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UV-C radiation induced conformational relaxation of pMTa4 DNA in *Escherichia coli* may be the cause of single strand breaks

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Abstract

Purpose: The biological consequences of initial physicochemical events following exposure of DNA to germicidal (254 nm) ultraviolet C (UV-C) radiation are not fully understood despite progress that has been made. In particular the cause of UV-C induced single strand breaks is not known. This question has been addressed in the present investigation.

Materials and methods: A plasmid construct, pMTa4, was exposed to UV-C *in vitro* as well as *in vivo* after transforming the plasmid into a repair proficient wild type and repair deficient, *recF*, mutant of *E. coli*. Following UV exposure *in vivo*, the plasmid was isolated under repair non-permissive and permissive conditions. The plasmid isolate and the pure super-coiled closed circular (CC) topological form of the plasmid were analyzed by agarose gel electrophoresis. The dependence of UV-C induced damage and conformational changes on the dose of radiation as well as on the duration of post-irradiation repair incubations was observed. The influence of UV-C on hyperchromic change and intercalation of ethidium bromide into plasmid DNA were also recorded.

Results: UV-C exposure of pMTa4 DNA *in vitro* and *in vivo* induced dose dependent, but sparsely placed, single strand breaks (SSB). While the wild type (AB1157) *E. coli* was able to repair SSB nearly completely under repair permissive condition, the *recF* (JC9239) mutant failed to do so. A dose-dependent relaxation of super-structure of CC form of pMTa4 DNA concomitant with enhanced ethidium bromide intercalation into the plasmid DNA was observed.

Conclusion: It is proposed that the conformational relaxation generated negative super-coiling strain on the DNA backbone of CC form of plasmid as well as exposed chemical bonds for hydrolytic cleavage. This might be the cause of the production of sparsely placed single strand breaks in pMTa4 upon exposure to low doses of UV-C.

Keywords: *UV-C, pMTa4, E. coli, conformational relaxation, single strand breaks in DNA*

Introduction

The understanding of DNA damage induced by germicidal (254 nm) ultraviolet C (UV-C) radiation is biologically relevant as its wavelength coincides with the absorption maxima of purine and pyrimidine nucleotides (NT) (reviewed by the World Health Organization [WHO] 1994, Ravanat et al. 2001). The DNA damaging potential of UV-C has been attributed to its ability to induce dimeric photoproducts (Douki et al. 2003a), oxidized pyrimidine and purine NT (Ravanat et al. 2001, Douki et al. 2003b) and single strand breaks (SSB) in DNA (Miguel & Tyrrell 1986, WHO 1994). The major lesions induced by germicidal UV-C are reported to be cyclobutane pyrimidine dimers (CPD) (Matsunaga et al. 1991) as well as varying amounts of pyrimidine (6-4) pyrimidone photoproducts ((6-4)PP), their Dewar isomers, other dimeric NT and adenine dehydrodimer (Haseltine 1986, Ravanat et al. 2001, Douki et al. 2003b). Relatively few SSB are reported to be induced by UV-C radiation (WHO 1994). Nonetheless, even a low level of strand breakage in DNA can potentially become a source of mutations, if misrepaired or left unrepaired. *E. coli* is capable of recovering from UV induced damage primarily utilizing the nucleotide excision repair (NER) system (Kurosaki et al. 2003). In visible or near-UV (310–480 nm) light, the pyrimidine NT dimers are also repaired utilizing DNA photolyase (Oguma et al. 2001, Burger et al. 2002, Schul et al. 2002). However, in repair deficient situations the unrepaired or misrepaired SSB, even at a low level, can cause significant metabolic problems. Furthermore, compared to ionizing radiation, the absorbed UV-C energy is unlikely to cause DNA strand breaks

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directly. Therefore, it becomes important to understand how strand breaks are induced by UV-C radiation. To the best of our knowledge this has not been elucidated.

Plasmid DNA is a convenient tool to study the molecular mechanism of radiation-induced damage and its repair (Humtsoe & Sharan 2004). Using pMTa4, a plasmid construct of 6,173 base pairs (bp), we have earlier studied the effects of either low and high linear energy transfer (LET) radiation on induction and processing of DNA damage *in vitro* and *in vivo* (Humtsoe et al. 1998, 2003, Humtsoe & Sharan 2004) or radiomimetic chemicals (Odyuo & Sharan 2005). In the present investigation, we have used the plasmid after transforming it into repair proficient wild type and repair deficient *recF* mutant of *E. coli* to study the low-dose UV-C induced damage to pMTa4 DNA and its repair *in vivo*. The aim was elucidation of the cause of induced strand breaks. After exposure of *E. coli* to UV-C *in vivo*, the plasmid DNA was isolated under repair non-permissive and permissive conditions, and analyzed. For comparison, an aqueous solution of pMTa4 was exposed to UV-C radiation *in vitro* and analyzed. The initial events following UV-C exposure *in vitro* and *in vivo* appear to introduce conformational relaxation in DNA super-structure with concomitant increase in SSB. While the wild type *E. coli* could repair the damage, the *recF* mutant failed to do so.

