration of the incision of uvDNA by Uvr(A)BC. The inability of Uvr(A)BC to incise highly positively supercoiled uvDNA is due to the inhibition of UvrAB-dependent incision intermediates associated with the DNA substrate conformational change (25). The results are discussed in terms of the influence of DNA topology on the process of Uvr(A)BC damage recognition and incision as well as in terms of the applicability of this in vitro topodynamic system for study of the topological aspect of NER and its interaction with other DNA topology-involved cellular processes.

EXPERIMENTAL PROCEDURES

Proteins—The UvrA, UvrB, and UvrC proteins were purified according to previously published procedures (26, 27). E. coli DNA topo I was purified according to the procedure provided by Dr. J. Wang (Harvard University). Gyrase A and B subunits of E. coli gyrase were kindly provided by Drs. B. Learn and R. McMacken (Johns Hopkins University). Calf thymus topo I was from BRL. The M. luteus UV endo (28) was purified according to the procedure published by Dr. J. Wang (Harvard University). Gyrase A and B subunits of E. coli gyrase were kindly provided by Drs. B. Learn and R. McMacken (Johns Hopkins University). Calf thymus topo I was from BRL. The M. luteus UV endo (28) was purified according to the procedure.
from Applied Biotechnology Co. The concentrations of all proteins were determined using a Bradford Protein Assay Kit (Bio-Rad) with BSA used as a standard.

Preparation of 3H-Labeled Plasmid DNA—Plasmid pTZ18R DNA was labeled by growing E. coli DH5α harboring this plasmid in M9 medium containing [3H]thymidine (29). The plasmid was further purified with a Quick Prep Kit (Qiagen) according to the manufacturer's recommendations. The specific activity of the labeled plasmid was 4.3 × 10^7 cpm/μg as measured by scintillation counting (Beckman LS 3801).

Preparation of Highly Positively Supercoiled DNA—The relaxed plasmid (100–200 ng) (pTZ18R cold or 3H-labeled) was incubated with 400 ng each of UvrA and UvrB, and 400 ng of E. coli DNA topo I in the supercoiling buffer (see below) for 60 min at 37 °C. The extent of ATP hydrolysis was measured as described under “Experimental Procedures.” The final reaction volume was about 20 μl.

One-third (8 μl) of reaction mixtures were loaded onto an agarose gel. The reaction mixtures (50 μl) were incubated using 5 ng or 10 ng each of UvrA and UvrB. There was no incubation initiated by the addition of UvrA protein and allowed to equilibrate at 37 °C for 30 min. The nucleoprotein complexes were trapped by filtration through 0.45-μm nitrocellulose filters (HAWP 025, Millipore) (29). The amount of [3H]DNA retained on the filter was determined by binding assay—The formation of UvrA nucleoprotein complexes was monitored by nitrocellulose filter binding (29). The reaction mixtures (50 μl) contained 6.7 fmol of negatively or 8.8 fmol of highly positively supercoiled 3H-labeled pTZ18R plasmid DNA circles, 25 mM MOPS (pH 7.6), 25 mM Hepes (pH 7.6), 0.1 μM KCl, 10 mM MgCl2, 1 mM DTT, 50 μg/ml BSA, 5% glycerol, and 2 mM ATP. The reactions were initiated by the addition of UvrA protein and allowed to equilibrate at 37 °C for 30 min. The nucleoprotein complexes were trapped by filtration through 0.45-μm nitrocellulose filters (HAWP 025, Millipore) (29). The amount of [3H]DNA retained on the filter was determined by scintillation counting. The amount of radioactivity was converted into the number of nucleoprotein complexes retained using Poisson distribution (30).

Uvr Protein Complex(es)-associated ATPase—The reaction mixtures (20 μl) contained 25 mM MOPS and HEPES, pH 7.6, 0.1 mM KCl, 10 mM MgCl2, 1 mM DTT, 50 μg/ml BSA, 5% glycerol, and 50 ng of UvrA or 50 ng of UvrB. The DNA (pTZ18R) concentration was 1.9 μg (base pairs). The reactions were initiated by the addition of ATP (final concentration 108 μM), containing [3H]ATP (final specific activity 1 Ci/mmol, ICN). The extent of ATP hydrolysis was measured as described (31).

