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Effect of DNA topology on plasmid DNA repair in vivo

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Abstract ***Escherichia coli*** **nucleotide excision repair (NER) is responsible for removing bulky DNA adducts by dual incisions of the UvrABC endonuclease. Although the activity of the UvrAB complex which can induce DNA conformational change is employed in NER, the involvement of DNA topology and DNA topoisomerases remains unclear. We examined the effect of topoisomerase inhibitions on a NER in vivo system. The repair analysis of intracellular plasmid revealed that the DNA damage on positive supercoils generated by gyrase inhibition remained unrepaired, whereas the DNA damage was repaired in topoisomerase I mutants. These results suggest that DNA topology affects the NER process and the removal of positive supercoils by gyrase is vital for the efficiency of the *E. coli* NER system.**

Key words: DNA topoisomerase; DNA topology; Nucleotide excision repair

1. Introduction

The nucleotide excision repair (NER) in *Escherichia coli* is catalyzed by the multisubunit excision endonuclease UvrABC that initiates the excision of a large number of different DNA adducts including pyrimidine dimers [1,2]. Studies of purified UvrABC proteins have demonstrated that the UvrAB complex can unwind undamaged duplex DNA and the DNA at a lesion and that dual incisions, 5′ and 3′ to the damaged sites, are generated by the UvrABC endonuclease [3–6]. The initial step of dual incision is the specific binding of the repair enzymes to damaged sites and an important aspect of this process is the mechanism by which these enzymes locate a damaged site within large domains of DNA. In particular, since a DNA helix-tracking activity of the UvrAB complex can generate positive and negative supercoiled domains ahead and behind moving complex, respectively, the activity is employed in scanning and/or pre-priming of damage by UvrABC rather than encountering damage by a random diffusional mechanism [7–9].

Although DNA topodynamics seems to be related with one or more steps of the NER process including DNA damage recognition through tracking along DNA of the UvrAB complex, the involvement of DNA topoisomerases or DNA topology in NER process has not been fully explored in vivo. However, there are several reports supporting the involvement of topoisomerases in DNA repair. The *E. coli* topoisomerase I and gyrase mutation as well as gyrase inhibitors (novobiocin and nalidixic acid) confer an increased susceptibility to UV irradiation, alter the degree of excision repair and post-replicational repair, and inhibit recovery of UV-irradiated non-replicative lambda phage [10–12]. A recent in vitro biochemical study showed that a highly positive supercoiled DNA, containing UV-induced photoproducts, is resistant to the incision by the UvrABC endonuclease, whereas the UvrABC endonuclease effectively incises negatively supercoiled and relaxed forms of UV-damaged DNA [13]. When *E. coli* gyrase or eukaryotic topoisomerase I was included in the reaction, the UvrABC-catalyzed incision was restored. However, other cellular processes generating topological domain such as transcription and replication have not been engaged in the reaction yet. Thus, it is necessary to use in vivo systems in which DNA topological states could be regulated to clarify the relation between DNA topology and the NER process. To control the DNA topological state in this study, *E. coli* cells were treated with novobiocin, DNA gyrase subunit B inhibitor, resulting in highly positive supercoiled plasmid and *E. coli* topoisomerase I mutant (Δ*topA*) was used. Since intracellular plasmids represent a population of discrete and identical DNA molecules, the completion of the repair of individual plasmids within UV-irradiated *E. coli* harboring pBR322 could be analyzed. This in vivo study shows that gyrase inhibitor-treated cells are sensitive to UV killing and that the DNA damage in the resulting supercoils is not repaired by the *E. coli* NER. However, in Δ*topA* mutant DNA repair occurs normally. These results suggest that the NER process could be affected by DNA topology.

2. Materials and methods

2.1. Strains and culture conditions

AB1157 (wild-type), AB1886 (*uvrA*$^-$), AS19 (*E. coli* B strain permeable to a number of antibiotics), N3137 (*uvrA*::Tn10), and DM800 (Δ*topA*, *gyrB225*, Δ*cysB*) are used in this work. *E. coli* BA9801 (*uvrA*::Tn10) was constructed by first transducing *E. coli* AS19 to tetracycline resistance using a P1vir preparation from *E. coli* N3137 [14]. For the experiments the strains were routinely grown in LB medium and/or with antibiotics. This medium was also used as the solid plating medium in survival experiments.