Materials and methods

Chemicals

High purity biochemicals obtained from different sources, as indicated below, were used in the study: Ampicillin (Duchefa, Haarlem, The Netherlands); Agarose (Genei, Bangalore, India); Ethidium bromide (EB) and sodium hydroxide (Merck, Darmstadt, FRG); Acetic acid, ethylenediaminetetraacetic acid (EDTA), sodium chloride (NaCl), acetic acid, sulfuric acid, glucose and sucrose (Qualigens, Mumbai, India); Tris (Boehringer, Manheim, FRG); sodium dodecyl sulphate (SDS) (Sigma, St. Louis, Missouri, USA); Luria-Bertani (LB) broth and LB-agar (Himedia, Mumbai, India); diphenylamine (Glaxo, Mumbai, India) and trichloroacetic acid (TCA) (SRL, Mumbai, India).

E. coli *strains and culture condition*

A repair proficient, wild type *E. coli* K12 strain, AB1157, and a repair deficient *recF* mutant, JC9239, were used in the study (Humtsoe & Sharan 2004). The *recF* gene product is involved in repair of UV-induced DNA damage. The *recF* mutant lacks the functional *recF* gene product and, thus, is deficient in repair of UV-induced damage. Single colonies picked up from Ampicillin$^+$-agar plates were grown overnight at 37°C in LB medium supplemented with 100 μg ml^{-1} Ampicillin. The cells were harvested in mid-log phase for experiment.

Plasmid pMTa4 and its isolation

The plasmid pMTa4 has been described earlier (Humtsoe et al. 1998, 2003, Humtsoe & Sharan 2004, Odyuo & Sharan 2005). It was isolated from the overnight culture of *E. coli* by the alkaline lysis method with minor modification (Sambrook & Russel 2001). The plasmid isolate was dissolved in sterile water to avoid any influence of chemical constituents of buffer, such as counterions, and stored refrigerated.

Transformation of E. coli *with pMTa4*

A standard transformation protocol (Sambrook & Russel, 2001) was used with some modifications. Briefly, to 200 μl of freshly prepared competent cells in a pre-cooled tube, $\approx$75 μl (300 ng DNA) of pMTa4 was added, gently mixed and kept on ice for 20 min. The tube was then incubated, in sequence, at 42°C for 30 s and on ice for 180 s. LB medium (500 μl, pre-warmed to 37°C) was added into the tube, gently mixed and incubated at 37°C for 60 min. The content (200 μl) was then plated on LB agar plates (with 100 μg ml^{-1} Ampicillin) at 10^{-3} and 10^{-5} dilutions and incubated overnight at 37°C. The control was LB-agar plates without Ampicillin.

UV source, dose and experimental design

UV-C germicidal tube (Philips Eindhoven, The Netherlands) fitted in a glass chamber in a dark room was used for the study. The source was approximately 46 cm above the irradiation table delivering 0.04 J $\cdot$ m^{-2} $\cdot$ s^{-1} as measured by UV 340 dosimeter (Biostep, Johnsdorf, Germany). Samples for exposure were placed in open sterile Petri plates and irradiated in the dark for varying time (0 – 150 s) to accumulate UV-C doses of 0, 1.2, 2.4, 3.6, 4.8 and 6 J $\cdot$ m^{-2}.

(a) Dose and time kinetic studies *in vivo*. The overnight cultures of *E. coli* (1.5 ml; 8×10^8 cells ml^{-1}) harboring pMTa4 were pre-cooled on ice and exposed to different doses of UV rays on ice in the dark. Plasmid was isolated from the UV exposed *E. coli* either immediately after exposure (repair non-permissive, R$^-$) or after a post-exposure repair incubation of 60 min at 37°C under fluorescent light (repair permissive, R$^+$). In a 3-point time kinetic study,

the plasmid was also isolated after 15 and 60 min of post-irradiation repair incubation (R^+). The plasmid isolates were subjected to agarose gel electrophoresis.

(b) Dose and time kinetic studies *in vitro*. Aqueous solution of pMTa4 (5 μl containing 8.5 μg DNA) was irradiated to different doses of UV rays at room temperature. They were subjected to agarose gel electrophoresis immediately or after 15 and 30 min of post-irradiation sham-repair incubation (R^+).

Agarose gel electrophoresis

Agarose (1%) gel electrophoresis was done using Tris-acetate-EDTA (TAE) buffer at 1.5 V cm^{-1} for 60 min. After electrophoresis, the gel was stained with EB at a concentration of 0.3 μg ml^{-1} for 15 min, de-stained in water for 30 min and EB-intercalated DNA bands visualized on an UV trans-illuminator (Bio-Rad, California, USA).

Preparation of CC form of pMTa4 DNA from plasmid isolate

A preparative agarose gel (1%) electrophoresis was done to separate out the CC and open circle (OC) forms of the plasmid DNA isolate. After electrophoresis, small piece of the gel with CC band was carefully excised out. The gel slice was placed in a sterile tube over a bed of sterile glass wool and centrifuged (8000 $\times$ *g* for 45 s). From the eluent, CC form of pMTa4 was purified by phenol-chloroform extraction. The extract was lyophilized. Typically, from a 200 μg isolate, 30–40 μg of CC form of plasmid was recovered. The purity of isolation of CC form was checked by agarose gel electrophoresis.