2 × SSC-resistant Complex—Uvr protein-[3H]DNA complexes were assembled under the conditions described under “DNA Binding Assay” using 5 ng or 10 ng each of UvrA and UvrB. The reaction mixtures were diluted into 0.5 ml of cold (4 °C) 2 × SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0) and filtered through 0.45-μm nitrocellulose filters (HAWP 025, Millipore) (30). The extent of nucleoprotein complex formation was calculated as described under “DNA Binding Assay.”

Incision Assay—The incision activity of UvrA/ABC or M. luteus UV endo was monitored by the conversion of covalently closed circular plasmid uvDNA (pTZ18R) into nicked forms. The incision reactions were initiated by the addition to supercoiling mixtures (see below) 2 μl (50–60 ng) UvrC or 0.5–1 μl (50–100 ng) of M. luteus UV endo and subsequent incubation for 5 min (incision step) at 37 °C. The reactions were stopped as described under Preparation of Highly Positively Supercoiled DNA. DNA products were analyzed on two-dimensional agarose gels (see below).

The Supercoiling Reactions in the Presence of Uvr Proteins—Supercoiling reactions were carried out according to previously described conditions (7, 8). The relaxed (by calf thymus topo I) pTZ18R plasmid DNA (40 ng), containing approximately 24 cyclobutane pyrimidine dimers per circle, was incubated at 37 °C in supercoiling buffer (30 mM Tris-Cl, pH 7.5, approximately 100 mM KCl, 10 mM MgCl2, 1 mM DTT, and 4 mM ATP) with UvrA (200 ng) and UvrB (200 ng) in the presence of 80–100 ng E. coli periplasmic DNA (as a standard for the UvrC or UvrB reaction or UvrA at concentrations ranging from 50 μM to 750 μM). Where indicated the reaction was terminated at 10 min of the incision step by adding an equal volume of 20 μl of 10 mM EDTA. The reactions were stopped by the addition of 95% cold ethanol and centrifuged at room temperature. DNA products were detected in the gel by 32P-labeled probe as described (4). The data were quantified using a PhosphorImager (Fuji BAS 1000) and MacBas 2.0 software.

RESULTS

Incision of uvDNA by Uvr(A)BC and Micrococcus luteus UV Endonuclease in the Absence of Topoisomerases—In order to detect the potential effect of topological reactions on the incision of uvDNA by Uvr(A)BC and M. luteus UV endo, the incision of the relaxed uvDNA was initially carried out in the absence of topoisomerases; that is, when the linking number of the ccDNA was invariant. It was found that, under the conditions of the supercoiling assay (see “Experimental Procedures”), the 60% and 70% of relaxed pTZ18R substrate DNA having about 24 photodimers per circle were converted into nicked species as a result of the 5-min incubation with Uvr(A)BC (200 ng of UvrA and UvrB and 50 ng of UvrC) and 50 ng of M. luteus UV endo (200 ng of UvrA and UvrB were also present in the reaction mixture, see “Experimental Procedures”), respectively (Fig. 1, A and B). This conversion was due to damage-dependent incision (Fig. 1, A, C, and DNA panels, and B). These conditions were used through all experiments involving the incision step. Some “smearing” of the topoisomer bands in the UV-irradiated samples was due to the presence of DNA photoproductions (33).

Highly Positively Supercoiled uvDNA Is Resistant to Incision by Uvr(A)BC—The incubation of relaxed uvDNA with UvrA and UvrB in the presence of E. coli topo I generates highly positively supercoiled DNA (7). Under the supercoiling assay conditions employed in this study, the conversion of relaxed pTZ18R uvDNA into the highly positively supercoiled species increased within the first 10–15 min of incubation and then leveled off (Fig. 2, A and C). As the time of incubation was increased, the nicked DNAs accumulated (Fig. 2, A and C), most probably, due to the occasional failure of the E. coli topo I to seal transient breaks in uvDNA strands (3). The addition of UvrC for 5 min (incision step) at various times during the supercoiling reaction resulted in a significant increase in nicked DNAs (Fig. 2, B and D). This increase, however, took place at the cost of relaxed species but not those highly positively supercoiled ones (Fig. 2, cf. C and D). The highly positively supercoiled uvDNA accumulated prior to the incision step was not incised by UvrA/ABC (Fig. 2, B and D).

The addition of relaxed undamaged DNA by UvrA/ABC in the conditions of supercoiling assay was not observed (data not shown).

