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Enhanced Sensitivity of *Escherichia coli uvr*B Mutants to Mitomycin C Points to a UV-C Distinct Repair for DNA Adducts

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Nucleotide excision repair (NER) in *Escherichia coli* repairs DNA by incising the damaged strand on the 3′ and 5′ sides of the lesion within pyrimidine dimers and DNA cross-linking adducts. Cross-linking adducts belong to a class of chemical damage to DNA that prevent strand separation, and thus, replication and transcription. For this reason, cross-linking agents such as mitomycin C (MC) have been used in cancer chemotherapy. The mechanisms involved in MC binding to DNA have already been defined; however, the repair of these lesions is not fully understood. Our goal was to study the repair of MC DNA lesions in *E. coli* cells. Several bacterial strains with specific mutations were tested for cellular inactivation and kinetics of DNA repair through analysis of DNA sedimentation profiles in alkaline sucrose gradients. The results obtained show that *uvr*B mutants are extremely sensitive to MC in contrast to the other isogenic *uvr*A and *uvr*C mutant strains. *uvr*B mutant strains are unable to repair DNA strand breaks produced by MC. Thus, UvrB might play a NER-uncoupled role in the repair of lesions induced by MC in vivo, different from its role on the repair of lesions produced by UV-C. Also it is suggested that a modified NER system is taking place in the repair of MC-adducts.

Introduction

Nucleotide excision repair (NER)[1] is the most important DNA repair systems, since it recognizes and processes a great variety of chemically distinct DNA lesions, including UV-induced photoproducts, adducts induced by some therapeutic drugs, and certain types of cross-links (*1*, *2*).

NER mechanism is conserved in all organisms and comprises five basic steps in *Escherichia coli*: damage recognition, incision of the damaged strand, excision of the lesion-containing oligonucleotide, resynthesis, and ligation (*3*−*5*). Sancar and Rupp (*6*) proposed a model in which at least six proteins were shown to be involved in the pathways named above, three of them responsible for the incision activity, namely, UvrA, UvrB and UvrC (*1*, *7*). The enzymatic action of the Uvr(A)BC system begins with the dimerization of UvrA in the presence of ATP. UvrA is a molecular matchmaker that recognizes damaged sites and delivers UvrB to the site (*2*, *7*, *8*). To do so, the $UvrA_2$ dimer interacts with UvrB to form an $UvrA_2B$ complex, which activates the UvrB-dependent helicase function to scan DNA in search of damage. The complex halts at the damaged site and, while binding to it, kinks the unwound region resulting in the dissociation of UvrA. Further recognition of the damaged strand by UvrB (*2*, *7*, *8*) is achieved by the formation of a stable interaction between UvrB and DNA (*9*). This enables the binding of UvrC, resulting in the assembling of the UvrBC−DNA pre-incision complex (*10*), which performs incisions at the fourth phosphodiester bond on the 3′ side of the damage by UvrB, and then at the eighth phosphodiester bond on the 5′ side of the damaged strand by UvrC (*7*, *8*).

The entire process of NER in *E. coli* has been exhaustively studied in vitro with purified Uvr proteins and defined DNA substrates. However, specifically in the repair of DNA adducts, information about how the process occurs in vivo is still missing.

Depending on the damage present in DNA, the 3′ incision site may differ from two to four phosphodiester bonds relative to that observed for cyclobutane pyrimidine dimer (CPD) (*11*). Using an in vitro model, Verhoeven et al. (*12*) proposed that the catalytic sites for 3′ and 5′ incisions are both located in UvrC. The recently discovered UvrC homologue, Cho, was shown to have a nuclease activity similar to that of UvrC. In fact, in vitro Cho can elicit the 3′ incision, but not the 5′ one (*13*). After the dual incisions, UvrC slowly and spontaneously dissociates. The subsequent binding of UvrD (DNA helicase II) to the nicked strand removes the oligomer and also facilitates the dissociation of the attached UvrC, while UvrB-gapped DNA complex is still stable until DNA polymerase I arrives. DNA polymerase I fills in the resulting gap, and finally, the remaining nick is closed by DNA ligase (*7*, *8*).