2.2. UV survival and streak test

A fresh LB inoculated with over-night culture was shaken until cells were well into a logarithmic phase. Aliquots from serial dilutions of the cultures were plated. UV damage was achieved by exposing the plates to various dose of UV (254 nm, germicidal lamp). UV dose was determined with a digital UV meter (UVX). The colonies were counted after an overnight incubation in the dark at 37°C. Streak tests were performed on LB-agar plates. Cells were grown in Luria broth to an apparent optical density of 0.8 at 600 nm. The grown cells (10 μl) were streaked cross the agar plate and then the plate was irradiated from one side to the opposite side with UV as indicated dose in the dark room. The plate was wrapped immediately with aluminum foil and incubated at 37°C for an overnight.

2.3. Analysis of plasmid DNA repair

E. coli harboring pBR322 plasmid DNA from overnight culture

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was grown freshly to optical density 0.6–0.7 at 600 nm and 37°C in 75 ml of Luria broth containing 100 μg/ml ampicillin. 5.5 ml of the culture was transferred to autoclaved glass petri dish on ice pack at the dark room. The transferred culture was subjected to UV and the UV-irradiated culture was partitioned into a new autoclaved 20 mm test tube (diameter) which was pre-warmed at 37 °C. After all the time points were taken, the samples were transferred to centrifuge tubes and the cultures were pelleted by centrifugation. Plasmids were isolated by alkali lysis method [15] and the plasmid isolates were reacted with purified *Micrococcus luteus* UV-endonuclease (see below). The generation of nicked form is diagnostic for unrepaired plasmid DNA containing DNA damage which is endonuclease-sensitive. The plasmids were analyzed by autography after electrophoresis separation in agarose gel (one dimension or two dimensions) (see below).

2.4. Two-dimensional (2-D) gel electrophoresis for analysis of DNA repair of positive supercoils

2-D gel electrophoresis was carried out in 0.8% agarose and TPE buffer. Electrophoresis in the first dimension (from top to bottom) was performed for approximately 18 h at room temperature. The gel was then soaked in 1.5 liter of the same buffer supplemented with 2.0 μg/ml chloroquine for 3 h with gentle shaking. Electrophoresis in the second dimension was then carried out in chloroquine-containing TPE buffer (from left to right) for 6 h. The plasmid DNA in the gel was detected by in situ Southern hybridization with ^{32}P-labeled probe as previously described [16]. The data were quantitated using the NIH image program.

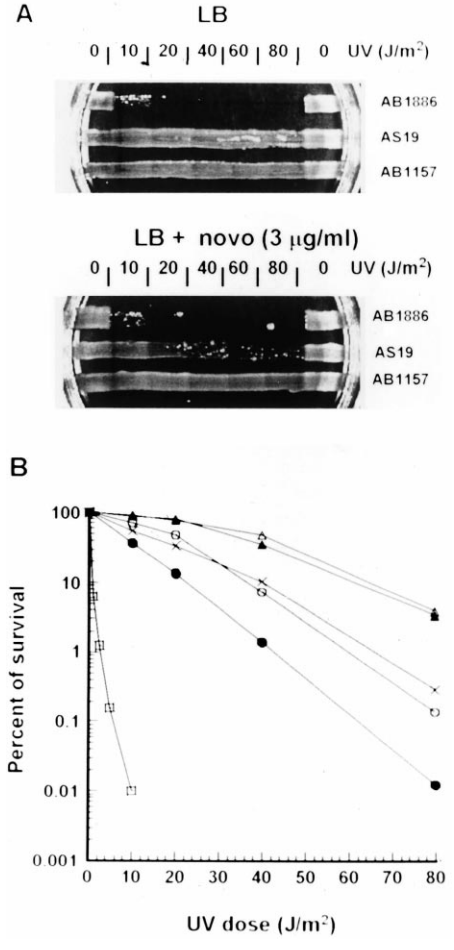

Fig. 1. The sensitivity of AS19 to UV-irradiation in the presence of novobiocin. A: UV streak test of *E. coli* strains. B: Survival curves of *E. coli* strains: Novo 1.0: 1.0 μg/ml of novobiocin, AS19 (open circle), AS19 treated with novo (closed circle), AB1157 (open triangle), AB1157 treated with novo (closed triangle), DM800 (X), BA9801 (open square).