The M. luteus UV Endo Can Incise Highly Positively Supercoiled uvDNA—In contrast to UvrA/ABC, the addition of M. luteus UV endo for 5 min to the supercoiling reaction resulted in efficient incision of not only relaxed uvDNA but also highly positively supercoiled uvDNA (Fig. 3C). In the above experiment, M. luteus UV endo was added after 10 min of supercoiling reaction. At this time, chosen also for supercoiling reactions including other topoisomerase, a substantial part of relaxed DNA was cleaved.

2 The ratio of gyrase subunits was suggested by Dr. B. Learn based on the ability of the enzyme to support λ phage DNA replication in the in vitro system.

O. I. Kovalsky, L. Grossman, and B. Ahn, unpublished observation.
DNA was converted into highly positively supercoiled species while the accumulation of E. coli topo I-related nicks (see above) was minimized.

Incision of Purified Highly Positively Supercoiled uvDNA by Uvr(A)BC and M. luteus UV Endo Correlates with That Observed under Supercoiling Assay Conditions—In order to rule out the immediate effect of E. coli topo I on the Uvr(A)BC functioning, the highly positively supercoiled DNA was rescued from the reaction mixture (see “Experimental Procedures”) and subjected to incision either by Uvr(A)BC or M. luteus UV endo. Consistent with the results obtained in the design of supercoiling assay, Uvr(A)BC was not able to incise purified highly positively supercoiled pTZ18R uvDNA, while M. luteus UV endo efficiently incised such DNA (Fig. 4A). The Uvr(A)BC, however, did incise similarly purified negatively supercoiled pTZ18R plasmid DNA irradiated with the same UV dose (Fig. 4B).

UvrA-DNA Complex Formation—In order to gain insight into the cause for the inability of Uvr(A)BC to incise highly positively supercoiled uvDNA, the formation of nucleoprotein intermediates in the incision of uvDNA by Uvr(A)BC was assayed with negatively and highly positively supercoiled DNA.

The UvrA protein is the only component of Uvr(A)BC endonuclease that can bind to double-stranded DNA by itself. It has a higher affinity for damaged versus undamaged DNA (30). From filter binding assays it was found that the UvrA protein is able to bind to undamaged negatively and highly positively supercoiled DNA with a similar affinity (Fig. 5). The extent of UvrA-DNA complex formation substantially increased with both UV-irradiated highly positively and negatively supercoiled DNA (Fig. 5). This finding suggests that UvrA can recognize damaged sites on the highly positively supercoiled DNA almost as well as on negatively supercoiled DNA.

2 × SSC-resistant Nucleoprotein Complex Formation—The resistance of nucleoprotein complexes formed in the presence of UvrA, UvrB, and uvDNA to chelators such as 2 × SSC reflects the formation of preincision intermediates (29). Both UvrA-uvDNA and UvrAB-DNA complexes do not survive the 2 × SSC challenge (29). The number of 2 × SSC-resistant complexes formed on negatively supercoiled uvDNA increased with increasing UvrAB concentration, indicating formation of specific complexes at damaged sites (29) (Fig. 6). However, significantly reduced levels of 2 × SSC-resistant complexes were formed with highly positively supercoiled plasmid uvDNA (Fig. 6). Furthermore, this residual amount of 2 × SSC-resistant com-
plexes can be attributed to the presence of a certain amount of nicked DUDNA in the presence of UvrAB and E. coli topoisomerase I after M. luteus UV endo-induced mock-incision step (B) or incision step (C) were added at 10 min of reaction. Note that incubation of the DUDNA with E. coli topoisomerase I alone (A) resulted in some accumulation of linear DUDNA (see “Results”). nick., nicked DUDNA; rel., relaxed DUDNA; lin., linear DUDNA; +s.c., highly positively supercoiled DUDNA.

**Fig. 3.** Incision of highly positively supercoiled DUDNA by M. luteus UV endo. Two-dimensional electrophoresis of the products of the supercoiling reactions in the presence of UvrAB and E. coli topoisomerase I after M. luteus UV endo-induced mock-incision step (B) or incision step (C) were added at 10 min of reaction. Note that incubation of the DUDNA with E. coli topoisomerase I alone (A) resulted in some accumulation of linear DUDNA (see “Results”). nick., nicked DUDNA; rel., relaxed DUDNA; lin., linear DUDNA; +s.c., highly positively supercoiled DUDNA.