One class of DNA damaging agent used to study NER in vitro is Mitomycin C (MC). MC is a natural antitumor drug produced by *Streptomyces lavendulae* (*14*) that has been used for cancer chemotherapy after being approved by the Food and Drug Administration in 1974 (*15*, *16*).

MC damages DNA by mono- and bifunctional alkylations at N^2 of guanines, leading to both MC−guanine monoadducts and MC−guanine biadducts, which can occur as interstrand or intrastrand cross-links (*17*). Both the monofunctional and bifunctional DNA lesions occur only after reduction of MC molecules, since nonreduced MC cannot bind to DNA (*18*). Active MC monoalkylates the $2\text{-}NH_2$ groups of guanines via its C1 carbon, but intra- and interstrand cross-links may also be formed by means of an additional reaction with the C10 position (*19*). The reactivity of guanines with MC depends on their sequence context, and CpG sequences are the preferred sites for monoalkylations (*20*) and cross-links. Cross-links do not occur with guanines in any other sequence combination,

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[1] Abbreviations: NER, nucleotide excision repair; CPD cyclobutane pyrimidine dimer; MC, mitomycin C; LB, Lysogeny Broth.

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Table 1. *E. coli* K-12 Strains Used in This Study

designation	relevant genotype	source
AB1157	Wild-type	P. Howard-Flanders, Yale University, CT
AB1885	[AB1157] *uvr*B5	P. Howard-Flanders, Yale University, CT
GY4763	[AB1157] *rec*A142	R. Devoret, CNRS, Gif-sûr-Yvette, France
JC3890	[AB1157] *uvr*BΔ301	R. Devoret, CNRS, Gif-sûr-Yvette, France
CS5540	Δ*cho*::Cam	N. Goosen, Leiden University, The Netherlands
RJF573	[AB1157] Δ*uvr*C::*Tn*10	our laboratory
RJF574	[AB1157] Δ*uvr*A::*Tn*10	our laboratory
RJF604	[AB1884] Δ*uvr*A::*Tn*10	our laboratory
RJF625	[AB1885] pNP83 (*uvr*B$^+$, Amp)	our laboratory
RJF756	[JC3890] pNP83 (*uvr*B$^+$, Amp)	our laboratory
RJF759	[CS5540] Δ*uvr*C::*Tn*10	our laboratory

although their monoalkylation may be observed (*21*).

Interestingly, Kenyon and Walker (*22*) found no induction of β-galactosidase in a *uvr*B::*lac*Z fusion strain in the presence of MC, while exposure of this strain to UV-C normally induced β-galactosidase. On the other hand, β-galactosidase was induced in the *uvr*A::*lac*Z fusion strain in the presence of either MC or UV-C. These findings show that *uvr*B might be differently sensitive to UV-C and MC.

So, in search for a better understanding on the possible role of NER on MC−DNA adducts, in vivo experiments were performed with *E. coli uvr* mutant strains. Our results suggest the existence of a NER-uncoupled role for UvrB in the repair of MC−DNA adducts.

Experimental Procedures

Bacterial Strains and Plasmids. The bacterial strains used in this work were derived from *E. coli* K-12 and are listed in Table 1.

RJF strains were constructed for this study and generated by standard P1*vir* transduction techniques using N3137 strain (*uvr*A::*Tn*10) and N3124 strain (*uvr*C::*Tn*10) provided by Prof. R. G. Lloyd (Genetics Division, Queens Medical Centre, Nottingham, U.K.) (*23*). The JC3890 *uvr*BΔ301 mutant was derived from AB1157 after a spontaneous loss of the entire *bio uvr*B region, as described in Kato et al. (*24*). CS5540 strain was provided by N. Goosen (Leiden University, Leiden, The Netherlands) and is described in Moolenaar et al. (*13*). Plasmid pNP83 was obtained from R. Fuchs (École Superieur de Biotecnologie, Strasbourg, France).