Monitoring hyperchromic shift to measure conformational relaxation in pMTa4

To monitor the conformational alteration in pMTa4 DNA molecules, its photochromic property was exploited (Berg et al. 2002). It is known that DNA molecules show maximum absorption at 260 nm and record increasing absorption (hyperchromic shift) with progressive denaturation and *vice versa*. This property of DNA was used to monitor UV-C induced conformational relaxation in pMTa4. Purified CC forms of the plasmid isolates (5.1 μg DNA in 1 ml H_2O) were taken in different tubes and their $A_{260\,nm}$ recorded (DU 530, Beckman, California, USA, UV/Vis spectrophotometer). The samples were then exposed to increasing doses of UV-C in the dark. Immediately after irradiation, the $A_{260\,nm}$ was recorded for each tube again. The difference of the two gave the hyperchromic shift induced by UV-C in pMTa4 since the quantity of pMTa4 DNA remained the same for each pair of measurements.

Monitoring ethidium bromide (EB) intercalation in pMTa4

The fluorescence of EB is known to increase 20–30 fold upon its intercalation into DNA helix (Smith et al. 1992) and the steady state fluorescence of EB is routinely used in visualization, detection and quantification of DNA. This property has been exploited to monitor alterations in the conformational state of pMTa4 following exposure to UV-C. In a relaxed conformational state, the same quantity of pMTa4 DNA would bind more of EB molecules than its native conformation. Equal amounts of purified CC form of pMTa4 isolates (5 μl containing 8.5 μg DNA) were taken and exposed to increasing doses of UV-C. Immediately afterwards, 2 μl of EB (40 ng ml^{-1}) was added to each irradiated sample and incubated for 30 min at room temperature. The mix was carefully loaded in wells (dot spots) punctured on freshly cast 1% agarose gel on glass slides. After 3 min incubation at room temperature, the gel was transferred onto a UV transilluminator to capture the emitting fluorescence from the wells.

Quantification of pMTa4 DNA by diphenylamine assay

DNA was estimated according to Burton (1956) using diphenylamine with slight modifications. The absorbance was recorded at 540 nm ($A_{540\,nm}$). Calf thymus DNA served as a standard.

Analysis

The images of electrophoresed or dot-spotted agarose gels were digitized (Kodak, New York, USA) immediately. Pixel densities of bands of the OC and CC topological forms of pMTa4 on electropherograms were quantified using 1D Image Analysis software (Kodak, New York, USA). Similarly, the total florescence emancipating from pMTa4 DNA in dot-spots on gels were also quantified.

Results and discussion

Plasmid (pMTa4) DNA has been used in this investigation as it offers a direct measure of induced SSB and double strand breaks (DSB) by acquiring different topological forms (OC and linear (L), respectively) from its native CC form that are clearly resolved on an agarose gel (Humtsoe et al. 1998, 2003, Humtsoe & Sharan 2004, Odyuo & Sharan 2005). Under *in vivo* conditions, the plasmid DNA exists in CC form. However, during its isolation, the methodological interventions invariably induce SSB.

The plasmid isolate in this study contained both CC and OC forms (Figures 1–3, lanes C) typically comprising 60% of CC and 40% of OC. The *in vitro* exposure of pMTa4 to increasing doses of UV-C radiation ($0-6\,J\cdot m^{-2}$) showed a progressive increase in SSB on pMTa4 DNA as the pixel density of the OC form of the plasmid increased correspondingly (Fig. 1). No L form band of pMTa4 was detected on the electropherogram (Figure 1A) showing the inability of UV-C to induce DSB in the dose range used in this investigation. Figure 1A further suggests that the SSB induced by UV-C in pMTa4 were sparsely placed; otherwise some of the proximal SSB could potentially get converted to DSB causing appearance of the L form of pMTa4 on the gel. It is known that UV-C exposure essentially induces NT photoproducts (Matsunaga et al. 1991, WHO 1994, Ravanat et al. 2001, Douki et al. 2003a, b). The energy being low, UV-C does not produce nicks or SSB by direct action. This is in contrast to low- and high-LET radiation (Humtsoe et al. 1998, 2003, Humtsoe & Sharan 2004) or free radical generating Fenton and Haber-Weiss radiomimetic chemical systems (Odyuo & Sharan, 2005), which have been shown to induce both SSB and DSB in pMTa4 DNA by direct as well as indirect means. This investigation was designed to look into the possible consequences of UV-C induced changes in DNA. No direct measure of the induced NT-photoproducts was made in the investigation as estimates are available in literature (WHO 1994, Ravanat et al. 2001). The action spectrum for induction of SSB in cultured cells was reported to be highest for UV-C. This sharply decreased with increasing wavelength and matched rather well with the action spectrum of CDP and (6-4)PP inductions by UV rays, especially in the germicidal wavelength (254 nm) and higher (WHO 1994).

Interestingly, the profile of UV-C dose dependent induction of SSB on pMTa4 *in vitro* (Figure 1A and B) was remarkably similar to those under *in vivo* exposure conditions in AB1157 (Figure 2A and B) as well as in JC9239 (Figure 3A and B) under repair non-permissive (R^-) condition. This was expected as both *in vitro* and repair non-permissive (R^-) *in vivo* conditions were quite similar. In both, due to different reasons, damage to DNA was not repaired. However, following post-exposure repair incubation (R^+), the profiles of SSB in AB1157 (Figure 2C and D) and JC9239 (Figure 3C and D) were significantly different. The repair proficient wild strain of *E. coli*, AB1157, nearly completely abolished at least the SSB type of pMTa4 damage (Figure 2D) while its *recF* mutant, JC9239, failed to do so (Figure 3D). The study was extended by performing a series of experiments to monitor the influence of duration of repair incubation (R^+) on repair of UV-C induced SSB *in vitro* as well as *in vivo* condition for wild and *recF* strains for all dose points. The *in vitro* samples being aqueous solution of pMTa4 did not have any repair system. Nonetheless, they were still sham-repair incubated to create identical conditions of repair incubation as *in vivo*. This ensured that any chemical changes taking place on pMTa4 DNA during repair incubation was equally applicable to the *in vitro* and *in vivo* systems used in the investigation. Accordingly, for *in vitro* investigation, plasmid isolates were exposed to UV rays, repair incubated (R^+) for 15 and 30 min and then analyzed by electrophoresis. For *in vivo* experiments, wild and *recF* cultures harboring pMTa4 were irradiated and subjected to post-irradiation repair incubation (R^+) for 15 and 60 min before isolation of plasmid. The plasmid isolate was analyzed by electrophoresis as described. A representative set of results of such experiments for $6\,J\cdot m^{-2}$ dose of UV is shown in