2.5. M. luteus UV endonuclease incision assay

The standard reaction mixtures contained 50 mM HEPES–NaOH (pH 7.6), 1 mM EDTA, 5 mM $MgCl_2$, and 10–20 μg/ml of DNA. Reactions were initiated by the addition of 1.0 μg of *M. luteus* UV endonuclease (provided by Dr. Grossman, The Johns Hopkins University) and then were subjected to subsequent incubation for 1 h at 37 °C. The reactions were terminated with stop solution (100 mM EDTA, 1% SDS, xylene cyanol and bromo phenol blue). The extent of reaction was monitored by electrophoresis in 1-D and 2-D agarose gel.

2.6. UvrABC incision assay

UvrA, UvrB and UvrC proteins were incubated with UV-damaged plasmid DNA in the standard reaction buffer (20 mM Tris–HCl, pH 7.6, 85 mM KCl, 1 mM DTT, 100 μg/ml BSA, 10 mM $MgCl_2$, 2 mM ATP) for 20 min at 37 °C. Reactions were terminated by the addition of the stop solution and then the plasmid DNA was analyzed on agarose gel and detected by ethidium bromide staining.

2.7. UvrD helicase assay

To detect UvrD helicase activity, blunt-ended duplex DNA substrate (121 bp) were prepared by *Bam*HI and *Pvu*II restriction enzyme digestion of pTZ18R followed by fill-in of T4 DNA polymerase and [α-^{32}P]dTTP. Purified UvrD proteins were incubated with the prepared DNA substrate in the standard reaction buffer (20 mM Tris–HCl, pH 7.6, 35 mM NaCl, 2.5 mM $MgCl_2$, 2 mM ATP, 4 mM β-mercaptoethanol) for 20 min at 37 °C and then the reactions were terminated with the stop solution. The DNA was analyzed on 9% polyacrylamide gel followed by autoradiography of X-ray film.

2.8. E. coli DNA polymerase I filling-in assay

The activity of DNA polymerase I was measured by filling-in of sticky-ended plasmid pTZ18R generated by the digestion with *Bam*H1. The *E. coli* DNA polymerase I (New England Biolabs, USA), dNTP including [α-^{32}P]dTTP, and the DNA template were incubated in the presence of indicated amount of novobiocin. The standard reaction buffer was provided by New England Biolabs. Reactions were subjected to incubation for 20 min at 37°C. The DNA was analyzed on 0.9% agarose gel and dried. The dried gel was exposed on X-ray film.

3. Results

3.1. Effect of gyrase inhibition on the susceptibility to UV irradiation

The susceptibility of *E. coli* AS19 strain to novobiocin, gyrase B subunit inhibitor, was examined. The AS19 showed 0.001% of survival at higher concentrations (greater than 10 μg/ml) and more than 20% of survival at lower concentrations (less than 3 μg/ml of novobiocin) (data not shown). This increased susceptibility of AS19 to novobiocin confirms previous findings [17,18]. To investigate the effect of DNA gyrase inhibition on DNA repair, the sensitivity of the *E. coli* AS19 cells to UV irradiation in the presence of novobiocin (1–3 μg/ml) was determined. The cultures were streaked or plated on agar plates and then exposed to various doses of UV (Fig. 1). As shown in Fig. 1A, novobiocin-treated AS19 conferred an increased sensitivity to UV irradiation compared to untreated AS19, whereas wild-type strain AB1157 was grown well but its isogenic *uvrA* mutant AB1886 was sensitive to UV irradiation regardless of novobiocin. The quantitation of viable colonies on agar plates revealed that the novobiocin-treated AS19 was more sensitive than the untreated AS19 (Fig. 1B). This UV sensitivity suggests an indicative role for gyrase function in the repair of UV-induced damage to DNA.

In order to regulate the DNA topological state of intracellular plasmid, *E. coli* AS19 harboring pBR322 was treated with novobiocin at 80 μg/ml for 30 min. A new cluster of positive supercoils appeared in the novobiocin-treated cells,

whereas negative supercoils were detected in untreated cells (data not shown). This generation of positive supercoils is consistent with previous observation [17,19]. Thus, this system would be amenable to regulate the DNA topological state in vivo.