Growth Conditions and Radioactive Labeling. Bacterial cultures were grown overnight in Lysogeny Broth (LB) medium (*23*) at 37 °C with shaking. For survival experiments, overnight cultures were diluted 1:40 in fresh LB medium and cultivated until the mid-exponential phase (2×10^8 cells per mL). Cellular washing, dilutions, and MC treatment were performed in M9 buffer (*23*). Whenever necessary, M9 was supplemented with 4 g of glucose/L, and supplemented with 2.5 mg of Casamino Acids/mL and 10 μg of thiamine/mL (M9S). Radioactive cultures were grown in M9S medium containing 10 μCi [methyl-^{3}H]-thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA)/mL and 250 μg of 2′-deoxyadenosine/mL until the initial exponential growth phase. Cells were then harvested, resuspended in cold M9S medium supplemented with 10 μg of thymidine/mL, and incubated for 30 min at 37 °C to chase the unincorporated [methyl-^{3}H]-thymidine from the intracellular pool.

Cellular Inactivation by MC. Cultures in the mid-exponential phase of growth were centrifuged (7700*g*, 4 °C, 10 min) and challenged with different concentrations of MC (Mitocin 5 mg, Bristol-Myers Squibb, Brazil S/A) and incubated for 30 min in M9 buffer at 37 °C, under rapid shaking. Aliquots were collected after incubation with MC. Samples were properly diluted in M9 buffer and spread onto LB medium solidified with 1.5% agar (Bacto-agar; Difco). Colony-forming units were scored after overnight incubation at 37 °C. Survival was calculated by dividing the number of remaining viable cells at each dose (N) by those of the titer (N_0). Surviving fractions were expressed as the mean and standard errors of at least three independent experiments. N/N_0 values were plotted in semilogarithmic graphs as a function of the indicated MC concentrations (μg/mL).

DNA Sedimentation Studies. The formation and disappearance of DNA breaks were analyzed by sedimentation of DNA in alkaline sucrose gradients as described by McGrath and Williams (*25*), with slight modifications. Radioactive cultures prepared as described above were treated with MC for 30 min at concentrations that brought cultures to 10% of survival (LD_{10}) as described in the previous item, centrifuged (7700*g*, 4 °C, 10 min), and resuspended in cold M9S medium. Cells were allowed to recover from DNA damage in nonradioactive M9S medium at 37 °C with shaking, and samples were collected at the indicated intervals of time. Undiluted 100-μL aliquots were added on top of 0.2 mL of lysing solution (0.5 M NaOH, 0.01 M EDTA, and 0.05% sodium dodecyl sulfate) layered on the top of a 4.2-mL sucrose gradient of 5−20% (w/v) in 0.4 M NaCl, 0.2 M NaOH, and 0.01M EDTA. The tubes were maintained for 30 min at room temperature and then centrifuged in a Beckman SW55.1 rotor for 120 min at 25 000 rpm and 20 °C. After centrifugation, 30 fractions were collected on paper strips (Whatman No. 17) presoaked with 5% trichloroacetic acid, using a peristaltic pump. The paper strips were washed once in ice-cold 5% trichloroacetic acid, once in 95% ethanol, and once in acetone. After drying, the radioactive content of each fraction was determined in a Beckman liquid scintillation counter. The average molecular weights were calculated according to the method described by Ley (*26*), and the number of DNA strand breaks per *E. coli* genome (2.5×10^9 Da) were calculated as described by Ananthaswamy and Eisenstark (*27*).

Results

Maximum bacterial cell inactivation by MC was obtained with an incubation period of 30 min, irrespective of the dose used (data not shown). Survival curves of each *uvr*-disrupted mutant and wild-type strain exposed to different concentrations of MC are shown in Figure 1. *uvr*A and *uvr*C genes were disrupted by insertion of a *tet* marker into each gene by P1 transduction, and an isogenic-deleted *uvr*B mutant was obtained as described previously (*23*).

*uvr*B mutant was highly sensitive to MC in contrast to the other isogenic *uvr* strains. A less pronounced sensitivity was observed for both the *uvr*C and *uvr*A mutants. The same differences in sensitivity have been detected using MC in a lower dose range (0.1−1 μg/mL). These results indicate that UvrB may play an important role in the repair of DNA lesions generated by MC, while the other two Uvr proteins seem to be less relevant, at least under the tested conditions.