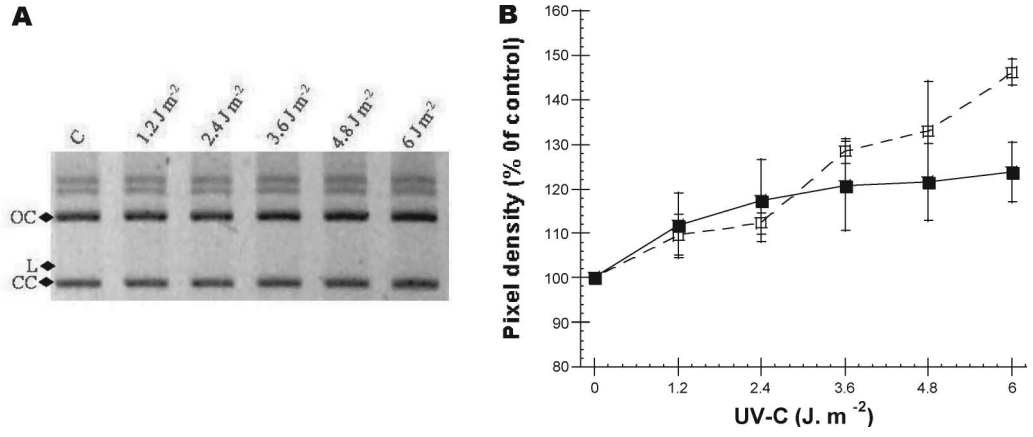

Figure 1. *In vitro* effect of UV-C radiation on pMTa4. The electropherogram (A) shows the resolved topological forms of pMTa4 as a function of increasing dose of UV-C. The pixel densities of CC (—■—) and OC (---□---) bands (mean ± SD) have been plotted (B) as a function of dose of UV-C ($n = 6$). L shows the expected position of linear band of pMTa4.