Uvr Nucleoprotein Complex-associated ATPase Activity—
The formation of UvrA2B1-DNA complexes results in a stimulation of the UvrAB-associated ATPase due to induction of the cryptic ATPase of UvrB. This stimulation further increases when DNA is damaged (34). It was consistently found that the level of ATP hydrolysis by the UvrAB complex is significantly stimulated in the presence of 1.9 μM undamaged or UV-damaged negatively supercoiled pTZ18R plasmid DNA (Fig. 7). However, significantly reduced levels of stimulation were observed with the same amount of undamaged or UV-irradiated highly positively supercoiled DNA (Fig. 7). Again, this residual stimulation can be attributed to the presence of some nicked DNA in the preparation of highly positively supercoiled DNA (cf. Fig. 4).

**Fig. 4.** Incision of purified highly positively supercoiled DUDNA by Uvr(A)BC and M. luteus UV endo. A, two-dimensional electrophoresis of gel-purified highly positively supercoiled DUDNA subjected to mock-incision (a), incision by M. luteus UV endo (b), and incision by Uvr(A)BC (c). B, gel-purified negatively supercoiled DUDNA subjected to mock-incision (a) and incision by UvrA2BC (b). nick., nicked DUDNA; +s.c., highly positively supercoiled DUDNA; rel., relaxed DUDNA; lin., linear DUDNA.

**Fig. 5.** The formation of UvrA nucleoprotein complexes with undamaged (−UV) and UV-irradiated (+UV) negative (−) and highly positively supercoiled DUDNA (+).

**Fig. 6.** The formation of UvrAB-dependent 2 × SSC-resistant nucleoprotein complexes with undamaged (−UV) and UV-irradiated (+UV) negative (−) and highly positively supercoiled (+) DNA.

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products of the supercoiling reactions in the presence of both *E. coli* topo I and gyrase and in the absence of incision step were found to be modestly negatively supercoiled uvDNA species (Fig. 8A). Upon the addition of UvrC protein, however, most of supercoiled uvDNA was converted into the nicked species (Fig. 8A). Most of the UV-irradiated ccDNA was also incised when the Uvr(AB)C-dependent incision step was included in the supercoiling reaction containing both calf thymus and *E. coli* topo I (Fig. 8B). It is worth noting that in the absence of the incision step the final product of the latter reaction is a population of slightly negatively supercoiled topoisomers (Fig. 8B). This is consistent with an earlier finding that the Uvr(AB)C-dependent formation of the preincision complex is accompanied by a decrease in the number of ccDNA helical turns (25).

The exclusive presence of gyrase in the supercoiling assay instead of *E. coli* topo I generated highly negatively supercoiled uvDNA (Fig. 8C). The extent of negative supercoiling was clearly greater than that generated in the presence of both *E. coli* topo I and gyrase (cf. Fig. 8, A and C). However, this highly negatively supercoiled DNA was efficiently incised upon the addition of UvrC protein into the reaction (Fig. 8C).

**DISCUSSION**

NER of damaged DNA can be accomplished *in vitro* by six highly purified *E. coli* proteins. The three components of UvrA/BC acting in a sequential and coordinated manner initiate repair by carrying out dual incision of the damaged strand. The incision step is a complex ATP-dependent process involving structural changes of protein and nucleoprotein intermediates. The combined action of UvrD (helicase II) and DNA polymerase I in the presence of dNTPs results in a displacement of postincision Uvr protein-incised fragment complex(es) and resynthesis of DNA. DNA ligase completes the repair by restoring the integrity of DNA (1, 2). This *in vitro* system neither requires any of the known *E. coli* topoisomerases nor takes into account a potential influence of the DNA topology on NER process. However, the reconstituted NER system underestimates the dynamic nature of DNA *in vivo* with respect to its topology. In particular, translocation-involved cellular processes, notably transcription and replication, generate domains of negative and positive supercoiling behind and ahead of a translocating complex (8–10, 13). Although this differential supercoiling may not be persistent in an *in vitro* reconstituted system, the large protein-, nucleoprotein-, and membrane-involved *in vivo* macromolecular assemblies are able to function as “anchoring” structures preventing or slowing down the fusion of opposite supercoils (9, 10). Given that the incision step of Uvr(AB)C-dependent NER utilizes DNA helix-tracking activity and proceeds through DNA conformational changes resulting in DNA unwinding (25), it is reasonable to expect that NER can be influenced by the topological waves of opposite supercoiling. This reasoning is strengthened by the phenomenon of transcription-coupled repair (37, 38). The transcription and repair macromolecular assemblies seem to be in a physical association during this process.4 Furthermore, the Uvr(AB)C complex seems to preferentially operate downstream of the transcription machinery (39, 40), that is, in the region of potential positive supercoiling. In supporting this suggestion, we found that highly positively supercoiled DNA, generated under conditions of supercoiling assay (7), is resistant to the incision by Uvr(AB)C. Such DNA is refractory to two of the UvrAB-dependent functions thought to be associated with the formation of obligatory intermediates in the Uvr(AB)C-catalyzed incision (1): (i) the robust stimulation of the UvrA,B1-associated ATPase is not observed and (ii) no stable damage-dependent nucleoprotein complexes are formed.