A true comparison between a *uvr*B-proficient and a *uvr*B-deficient background was made possible by the construction of a *uvr*A−*uvr*C double mutant strain. The *uvr*A::*Tn*10 marker was introduced into AB1884 *uvr*C34 strain by P1 transduction (Table 1), with the resulting strain switching its survival from the original less UV-sensitive *uvr*C34 strain to superimpose that of a *uvr*A strain (data not shown). Notably, the *uvr*A−*uvr*C

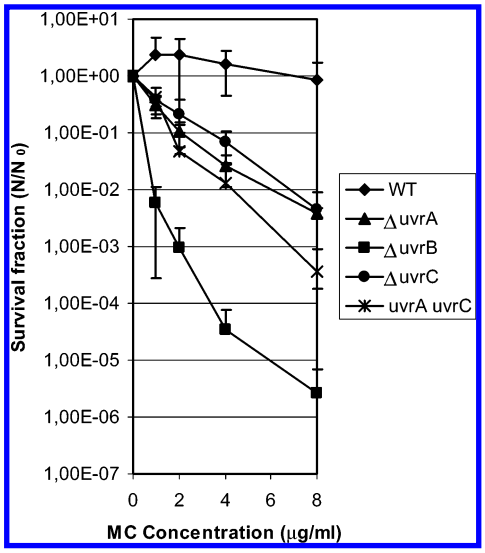

Figure 1. Survival of *E. coli* wild-type and *uvr*-deficient strains. Cultures in the mid-exponential phase of growth were treated with different concentrations of MC in M9 buffer for 30 min. AB1157, wild-type (◆); RJF573, *uvr*A::*Tn*10 (▲); JC3890, *uvr*BΔ301 (■); RJF574, *uvr*C::*Tn*10 (●); and RJF604, *uvr*A::*Tn*10 *uvr*C34 (×). Plots represent the mean ± standard errors.

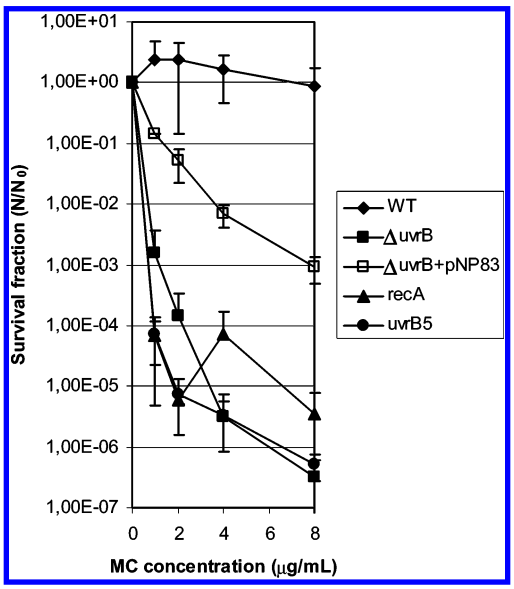

Figure 2. Survival of *E. coli* wild-type, *uvr*B-deficient, and *rec*A142 recombination-deficient strains. Cultures in the mid-exponential phase of growth were treated with different concentrations of MC in M9 buffer for 30 min. AB1157, wild-type (◆); JC3890, *uvr*BΔ301 (■); RJF756, *uvr*BΔ301 + pNP83 (□); GY4763, *rec*A142 (▲); and AB1885, *uvr*B5 (●). Plots represent the mean ± standard errors.

double mutant strain was as resistant as the original *uvr*A and *uvr*C single ones for all MC concentrations employed (Figure 1).

The same experimental procedure was done using point-mutated *uvr* mutants. The *uvr*B5 mutant was even more sensitive to lower MC concentrations than the *uvr*B deletion mutant, while the other *uvr* mutants presented similar sensitivities no matter whether the genes were point-mutated or deleted (data not shown). However, at higher MC concentrations, both uvrB mutants were equally sensitive (Figure 2).

To ensure that the differential sensitivity of the *uvr*B mutant was specifically related to MC, all the *uvr* strains used were also challenged with UV-C, for which these strains are known to be indistinctly sensitive. The expected sensitivity was observed for all three *uvr* mutants (Table 2).

To further ascertain the involvement of *uvr*B in the repair of MC-induced damage, the *uvr*B deletion mutant was transformed with an *uvr*B-harboring plasmid (pNP83). Our results show that this strain was more resistant to MC than the original one (Figure 2).