3.2. *In vivo plasmid DNA repair under inhibition of DNA gyrase*

To determine the formation of DNA damage in plasmid pBR322 in *E. coli* AS19 cells, the cells were irradiated with various doses (200, 450, 900 and 1200 J/m^2) of UV. The plasmid isolates digested with or without a *M. luteus* UV-endonuclease were resolved by electrophoresis. The conversion of supercoiled plasmids to nicked forms increased with increasing UV dose and a plateau level of nicked forms was observed at near 900 J/m^2 (data not shown). The kinetics of the accumulation of repaired plasmid molecules in UV-irradiated *E. coli* AS19 cells is shown in Fig. 2. The accumulation of *M. luteus* UV-endonuclease-resistant DNA (denoted by SC) was detected in novobiocin-untreated cells (Fig. 2A, Novo(−)) and about 70% of DNA damage was removed in the first 30–40 min (Fig. 2B). This profile is consistent with the previous observation in *E. coli* genomic DNA repair of UV damage [20]. When *E. coli* AS19 cells were allowed to repair DNA in presence of novobiocin (100 μg/ml), the extent of plasmid DNA repair was reduced to 85% in 30 min (Fig. 2A Novo(+) and Fig. 2B). However, it is likely that DNA repair seems to occur gradually when further incubated. This observation is discussed.

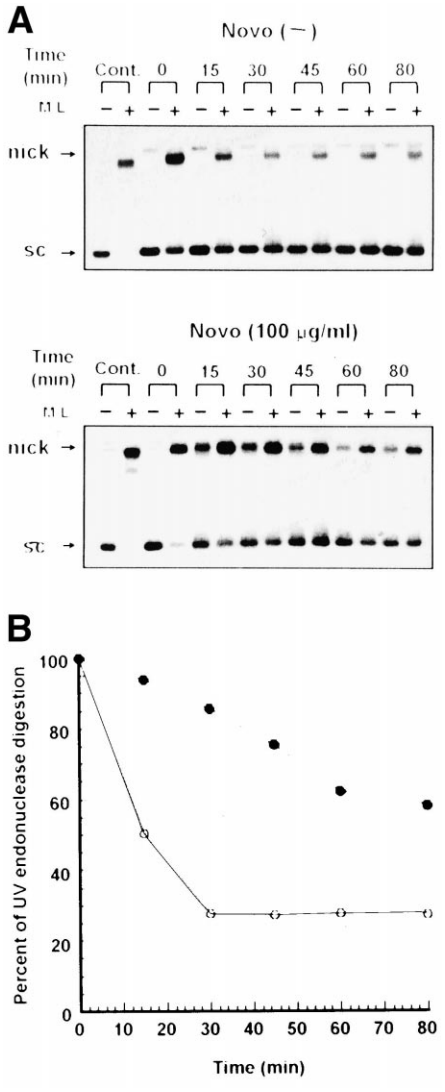

Fig. 2. Kinetic analysis of in vivo plasmid DNA repair. A: The digestion of the plasmid isolates with *M. luteus* UV-endonuclease. novo(−): no novobiocin. novo: 100 μg/ml of novobiocin was added after 900 J irradiation. Time (min): the incubated time after 900 J irradiation. M.L.−: no treatment with *M. luteus* UV-endonuclease. M.L.+: treatment with *M. luteus* UV-endonuclease. Cont.: purified plasmid pBR322 was irradiated in vitro as a control DNA. B: Quantitation of accumulation of UV-endonuclease-resistant form DNA. AS19 (open circle), AS19 novo (closed circle).