It was found earlier that these obligatory intermediates are associated with the DNA conformational changes resulting in the decrease of a number of helical turns in ccDNA (25). Such a decrease should be compensated by an increase in the number of superhelical turns in order to maintain a constant linking number of ccDNA (32). In cases of positively supercoiled DNA, this compensation would result in an unfavorable free energy change (41). The energy input produced by the UvrAB-

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4 It was shown that the β-subunit of the *E. coli* RNA polymerase and UvrA protein can be reversibly cross-linked *in vivo* (C.-L. Lin, O. I. Kovalsky, and L. Grossman, manuscript in preparation).
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associated ATPase, which probably provides for power stroke during the above conformational change (1), may not be sufficient to overcome that unfavorable free energy change. As a result, obligatory intermediates in this process are unable to form on highly positively supercoiled DNA. In cases of highly negatively supercoiled DNA, produced when gyrase substitutes for \( E. \text{coli} \) topo I in the supercoiling assay, a decrease in the number of helical turns should be energetically favorable (41). Hence, the formation of intermediates in question should be facilitated. Consistently, this DNA was found to be efficiently incised by Uvr(A)BC.

It was shown that in the absence of UvrB, UvrA protein recognizes damage by random diffusion mechanism, and its binding does not result in a significant DNA unwinding (25, 30). Consequently, the UvrA protein was able to recognize and bind damaged sites on highly positively supercoiled uvDNA. This further supports the earlier observations that affinity of UvrA for damage does not correlate with the efficiency of incision (42, 43) and that UvrAB complex provides for a more productive DNA-binding intermediate (39, 40). Notably, \( M. \text{luteus} \) UV endo which by analogy with T4 phage UV endonuclease seems to directly recognize cyclobutane pyrimidine dimers and does not induce extensive DNA unwinding (28, 44) efficiently incised uvDNA regardless of its topological state.

The problem of incising highly positively supercoiled uvDNA by UvrA/BC is reminiscent of transcription and replication encountering accumulated positive supercoils (45–47). \( E. \text{coli} \) possesses two major topoisomerases responsible for maintaining adequate superhelical stress: topo I removes negative supercoils whereas gyrase introduces negative supercoils. The presence of both activities in the supercoiling assay abolished accumulation of highly positively supercoiled uvDNA and restored incision by UvrA/BC. The eukaryotic (calf thymus) topo I, capable of removing positive supercoils, was able to substitute for gyrase in restoring UvrA/BC incision. Hence, the restoration of incision seems to take place mainly due to prevention of the accumulation of positive supercoils. In support of the existence of similar topological problems in vivo, both \( E. \text{coli} \) gyrase and topo I are recruited in a damage-dependent manner (among other NER-related and seemingly unrelated proteins) to the inner membrane of the UV-irradiated cells as a part of SOS response. These topoisomerases may be required to regulate DNA topology for optimum repair in such membrane-attached configuration.

The topodynamic in vitro system described in this report may be a prototype for studying different aspects of the interrelation of NER and DNA topology. By using specific ccdNA templates, different combinations of topoisomerases, and other macromolecular entities in the system, it is feasible to gain insights into the interaction of NER and other DNA topology-involved cellular processes.

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\[ ^5 \text{C.-L. Lin, O. I. Kovalsky, and L. Grossman, manuscript in preparation.} \]