Homologous recombination has been shown to cooperate with NER in the repair of cross-links (*28*); thus, we tested the survival to MC in an SOS-proficient, recombination-deficient *rec*A142 mutant strain. This strain appeared to be as sensitive to MC as the *uvr*B5 strain under low MC concentrations (data not shown), and at higher MC concentrations, the *rec*A142 mutant was as sensitive as both *uvr*B mutant strains (Figure 2).

UvrB could bind to a MC adduct and recruit a "downstream" endonuclease. To see if this endonuclease is the UvrC homologue, Cho protein, we challenged either a *cho*-deficient, and an *uvr*C−*cho*-deficient double mutant strain with the same MC concentrations. The *cho* strain is as resistant as the wild-type strain, while we observed matching sensitivities for both, *uvr*C and *uvr*C−*cho*-mutant strains (Figure 3).

Subsequently, analysis of DNA sedimentation on alkaline sucrose gradients was employed to further investigate the participation of UvrB in the repair of MC-induced lesions. UvrB-proficient (wild-type, *uvr*A−*uvr*C double mutant, and *uvr*B5 pNP83) and -deficient (*uvr*B5 mutant) strains were compared. The kinetics of the repair process was evaluated by following the average molecular weight of DNA in each strain after MC treatment. The bars shown in Figure 4 refer to the numbers of DNA strand breaks per genome calculated after increasing recovery periods The results indicate that high molecular weight DNA was not recovered in the *uvr*B strain, as expected from the survival results; instead, increasing amounts of DNA strand breaks appear after MC treatment. On the other hand, the *uvr*A−*uvr*C double mutant, *uvr*B5 mutant with plasmid pNP83, and wild-type strains were all able to recover high molecular weight DNA, although the double mutant took more time to do so. In fact, in the double mutant, the whole kinetic process seems to proceed in a slower fashion when compared to the wild-type strain and to the *uvr*B$^+$-transformed *uvr*B5 mutant.

Hence, as determined by the results of DNA sedimentation analysis using alkaline sucrose gradients and the survival experiments, UvrB appears to play a NER-independent role in the repair of MC-induced DNA lesions in *E. coli* in vivo. Once DNA strand breaks appear but are not sealed in the *uvr*B strain after treatment with MC, it is inferred that the resynthesis/ligation step is somehow affected by this mutation.

Discussion

Our results indicate that part of the induced MC damage in vivo appears to require some specific UvrB-dependent action, uncoupled from the other two Uvr proteins, UvrA and UvrC. It seems plausible that UvrB could reach MC-damaged sites without the molecular matchmaking of UvrA. Zou et al. (*2*) observed that DNA damage recognition plays a central role in NER and defined different kinetic rates for the repair process pathway depending on the conformational changes introduced by each damaging agent. Bubble substrates produce a dramatic deformation in DNA structure, which can be efficiently incised by UvrB, even in the absence of UvrA (*2*). The pre-existing strand separation caused by these bubbles is thought to allow the initial UvrA-dependent recognition step to be skipped and to provide direct access to UvrB and UvrBC to the damaged nucleotide. Other proteins involved in DNA metabolism might also facilitate UvrB binding to such DNA structures in an analogous manner as that mediated by UvrA (*29*). Additionally,

Table 2. Survival of *E. coli*, Wild-Type and *Uvr*-Deficient Strains[a]

UV-C (J/m^2)	AB1157 (WT)	RJF574 (Δ*uvr*A)	GY5564 (Δ*uvr*B)	RJF573 (Δ*uvr*C)
0	1.00E+00 ± 0.00	1.00E+00 ± 0.00	1.00E+00 ± 0.00	1.00E+00 ± 0.00
2.5	7.74E−01 ± 0.13	6.01E−01 ± 0.38	1.23E−01 ± 0.00	2.25E−01 ± 0.55
5	7.83E−01 ± 0.13	4.47E−04 ± 0.26	2.95E−02 ± 0.11	5.89E−02 ± 0.01
10	8.16E−01 ± 0.29	1.82E−04 ± 0.00	6.81E−04 ± 0.01	1.28E−03 ± 0.00

[a] Cultures in the mid-exponential phase of growth were treated with different doses of UV-C in M9 buffer. AB1157, wild-type; RJF574, *uvr*A::*Tn*10; JC3890, *uvr*BΔ301; RJF573, *uvr*C::*Tn*10. Numbers represent the mean ± standard errors.