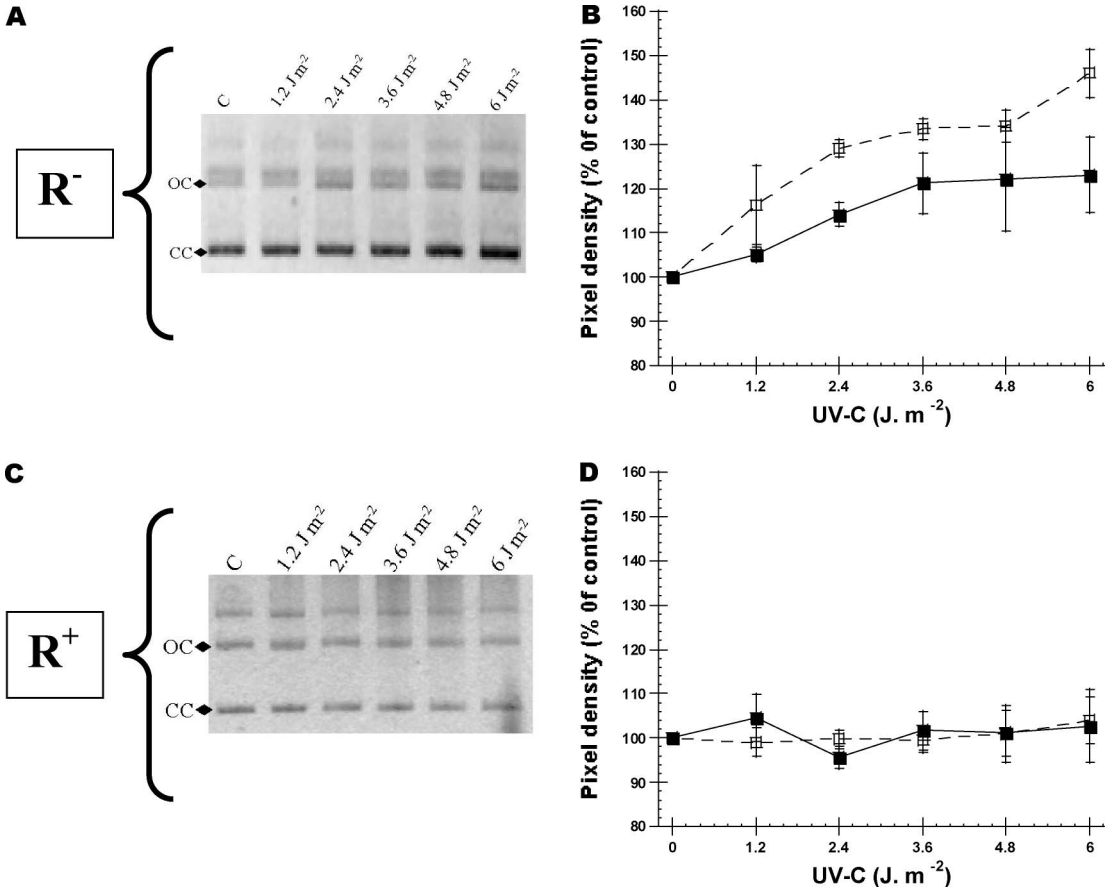

Figure 2. *In vivo* effect of UV-C radiation on AB1157 strain of *E. coli* harboring pMTa4. The electropherograms (A and C) show the resolved topological forms of pMTa4 isolated from AB1157 (wild) *E. coli* as a function of increasing dose of UV-C under repair non-permissive (R^-; top panel) and permissive (R^+; bottom panel) conditions. The corresponding pixel densities of the CC (—■—) and OC (---□---) bands (mean ± SD) have been plotted (B and D, respectively) as a function of dose of UV-C ($n = 5$).

Figure 4. As expected, under *in vitro* exposure condition (Figure 4A), both CC and OC forms remained invariant for increasing period of R^+ incubation indicating that no repair of SSB occurred. Under *in vivo* exposure condition, the wild type strain showed a sharp decline in pixel density of OC form in 15 min of R^+ incubation (Figure 4B). The results suggest that the pace of repair of inflicted SSB was rather fast in the wild type and, perhaps, all SSB were completely repaired in 15 min of R^+ only. In the *recF* mutant, on the other hand, the pixel density of OC declined only marginally after 15 min of R^+ and maintained the level for up to 60 min (Figure 4B) suggesting poor repair of SSB in *recF* mutant in line with the earlier observation (Figure 3). The trend was similar for all lower doses of UV-C for *in vitro* and for wild and *recF* mutant under *in vivo* condition (results not shown).

The UV hypersensitivity of JC9239 is known. We also observed clonogenic survival of AB1157 and JC9239 strains against UV-C wherein only JC9239 had a highly compromised survival (≈20 %) at the maximum dose. Repair of UV-C induced damage in *E. coli* is understandably complex, involving interplay of several repair pathways (Haseltine 1986, Miguel & Tyrrell 1986, Oguma et al. 2001, Burger et al. 2002, Schul et al. 2002, Kurosaki et al. 2003). Nonetheless, the nucleotide excision repair (NER) pathway is proposed to be the main machinery for repair of UV-C induced photoproducts (Kurosaki et al. 2003). Since the JC9239 mutant lacks *recF* protein and it showed persistence of SSB even under R^+ condition (Figure 3B, D), it is logical to assume that UV-C induced photoproducts were not repaired. The wild type, AB1157, which has all repair systems including *recF* protein mediated repair, showed total abolition of SSB under R^+ conditions. Therefore, it is apparent that in the presence of *recF* protein, AB1157 was able to completely repair UV-C induced damage on DNA manifesting as SSB under R^+ condition. In contrast, the JC9239 strain, lacking *recF* proteins, failed to do so and continued with SSB even after post-exposure repair incubation (Figure 3). These facts strongly indicate that persistence of UV-C NT-photoproducts in JC9239 might have been the cause of the observed SSB in pMTa4 DNA (Figures 3 and 4).