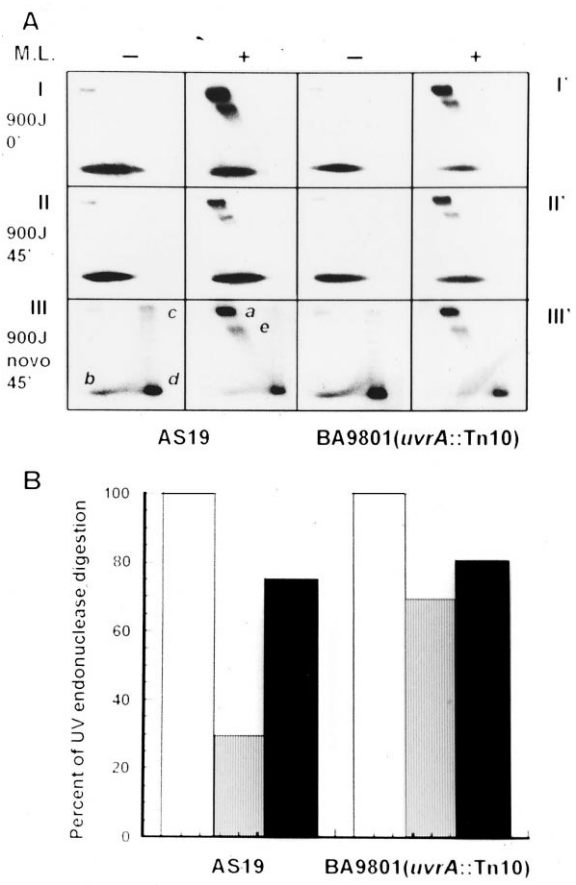

Fig. 3. Analysis of DNA repair of negatively and positively supercoiled plasmid. A: 2-D electrophoresis of plasmids treated with UV-endonuclease. a: nick circular DNA, b: negatively supercoiled DNA, c: closed circular DNA, d: positively supercoiled DNA, e: linear DNA. I (AS19, 900J irradiation, 0 min), II (AS19, 900 J, 45 min incubation), III (AS19, 900 J, 100 μg/ml of novobiocin, 45 min), I′ (BA9801, 900 J irradiation, 0 min), II′ (AS19, 900 J, 45 min), III′ (AS19, 900 J, 100 μg/ml novobiocin, 45 min). B: Quantitation of accumulation of UV-endonuclease-resistant positive supercoils on (A). 0 min (open bar), 45 min (gray bar), novo(+) and 45 min (closed bar).

To determine whether the reduced DNA repair in the presence of novobiocin depends on the DNA topological state, we carried out DNA repair in AS19 and its isogenic *uvrA* mutant BA9801. The isolated supercoiled plasmids were treated with *M. luteus* UV-endonuclease followed by two-dimensional gel electrophoresis (Fig. 3). At time 0 min, that is, just after UV irradiation, the plasmid isolates from both AS19 and BA9801 strains were negative supercoils and were *M. luteus* UV-endo-

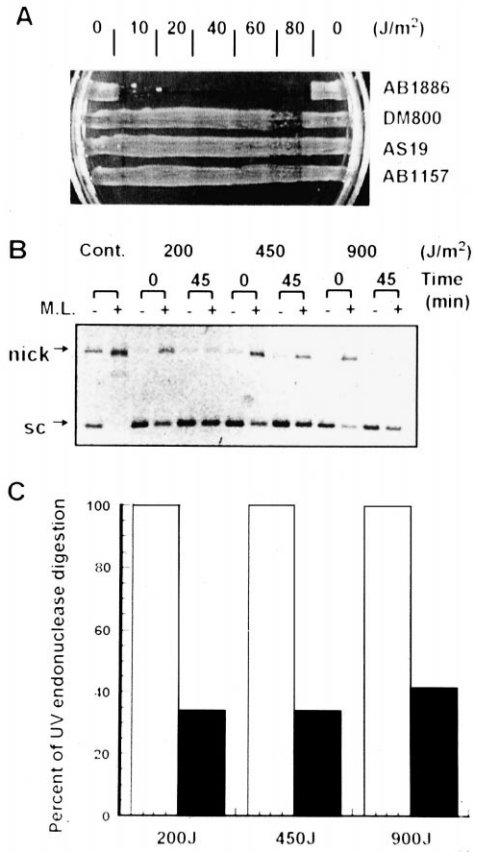

Fig. 4. Effect of topoisomerase I deletion mutation on DNA repair. A: UV streak test of different *E. coli* strains. B: The plasmid DNA repair in DM800: nick: nicked circular plasmid. SC: supercoiled circular plasmid. C: Quantitation of accumulation of UV-endonuclease resistant from DNA in (B). 0 min (open square), 45 min (closed square).

nuclease-sensitive (Fig. 3A-I, ML(+) and I′, ML(+)). When the irradiated *E. coli* AS19 cells were incubated for 45 min in the absence of novobiocin, the resulting plasmids were negative supercoils and the photodimers on the given plasmids were repaired to 70% in 45 min (Fig. 3A-II and Fig. 3B), whereas when the irradiated AS19 cells were allowed to repair in the presence of novobiocin, the resulting plasmids were positive supercoils (Fig. 3A-III, 'd') and the extent of plasmid repair greatly reduced to 75% (Fig. 3A-III and Fig. 3B). In contrast, in repair-deficient strain BA9801, most of the photodimers remained regardless of novobiocin treatment and the extent of DNA repair in the absence of novobiocin was similar to that in the novobiocin-treated AS19 (Fig. 3A-II′, III′ and Fig. 3B). These results suggest that DNA repair is affected by DNA topology and gyrase activity, removing positive supercoils may be required for the DNA repair.