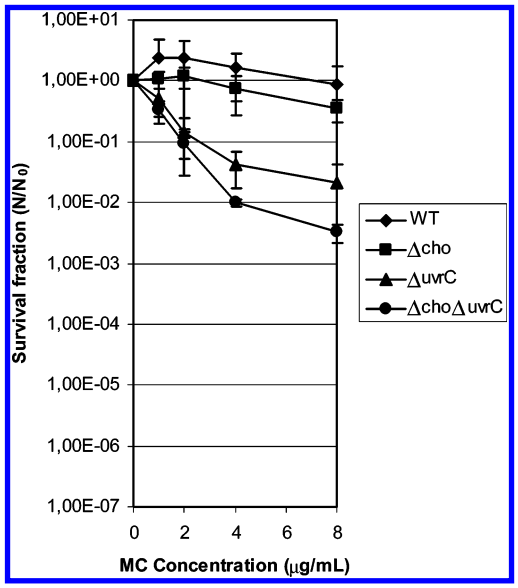

Figure 3. Survival of *E. coli* wild-type, *uvr*C-deficient, and *cho*-deficient strains. Cultures in the mid-exponential phase of growth were treated with different concentrations of MC in M9 buffer for 30 min. AB1157, wild-type (◆); CS5540, Δ*cho* (■); RJF573, Δ*uvr*C (▲); and RJF759, Δ*cho* Δ*uvr*C (●). Plots represent the mean ± standard errors.

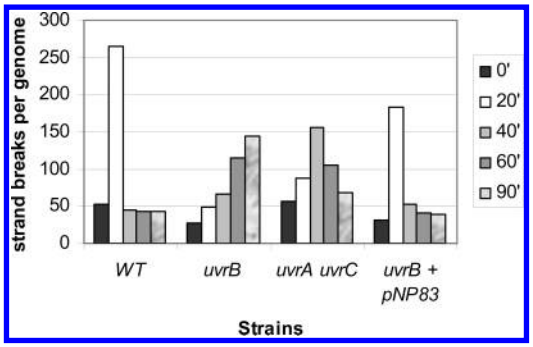

Figure 4. Bar diagram representing the number of DNA strand breaks per genome generated by treatment with MC (at concentrations of their LD_{10}) and after different postincubation times, in different *E. coli* strains. AB1157, wild-type; AB1885, *uvr*B5; RJF604, *uvr*A::*Tn*10 *uvr*C34; and RJF625, *uvr*B5 with pNP83 plasmid.

Moolenaar et al. (*30*) observed that UvrB alone was able to recognize a single cholesterol adduct in vitro, when it is located near to the 5′ end of a double-stranded oligonucleotide. Taking into consideration the results obtained herein under in vivo conditions, MC-induced damages can be directly recognized by UvrB, and then incised by another endonuclease partner different from UvrC, according to the alkaline gradient profile seen in the *uvr*B mutant. The possibility of participation of another endonuclease is supported by the profiles obtained for the *uvr*B-proficient, but *uvr*A−*uvr*C-deficient strain after MC treatment, in which we could find incisions and assemblage of high molecular weight DNA processes taking place. Such endonucleolytic activity has been shown to be absent in the *uvr*B5 mutant strain after irradiation with UV-C (*31*, *32*). The role of UvrB on DNA resynthesis can be supported by the fact that UvrB binds to single-stranded DNA, playing an Ssb-like role, and remains attached to the gapped-DNA until DNA polymerase I arrives (*33*). Besides, there is evidence that a *pol*A−*uvr*B double mutant is not viable (*34*, *35*). Thus, UvrB could recognize the lesion site without UvrA and develop an alternative role in finishing the repair of MC-damaged sites.