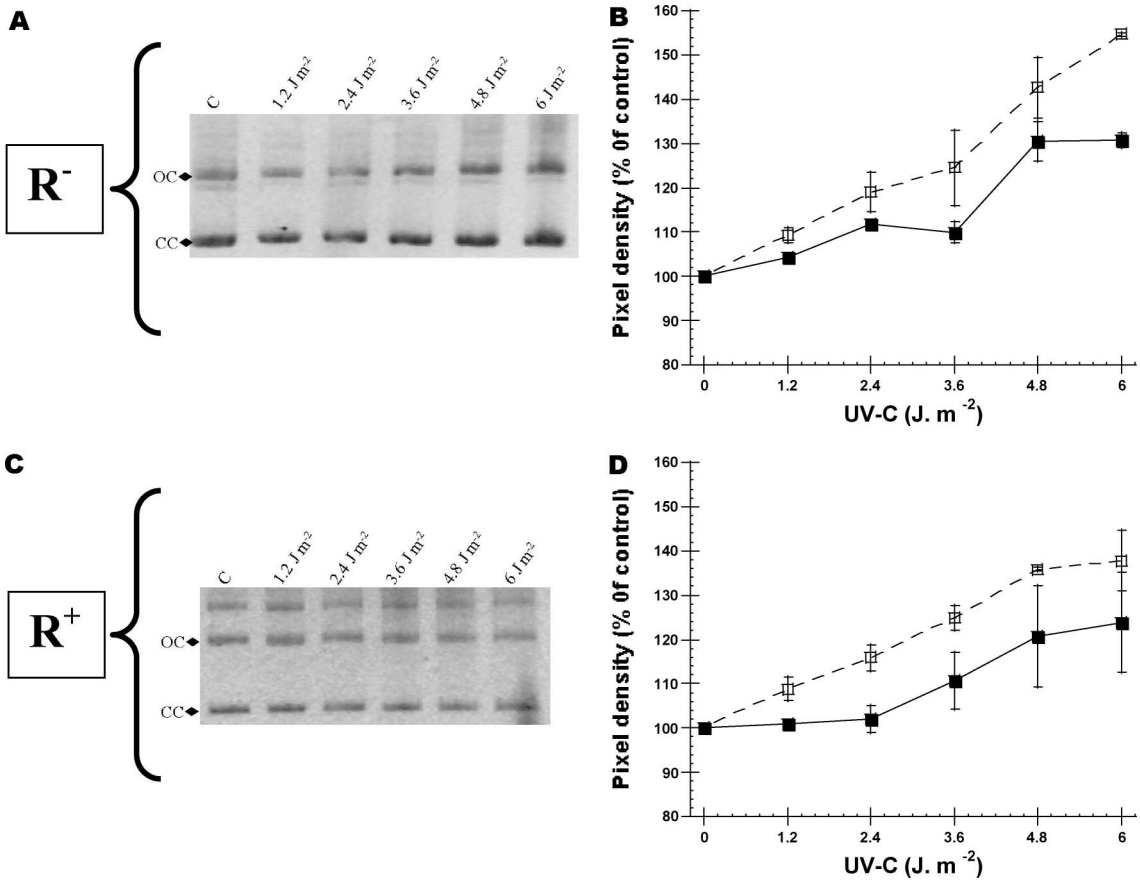

Figure 3. *In vivo* effect of UV-C radiation on JC9239 strain of *E. coli* harboring pMTa4. The electropherograms (A and C) show the resolved topological forms of pMTa4 isolated from JC9239 (*recF*) *E. coli* as a function of increasing dose of UV-C under repair non-permissive (R^-; top panel) and permissive (R^+; bottom panel) conditions. The corresponding pixel densities of the CC (—■—) and OC (---□---) bands (mean ± SD) have been plotted (B and D, respectively) as a function of dose of UV-C ($n = 5$).

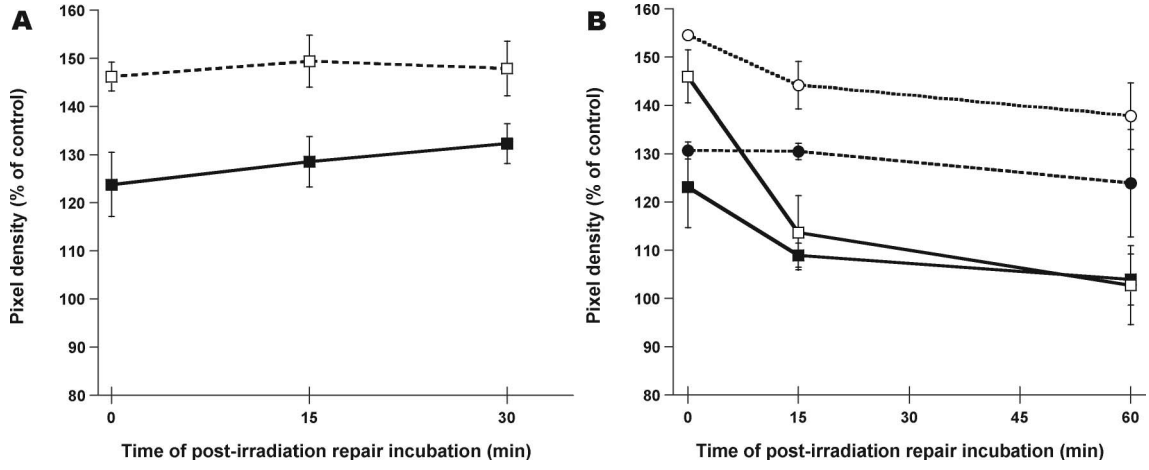

Figure 4. Effect of time of repair incubation on repair of induced SSB by 6 $J.m^{-2}$ dose of UV-C. *In vitro* (A) Plots of pixel densities of CC (—■—) and OC (---□---) bands (mean ± SD) of pMTa4 at 0 (control), 15 and 30 min of R^+. *In vivo* (B) Plots of pixel densities pMTa4 bands (mean ± SD) in wild (AB1157) strain [CC (—■—) and OC (---□---)] and *recF* mutant [CC (—●—) and OC (---○---)] at 0 (control), 15 and 60 min of R^+.

However, we also noticed a dose-dependent increase in the CC band of the plasmid exposed to UV-C *in vitro* (Figure 1) and *in vivo* in both AB1157 (Figure 2) and JC9239 (Figure 3) strains of *E. coli* under R^- condition. This is in contrast to γ-radiation induced or radiomimetic chemical strand breaks in

pMTa4 *in vitro* reported earlier wherein progressive increases in the pixel densities of OC and L bands on the agarose gel were accompanied by progressive decreases in CC form (Humtsoe et al. 1998, Humtsoe & Sharan 2004, Odyuo & Sharan 2005). Since UV-C induced increases in the pixel density of CC form of pMTa4 were remarkably similar under *in vitro* (Figure 1) and repair-non-permissive *in vivo* condition for wild type (Figure 2) and *recF* mutant (Figure 3) strains, it is logical to assume existence of a common cause for this. Under the *in vitro* conditions of our investigation, a fixed amount of pMTa4 preparations were exposed to UV-C and then analyzed by agarose gel electrophoresis (Figure 1). Part of the CC form of the plasmid DNA, upon sustaining SSB following UV-C exposure, should migrate with OC band on the gel (Figure 1). Therefore, with an increase in pixel density of the OC band, a corresponding decrease in CC band was expected. Since our results show otherwise, it can only be explained by hypothesizing that the observed increase in the pixel density of CC band on the gel was due to increased EB intercalation into the CC form of pMTa4 following UV exposure. In other words, the increase in observed pixel density of CC band following UV-C exposure was not due to an increase in the quantity of DNA in the CC band but due to an increase in intercalation of EB. EB binding affinity to relaxed forms (e.g., OC or L forms) is reported to increase up to 1.4 fold compared to the compact form (i.e., the CC form) of SV40 DNA (Jones et al. 1993, Gulston et al. 2002). Therefore, it appears that UV-C exposure was also inducing conformational relaxation in the CC form of pMTa4 DNA.