3.3. Plasmid DNA repair in E. coli topA mutant

To determine the effect of topoisomerase I mutation on DNA repair, the UV sensitivity of *E. coli* DM800 and its repair capacity were analyzed. The sensitivity of the *E. coli* DM800 to UV was similar to that of AS19 (Figs. 1B and 4A). The *E. coli* DM800 harboring pBR322 was irradiated with various doses of UV and then the plasmids isolated at the indicated time points were incubated with *M. luteus* UV-endonuclease. The incubated plasmids were analyzed on gel electrophoresis (Fig. 4B). 35% of DNA damage remained (Fig. 4C) and this extent was similar to that in novobiocin-untreated AS19 (Fig. 3B). In addition, the extent of DNA repair was not significantly different at various UV doses. It is thought that the capability of *topA* mutants to repair DNA damage may be accounted for by highly negatively supercoiled forms of plasmid generated in the cells lacking functional DNA topoisomerase I [19].

3.4. Effect of novobiocin on the activities of repair proteins and DNA polymerase I

As it is known that novobiocin effectively inhibits both chromosomal and plasmid replication and affects gene expression of *E. coli* [21], it is necessary to examine whether novobiocin affects the activities of the proteins: UvrABC endonuclease, UvrD helicase, and DNA polymerase I involved in NER process. As depicted in Fig. 5A, the extent of the conversion of UV-damaged negative supercoils to nicked forms by UvrABC endonuclease was not changed with up to 100 μg/ml of novobiocin. The filling-in activity of DNA polymerase I for overhangs produced by restriction enzyme digestion was unchanged by the novobiocin (Fig. 5B) and the unwinding of labeled duplex DNA (121 bp) by UvrD was detected similarly even in the presence of novobiocin as well (Fig. 5C). These results indicate that the activities of the proteins involved in the NER process are not affected by novobiocin.

4. Discussion

Studies of NER reaction with purified proteins have demonstrated that DNA damage on linear DNA, relaxed circular DNA, and negatively supercoiled DNA could be incised only by UvrABC endonuclease but no other proteins are required. However, it was reported previously that the incision of UV-

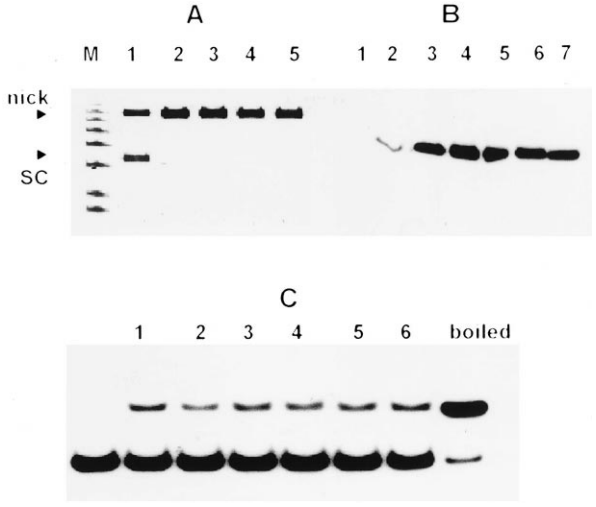

Fig. 5. Effect of novobiocin on the incision by UvrABC, the filling-in by *E. coli* DNA pol I, and unwinding by UvrD helicase. A: UvrABC incision assay. M: 1 kb ladder, lane 1: UV-damaged DNA. Novobiocin concentrations were: lane 2: none, lane 3: 10.0 μg/ml, lane 4: 40.0 μg/ml, lane 5: 80.0 μg/ml. B: The filling-in assay of the *E. coli* DNA polymerase I. Lane 1: no [α-^{32}P]dTTP, lane 2: only [α-^{32}P]dTTP. Novobiocin concentrations were: lane 3: none, lane 4: 3.0 μg/ml, lane 5: 10.0 μg/ml, lane 6: 40.0 μg/ml, lane 7: 80.0 μg/ml. C: UvrD helicase assay. Left lane: no protein. Novobiocin concentrations were: lane 1: none, lane 2: 1.0 μg/ml, lane 3: 3.0 μg/ml, lane 4: 10.0 μg/ml, lane 5: 40.0 μg/ml, lane 6: 80.0 μg/ml.