The intact N-terminus, 126-amino acid sequence of UvrB5 protein (*36*) could still hold the ability to couple a downstream endonuclease, but unable to proceed to the resynthesis step, what might explain the DNA sedimentation profiles obtained for the *uvr*B5 mutant. A true null *uvr*B mutant (*uvr*BΔ301 strain), which expresses no UvrB protein, is relatively more resistant than the *uvr*B5 one is to MC concentrations below 1 μg/mL (data not shown). These findings suggest that UvrB could be part of more than one multiprotein complex that participate in DNA repair; thus. the lack of UvrB is not as harmful as the presence of a truncated form that could compete with normal proteins to their binding sites.

The participation of UvrC or its homologue Cho in the incision of DNA in the *uvr*B mutant has been ruled out by the findings that the lack of both UvrC and Cho do not lead to a higher sensitivity to MC, suggesting that neither UvrC nor Cho may be the endonucleases acting in MC repair, after the binding of UvrB to a MC adduct.

The importance of UvrB in repairing MC lesions has already been quoted in Kenyon and Walker's previous report (*22*). While searching for LexA-controlled genes, they used *uvr*A and *uvr*B fusion strains with the *lac*Z reporter gene. Several DNA-damaging agents, including MC, were shown to induce β-galactosidase expression in the *uvr*A::*lac*Z fusion strain. They have also isolated two putative *uvr*B::*lac*Z fusion strains, and those showed little induction by MC, despite displaying a normal increased response after UV or nalidixic acid as compared to the *uvr*A::*lac*Z fusion strains. In their study, MC concentrations started from 1 μg/mL that is able to score for very low survival fractions in *uvr*B mutants. Thus, the lack of β-galactosidase induction by MC in the *uvr*B::*lac*Z fusion strains might be explained by a low survival rate.

The homologous recombination pathway has also been proposed to repair interstrand cross-links (*28*), where the excision of the damage from one strand results in a yet attached monoadduct to the opposite strand. This biadduct could then be repaired by the combined action of the recombinational repair system and NER. Our experiments using a recombination-deficient, SOS-proficient, *rec*A142 mutant strain show that it is as sensitive to MC as the *uvr*B mutant. Thus, a new scenario in the repair of MC damage is proposed, indicating a complementary role for UvrB and RecA in cell survival, the first one recognizing damaged sites, and the second one being responsible by recombination events to overcome obstructive MC-induced damage, specially the cross-links. To test this hypothesis, a double mutant *uvr*B-recombination deficient, SOS-proficient *rec*A142 mutant, might be studied in order to measure the sensitivity of this double mutant to MC.

NER has been extensively characterized in terms of repairing UV-C-induced damage, with the three purified Uvr proteins acting on isolated DNA (*6*). However, collected in vivo evidence from our group has pointed to different pathways in the repair of non-CPD DNA adducts (*37*): a UV-C-like one repairing Nitrogen-Mustard-induced (HN2) guanine adducts (*38*), and an UvrB action uncoupled from UvrA and UVrC, as shown herein.

Despite sharing the same base targets in DNA, different damaging agents may require particular forms of repair, according to the resulting distortions in the double helix. MC-induced damage has already been shown to be subjected to the action of the purified UvrABC complex in vitro (*39*). In the present paper, we show in vivo evidence of a new form of NER in MC-treated cells.

So far, the NER machinery was considered as driving removal of a broad range of substrates in vitro (*40*). In fact, such expected NER phenotype can be seen in this paper in terms of sensitivity to UV-C in vivo. In fact, the sensitivity of *uvr* mutants to UV-C and 4NQO are similar (data not shown), as predicted by the in vitro results, while this does not seem to be the case for MC damage. One hypothesis to explain such differences in phenotype regards the different adducts induced by MC; the monoadduct form could be the one to induce the UvrB-dependent mechanism, as already pointed out by our group (*37*). Other adducts, probably the cross-links, would require the complete NER pathway, similarly to what was seen for HN2-induced lesions (*38*).

Although the inherent cytotoxicity of DNA cross-links is a widely held paradigm, the relative importance of MC cross-links and monofunctional adducts has not been satisfactorily addressed so far. Thus, experiments are being carried out to further correlate the UvrB-dependent mechanism with any specific structural DNA damage induced by MC, whether cross-links and/or monoadducts.

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