To verify whether or not UV-C exposure caused conformational relaxation in pMTa4 under our experimental conditions, we needed pMTa4 DNA only in the CC form. It was prepared (see above) and the product was confirmed as 100% pure CC form of pMTa4 isolate by agarose gel electrophoresis (result not shown). Hyperchromic shift in the CC form of plasmid DNA was monitored (see above) as a function of UV-C dose *in vitro* (Figure 5). The progressive increase in the absorption essentially suggests that UV-C induced a dose-dependent relaxation in the conformation of CC form of pMTa4 DNA by taking the positively super-coiled DNA into more negatively super-coiled or relaxed state. The negative super-coiling is likely to relax essentially the CC form of the plasmid as this is the only topological form of a plasmid, which has no free ends on the DNA backbone, to release torsional energy. For the OC forms, the situation was slightly different as it had free ends of the DNA backbone to release torsional energy. The same was true for the L form. Due to this, the negative super-coiling is unlikely to result in

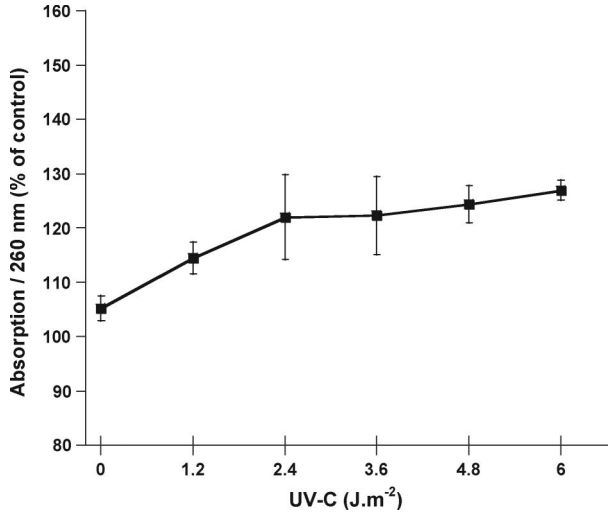

Figure 5. Effect of UV-C radiation on induction of hyperchromic shift in pMTa4. Absorbance (260 nm) of pMTa4 DNA has been plotted as a function of dose of UV-C ($n = 4$) to monitor induced hyperchromic shift (see text for details).

conformational relaxation of OC form of pMTa4. Thus, the cause of the observed increase in pixel density of CC bands on gels as a function of UV-C dose (Figures 1, 2A and B, 3A and B) seems to be a UV-C induced negative super-coiling or relaxation of the CC form of pMTa4 DNA.

We assume UV-C induced photoproducts to be the main cause of the resulting negative super-coiling or relaxation of the CC form of pMTa4 DNA (Figure 5). This assumption derives support from the results of experiments dealing with the kinetics of repair of the plasmid DNA following UV-C exposure (Figure 4). We did not observe any significant modification in the proportions of OC and CC forms in JC9239 cells as a function of duration of repair incubation, but found a rapid decrease in the amount of OC form in AB1157 (Figure 4B). Interestingly, the pixel density of CC form in AB1157 did not increase as a function of repair time, as was expected, because the repair of the OC form leads to regeneration of the CC form of the plasmid. This observation is in agreement with our proposal of an increase in EB staining induced by the presence of photoproducts and consequent relaxation of plasmid DNA that would be lost in AB1157 during repair incubation because of efficient removal of base damage in the CC form.

To further verify whether or not the CC form of the plasmid in a relaxed conformational state intercalated more EB, precisely measured equal amounts of the purified CC fraction of pMTa4 isolate were exposed to increasing doses of UV-C radiation. After exposure, EB intercalation was monitored for a fixed amount of EB and intercalation time (see above). The results showed a dose-dependent increase in the pixel density of DNA-intercalated EB (Figure 6). Since the

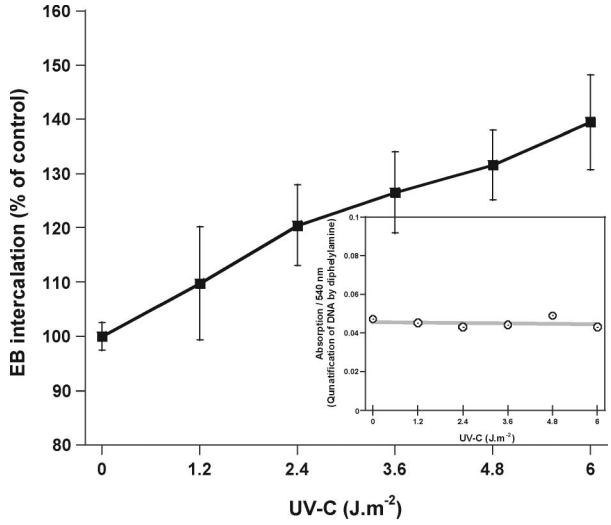

Figure 6. Effect of UV-C radiation on intercalation of ethidium bromide in pMTa4. The fluorescence of DNA-intercalated ethidium bromide in CC form of pMTa4 has been plotted as a function of dose of UV-C ($n = 4$). The inset shows plot of DNA quantification by diphenylamine assay for the samples used for EB intercalation experiment. See text for details.