damaged supercoils by UvrABC endonuclease was greatly inhibited when an in vitro NER reaction of closed circular DNA is operated in the presence of *E. coli* topoisomerase I [13]. This suggests that DNA topology may affect repair and that DNA topoisomerases regulating DNA topology may be required for the efficiency of the *E. coli* NER system. However, a virtual in vivo system has been utilized yet. We used the conditions of inactivation of gyrase and absence of topoisomerase I to facilitate change of DNA topology in vivo. In fact, in novobiocin-treated cells, most of plasmid DNA population was the highly positive supercoiled form. The data presented here show that the inhibition of DNA gyrase confers increased susceptibility to the toxic effect of UV irradiation and that the *E. coli* NER system is affected by DNA topology.

E. coli AS19 which is permeable to a number of antibiotics including novobiocin did not grow at higher concentrations of novobiocin due to inhibition of gyrase resulting in blocking DNA replication. However, novobiocin-treated cells should grow to examine the effect of novobiocin on UV sensitivity of cells. Fig. 1 showed that the AS19 cells could grow at less than 3 μg/ml of novobiocin and were sensitive to UV under this conditions, consistent with previous observations where *gyrA* or *gyrB* mutants conferred an increased susceptibility to UV irradiation [12]. This UV sensitivity supports a potential role for DNA gyrase in DNA repair. Although AS19 cells in the presence of 1.0 μg/ml of novobiocin are less sensitive to UV than those of the *uvrA* mutant, the UV sensitivity of cells treated with higher concentrations of novobiocin could be clarified by the analysis of plasmid DNA repair. Kinetic analysis of plasmid DNA repair revealed that the plasmid DNA repair was greatly reduced and DNA damage in the positive supercoils generated by the novobiocin treatment was not greatly repaired (Fig. 3A-III, III′ and Fig. 3B). However, when the treated cells were incubated further, DNA repair seems to occur gradually (Fig. 2A,B). Although most of DNA damage seems to be cleaved by *M. luteus* UV-endonuclease regardless of DNA topology, the dependence of the efficiency of endonuclease-induced cleavage on the superhelical density has not been studied yet. Thus, it is possible that even the remained positive supercoils contain DNA damage, the positive supercoils may not be digested because of the very highly positive supercoiled form (detected in Fig. 3A-III, and -III′). The nature of the undigested-positive supercoils needs to be further analyzed.

Since novobiocin does not induce the SOS DNA repair response in *E. coli* [22], novobiocin may not have any effect on the induction of NER proteins. Indeed, any inhibitory effect of novobiocin on repair proteins was not detected (Fig. 5), indicating the repair proteins pre-existing in cells before SOS induction or the induced protein after SOS may act normally. However, it is hard to rule out that novobiocin does not affect the expression of repair genes because blocking DNA gyrase results in drastic changes in *E. coli* gene expression. Even if there may be any regulation of repair genes by novobiocin, the repair proteins expressed before the addition of novobiocin may perform NER because early NER of total genomic DNA is catalyzed primarily by constitutive Uvr proteins [23].

Studies of purified UvrA, UvrB, and UvrC proteins indicate that the UvrAB complex containing a DNA helix-tracking activity generates domains of positive and negative supercoils ahead and behind the moving complex, respectively. With respect to DNA superhelical tension if the energy input produced by the UvrAB-associated ATPase may not be sufficient to overcome unfavorable free energy change within positively supercoiled domain, the capacity of the UvrAB complex could be regulated by cellular factors such as other site-specific DNA interactive proteins and DNA topological state. In this regard, our present results suggest that DNA topology affects NER and the prevention of the accumulation of positive supercoils may be required for optimum NER. Hence, the maintenance of adequate superhelical stress may be required for survival in the context of DNA damage recognition. Furthermore, although very little is known about the effect of DNA on eukaryotic systems, eukaryotic chromosomes should be remodeled to repair chromosomal DNA. Hence, this study may lead to advance in our understanding of DNA repair related to DNA topodynamics.

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