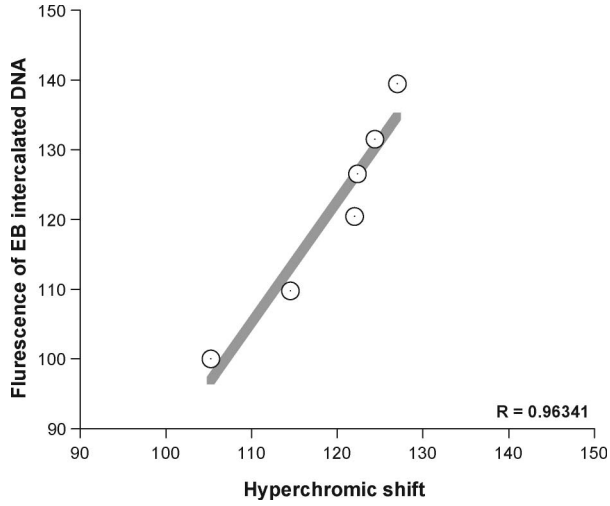

Figure 7. Correlation between hyperchromic shift and ethidium bromide intercalation in pMTa4. The data for hyperchromic shift and ethidium bromide intercalation for increasing doses of UV-C radiation were plotted to find correlation between the two. The correlation coefficient, R, of the slope of linear fit (solid bar) is 0.96341.

increase in pixel density of EB intercalated DNA also indicates a quantitative increase in DNA, it was important to reconfirm the equality of DNA samples in this experiment by an independent assay. Therefore, diphenylamine based chemical quantification of the amount of DNA in each sample was also done (Burton 1956). The result shows an invariant quantity of pMTa4 DNA (Figure 6: inset) in each sample used for the EB intercalation experiment. The chemical assay of DNA was preferred over possible assays using radiolabeling to avoid interference of another quality of radiation with UV-C radiation effects. The plot of EB fluorescence (Figure 6) versus hyperchromic shift in absorbance (Figure 5) shows a correlation coefficient of 0.96314 (Figure 7) – a near linear correlation for the dose range of UV-C used in this investigation. These results strongly suggest that (a) an UV-C induced conformational relaxation in the CC form of pMTa4 DNA occurred, and (b) in a relaxed conformation the CC form of plasmid DNA intercalated more EB.

Based on the results presented here it is hypothesized that the UV-C induced conformational relaxation of pMTa4 DNA might be the cause of induction of SSB. The conformational relaxation is not likely to be the effect of UV-C induced strand break as neither γ-rays (Humtsoe et al. 1998), lithium swift ion (Humtsoe et al. 2003) nor radiomimetic chemicals (Odyuo & Sharan 2005) induced any conformational relaxation in pMTa4 despite strand breaks. Furthermore, UV-C exposure *in vitro* (Figure 1) and *in vivo* (Figures 2 and 3) produced similar effects on the pMTa4 DNA. Lastly, we have directly measured UV-C induced conformational relaxation in CC form of pMTa4 DNA (Figures 5, 6). Kurosaki et al. (2003) have also observed tertiary structural changes in the genome of M13 virion after UV-C exposure. Isaacs and Spielmann (2004) have suggested possible induction of alternative DNA conformations by covalent damage and NT mismatch. DNA double helix unwinding by $\approx 9^\circ$ and bending of the helical axis by $\approx 30^\circ$ due to pyrimidine photodimerization have been directly measured in crystal structures (Park et al. 2002). UV-C induced inter- and intra-strand cross-links of NT (Douki et al. 2003b) are also likely to contribute to DNA unwinding. The conformational relaxation, negative supercoiling or unwinding is likely to generate significant torsional strain on the DNA backbone of the CC form of the plasmid pMTa4 as this form alone has no free end to release the generated torsional energy. This could lead to induction of sparsely placed SSB. However, a contribution of other chemical processes in the induction of SSB cannot be ruled out. For instance, oxidative damage to base or sugars moieties of DNA may induce hydrolytic cleavage of phosphodiester bonds (Cowan 2001, Zeng & Sheppard 2004). This may be achieved without degradation of the 2-deoxyribose moiety or loss of base. In contrast, cleavage may also involve H· atom abstraction of the sugar moiety leading to partial decomposition of 2-deoxyribose unit and release of a base (Gurzadyan & Gorner 1992). Additionally, UV-C induced modifications of nucleosides are also reported to exhibit labile N-glycosidic bonds. These, upon hydrolysis, might produce unstable abasic sites that through I^2-elimination result in breaks in phosphodiester bonds. Involvement or contribution of these processes in the

observed induction of strand breaks has not been ascertained in this work and should be studied further. Nonetheless, the results of the present investigation suggest that the immediate effect of UV-C exposure of pMTa4 DNA is a relaxation of the DNA super-structure. The resulting strain on the DNA backbone or consequent availability of vulnerable bonds for hydrolytic cleavage might be the cause of the induction of sparsely placed SSB on pMTa4 upon exposure to these low doses of UV-C radiation.

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